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Functional properties of a heterozygous mutation ($Arg^{1174} \rightarrow Gln$) in the tyrosine kinase domain of the insulin receptor from a Type A insulin resistant patient

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Abstract We analysed the biochemical properties of insulin receptors of a Type A insulin resistant patient with a single heterozygous point mutation substituting Gln for Arg^{1174}. Insulin binding capacity and affinty to Epstein-Barr virus transformed lymphocytes was normal. Quantitative analysis of autophosphorylation and substrate phosphorylation of soluble insulin receptors isolated from patient cells revealed no differences in the basal state whereas in the presence of insulin autophosphorylation activity was only 30% of control receptors. The stimulation of substrate phosphorylation and down-regulation of receptors on patient cells after chronic exposure to insulin was diminished when compared to controls. We conclude that the heterozygous Arg^{1174} mutation does not perturb basal kinase activity but specifically interferes with the kinase activation by insulin and that the mutation has a dominant negative effect on the wild type kinase.

Key words: Receptor; Insulin; Protein tyrosine-kinase; Down-regulation; Mutation

1. Introduction

Insulin resistance plays an important role in the pathogenesis of non-insulin-dependent diabetes mellitus (NIDDM). The insulin receptor is the first site of interaction of insulin with the cell and has, therefore, been a major focus of the study of genetic causes of insulin resistance. Indeed, many mutations of the insulin receptor gene have been identified over the past few years in patients with insulin resistance. Inherited defects of the insulin receptor gene may cause diminished receptor expression [1–6] or impaired receptor function, i.e. insulin binding [7,8] or tyrosine kinase activity [9–11].

The insulin receptor is an allosteric enzyme consisting of two disulfide linked $\alpha\beta$ receptor halves ($\alpha_2\beta_2$). Each $\alpha\beta$ receptor half is derived from one gene product and is processed into α and β-subunits during holoreceptor formation [12]. Insulin binding to the α-subunit induces autophosphorylation of tyrosine residues on the cytoplasmic domain of the β-subunit and this results in the activation of the tyrosine specific kinase. The phosphorylation of the sites Tyr^{1158}, Tyr^{1152} and Tyr^{1163} is critical for the activation of the kinase [13]. In this report we describe a heterozygous point mutation ($Arg^{1174} \rightarrow Gln$) located close to the regulatory Tris-phosphorylation site in a patient with Type A insulin resistance. Remarkably, the same heterozygous mutation $Arg^{1174} \rightarrow Gln$ was recently detected in another patient with type A insulin resistance [14]. The appearance of the same point mutation in two unrelated patients as well as the proximity of the mutation to the major regulatory autophosphorylation sites suggests that this site is critical for proper functioning of the insulin receptor kinase. We therefore, characterize the hiherto unknown functional consequences of this mutation with particular emphasis on the evaluation of the insulin receptor kinase and possible dominant negative effects of the mutant allele on the wild type allele.

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2. Experimental

2.1. Subjects

The patient, a 19-year-old girl of spanish origin, was severely virilized and exhibited a moderate degree of acanthosis nigricans. Both fasting blood glucose (9.6 ± 1.4 mmol/l), and serum insulin concentrations (3020 ± 398 pmol/l) as well as haemoglobin-A_{1C} (13.8%) were markedly elevated [15]. The patient's resistance towards intravenously administered insulin and the absence of an insulin receptor antibody had previously been demonstrated [16]. Control subjects were 6 healthy laboratory staff members.

2.2. Materials

Purified sperm wale insulin was a gift from Shimizu Pharmaceutical (Shizuoka, Japan). Cyclosporin A was provided by Sandoz Pharma Ltd. (Basel, Switzerland). Monoclonal anti-insulin receptor antibody (code RPN.538), ^{125}I-insulin, [γ-^{32}P]ATP, [α-^{33}P]ATP and [γ-^{33}P]ATP and ^{14}C-labelled electrophoresis molecular weight markers (Rainbow, code CFA.756) were purchased from Amersham (Amersham, England). Wheat-germ-agglutinin (WGA)-agarose gel was purchased from E-Y Labs, Inc. (San Mateo, CA) and bovine serum globulin, bovine serum albumin, *N*-acetyl-glucosamine and poly Glu,Tyr (4:1) from Sigma Chemicals Co. (St. Louis, MO). Protein G agarose was obtained from Calbiochem corporation (La Jolla, CA). Primers were synthesised by Microsynth (Windisch, Switzerland) and the PCR reagent kit was purchased from Perkin Elmer Cetus (Norwalk, CT). Sephaglas band prep kit was purchased from Pharmacia LKB (Milwaukee, Wisconsin) band the Cyclist DNA sequencing kit from Stratagene (La Jolla, CA).

2.3. Methods

2.3.1. Transformed lymphocyte culture. EBV-transformed lymphocyte cell lines were established from peripheral blood lymphocytes from the patient and 6 normal subjects as described elsewhere [17]. The incubation of freshly isolated lymphocytes with conditioned medium of marmoset B95/8-cells was performed in the presence of 600 ng/ml Cyclosporin A.

2.3.2. Insulin binding. ^{125}I-insulin binding to transformed lymphocytes was performed at RT for 2 h as previously described [17]. Insulin binding to lectin purified insulin receptors was measured as described before [18]. Specific binding was calculated by subtracting non-specific from total binding. Non-specific binding, determined with 1 μM unlabelled insulin, was less than 2% of total binding. Data were analysed using the LIGAND program [19].

2.3.3. Preparation of partially purified insulin receptors. EBV-cells were washed with phosphate-buffered saline (PBS) and solubilized at

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4°C during 2 h in a solution of 50 mmol/l HEPES buffer containing 5 mmol/l EDTA, 5 mmol/l EGTA, 2% Triton X-100, 1 mmol/l phenyl methyl sulfonyl fluoride, 50 μg/ml leupeptin and 10 μg/ml aprotinin. The soluble fraction was chromatographed over WGA-agarose column to isolate the glycoprotein fraction [20].

2.3.4. Autophosphorylation assay. Lectin purified insulin receptors were adjusted for equal insulin binding (4% specific binding at a ^{125}I-insulin concentration of 1 ng/ml) and incubated with or without 100 nM insulin, with 10 mM $MgCl_2$and with 4 mM $MnCl_2$ for 30 min at RT. The phosphorylation reaction was initiated by the addition of 30 μCi [γ-^{32}P]ATP and unlabelled ATP (50 μM final concentration) and stopped after 6 min at room temperature by adding EDTA and Na_3VO_4 (final concentration 50 mM and 3 mM, respectively). After immunoprecipitation with monoclonal anti-insulin receptor antibody (20 μg/ml) and protein G agarose, the receptors were analysed by reducing 7.5% polyacrylamide gel electrophoresis and autoradiography. The ^{32}P content of the β-subunit was assessed by excising the band from the dried gel and counting its radioactivity. The background as determined by excising another area of each lane was subtracted.

2.3.5. Exogenous substrate phosphorylation. WGA-purified insulin receptors, adjusted for equal insulin binding, were incubated with 0.6 μM insulin, 20 mM $MgCl_2$ and 8 mM $MnCl_2$ for 30 min at RT. 40 μM ATP containing 10 μCi [γ-^{32}P]ATP and poly Glu,Tyr (4:1) (final concentration 2.5 mg/l) were added for 10, 20 and 30 min at RT (during this time the reaction kinetics was linear). Aliquots were spotted on discs of Whatman paper (3MMChr) and immediately washed in 10% TCA containing 10 mM sodium phosphate and the radioactivity incorporated in the exogenous substrate was determined by liquid scintillation counting.

2.3.6. Insulin receptor down-regulation. EBV-transformed lymphocytes from the patient and control subjects were cultured for at least 15 h in the absence or presence of 0.3 μM insulin [21]. The cells were chilled on ice and washed twice with acidic binding buffer (120 mM NaCl, 25 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 1 mM EDTA, 50 mM HEPES, 15 mM Na-acetate, BSA 10 mg/ml, pH 4.5) and two times with neutral binding buffer (pH 7.8). Specific ^{125}I-insulin binding was then determined for 4 h at 4°C [21] and down-regulation was expressed as the percentage of insulin binding to cells not chronically treated with insulin.

2.3.7. Direct sequencing of amplified genomic DNA. Exon specific primers described by Seino et al. [22] were used to amplify exon 1–22 of the insulin receptor gene from genomic DNA of our patient and controls. The PCR products of exon 1–22 were excised from a 1% LMP-agarose gel and subjected to direct sequencing using the Cyclist DNA sequencing kit. The oligonucleotides used to amplify the DNA fragments were end-labelled with [γ-^{32}P]ATP and served as sequencing primers for the (+) and (−) strand. Amino acids of the insulin receptor are numbered according to Ebina et al. [23].

3. Results

3.1. Binding studies

Maximal specific insulin binding to transformed lymphocytes from the patient was 70% of mean control value and well within the normal range (Fig. 1, Table 1). Scatchard analysis of competition binding data revealed no affinity differences of the high-affinity binding site of control and patient receptors (Table 1). We found a high degree of variability of insulin receptor sites on cells of control subjects (3,350 ± 1,600, mean ± S.D.). The apparent number of receptors on patients lymphocytes was (1,500 ± 650).

Table 1
Scatchard analysis of insulin binding data from EBV-transformed lymphocytes

Cell line	^{125}I-insulin bound (%/2 · 10^6 cells)	K_d (nM)	Receptors/cell
Controls	13.8 ± 6.4	0.16–0.42	3,357 ± 1,620
Patient	9.7 ± 1.4	0.08–0.26	1,479 ± 644

Binding data were analysed using the LIGAND program. Curve fitting and parameter estimation was carried out applying the two site binding model. Values for the dissociation constants (K_d) and cell surface receptor numbers are only indicated for the high-affinity site. Data are means ± S.D. from 6 normal control subject cell lines and three independent experiments with the cell line of the patient.

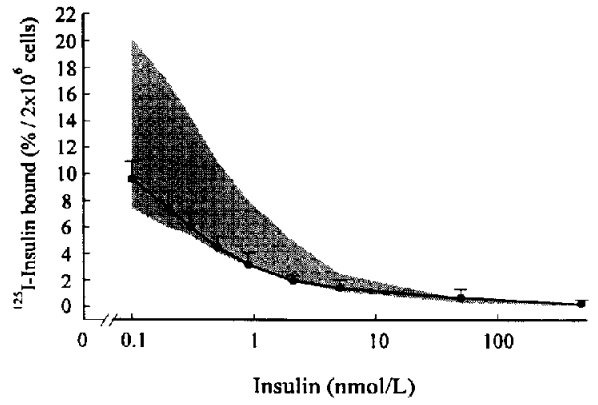

Fig. 1. Insulin binding studies with EBV-transformed lymphocytes from the patient. Values are plotted as the mean ± S.D. of three separate experiments. The shaded area represents the mean ± S.D. of insulin binding in transformed cell lines from 6 normal subjects.

3.2. Phosphorylation studies

Table 2 shows that the maximal autophosphorylation capacity of the patient's WGA purified insulin receptors in the presence of 0.1 μM insulin was only 30% compared to the mean autophosphorylation capacity of normal control receptors, whereas the basal unstimulated autophosphorylation was similar. The resulting stimulation factor at 10^{-7} M insulin for the patient's receptors was therefore 5 times lower than that of the control receptors. A representative autoradiogram is shown in Fig. 2. The immunoprecipitation efficiency of the phosphorylated insulin receptors had minimal variation within an experiment.

Unstimulated phosphorylation of the synthetic substrate poly Glu,Tyr (4:1) by insulin receptors of the patient was nearly identical (101% of the mean control value) to the basal exogenous kinase activity of control receptors as shown in Table 2. In contrast, the stimulation of substrate phosphorylation in the presence of 0.6 μM insulin was 2.03-fold with control receptors and only 1.37-fold with patient receptors.

3.3. Insulin receptor down-regulation

Since kinase activity was shown to be necesarry for insulin receptor internalization [21,24–26] we investigated the down-regulation capability of the patients EBV-transformed lymphocytes upon chronic exposure (at least 15 h) to 0.3 μM insulin. Several incubations with insulin treatments ranging from 15 to 48 h were performed. Down-regulation of cell surface insulin receptors after incubation with insulin for 48 h was 65.6 ± 7.8% (mean ± S.D., $n = 3$) for control cells and 33.7 ± 10.6% for patient cells (data not shown). No differences were noted with shorter (15 h) exposures to insulin. Also, there is more TCA-precipitable ^{125}I-insulin in supernatants of patient cells than of control cells (data not shown), suggesting that there is diminished internalization and subsequent degradation of receptor-bound insulin.

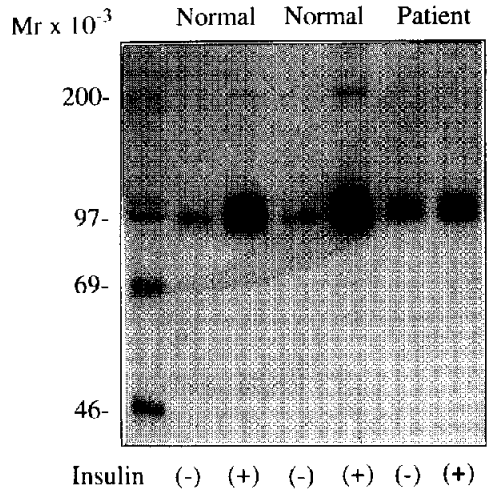

Fig. 2. Representative autoradiogram of autophosphorylated soluble insulin receptors in the absence and presence of insulin from two control subjects and the patient. WGA-purified insulin receptors from EBV-transformed cells of the patient and control subjects were adjusted for equal insulin binding capacity (4% of total binding) and autophosphorylation was assayed with [γ-^{32}P]ATP in the absence (−) and presence (+) of 0.1 μM insulin as described in section 2. After immunoprecipitation with monoclonal anti-insulin receptor antibody the precipitated protein were analysed by 7.5% SDS-PAGE.

3.4. Sequencing

By subsequent direct sequencing of all 22 exons of the patients insulin receptor gene we detected four known silent polymorphisms, each of them in one of the two alleles: Asp^{519} (GAT/C) and Ala^{523} (GCG/A) in exon 8, Phe^{642} (TTC/T) in exon 9 and Tyr^{984} (TAC/T) in exon 17. The sequence pattern of exon 20 of the patient revealed two bands at the second nucleotide in codon 1,174 representing a heterozygous state for the point mutation CAG → CGG which results in the substitution of glutamine for arginine (Fig. 3).

4. Discussion

In this report we characterize some of the functional properties of a mutation in the insulin receptor gene of a patient with the type A syndrome of insulin resistance. This mutation leads to the substitution of Gln for Arg^{1174} in the β-subunit of the insulin receptor and is present in a heterozygous state. Arg^{1174} is located in the proximity to the major regulatory autophosphorylation site (Tyr^{1158}, Tyr^{1162} and Tyr^{1163}) and is a highly conserved residue in the family of insulin receptor genes. In all other tyrosine kinases the corresponding site is also occupied by a basic amino acid, namely a lysine residue [27]. Several other mutations in the regulatory domain of the insulin receptor tyrosine kinase have been reported to alter tyrosine kinase activity: Ala^{1134} → Thr [28], Ala^{1135} → Glu [29], Met^{1153} → Ile [30], Arg^{1164} → Gln [31], Trp^{1193} → Leu [32] and Trp^{1200} → Ser [33,34].

Two different experimental systems have mainly been used to examine the functional properties of mutated receptors: (i) Different cell lines (i.e. CHO, NIH-3T3 and COS 7) transfected with the mutated insulin receptor [28–30,35–38] and (ii) Epstein-Barr virus transformed patient lymphocytes [9,11,31,32,39]. The two systems differ profoundly in that the mutated receptor gene in transfected cells is present in a 'homozygous' state so that only mutated receptors are synthesised. The homogeneity of the receptors subsequently facilitates the biochemical analysis of receptor functions. Therefore, in most recent studies the transfection system was used. However, the Arg^{1174} mutation, as well as the majority of all receptor kinase mutations are in a heterozygous state and therefore, both, mutated (mt) and wild type (wt) alleles, are expected to be expressed. Furthermore, the mature receptor is formed by the assembly of two gene products, which have to interact during the allosteric activation of the receptor kinase by insulin [20]. If both alleles are equally expressed, three possible receptor species, wt/wt, wt/mt hybrid and mt/mt receptors could form in a expected ratio of 1:2:1. Within hybrid receptors, the mt gene product could impair the wt gene product or the wt could largely compensate for a defective mt gene product. In a 'homozygous' transfection system, this issue cannot be addressed and we therefore, chose to analyse the functional properties of the Arg^{1174} mutation using EBV transformed patient cells. The expected inhomogeneity of receptors necessitates a careful quantitative analysis of receptor functions.

Five parameters were examined: (i) insulin receptor number, (ii) affinity of insulin receptor binding, (iii) insulin receptor autophosphorylation, (iv) substrate phosphorylation and (v) insulin receptor down-regulation.

Scatchard analysis of competition binding of insulin to EBV transformed cells from the patient revealed no significant differences in receptor affinity and receptor number. The insulin receptor number of patient cells was lower than the mean value of normal control subjects, but it was still within the normal range. The observed high degree of variability of insulin receptor numbers on EBV-transformed cells of normal subjects has already previously been documented [17,40]. The finding of

Table 2
Comparison of autophosphorylation and substrate phosphorylation activities

	Autophosphorylation (P_i/receptor)		Stimulation factor	Substrate phosphorylation (pmol $P_i \cdot min^{-1} \cdot mg^{-1}$ substrate)		Stimulation factor
	− insulin	+ insulin		− insulin	+ insulin	
Controls	0.35 ± 0.08	2.72 ± 0.77	7.8	0.19 ± 0.06	0.37 ± 0.10	2.03
Patient	0.53 ± 0.16	0.81 ± 0.26[a]	1.5	0.19 ± 0.03	0.26 ± 0.05[a]	1.37

Autophosphorylation and substrate phosphorylation studies of partially purified insulin receptors were performed as described in section 2.3. Receptors were normalized for insulin binding activity and incorporation of phosphate in the absence and presence of insulin was quantified by dry β-counting of the excised bands from the polyacrylamide gel representing the 92 kDa β-subunit of the insulin receptor. Phosphorylation data of the synthetic substrate poly Glu,Tyr (4:1) were assessed by liquid scintillation counting of the dried filter disks containing aliquots of the reaction. Data represent the means ± S.D. in 8 (3 control subjects) and 4 (patient) experiments for the autophosphorylation and 3 (2 control subjects) and 3 (patient) experiments for the substrate phosphorylation. [a] $P < 0.001$ vs. control subjects.

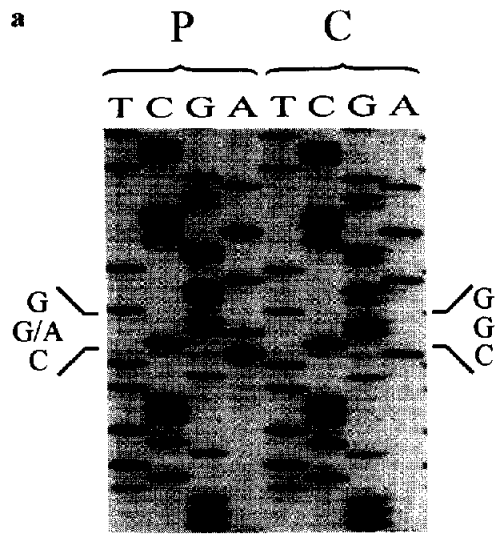

Fig. 3. Partial nucleotide sequence of exon 20. Genomic DNA of the patient (P) and one control subject (C) was amplified using primers which flank exon 20. Sequencing was performed as described in section 2.3. (a) For the patient, the sequencing ladder shows a mutation in codon 1,174 at a heterozygous state. (b) The sequences of the normal and mutant alleles from codon 1,172–1,176 are indicated.

normal binding parameters as well as of normal insulin receptor mRNA levels (results not shown) suggests that the 1,174 mutation neither impairs receptor synthesis nor insulin binding.

To analyse the kinase activity of insulin receptors from the patient equal numbers of partially purified insulin receptors were subjected to autophosphorylation and substrate phosphorylation in the presence or absence of insulin. In the absence of insulin neither autophosphorylation nor substrate phosphorylation of patient receptors was impaired. However in the presence of insulin a marked reduction of autophosphorylation and substrate phosphorylation was noted when compared to receptors from control cells. Thus the 1,174 mutation appears to interfere predominantly with the insulin mediated kinase activation process, but does not seem to perturb the kinase in its basal state. As emphasized before the heterozygosity of the mutation should lead to the formation of 25% fully functional wt/wt receptors which in turn should contribute 25% to the autophosphorylation capacity of patient receptors when compared to receptors from control cells. The finding of only 30% autophosphorylation activity in patient receptors when compared to controls shows that in both mt/mt receptors as well as mt/wt hybrid receptors, the insulin stimulated autophosphorylation capacity must be severely reduced. If the wt gene product is not influenced by the mt gene product, the expected autophosphorylation capacity of patient receptors would be 50% of control receptors. In all experiments the autophosphorylation capacity of patient receptors was well below 50% of controls and therefore, the mt kinase negatively affects the wt kinase, most likely within the hybrid receptor structure (mt/wt). This interpretation is consistent with the observation that most of those kinase mutations are inherited as a dominant trait [9,10,31,32,38]. Furthermore, the in vitro assembly of kinase defective receptor halves with wt receptor halves also results in a dominant inhibition of the kinase activity [41,42].

The functional impairment of the patient receptors with the 1174 mutation, the lack of additional mutations in the insulin receptor coding sequence as well as the appearance of the same heterozygous mutation in an unrelated patient with a similar degree of insulin resistance as our patient suggest that this mutation is likely to be involved in the appearance of insulin resistance. However, the heterozygous state of the 1,174 mutation and of all other heterozygous kinase mutations described previously, should result in the synthesis of 25% kinase active (wt/wt) receptors. How such a quantitative change of receptor should lead to such a severe insulin resistance is subject of speculations. We envisage mainly two possibilities: (i) patients with a heterozygous insulin receptor mutation may carry an additional mutation in a gene encoding a protein involved in the intracellular signalling pathway of the receptor or (ii) the extremely high plasma insulin levels of the patient (3,020 pmol/l) may aggravate insulin resistance by down-regulating any residual functional receptors. The kinase defective receptors may preferentially accumulate at the cell surface, since kinase activity appears to be required for receptor internalization. Though there have been conflicting data on this issue [43], the majority of the reports point to a critical role of the kinase for receptor mediated endocytosis and down-regulation [21,24–26,44]. In accordance, we find a reduced insulin receptor induced down-regulation in EBV-cells with the kinase mutated receptor when compared to EBV-cells with normal receptors. Another hint that insulin levels may influence the degree of insulin resistance in this patient comes from the observation that treatment of our patient with rhIGF I resulted in a pronounced decrease of insulin synthesis and an improvement of hyperglycaemia [15]. A decreased plasma insulin concentration might lead to a reduced down-regulation and the appearance of functional insulin receptors at the surface of insulin target cells and therefore, result in a increased insulin sensitivity. In any case, both, physiological mechanisms as well as potential presence of additional mutations, may contribute to the so far unexplained clinical heterogeneity of carriers of insulin receptor mutations.

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