Brief Genetics Report

Naturally Occurring Amino Acid Substitutions at Arg1174 in the Human Insulin Receptor Result in Differential Effects on Receptor Biosynthesis and Hybrid Formation, Leading to Discordant Clinical Phenotypes

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Missense mutations in the tyrosine kinase domain of the human insulin receptor frequently result in a dominantly inherited form of insulin resistance. We noted a marked disparity in the clinical phenotypes of our study subjects with different missense mutations at the same residue (Arg1174) of the insulin receptor. Subjects with a tryptophan substitution (W) were only moderately hyperinsulinemic, whereas those with a glutamine substitution (Q) had severe clinical and biochemical insulin resistance. Studies were undertaken to explore the molecular mechanisms underlying these differences. Both W and Q mutant receptors bound insulin normally but were kinase inactive. The W mutation resulted in more rapid degradation of newly synthesized mutant receptor, which contrasted with the near-normal biosynthesis of the Q receptor. The propensity of the W receptor to form hybrids with the cotransfected wild-type (WT) receptor was also markedly impaired compared with the Q receptor, to an extent greater than could be explained by lower steady-state expression. Thus, the more clinically benign consequences of the heterozygous W mutant receptor are likely to relate to its impaired biosynthesis and/or reduced capacity to form hybrids with WT receptors. In addition to providing an explanation for the milder phenotype of 1174W versus 1174Q carriers, these studies provide further support for the notion that the dominant-negative effect of insulin receptor tyrosine kinase mutations involves the competition between inactive mutant homodimers and WT/mutant hybrids with active WT homodimers for both ligands and intracellular substrates. *Diabetes* 49:1264–1268, 2000

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CHO, Chinese hamster ovary; hIR, human insulin receptor; PVDF, polyvinylidene fluoride; Q, glutamine substitution; W, tryptophan substitution; WT, wild-type.

Naturally occurring mutations in the human insulin receptor are a rare cause of inherited insulin resistance in humans (1). However, to date, mutations in the insulin receptor and peroxisome proliferator-activated receptor-γ remain the only known monogenic forms of severe human insulin resistance, and detailed study of these monogenic forms has led to important insights into the normal biology of insulin action (2–4). A genotype/phenotype correlation is emerging, with Donohue's syndrome (leprechaunism) being the result of 2 null or similarly devastating mutations in the receptor and Rabson-Mendenhall syndrome ensuing when a small amount of residual receptor function remains. Mutations in the tyrosine kinase domain of the receptor result in dominantly inherited insulin resistance, with a wide range of severity of phenotypes both between and within families (3). To date, however, there have been few attempts to explain in molecular terms the variation in clinical severity associated with this subtype of insulin receptor mutation. We recently observed that subjects with insulin resistance due to missense mutations at Arg1174 of the insulin receptor appeared to have markedly different severity of clinical and biochemical phenotypes depending on the precise residue that replaced the arginine. Our current studies were designed to address the molecular basis for this phenotypic difference.

Table 1 shows the clinical and biochemical features of subjects heterozygous for either the Arg1174Trp (tryptophan substitution [W]) or Arg1174Gln (glutamine substitution [Q]) mutant. The W mutation was initially found in a compound heterozygous subject with Donohue's syndrome who had a missense mutation in the other allele, which resulted in dramatically decreased receptor expression and binding (5). Of the family members available for study, 1 female and 1 male subject were heterozygous for the W mutation, and both were clinically normal. Both had normal glucose tolerance in association with moderate fasting and postprandial hyperinsulinemia. The Q mutation was originally reported in a 16-year-old female with the type A syndrome of insulin resistance, and

TABLE 1
Clinical and biochemical features of heterozygous patients with R1174W and R1174Q insulin receptor mutations

IR-1174 (Ref.)	Sex	Age (years)	Ethnicity	Acanthosis nigricans	Glucose (fasting/2 h) (mmol/l)	Insulin (fasting) (pmol/l)	Clinical phenotype
W (5)	M	35	Macedonian	–	3.8/6.9	432	None
W (5)	F	10	Macedonian	–	3.8/4.8	186	None
Q (6)	F	16	African-American	+	8.2/16.2	1,100	Type A
Q (6)	F	18	African-American	+	8.2/16.2	1,100	Type A
Q (7,15)	F	19	Spanish	+	9.6/17.0	3,020	Type A

all carriers of the mutation in that family were clinically affected and/or had extreme hyperinsulinemia (Table 1) (6). Subsequently, the same mutation was described in an unrelated patient with type A insulin resistance (7). The functional properties of both these mutations have to some extent been studied previously by ourselves and others (3,5,7,8). Although African-American and Spanish ethnicity might confer additional predisposition to type 2 diabetes in the heterozygous carriers for the Q mutation, the marked clinical discrepancy between subjects with mutations at the identical codon of the receptor prompted us to undertake further studies involving a more detailed comparison of receptor properties under identical experimental conditions.

When corrected for receptor expression, specific binding of radiolabeled insulin tracer to transfected Chinese hamster ovary (CHO) cells was indistinguishable among wild-type (WT), W, and Q receptors (data not shown). Both W and Q receptors, when immunocaptured, were equally defective in their ability to phosphorylate artificial peptide substrates based on the sequences of the insulin receptor autophosphorylation site and the YMXM motif centered around Tyr613 of insulin receptor substrate 1. In addition, consistent with previous reports, autophosphorylation of W and Q mutant receptors was undetectable by antiphosphotyrosine immunoblotting (data not shown).

The steady-state levels of mutant and WT receptors were examined by immunoblotting cell lysates of transient transfected CHO cells. The amount of W receptor detected was consistently low—only 18% of WT ($P < 0.0001$). The Q receptor levels were also reduced, but to a lesser extent (62% of WT, $P < 0.0001$) (Fig. 1). Pulse-chase experiments were performed to further characterize the altered expression of both mutant receptors. Processing of the WT receptor from the unprocessed proreceptor to the α- and β-subunits occurred in the "chase" period from 0 to 6 h (Fig. 2). As the amount of WT proreceptor decreased, there was a simultaneous increase in the amounts of α- and β-subunits. As previously reported (5), the W receptor showed a marked impairment in receptor biosynthesis and stability. The disappearance of proreceptor occurred marginally faster than that of WT; however, there was a markedly reduced appearance of the mature α- and β-subunits. In contrast, the behavior of the Q mutant, although showing some impairment in the appearance of α- and β-subunits, was much closer to that of the WT receptor. This finding was consistent with the steady-state expression levels shown in Fig. 1. To examine whether the mutant receptors were targeted for degradation by the proteasome, experiments were undertaken using the proteasome inhibitor lactacystin. Transfected CHO cells were treated with clasto-lactacystin-β-lactone at a final concentration of 10 μmol/l, such that >95% of proteasome activity would be irreversibly inhibited (9,10). Lactacystin did not influence expression levels of the WT insulin receptor β-subunit but led to a significant increase of W and Q insulin receptor β-subunits (Fig. 3). With the W receptor, β-subunit levels were rescued to a greater extent than the β-subunit of the Q receptor (3.6- vs. 1.6-fold, $P = 0.02$). This result is consistent with the processed W receptor being more susceptible to proteasomal degradation than the Q receptor.

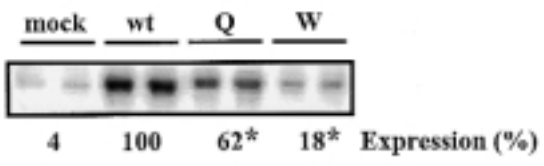

FIG. 1. Insulin receptor expression levels. CHO cells were transfected with expression vectors (pRc.CMV) encoding WT, Q, or W insulin receptors. An empty vector was used for mock transfected cells. Total cell lysates were immunoblotted using an anti-hIR (Carl10), and β-subunits were visualized and quantified using a Fujix BAS 2000 PhosphorImager. The panels shown are representative of 6 experiments performed in duplicate. Expression levels are given as the percentage of expression compared with the WT receptor. *$P = 0.0001$, W or Q vs. WT.

The dominant-negative effects of heterozygous mutations in the insulin receptor tyrosine kinase domain have long been suggested to be due to the formation of kinase-inactive hybrids between WT and mutant half-receptors (11–14). To directly examine the propensity of the W and Q mutant receptors and to form such hybrids, 4 COOH-terminally epitope-tagged constructs were made: WT-myc, WT-EE, W-myc, and Q-myc. Addition of either the c-myc- or the EE-epitope tags had no discernible effect on receptor expression or function, as determined by comparison of WT-tagged and nontagged receptor expression, processing, insulin binding, autophosphorylation, and tyrosine kinase activity (data not shown). The epitope-tagged receptors were recognized only by the appropriate anti-epitope antibodies in immunoblotting and immunoprecipitation studies, whereas anti–insulin receptor antibodies recognized all receptors (Fig. 4; data not shown). The formation of hybrids, consisting of WT and mutant half-receptors, was assessed by immunoblotting of total cell lysates and anti-EE immunoprecipitates with anti–c-myc antibody and vice versa (Fig. 4*A*) as well as by immunoblotting sequential anti-EE and anti–c-myc immunoprecipitates (data not shown). When EE-tagged and c-myc–tagged WT insulin receptors were cotransfected, 20% of receptor immunoreactivity was found in hybrids. In contrast, significantly reduced levels of hybrids

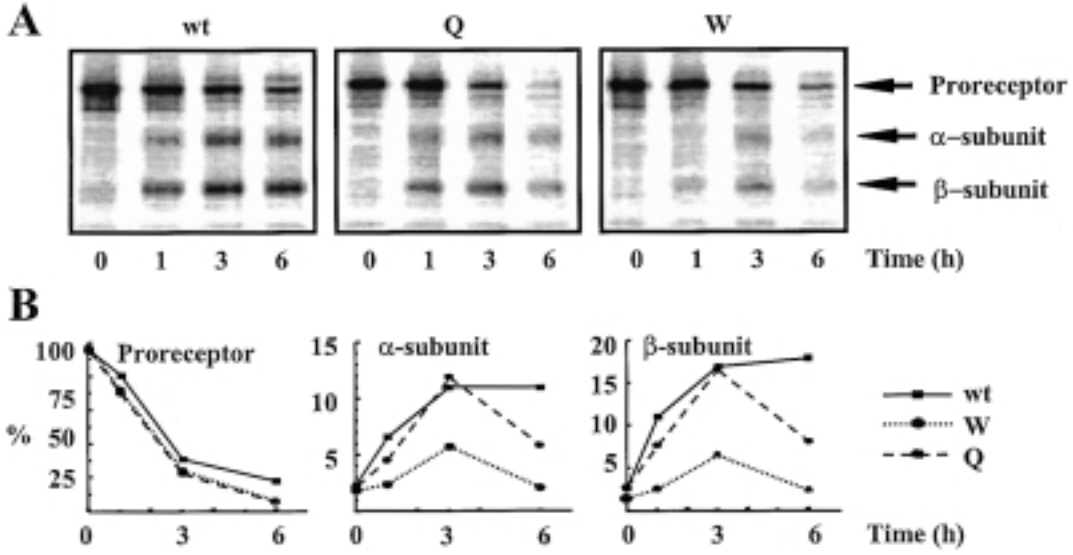

FIG. 2. Pulse chase analysis of WT, Q, and W insulin receptors. CHO cells were transfected with expression vectors encoding WT, Q, or W insulin receptors. Cells were metabolically labeled for 1 h and harvested at the indicated times. Lysates were immunoprecipitated using anti-hIR antibody (83-14). Immunoprecipitates were subjected to SDS-PAGE and visualized by autoradiography (*A*). Panels shown are representative of 4 experiments. Quantification of *A* was performed using a Fujix BAS 2000 PhosphorImager (*B*). Results are presented as incorporation of radiolabel into total receptor (total receptor = proreceptor + α-subunit + β-subunit) at 0 h, standardized to 100% for each receptor. The proportion of radiolabel in the proreceptor, α-subunit, and β-subunit forms are shown in separate panels.

were identified in W/WT cotransfections, with only 2% of total receptors being in hybrids ($P = 0.006$ for WT/WT vs. WT/W). The Q mutant receptors formed hybrids at an intermediate level (14%) but were significantly ($P = 0.001$) greater than the W receptors. The degree of impairment of hybrid formation with the W receptor (7-fold less than Q/WT) was greater than could be explained by the reduced steady-state expression levels of the W receptor (3.4-fold less than the Q receptor), suggesting that the W mutation, in addition to being susceptible to proteasomal degradation, is also less able to form dimers with coexpressed WT receptors. Because the ultimate insulin sensitivity of a cell is likely to be determined by cell surface receptors rather than total receptors, we studied the formation of cell-surface hybrids by cell-surface biotinylation, sequential immunoprecipitation with anti-EE and anti–c-myc antibodies, and blotting with labeled streptavidin. The results were qualitatively similar to those seen in whole-cell lysates with hybrid formation higher in Q/WT than in W/WT (data not shown).

Transiently cotransfected cells were exposed to insulin, and receptor autophosphorylation was examined by antiphosphotyrosine blotting. As expected, clear evidence for autophosphorylation was observed in total cell lysates expressing WT receptors in the presence or absence of either W or Q mutant receptors (Fig. 4*C*). After immunoprecipitation of receptors using the anti-EE antibody, with WT homodimers and mutant/WT hybrids selectively immunoprecipitated, receptor autophosphorylation was again detected in samples stimulated with insulin. However, after immunoprecipitation using anti–c-myc antibody, which selectively captures mutant homodimers and mutant/WT hybrids, no evidence of insulin-stimulated receptor autophosphorylation was seen, which suggests that the mutant/WT hybrids are incapable of undergoing even limited autophosphorylation.

These studies emphasize the importance of considering the effects of a mutation on both the enzymatic and cell biologic properties of the insulin receptor to gain a fuller understanding of the genotype/phenotype relationship. Whereas the substitution of arginine by either tryptophan or glutamine at position 1174 produces an equally deleterious effect on the kinase activity of the receptor, the metabolic and clinical consequences of the 2 substitutions are markedly different. Our studies demonstrate that this difference is likely to be due to 2 phenomena. First, the W substitution results in a receptor that

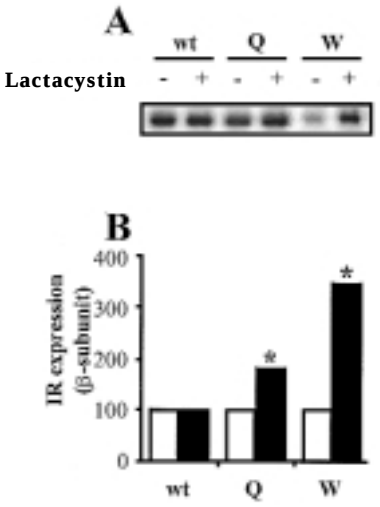

FIG. 3. Effects of proteasome inhibition on receptor expression. Transfected CHO cells were treated with vehicle (DMSO, open bars) or vehicle containing lactacystin (10 μmol/l final concentration, black bars) as indicated. Total cell lysates were analyzed by immunoblotting using anti-hIR antibody (Carl10). Results were visualized and quantified using a Fujix BAS 2000 PhosphorImager (*A*). Panel shown is representative of 4 separate experiments. Quantitative effects of proteasome inhibition (from *A*) on the levels of receptor β-subunit expression are presented graphically in *B*. β-Subunit expression was standardized to 100% for each receptor in the absence of lactacystin. **P* = 0.02 for treated vs. untreated. IR, insulin receptor.

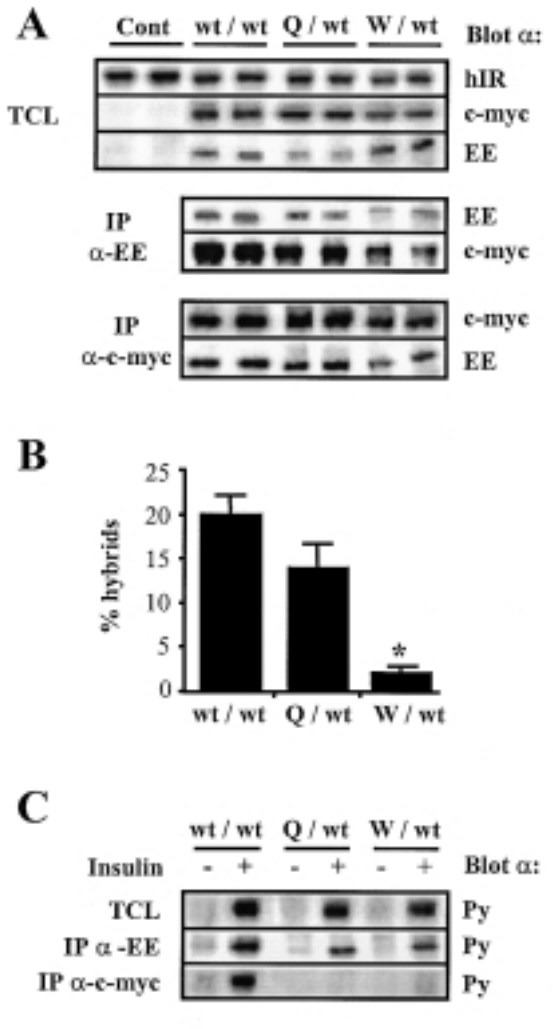

FIG. 4. Hybrid receptor formation and absence of insulin-stimulated hybrid phosphorylation in cotransfected CHO cells. *A*: CHO cells were transiently transfected with a vector encoding native insulin receptor (Cont), cotransfected with vectors encoding EE- and myc-tagged WT receptors (wt/wt) or cotransfected with EE-tagged WT receptors and myc-tagged Q (Q/wt) or W (W/wt) receptors. The upper panel demonstrates the specificity of the immunoblotting antibodies used for the epitope tags (no signal in Cont lane) and broadly comparable levels of expression between cotransfection experiments. The middle panel shows the same lysates subjected to immunoprecipitation by anti-EE antibodies and blotted with anti–c-myc or anti-EE, and the lower panel displays the reciprocal experiment of immunoprecipitation with anti–c-myc and immunoblotting with anti-EE or anti–c-myc. Both panels show lower amounts of hybrid formation with the W/WT than with either the WT/WT or WT/Q cotransfections, and this result was quantitated and plotted in *B*. **P* = 0.006 for W/WT vs. WT/WT. Values are means ± SE. *C*: After overnight serum starvation, cells were stimulated with 100 nmol/l insulin for 2 min where indicated. Receptor autophosphorylation in total cell lysates (TCL) and anti-EE and anti–c-myc immunoprecipitates (IP) was determined by antiphosphotyrosine immunoblotting and visualization using a Fujix BAS 2000 PhosphorImager. The panels shown are representative of 6 experiments. There is no evidence of transphosphorylation of the mutant myc-tagged receptors by cotransfected WT receptors. Py, phosphotyrosine.

is recognized as abnormal by the proteasomal editing system and is degraded at a much faster rate than either WT or Q receptors. Second, the propensity of the W receptor to form hybrids with WT receptors appears to be markedly reduced to an extent not entirely explained by its lower steady-state levels of expression. Thus, in human tissues in which mutant and WT alleles are coexpressed, homozygous WT receptors will predominate at the cell surface in subjects heterozygous for the W receptor, whereas kinase dead WT/mutant and mutant/mutant receptor dimers will be formed to a much greater extent in subjects heterozygous for the Q mutation. As a consequence, a higher number of inactive holoreceptors are present and able to compete with WT homodimers for extracellular ligands and intracellular substrates.

In addition to providing an explanation for the genotype/phenotype relationship in a human disease state, these studies have demonstrated for the first time that differential COOH-terminal epitope tagging of the insulin receptor is a powerful technique for the study of hybrid formation and functional activity. The studies have also shown that structural alterations in the intracellular β-subunit of the insulin receptor can result in differentially enhanced proteasomal degradation of receptors, depending on the specific mutation. Finally, these studies are consistent with the notion that the dominant-negative effects of insulin receptor tyrosine kinase mutations involve the competition between inactive mutant homodimers and WT/mutant hybrids with active WT homodimers for both ligand and intracellular substrates.

RESEARCH DESIGN AND METHODS

Chemicals and reagents. All chemicals and reagents were purchased from Sigma (Dorset, U.K.) unless otherwise stated. The monoclonal antibody 4G10 (antiphosphotyrosine) was purchased from Upstate Biotechnology (NY), the monoclonal antibodies 9E10 (anti-c-myc) and Glu-Glu (anti-EE) from BabCO (CA), and the polyclonal rabbit anti–c-myc antibody from Research Diagnostics (NJ). The monoclonal mouse anti–human insulin receptor (hIR) antibodies 83-7 and 83-14 as well as the polyclonal rabbit anti-hIR antibody Carl10 were gifts from Prof. K. Siddle (Cambridge, U.K.).

Construction of epitope-tagged WT and mutant insulin receptors. All routine DNA manipulations were performed using standard protocols (16). The pRc. CMV expression vectors (Invitrogen, Leek, the Netherlands) encoding full-length WT, R1174W, or R1174Q hIR have been described previously (5,8). To enable differential immunoprecipitation and immunoblotting of WT and mutant hIRs, the c-myc(EQKLISEEDL-EQKLISEEDL) and EE(EYMPME) epitope tags were introduced at the penultimate codons of the WT and mutant receptor cDNAs by polymerase chain reaction–based methods.

Transient transfection of cells. CHO.K1 cells were grown in Nutrient F-12 HAM medium (Sigma, Dorset, U.K.) supplemented with 10% fetal bovine serum, 10^5 U/l penicillin, 0.1 g/l streptomycin, and 2 mmol/l L-glutamine. Cells were grown at 37°C under an atmosphere of 95% air and 5% CO_2. Transfections were routinely performed on cells grown in 30-cm^2 wells using 1 μg DNA/well and Lipofectamine (Gibco, Paisley, U.K.) in accordance with the manufacturer's instructions. For cotransfections, 0.5 μg WT and 0.5 μg mutant insulin receptor cDNA containing plasmids were used. Mock transfections were performed using 1.0 μg empty plasmid. Experiments were performed ~24 h after transfection. Cells were serum starved for ~12 h before stimulation with 100 nmol/l insulin for 2 min.

Immunoprecipitation and Western blotting. Immunoprecipitation and Western blotting were performed as described previously (8). In brief, for determination of insulin receptor expression levels, cells were rinsed once with ice-cold phosphate-buffered saline, lysed, harvested, and precleared by centrifugation. Insulin receptors were immunoprecipitated from samples using 83-14, 9E10, or Glu-Glu antibody (diluted 1 in 100) and 50 μl of a 5% slurry of protein G-agarose. For sequential immunoprecipitation, after the first round of immunoprecipitation, proteins were released from the protein G-agarose by boiling for 4 min (1% SDS, 0.2% Tris, pH 6.8) and centrifuged for 1 min. Supernatants were diluted 1 in 10 in lysis buffer, and the second immunoprecipitation was performed. Immunoprecipitates, or total cell lysates, were resolved by 8% SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) unless otherwise stated. Blots were probed with Carl10, anti–c-myc, Glu-Glu, or 4G10 antibodies followed by [^{125}I]-labeled goat anti-rabbit or anti-mouse antibodies (17). Proteins were visualized using a Fujix BAS 2000 PhosphorImager. For detection of biotinylated proteins, PVDF membranes were blotted against horseradish peroxidase–labeled streptavidin and visualized by enhanced chemiluminescence (Amersham Life Science, Little Chalfont, U.K.).

Pulse chase studies. Transfected cells were preincubated in prewarmed methionine- and cysteine-free Dulbecco's modified Eagle's medium for 1 h. Then, 100 µCi [^{35}S] EasyTag Express Methionine/Cysteine Protein Labelling Mix (DuPont-NEN, Hertfordshire, U.K.) was added to each well, and cells were further incubated for 1 h. Cells were then washed once using complete HAMS F12 medium and incubated in the same mix. At the appropriate time points, media and cells were harvested, and insulin receptors were immunoprecipitated from cell lysates using the monoclonal antibody 83-14. Immunoprecipitates were resolved by 8% SDS-PAGE and analyzed using a Fujix BAS 2000 PhosphorImager.

Proteasome inhibition. Transfected cells were untreated, treated with vehicle alone (DMSO at a final concentration of 0.25%), or treated with vehicle containing the irreversible proteasome inhibitor clasto-lactacystin β-lactone (Calbiochem-Novabiochem, CA) at a final concentration of 10 µmol/l for 16 h. Total cell lysates or immunoprecipitates were analyzed by Western blotting as described.

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