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Mutation in a Conserved Motif Next to the Insulin Receptor Key Autophosphorylation Sites De-regulates Kinase Activity and Impairs Insulin Action*

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We have recently reported two non-insulin-dependent diabetic patients exhibiting a heterozygous point mutation (R^{1152}-Q) next to the key tyrosine autophosphorylation sites (Y^{1146}, Y^{1150}, Y^{1151}) of the insulin receptor. In the present study, we demonstrate that the Q^{1152} mutation alters a previously unrecognized consensus sequence in the insulin receptor family of tyrosine kinases. To define the effect of this alteration on insulin receptor function, the mutant insulin receptor (Q^{1152}) was constructed and overexpressed in NIH-3T3 cells. In spite of normal insulin binding, "*in vivo*" and "*in vitro*" autophosphorylation as well as transphosphorylation by the wild-type receptor (WT) were deficient in Q^{1152} as compared with the transfected WT receptors. Insulin-stimulated kinase activity toward poly(Glu, Tyr) 4:1 and the endogenous substrates p120 and p175 were also impaired in Q^{1152}. However, insulin-independent kinase activity of Q^{1152} was 2–5-fold higher than that of WT. While insulin stimulated 2-deoxyglucose uptake and glycogen synthase activity in WT-transfected cells with a sensitivity proportional to receptor number, no insulin stimulation was observed in Q^{1152} cells. Similar to the kinase, insulin-independent glycogen synthase activity and 2-deoxyglucose uptake were 2-fold higher in Q^{1152} than in either WT or parental cells. We conclude that the Q^{1152} mutation de-regulates insulin receptor kinase and generates insulin insensitivity in cells. Alterations in this highly conserved region of the insulin receptor may contribute to non-insulin dependent diabetes mellitin pathogenesis in humans.

Insulin biological effects are mediated by a transmembrane receptor composed of two extracellular α-subunits linked by disulfide bonds to two transmembrane β-subunits (1). Upon insulin binding to the α-subunit, the cytoplasmic tyrosine kinase encoded by the β-subunit undergoes autophosphorylation (2, 3). This immediate event activates receptor phosphotransferase activity (4–6). Biochemical studies have correlated activation of the receptor kinase with autophosphorylation of tyrosine residues 1146, 1150–1151[1] (9–18). Single point mutation of tyrosine 1146 or mutations of both tyrosines 1150–1151 result in partially defective insulin receptor (19–21), whereas simultaneous mutation of all of these residues completely inhibit insulin-dependent biological activity of the receptor (22). In contrast, all of these mutations increase insulin-independent (basal) kinase activity (20, 22). Also, when these mutant receptors are expressed in cells, higher levels of basal kinase-dependent biological effects are observed (20, 22, 23). Based on this evidence, it is believed that the region of the receptor encompassing tyrosines 1146–1151 serves as a regulatory domain for the kinase (22). However, the molecular details of the function of the insulin receptor regulatory domain have not been elucidated yet.

In a recent report (24), we have described a genetic defect in two consanguineous patients with non-insulin-dependent diabetes mellitus due to a single base substitution (CGG-CAG) in the insulin receptor gene. This defect led to substitution of the arginine immediately following tyrosine 1151 with a glutamine in the insulin receptor β-subunit (Q^{1152} mutation). Studies "*in vivo*" (24) and in cultured cells from the patients[2] indicated that the Q^{1152} mutation impaired the biological function of insulin receptor and insulin action. We suggested therefore that, in addition to the key autophosphorylation tyrosines, the insulin receptor region adjacent to these sites may also play a critical role in kinase regulation.

In the present work, we have directly addressed the role of this mutation in impairing insulin receptor biological functions by transfecting the mutant receptor cDNA in NIH-3T3 cells. We show that one or more basic amino acid following tyrosine 1151 are highly conserved in the insulin receptor family of tyrosine kinases. The Q^{1152} mutation alters this conserved motif and de-regulates insulin receptor kinase in cells.

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[1] The numbering of amino acids in this paper corresponds to the sequence of the receptor of Ullrich *et al.* (7). These differ from that of Ebina *et al.* (8) by being 12 lower.

[2] A. Petruzziello, G. Riccardi, P. Formisano, C. Miele, B. Di Finizio, A. M. Ferrara, L. Beguinot, and F. Beguinot, submitted for publication.

MATERIALS AND METHODS

General—Preparation of plasmid DNA, agarose gel electrophoresis, restriction enzyme digestion, bacterial transformation, and DNA sequencing were performed by standard methods (25). Enzymes were from Boehringer Mannheim (Kvistgard, Denmark), or Pharmacia LKB Biotechnology A/S (Hillerod, Denmark). All oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. All radiochemicals as well as monoclonal IG2 phosphotyrosine antibodies were from Amersham (Milano, Italy). Monoclonal Ab-3 insulin receptor antibody was obtained from Oncogene Science (Manhasset, NY), whereas polyclonal B9 insulin receptor antibody was a generous gift of Dr. C. R. Kahn (Joslin Diabetes Center, Boston). Media and serum for tissue culture were from GIBCO.

Mutant Construction, Transfection, and Cell Culture—The human insulin receptor cDNA (26) subcloned in pSP65 was kindly provided by Dr. Steen Gammeltoft (Bispebjerg Hospital, Copenhagen, Denmark). Wild-type hIR cDNA (5.2-kilobase pair *Sal*I fragment) was subcloned by linker insertion in the *Sac*II-*Xho*I site of pCO 11 expression vector containing the neo^r-selectable marker (27). To substitute Arg^{1152} with Gln (Q^{1152} mutant), the hIR cDNA fragment, *Bam*HI-*Sal*I (1926–5200), derived from pSP65-hIR was subcloned in M13 mp19. Single-stranded template was prepared and point mutation obtained by oligonucleotide-directed mutagenesis using the following primer 5′-ACGGATTACTACCAGAAAGGGGGCAAGGGT-3′. Mutagenesis was performed according to Taylor *et al.* (28) and confirmed by M13 dideoxy sequencing (29). The *Hinc*II fragment (3187–3871) encoding the Q^{1152} mutation was cloned back in the pSP65-hIR and the mutant hIR-Q^{1152} cDNA cloned into pCO 11 vector. The final construct was resequenced to confirm the presence of the mutation.

NIH-3T3 cells were grown in Dulbecco's modified minimum essential medium (DMEM)[3] supplemented with 10% newborn calf serum. Transfections were carried out by the calcium phosphate method (30) as described previously (31). G-418 (GIBCO) was used at the effective dose of 0.3 mg/ml. Individual G-418-resistant clones were isolated and screened by ^{125}I-insulin binding.

Metabolic Labeling—Confluent monolayers (approximately 1×10^7 cells were incubated in 4 ml of methionine-free DMEM, 0.5% dialyzed BSA for 1 h. Cells were labeled with [^{35}S]methionine (1000 Ci/mmol, 50 μCi/ml) for 16 h in 4 ml of methionine free DMEM with 10% fetal calf serum and glutamine. Cells were washed with phosphate-buffered saline and then prepared for immunoprecipitation by solubilization in 0.5 ml of S buffer (50 mM HEPES, pH 7.4, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 μg/ml pepstatin). After incubation at 4 °C for 2 h, cell extracts were clarified by centrifugation at 4 °C for 20 min at 100,000 × *g*. Insulin receptors were immunoprecipitated from the supernatants with 2 μg of B9 insulin receptor antibody. Immune complexes were washed with 60 volumes of 50 mM HEPES, pH 7.4, 0.1% Triton X-100, and 150 mM NaCl and prepared for SDS-PAGE (32). The gel was treated for 30 min with ENHANCE (Du Pont-New England Nuclear, Firenze, Italy). Radiolabeled receptors were detected following autoradiography at −70 °C for 16 h.

Receptor Purification and Insulin Binding—Confluent monolayers (corresponding to 6–8 × 10^7 cells) were solubilized in 1% Triton X-100, 50 mM HEPES, pH 7.6, 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml bacitracin, and 1 mM phenylmethylsulfonyl fluoride. The insoluble material was separated by ultracentrifugation at 100,000 × *g* for 1 h at 4 °C. The supernatant was applied to a wheat germ agglutinin (WGA)-Sepharose column pre-equilibrated with buffer containing 0.1% Triton X-100, 50 mM HEPES, pH 7.6, 150 mM NaCl and the protease inhibitors described above. The column was extensively washed using the same buffer, and bound glycoproteins were eluted in the same buffer containing 0.3 M *N*-acetylglucosamine.

Insulin binding activity in the WGA eluate was determined by incubating 40 μl of WGA eluate with 1 ml of binding buffer (DMEM, 0.5% dialyzed BSA, 50 mM HEPES, pH 7.5, and ^{125}I-insulin (45,000 cpm/ml, 100 Ci/g) for 16 h at 4 °C. Nonspecific binding was determined in the presence of 1 μM unlabeled insulin (<10% of specific binding). Insulin binding activity was quantitated by addition of ice-cold 25% polyethylene glycol 6000 using 0.3% human γ-globulin as carrier (33). Cell surface insulin binding assays were performed in triplicate on confluent monolayers of cells (approximately 1×10^6 cells/plate, 60-mm plates). Cells were incubated in 3 ml of binding buffer (15,000 cpm/ml) for 3 h at 15 °C in the presence of 0–1 μg/ml unlabeled insulin. Unbound radioactivity was rapidly eliminated by repeated washes with 5 ml of ice-cold phosphate-buffered saline, and cells were solubilized in 1 ml of 0.1% SDS. Radioactivity in the lysates was quantitated in a γ counter. Binding data were analyzed using the LIGAND program for curve fitting and parameter estimation (34).

"In Vitro" Phosphorylation—Aliquots of WGA-purified receptors (20 fmol of insulin binding activity) were incubated in the absence or the presence of 100 nM insulin for 1 h at room temperature. Thereafter, phosphorylation was initiated by the addition of 10 μCi of [γ-^{32}P]ATP in the presence of 3 mM manganese acetate, 1 mM CTP, and 10 μM ATP. After 20 min at room temperature, the reaction was stopped by the addition of 800 μl of ice-cold stopping solution (50 mM HEPES, pH 7.4, 10 mM $Na_4P_2O_7$, 100 mM NaF, 4 mM EDTA, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml aprotinin (14 trypsin inhibitor units/mg) and 1% Triton X-100) and insulin receptors immunoprecipitated as described previously (35) using either phosphotyrosine or monoclonal insulin receptor antibody. Immunoprecipitated phosphoproteins were separated on 7.5% polyacrylamide gels and detected by autoradiography. Phosphorylation of exogenous substrates was carried out as described above except that the synthetic peptide poly(Glu, Tyr) 4:1 was present at a concentration of 2.5 mg/ml, and 3 mM magnesium acetate was substituted for manganese acetate. After 20 min at 22 °C, reactions were stopped by spotting 40-μl aliquots onto Wattman No. 3MM paper (Clifton, NJ). The paper was extensively washed in a 10% trichloroacetic acid, 10 mM sodium pyrophosphate solution and dried; the incorporated radioactivity was determined by liquid scintillation counting. A correction was made for nonspecific adsorption of ^{32}P to the filter paper by subtracting the radioactivity bound to the filter at zero time.

Phosphorylation in Intact Cells—For *in vivo* phosphorylation experiments, 100-mm confluent cell dishes were incubated with serum-free medium for 16 h and then 100 nM insulin was added for 2 more min. Phosphorylation reactions were rapidly quenched by freezing the cells with liquid nitrogen, and cells were solubilized with 1 ml/dish of a solution containing 50 mM HEPES, pH 7.4, 10 mM $Na_4P_2O_7$, 100 mM NaF, 4 mM EDTA, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml aprotinin (14 trypsin inhibitor units/mg), and 1% SDS. Lysates were reduced with 5% 2-mercaptoethanol and proteins separated by 7.5% PAGE and transferred to nitrocellulose paper (Schleicher & Schuell No. 00790, pore size 0.2 μm) at 750 mA, constant current, for 2.3 h. Blotted phosphoproteins were probed by incubating filters in TBS buffer (0.01 M Tris, 0.15 M NaCl, 0.01% NaN_3, pH 7.8, 5% BSA) with 5 μg/ml phosphotyrosine antibody at 4 °C for 16 h. Fliters were further incubated with TBS buffer containing 0.05% Nonidet P-40 and 1 μCi/ml ^{125}I-protein A. Tyrosine-phosphorylated proteins were identified by autoradiography of dried filters and quantitated by laser densitometry.

Determination of 2-Deoxy-D-glucose Uptake—2-Deoxyglucose (2-DG) uptake was measured as described previously (36). Briefly, cells were washed twice with a buffer containing 118 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$ 1.2 mM KH_2PO_4, 10 mM Na_2HPO_4, 2% albumin, pH 7.4 (incubation buffer) and further incubated 45 min at room temperature in the same buffer with different concentrations of insulin. Uptake of 2-DG was measured by adding 100 μl of incubation buffer containing [^{14}C]2-DG (final concentration, 0.2 mM). After incubating for 10 min at room temperature, cells were washed rapidly with ice-cold 0.9% NaCl and lysed with 1 N NaOH. 2-DG uptake was then determined by liquid scintillation counting. Aliquots of the solubilized cells were kept for protein determination. Cytochalasin B (50 μM) was used to estimate carrier-independent uptake.

Determination of Glycogen Synthase Activity—Glycogen synthase was assayed in cell extracts as described by Mandarino *et al.* (37). Reactions were initiated by addition of 20 μl of cell extracts (equivalent to 2 × 10^6 cells) to 40 μl of a reaction mixture composed of 50 mM Tris-HCl, pH 7.4, 25 mM NaF, 20 mM EDTA, 10 mg/ml glycogen, 6.7 mM [^{14}C]uridine diphosphoglucose (specific activity, 0.56 μCi/nmol). Reaction mixture contained either 0.3 or 9 mM glucose 6-phosphate for determinations of the independent and the dependent forms of glycogen synthase, respectively. After a 20-min incubation at 30 °C, reactions were terminated by precipitating aliquots of the incubation mixture on 2 × 2-cm squares of filter paper dropped into cold 66% ethanol. After 30 min, the filter paper were washed twice for 45 min each in cold 66% ethanol, once for 5 min in acetone, dried, and counted for radioactivity by liquid scintillation. Enzyme activity

[3] The abbreviations used are: DMEM, Dulbecco's modified minimum essential medium; BSA, bovine serum albumin; hIR human insulin receptor; PAGE, polyacrylamide gel electrophoresis; *src*, oncogene product from Rous avian sarcoma virus; WGA, wheat germ agglutinin; DG, 2-deoxyglucose.

was expressed as percent independent form (activity measured with 0.3 mM glucose 6-phosphate/activity measured with 9 mM glucose 6-phosphate × 100).

Determination of Thymidine Incorporation—Six-well plates were seeded with 10^5 cells/plate in 1 ml of DMEM supplemented with 10% fetal bovine serum. After incubation for 24 h at 37 °C, the medium was removed and replaced with DMEM containing 0.05% BSA and no serum. After an additional 24 h, the medium was removed again and replaced with DMEM, 0.05% albumin, and different concentrations of insulin ($0–10^{-6}$ M). Incubation was prolonged for an additional 16 h, and the incubation media replaced with the same media supplemented with [^{3}H]thymidine (500 nCi/ml). After a 1-h incubation, media were removed and cells washed three times with ice-cold phosphate-buffered saline. The cell monolayers were solubilized in 1 ml of SDS (0.1%) solution for 30 min at 37 °C. An equal volume of 20% trichloroacetic acid was added to the detergent extract and radioactivity in the trichloroacetic acid precipitate was measured by liquid scintillation counting after solubilization of the pellet in 1 N NaOH (0.3 ml).

RESULTS

Expression of Mutant Receptors—The naturally occurring Arg^{1152}-Gln substitution (Q^{1152} mutation) in the insulin receptor immediately follows the key autophosphorylation sites of the kinase (7), thus altering its regulatory domain. To define the effects of the mutation on insulin receptor function, NIH-3T3 cells were stably transfected with either wild-type or mutant Q^{1152} hIR cDNAs and clonal cell lines screened for expression of transfected receptors by ^{125}I-insulin binding. Several cell clones were isolated. In these cells, similar to previous observations in Rat-1 fibroblasts (38), insulin sensitivity increased almost linearly with the increase in receptor number up to about 2×10^4 receptors/cell. Linearity disappeared with higher insulin receptor expression (data not shown). Therefore, two clones expressing 1×10^4 (WT_1) and 2.5×10^4 (WT_2) wild-type receptors and two clones expressing 1.2×10^4 (M_1) and 2.3×10^4 (M_2) Q^{1152} mutant receptors/cell have been studied in detail. Based on Scatchard analysis (39) of equilibrium [^{125}I]insulin binding data all of these clones displayed dissociation constants (K_D) for insulin between 0.62 and 0.72 nM (Table I). This is similar to the K_D of the endogenous insulin receptor measured in untransfected NIH-3T3 cells (which express 1.5×10^3 insulin receptors/cell). Thus, these transfected wild-type and mutant receptors exhibited normal insulin binding affinities in addition to comparable receptor levels.

To ensure that insulin receptor was properly processed and transported to the cell surface, extracts were prepared from cells metabolically labeled with [^{35}S]methionine. The radiolabeled insulin receptors were then immunoprecipitated with B9 polyclonal anti-insulin receptor antibodies. In all of the cell lines expressing transfected wild-type (WT_2, WT_1) and mutant (M_2, M_1) receptors, these antibodies immunoprecipitated two proteins migrating at M_r = 130,000 and 92,000, which corresponded to insulin receptor α- and β-subunits, respectively (Fig. 1, *lanes B–E*, indicated by *arrows*). Based on laser densitometry, the intensity of these bands correlated well with the number of cell surface receptors as measured by insulin binding (Fig. 1). Consistently, α and β insulin receptor subunits were barely visible in untransfected NIH-3T3 cells which express a very low number of endogenous insulin receptors (Fig. 1, *lane A*). Additional bands with $M_r \geq 200{,}000$ and <72,000 were also noted in some of the experiments. These bands might represent precursors and degraded receptors, respectively, since, like receptor subunits, they were more abundant in transfected cells. The rate of insulin receptor biosynthesis was also examined by labeling the cells with [^{35}S] methionine for 15 min followed by a chase for varying lengths of time. Both the wild-type and the mutant receptors were synthesized at approximately the same rate with no significant difference with respect to the endogenous receptors (data not shown).

Autophosphorylation and Transphosphorylation of the Mutant Receptor—In vitro autophosphorylation of the mutant receptors was compared with that of the wild-type receptors using equal amounts of partially purified receptors from WT_2 and M_2 cells. Consistent with our previous findings with receptors from cultured fibroblasts of patients bearing the Q^{1152} mutation, insulin-stimulated autophosphorylation of mutant receptor was 3-fold decreased compared with wild-type (Fig. 2). Reduced autophosphorylation was observed both at maximally effective (1 μM) and at submaximal (1 nM) insulin concentrations indicating that the defect involved transfected insulin receptors rather than endogenous insulin-like growth factor I receptors. Insulin-independent (basal) autophosphorylation was also lower in the mutant than in wild-type receptor. Same results were obtained by comparing normal and mutant receptor preparations from other transfected clones (data not shown).

Intermolecular phosphorylation of insulin receptors (transphosphorylation) has been recently proposed to play a role in mediating insulin action (40). In cells expressing both normal and mutant receptors such as those of the heterozygous patients bearing this defect (24), transphosphorylation by the normal insulin receptor may allow insulin signaling through

TABLE I

Scatchard analysis of insulin binding data

Equilibrium insulin binding assays were performed as indicated under "Material and Methods." Binding data were analyzed using the Ligand program for curve fitting and parameter estimation. In all of the described cell clones, values for the dissociation constants (K_D) and cell surface receptor number are provided for the high affinity binding sites only. NIH refers to untransfected cells WT_1 and WT_2 and M_1 and M_2 to two different clones of cells transfected with wild-type or Q^{1152} mutant insulin receptor cDNAs, respectively.

Cell line	K_D	Receptors/cell
	nM	
NIH	0.62	1,500
WT_1	0.72	10,000
WT_2	0.60	25,000
M_1	0.68	12,000
M_2	0.70	23,000

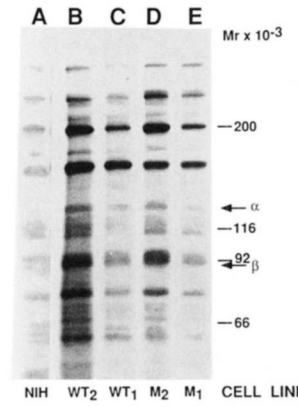

FIG. 1. **Immunoprecipitation of [^{35}S]methionine-labeled insulin receptors.** Cells overexpressing wild-type or Q^{1152} mutant insulin receptors were labeled for 18 h with [^{35}S]methionine as described under "Materials and Methods." Cell extracts were prepared and then immunoprecipitated with the B9 anti-insulin receptor antibody. Immunoprecipitates were subjected to 7.5% SDS-PAGE under reducing conditions and analyzed by autoradiography. *Arrows* indicate positions of the 130-kDa α-subunit and the 92-kDa β-subunit. The autoradiogram shown was exposed at −70 °C for 36 h.

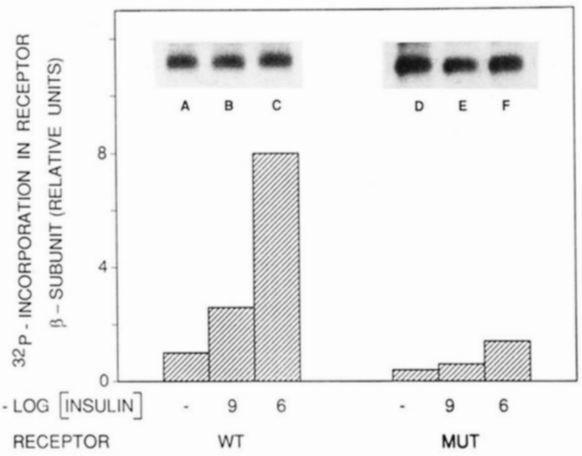

FIG. 2. **Autophosphorylation of partially purified insulin receptors.** WGA-purified insulin receptors (20 fmol of insulin binding activity) were assayed for autophosphorylation with [γ-^{32}P]ATP in the presence of the indicated concentrations of insulin as described under "Materials and Methods." Autophosphorylation reactions were performed for 20 min at 22 °C and receptors immunoprecipitated with agarose-coupled phosphotyrosine antibody in the presence of phosphatase and kinase inhibitors. Precipitated proteins were analyzed by 7.5% SDS-PAGE and autoradiography. *Bar graphs* represent densitometric analysis of the 92-kDa receptor bands. This quantitation was also confirmed by Cerenkov counting of the bands (not shown in the figure). To ensure that equal amounts of receptors were added in each assay, one-half of each incubation mixture was analyzed by immunoblotting with Ab-3 antireceptor antibody and visualized by ^{125}I-protein A. The autoradiograph of blotted receptors is shown in the *inset*. *WT* and *MUT* indicate receptor preparations from the WT_2 and the M_2 cell lines, respectively.

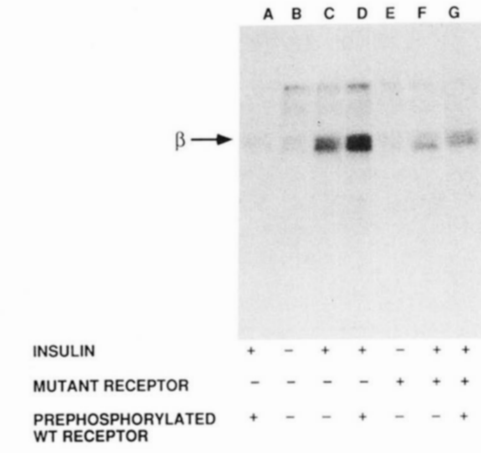

FIG. 3. **Transphosphorylation of the mutant receptor.** Partially purified wild-type receptors were incubated with insulin (1 μM) for 1 h at 22 °C and then prephosphorylated in the presence of unlabeled ATP (5 μM) and manganese acetate (3 mM) for 30 min at 22 °C. Thereafter, the prephosphorylated receptor preparation (80 fmol of insulin binding activity) was added to 80 fmol of the wild-type (*WT, lane D*) or the Q^{1152} mutant (*lane G*) receptor and autophosphorylation conducted in the presence of 2 μCi of [γ-^{32}P]ATP. The same amount of labeled ATP was also added to the remaining (80 fmol) of the prephosphorylated wild-type receptors (*lane A*). *Lanes B* and *C*, autophosphorylation of 80 fmol of the wild-type receptor in the absence or the presence of 1 μM insulin, respectively. *Lanes E* and *F*, autophosphorylation of 80 fmol of the Q^{1152} receptor in the absence or the presence of insulin, respectively.

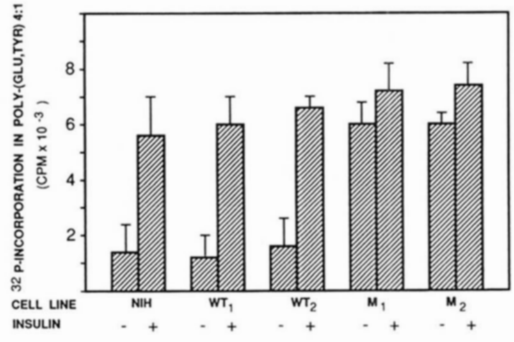

FIG. 4. **Phosphorylation of poly(Glu, Tyr) 4:1 by transfected receptors.** Insulin receptors were purified from untransfected cells (*NIH*) or different clones of cells transfected with either wild-type (WT_1, WT_2) or Q^{1152} mutant insulin receptors (M_1, M_2) as described under "Materials and Methods." Receptor aliquots were normalized for insulin binding activity and substrate phosphorylations initiated by the addition of poly(Glu, Tyr) 4:1 (10 mg/ml) in the presence or the absence of 1 μM insulin, as indicated. Upon 20 min at 22 °C, reactions were quenched on Wattmann No. 3MM paper, and the trichloroacetic acid-precipitable radioactivity was determined by liquid scintillation counting. *Bars* represent the means ± S.D. of four triplicate experiments.

the Q^{1152} as well as the normal receptors. To address this possibility, we studied phosphorylation of the Q^{1152} receptors by the wild-type receptors. The wild-type receptor was exposed to insulin and unlabeled ATP (10 μM) in order to prephosphorylate the receptor and activate its kinase activity and to block tyrosine phosphorylation sites so that they would not be further labeled by radiolabeled ATP. Accordingly, when [γ-^{32}P]ATP was added to the prephosphorylated wild-type receptor in the presence of insulin, no incorporation of ^{32}P was detected (Fig. 3, *lane A*). However, as expected, insulin stimulated autophosphorylation of the wild-type receptor that had not been prephosphorylated (Fig. 3, *lanes B* and *C*). Addition of the prephosphorylated wild-type receptors increased by 2-fold phosphorylation of the unphosphorylated wild-type receptors (Fig. 3, *lane D*). In contrast, when the prephosphorylated wild-type receptors were added to the Q^{1152} receptor, transphosphorylation by the wild-type receptor was not evident (Fig. 3, *lane G*). As shown before (Fig. 2), autophosphorylation of the Q^{1152} receptor in response to insulin was also markedly impaired (Fig. 3, *lanes E* and *F*). This suggested that the same phosphorylation sites which did not undergo insulin-dependent autophosphorylation in the Q^{1152} receptor were also unavailable for transphosphorylation by the wild-type receptor.

Tyrosine Kinase Activity of the Mutant Receptor—Tyrosine kinase activity of the Q^{1152} receptor was first examined *in vitro* using the synthetic peptide poly(Glu, Tyr) 4:1 as a substrate. As shown in Fig. 4, preincubation of endogenous and wild-type receptors with insulin increased by 3–4-fold substrate phosphorylation (Fig. 4, *NIH, WT_1, WT_2*). By contrast, little insulin stimulation of ^{32}P incorporation into the poly(Glu, Tyr) was detected with the mutant receptors (Fig. 4, M_1, M_2). Interestingly, however, basal levels of kinase activity were 3–4-fold higher in mutant than in wild-type receptors. Same results were obtained using the histone 2-B as substrate (data not shown). Thus, Q^{1152} receptors exhibited impaired autophosphorylation and insulin-dependent kinase activity but increased insulin-independent (basal) kinase activity toward exogenous substrates.

Insulin-stimulated tyrosine phosphorylation of insulin receptor substrates was also analyzed *in vivo*. Transfected cells were stimulated with insulin and lysates separated by SDS-PAGE, Western blotted, and probed with phosphotyrosine antibody. In WT_2 cells, insulin induced phosphorylation of a protein migrating at M_r = 94,000 upon reduction (Fig. 5, *lanes A* and *B*, I_R). This species was also detected using specific insulin receptor rather than phosphotyrosine antibodies (not

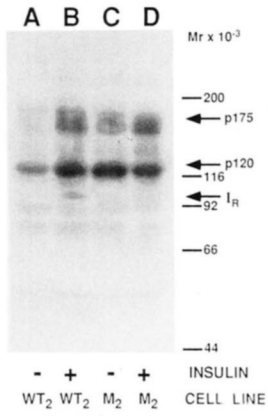

FIG. 5. **Phosphorylation of insulin receptor substrates in intact cells.** NIH-3T3 cell clones expressing wild-type (WT_2) or Q^{1152} mutant receptors (M_2) were stimulated with 100 nM insulin and solubilized as described under "Materials and Methods." Western blots of total cell lysates were probed with phosphotyrosine antibodies and tyrosine phosphorylated proteins revealed by ^{125}I-protein A. The autoradiograph shown is from one representative experiment and was obtained by exposing the dried filter for 24 h at −70 °C.

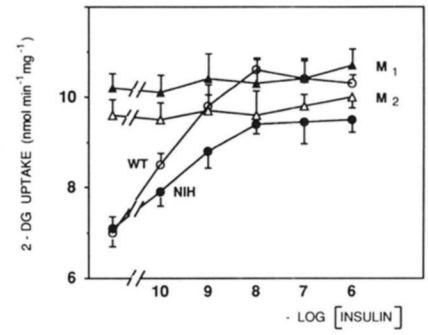

FIG. 6. **Insulin stimulation of 2-deoxyglucose uptake.** Cells were incubated with transport buffer containing the indicated concentrations of insulin for 60 min as indicated under "Materials and Methods." 2-Deoxyglucose uptake was then initiated by adding [^{14}C] 2-deoxyglucose and unlabeled 2-deoxyglucose at a final concentration of 150 μM in the presence or absence of 50 μM cytocalasin B. The cells were then rapidly washed with cold phosphate-buffered saline and lysed with 1 N NaOH, and 2-deoxyglucose uptake was determined by liquid scintillation counting. Each point is the mean ± S.D. of duplicate determinations in four experiments. *NIH* refers to the untransfected cells, *WT* to the transfected cells of the WT_2 clone, and M_1 and M_2 to transfected cells of the M_1 and M_2 clones, respectively.

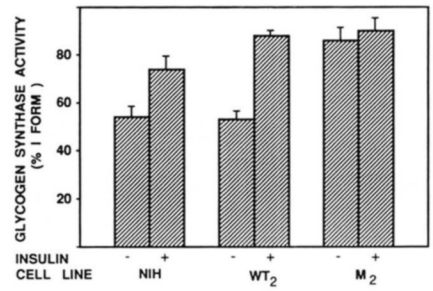

FIG. 7. **Insulin stimulation of glycogen synthase activity.** Confluent monolayers of untransfected (*NIH*), WT_2 and M_2 cells in 100-mm dishes were incubated with 100 nM insulin at 37 °C for 30 min and then cells were harvested as described under "Materials and Methods." Glycogen synthase activity was measured in broken cells at 0.3 and 6 mM glucose 6-phosphate concentrations as described. "% *independent*" activity (*I form*) was calculated by dividing percent [^{14}C]glucose incorporated into glycogen in the presence of 0.3 and 6 mM glucose 6-phosphate as described under "Materials and Methods." Values represent the means ± S.D. of three duplicate experiments.

shown) and therefore was identified as the insulin receptor β-subunit. Two additional phosphoproteins with M_r = 120,000 and 175,000 also underwent tyrosine phosphorylation upon insulin stimulation of the cells. These species were not recognized by insulin receptor antibodies but were phosphorylated *in vitro* by the insulin receptor (data not shown). Therefore, the molecular size and insulin-dependent tyrosine phosphorylation of the 120- and 175-kDa proteins suggested they represent the p120 and the p175 endogenous substrates in these cells, respectively (41, 42), rather than receptor precursor or degradation products. Similar to the *in vitro* phosphorylation experiment shown in Fig. 2, insulin-dependent receptor autophosphorylation was less evident in cells expressing mutant receptors (*lanes C* and *D*). Interestingly, in these cells, basal phosphorylation of the p120 substrate was almost 2-fold higher than in wild-type cells (Fig. 5, *lanes C* and *A*). Also, as was the case for phosphorylation of the exogenous substrates by the Q^{1152} receptors, no further increase in p120 phosphorylation was noted upon insulin stimulation of mutant cells (Fig. 5, *lane D*). Similar to p120, p175 displayed 2-fold increased basal phosphorylation and little insulin stimulation in cells expressing mutant receptors. Thus, the two major insulin receptor endogenous substrates observed in these cells appeared to be similarly affected by the abnormal kinase activity of Q^{1152} receptors.

Biological Activity—Insulin stimulation of 2-deoxyglucose uptake was examined by treating the cell lines with various concentrations of insulin for 30 min. The incorporation of [^{14}C]2-deoxyglucose over a 10-min period was then evaluated. As shown in Fig. 6, at each concentration, insulin exhibited a greater effect in cells transfected with the wild-type receptors (WT) than in parental cells (NIH), consistent with the expression of a higher receptor complement. Insulin ED_{50} was 0.2 nM in the WT_2 clone expressing about 2.5×10^4 wild-type receptors/cell and 1 nM in the parental NIH-3T3 cells expressing about 1.5×10^3 receptors/cell. Thus, in these cells, insulin sensitivity was approximately proportional to the number of insulin receptors. In contrast, almost no insulin stimulation of glucose uptake was detected in the two mutant clones (1.2×10^4 and 2.3×10^4 Q^{1152} receptors/cell). Interestingly, however, in these cells, insulin-unstimulated (basal) 2-deoxyglucose uptake was comparable with the maximally insulin stimulated uptake in both parental cells and in those expressing wild-type insulin receptors. Very similar results were obtained by studying insulin effect on glycogen synthase activity. In both parental and wild-type cells (NIH, WT), maximally effective concentrations of insulin increased the I form of glycogen synthase activity by 40–70% (Fig. 7, *bars*). Both in the absence and in the presence of insulin stimulation, cells expressing Q^{1152} receptors (M^2) displayed same levels of glycogen synthase activity as insulin-stimulated wild-type and parental cells. Thus, once transfected in NIH-3T3 cells, the Q^{1152} insulin receptor induced higher basal glucose uptake and glycogen synthase activity as well as lack of further insulin responses. This was coincident with the increased basal levels of the receptor kinase activity toward substrates.

The mitogenic signaling properties of the Q^{1152} receptor were also investigated. Insulin, at 1 nM, increased [^{3}H]thymidine incorporation by 15% in untransfected NIH-3T3 cells (Fig. 8, *bar A*). In these cells, however, 90% of maximal insulin effect occurred at 100 nM. In cells transfected with the wild-type receptor (WT_1, WT_2), 1 nM insulin elicited 30–40% of the maximal effect, whereas maximal increase in thymidine incorporation was evident at 100 nM (Fig. 8, *bars C–F*). This increase in insulin sensitivity suggested that transfected wild-

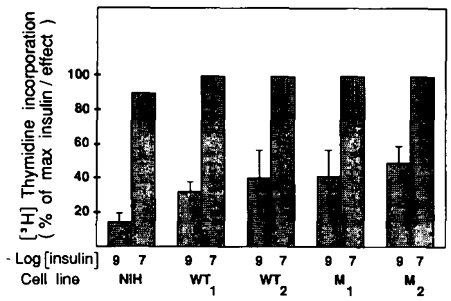

FIG. 8. **Insulin stimulation of thymidine uptake.** Cells were starved from serum, stimulated with the indicated concentrations of insulin, and further incubated with 500 nCi/well of [^{3}H]thymidine as described under "Materials and Methods." Monolayers were then solubilized, trichloroacetic acid-precipitated, and thymidine uptake determined by liquid scintillation counting of precipitates. In this figure, insulin-stimulated thymidine uptake is described as the percent of maximal insulin effect (measured at 3 μM insulin) above basal uptake (no insulin stimulation). Basal uptake (means ± S.D.) were 5300 ± 800 cpm for the NIH-3T3 cells, 5000 ± 600, 5600 ± 300, 4000 ± 600, and 4500 ± 700 cpm for the WT_1, WT_2, M_1, and M_2 cells, respectively. Values in the figure represent the means ± S.D. of three duplicate experiments.

INS R	MTRDIYETDY YRKG
IGFI R	MTRDIYETDY YRKG
DIL R	MTRDIYETDY YRKG
ROS	LARDIYKNDY YRKR
7LESS	LARDIYKSDY YRKE
TRK	MSRDIYSTDY YRVG

FIG. 9. **Partial amino acid sequence alignment of tyrosine protein kinases of the insulin receptor subfamily.** *INS R*, insulin receptor human placenta cDNA (26); *IGF I R*, insulin-like growth factor I receptor human placenta cDNA (43); *DIL R*, *Drosophila* gene product related to insulin receptor human placenta cDNA (44); *Ros*, cellular homolog of oncogene product from UR2 avian sarcoma virus from human placenta genomic DNA (45); *7LESS*, *Drosophila* sevenless gene product from *Drosophila melanogaster* eye imaginal disc cDNA (46); *TRK*, colon carcinoma oncogene product from human tumor cell DNA (47). Sequences have been aligned to maximize the homology in the region of the tyrosines corresponding to residues 1146, 1150, and 1151 in the human insulin receptor (*underlined*). The *boxed area* highlights the arginine-lysine residues following the tyrosine triplet which are conserved in six out of seven members of the family. The single letter amino acid code is used.

type receptors initiated mitogenic responses in NIH-3T3 cells. Similar to the WT cells, cells transfected with the Q^{1152} receptor (M_1, M_2) also exhibited increase sensitivity to insulin mitogenic effect compared with the untransfected cells (Fig. 8, *bars G–L*). It appeared therefore that, at variance with the metabolic responses, the Q^{1152} mutant receptor is able to mediate insulin mitogenic effect.

Comparison of the Mutation Site in the Insulin Receptor Family of Tyrosine Kinases—To further analyze the biological relevance of the Q^{1152} insulin receptor mutation, the site of mutation has been compared in different members of the insulin receptor family of tyrosine kinases. Consistent with an invariant function, tyrosines corresponding to residues 1146, 1150–1151 in the insulin receptor are present in all of these kinases (*underlined* in Fig. 9). In the insulin receptor, phosphorylation of these sites is critical for kinase activation (9–22). Interestingly, the tyrosine triplet is followed by a couple of basic residues (Arg-Lys) which also appeared to be highly conserved in the insulin receptor family of tyrosine kinases (Fig. 8, *boxed area*). In the Q^{1152} insulin receptor, the first of these residues was substituted with a glutamine. Concomitantly, kinase activity and metabolic effects were increased independently on insulin while insulin sensitivity was depressed. This suggests that, in the insulin receptor family of kinases, some biological functions might critically depend on the presence of a basic motif following the key phosphorylation sites.

DISCUSSION

The kinase domain of the insulin receptor exhibits close structural homologies with a number of related tyrosine kinases (48). Sequence homologies have been described in the ATP binding site and also in a region of the kinase domain displaying a triplet of highly conserved tyrosine residues (48). In the case of the human insulin receptor, these correspond to residues 1146, 1150, and 1151 and are key autophosphorylation sites whose simultaneous mutation completely inactivate the receptor (22). In the present work, we describe an arginine-lysine motif (RK motif) which is immediately adjacent to tyrosine 1151 and highly conserved within the insulin receptor family of tyrosine kinases. Conservation within these tyrosine kinases suggests that the RK motif is relevant to the function of the insulin receptor. However, at variance from the ATP binding consensus and the tyrosine triplet, its role has not been addressed.

In a previous report, we have described a heterozygous point mutation in the coding region of the insulin receptor gene in two consanguineous patients with a clinically common form of non-insulin-dependent diabetes mellitus (24). The mutation alters the RK motif since it substitutes arginine 1152 for glutamine next to the tyrosine triplet in receptor β-subunit (Q^{1152} mutation). *In vivo* (24) and *in vitro*[2] studies suggested that the Q^{1152} mutation impaired insulin receptor function and insulin action in the patient cells (erythrocytes and fibroblasts). However, neither the presence of other mutations in the Q^{1152} receptor or the possibility of a second alteration in the patient cells can be rigorously excluded. Thus, the primary effect of the mutation on insulin receptor function needed to be further substantiated.

In the present work, we directly addressed these issues by mutating arginine 1152 with glutamine in the human insulin receptor cDNA. Cell lines transfected with either the wild-type or the mutant cDNAs were isolated and shown to express similar numbers of insulin receptors. The mutation did not affect the affinity of the receptor for insulin, the rate of receptor synthesis, or its transport to the cell surface. However, consistent with our previous findings in cultured fibroblasts from the Q^{1152} patients[2] the mutation impaired insulin-dependent receptor autophosphorylation and kinase activity. Thus, the arginine with glutamine substitution in the RK motif of the insulin receptor kinase is by itself responsible for these functional alterations of the patient receptor. Most recently, Cama *et al.* (49) reported that the naturally occurring mutation of a conserved methionine residue, located 5 amino acids upstream in the insulin receptor, impairs receptor kinase activity and signaling. Based on these findings the authors suggested that methionine 1141 is relevant to the function of the tyrosine triplet. The same may also be true for arginine 1152, suggesting that the integrity of the region surrounding tyrosines 1146, 1150, and 1151, in addition to that of the triplet itself, is important for normal functioning of insulin receptor kinase.

In contrast to insulin-stimulated kinase activity, insulin-independent (basal) kinase activity of the Q^{1152} receptor toward the p120 and p175 endogenous substrates and several

exogenous substrates was 2–6-fold higher in mutant than in wild-type receptors. Since basal receptor autophosphorylation is decreased in this mutant while basal kinase activity toward substrates is increased, it is unlikely that the arginine-glutamine substitution impaired ATP interaction with the kinase. More likely, the mutation alters receptor conformation preventing phosphorylation of specific tyrosine sites. Consistent with this hypothesis, we show that transphosphorylation of the mutant by the wild-type receptor was also impaired.

In transfected cells, Q^{1152} receptors also increased glucose and amino acid uptake to the same levels achieved trough maximal insulin stimulation of normal insulin receptors. As was the case for receptor kinase activity, insulin addition to cells expressing the mutant receptor did not elicit further increase in glucose and amino acid uptake in spite of the presence of a small complement of endogenous murine insulin receptors. Although additional mechanisms cannot be presently excluded, it seems likely that the ligand-independent kinase of Q^{1152} receptor constitutively activate processes which are normally induced by insulin. Same as in the transfected cells, in cultured fibroblasts from Q^{1152} patients we found high glucose and amino acid uptake and glycogen synthase activity but no insulin stimulation of these processes.[2] Since Q^{1152} patients are heterozygous for the mutation (24), one may predict a much higher ratio of normal *versus* mutant receptors in patient fibroblasts than in transfected cells. The same lack of insulin sensitivity in the transfected and in the patient cells, supports the conclusion that the Q^{1152} receptors impair normal insulin regulation mainly by maximally activating insulin-sensitive processes in the absence of insulin.

In previous reports (20, 22, 23), mutations in the tyrosine triplet preceding arginine 1152 have also been shown to increase basal insulin receptor kinase activity, indicating that, when dephosphorylated, these tyrosines negatively control the activity of the kinase (50). As is the case with cells transfected with constitutively active kinases (51) basal glucose uptake was increased in cells expressing insulin receptors mutated in the triplet tyrosines. The possibility was raised therefore that amino acid substitutions of these tyrosines generates a constitutively active insulin receptor (22). This may also have been occurred in the Q^{1152} mutant receptor since expression of this insulin-insensitive receptor is accompanied by enhanced kinase-dependent metabolic effects. Thus, very much like the unphosphorylated tyrosines of the triplet, the arginine residue in the RK motif appears to negatively control insulin receptor signaling. Similar to insulin-dependent phosphorylation of the triplet tyrosines in the normal insulin receptor (9–18, 50), the neutral for basic amino acid substitution in the RK motif of the Q^{1152} receptor might weaken this inhibitory action and constitutively activate the kinase mimicking the effect of triplet tyrosine simultaneous mutation. The modulatory role of basic amino acids close to regulatory tyrosine phosphoacceptor sites in tyrosine kinases is also supported by previous observations in c-*src*. In this kinase, a naturally occurring substitution of an arginine residue following tyrosine regulatory phosphoacceptor sites has been reported (52). Same as the Q^{1152} mutation in the insulin receptor, this mutation is sufficient to activate c-src (52, 53). Work is in progress in our laboratory to further evaluate the role of the RK motif in the insulin receptor function.

Differently from the metabolic responses, Q^{1152} and wild-type receptor-transfected cells exibited comparable increases in sensitivity to insulin for mitogenic effects. This suggests that, although unable to mediate insulin metabolic effects, the Q^{1152} receptor mediates mitogenic responses. Insulin metabolic and proliferative responses may be evoked through different mediators interacting with separate receptor phosphorylation sites. These may be differently affected by the Q^{1152} mutation so that the constitutive increase in Q^{1152} receptor kinase needs not to alter all of the insulin bioeffects.

In conclusion, in the present work, we have addressed the role of a sequence homology adjacent to the three key tyrosine autophosphorylation sites of the insulin receptor and highly conserved in the insulin receptor family of tyrosine kinases. A mutation in this region appears to de-regulate insulin receptor activity presumably by impairing normal function of the regulatory domain of the kinase. This defect generates cell insensitivity to insulin metabolic effects and may significantly contribute to the pathogenesis of non-insulin-dependent diabetes mellitus in humans.

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REFERENCES

1. Goldfine, I. D. (1987) *Endocr. Rev.* **8,** 235–255
2. Kasuga, M., Karlsson, F. A., and Kahn, C. R. (1982) *Science* **215,** 185–187
3. Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M., and Kahn, C. R. (1982) *Nature* **298,** 667–669
4. Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M., and Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3237–3240
5. Yu, K.-T., and Czech, M. P. (1986) *J. Biol. Chem.* **261,** 4715–4722
6. Klein, H. H., Freidenberg, G. R., Kladde, M., and Olefsky, J. M. (1986) *J. Biol. Chem.* **261,** 4691–4697
7. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., and Ramachandran, J. (1985) *Nature* **313,** 756–761
8. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graft, L., Clauser, E., Ou, J.-h., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., and Rutter, W. J. (1985) *Cell* **40,** 747–758
9. Herrera, R., and Rosen, O. M. (1986) *J. Biol. Chem.* **261,** 11980–11985
10. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A., and Rutter, W. J. (1986) *Cell* **45,** 721–732
11. Goren, H. J., White, M. F., and Kahn, C. R. (1987) *Biochemistry* **26,** 2374–2382
12. Tornqvist, H. E., Pierce, M. W., Frackelton, A. R., Nemenoff, R. A., and Avruch, J. (1987) *J. Biol. Chem.* **262,** 10212–10219
13. White, M. F., Shoelson, S. E., Keutmann, H., and Kahn, C. R. (1988) *J. Biol. Chem.* **263,** 2969–2980
14. Tornqvist, H. E., and Avruch, J. (1988) *J. Biol. Chem.* **263,** 4593–4601
15. McClain, D. A., Maegawa, H., Levy, J., Hueckstedt, T., Dull, T. J., Lee, J., Ullrich, A., and Olefsky, J. M. (1988) *J. Biol. Chem.* **263,** 8904–8911
16. Tornqvist, H. E., Gunsalus, J. R., Nemenoff, R. A., Frackelton, A. R., Pierce, M. W., and Avruch, J. (1988) *J. Biol. Chem.* **263,** 350–359
17. Tavaré, J. M., and Denton, R. M. (1988) *Biochem. J.* **252,** 607–615
18. Flores-Riveros, J. R., Sibley, E., Kastelic, T., and Lane, M. D. (1989) *J. Biol. Chem.* **264,** 21557–21572
19. Wilden, P. A., Backer, J. M., Kahn, C. R., Cahill, D. A., Shroeder, G. J., and White, M. F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 3358–3362
20. Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, M. A., and Rutter, W. J. (1986) *Cell* **45,** 721–732
21. Debant, A., Clauser, E., Ponzio, G., Filloux, G., Auzan, C., Contreres, J. O., and Rossi, B. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8032–8036
22. Murakami, M. S., and Rosen, O. M. (1991) *J. Biol. Chem.* **266,** 22653–22660
23. Zhang, B., Tavaré, J. M., Ellis, L., and Roth, R. A. (1991) *J. Biol. Chem.* **266,** 990–996
24. Cocozza, S., Porcellini, A., Riccardi, G., Monticelli, A., Condorelli, G. L., Ferrara, A. M., Pianese, L., Miele, C., Capaldo, B., Beguinot, F., and Varrone, S. (1992) *Diabetes* **41,** 521–526
25. Davis, L. A., Dibner, M. D., and Battey, J. F. (1986) *Basic Methods in Molecular Biology,* Elsevier/North-Holland, New York
26. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., and Ramachandran, J. (1985) *Nature* **313,** 756–761
27. Helin, K., Velu, T., Martin, P., Vass, W. C., Allevato, G., Lowy, D. R., and Beguinot, L. (1991) *Oncogene* **6,** 825–832
28. Taylor, J. W., Ott, J., and Eckstein, F. (1985) *Nucleic Acids Res.* **13,** 8765–8785
29. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
30. Graham, F. L., and van der Eb, A. J. (1973) *Virology* **52,** 456–467
31. Velu, T. J., Beguinot, L., Vass, W. C., Zhang, K., Pastan, I., and Lowy, D. R. (1989) *J. Cell. Biochem.* **39,** 153–166
32. Laemmli, U. K. (1970) *Nature* **227,** 680–685
33. Cuatrecasas, P. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 318–322
34. Munson, L., and Rodbard D (1980) *Anal. Biochem.* **107,** 220–239
35. Beguinot, F., Smith, R. J., Kahn, C. R., Maron, R., Moses, A. C., and White, M. F. (1988) *Biochemistry* **27,** 3222–3228

36. Beguinot, F., Kahn, C. R., Moses, A. C., and Smith, R. J. (1986) *Endocrinology* **118,** 446–455
37. Mandarino, L. J., Madar, Z., Kolterman, O. G., Bell, J. M., and Olefsky, J. M. (1986) *Am. J. Physiol.* E489–E496
38. McClain, D. A., and Olefsky, J. M. (1989) in *Molecular and Cellular Biology of Diabetes Mellitus* (Draznin, B., Melmed, S., LeRoith, D., eds) Vol. 2, pp. 53–64, Alan R. Liss, New York
39. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51,** 660–672
40. Accili, D., Mosthaf, L., Ullrich, A., and Taylor, S. (1991) *J. Biol. Chem.* **266,** 434–439
41. Rees-Jones, R. W., and Taylor, S. I. (1985) *J. Biol. Chem.* **260,** 4461–4467
42. Beguinot, F., Kahn, C. R., Moses, A. C., White, M. F., and Smith, R. J. (1989) *Endocrinology* **125,** 1599–1605
43. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., and Fujita-Yamaguchi, Y. (1986) *EMBO J.* **5,** 2503–2512
44. Nishida, Y., Hata, M., Nishizuka, Y., Rutter, W. J., and Ebina, Y. (1986) *Biochem. Biophys. Res. Commun.* **141,** 474–481
45. Matsushime, H., Wang, L.-H., and Shibuya, M. (1986) *Mol. Cell. Biol.* **6,** 3000–3004
46. Hafen, E., Basler, K., Edstroem, J. E., and Rubin, G. M. (1987) *Science* **236,** 55–63
47. Martin-Zanca, D., Hughes, S. H., and Barbacid, M. (1986) *Nature* **319,** 743–748
48. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* **241,** 42–52
49. Cama, A., Quon, M. J., de la Luz Sierra, M., and Taylor, S. (1992) *J. Biol. Chem.* **267,** 8383–8389
50. Avruch, J. (1989) in *Molecular and Cellular Biology of Diabetes Mellitus* (Draznin, B., Melmed, S., and LeRoit, D., eds) Vol. 2, pp. 65–77, Alan R. Liss, New York
51. Flier, J. S., and Matsouka (1989) in *Molecular and Cellular Biology of Diabetes Mellitus* (Draznin, B., Melmed, S., and LeRoit, D., eds) Vol. 2, pp. 175–182, Alan R. Liss, New York
52. Grandori, C. (1989) *Nature* **338,** 467
53. Potts, W. M., Reynolds, A. B., Lansing, T. J., and Parsons, T. J. (1989) *Oncogene Res.* **3,** 343–355