

A Naturally Occurring Mutation of Insulin Receptor Alanine 1134 Impairs Tyrosine Kinase Function and Is Associated with Dominantly Inherited Insulin Resistance*

(Received for publication, March 15, 1990)

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We have identified a previously undescribed genetic variant of the insulin receptor (Ala¹¹³⁴ → Thr¹¹³⁴) in a family with the Type A syndrome of insulin resistance. Using the polymerase chain reaction to amplify insulin receptor cDNA and genomic DNA (exon 19), this mutation was detected in 1/2 alleles in the proband, her two affected sisters, and her affected father. Two normal alleles were present in the unaffected mother. No additional structural changes were encoded by the remainder of the proband's receptor cDNA.

The Ala¹¹³⁴ mutant receptor was expressed in Chinese hamster ovary cells. The expressed mutant receptors were processed normally and displayed normal affinity of insulin binding but were markedly deficient in insulin-stimulated autophosphorylation. The mutant receptor was unable to catalyze the phosphorylation of the endogenous substrate, pp185, and insulin-stimulated kinase activity toward an exogenous substrate *in vitro* also was markedly impaired.

Ala¹¹³⁴ is a highly conserved residue located in a consensus sequence found in most tyrosine kinases. It is likely that this previously uncharacterized residue and/or the immediate region surrounding it are important for normal kinase function in other members of this receptor family. This study also demonstrates that severe insulin resistance with dominant inheritance may be caused by a missense mutation in one allele of the insulin receptor gene.

The insulin receptor is a tyrosine-specific protein kinase which undergoes autophosphorylation as a consequence of insulin binding (1). Autophosphorylation of the β -subunit cytoplasmic domain activates the intrinsic tyrosyl kinase activity of the receptor which catalyzes phosphorylation of cellular protein substrates (2-4). There is substantial evidence that this tyrosine kinase activity is required for most aspects of insulin receptor signal transduction (5-9).

Studies of patients with severe acquired or familial forms of insulin resistance have advanced our understanding of insulin receptor function. A particularly valuable group in

this regard are those patients with severe insulin resistance that is genetically determined. In recent years, several such patients have been shown to have mutations in the coding region of the insulin receptor gene (10-16). We have studied the relation between insulin receptor structure and function in one such family, several of whose members have moderately severe insulin resistance. Insulin receptors derived from freshly obtained and cultured cells of the proband BI-1, one of three affected sisters, bind insulin normally but display defective insulin-stimulated autophosphorylation and tyrosine kinase activity (17). On the basis of this data, we predicted the existence of a mutation in the β -subunit of the receptor (17). Using the polymerase chain reaction (PCR)¹ to amplify cDNA and genomic DNA, we have identified a single base substitution in exon 19 of one allele of the proband's insulin receptor gene.

This sequence encodes a receptor in which Ala¹¹³⁴ (see Footnote 2) is replaced by Thr in the tyrosine kinase domain of the β -subunit. Both normal and abnormal alleles were detected in BI-1, her two affected sisters, and the affected father, demonstrating heterozygosity at this locus in all affected individuals. Two normal alleles were present in the unaffected mother.

To study the effect of Thr¹¹³⁴, this mutant insulin receptor was expressed in Chinese hamster ovary (CHO) cells. Insulin receptor processing, expression on the cell surface, and affinity of insulin binding of the mutant receptors were normal, but the ability of insulin to stimulate autophosphorylation and tyrosine kinase activity was markedly impaired. These studies indicate an important role for Ala¹¹³⁴ in the function of the insulin receptor kinase, and demonstrate that dominantly inherited insulin resistance can be caused by a mutation in one allele of the insulin receptor gene.

EXPERIMENTAL PROCEDURES

Subjects—The proband, patient BI-1, is a female who presented at age 15 with mild but typical features of the Type A syndrome of insulin resistance (18) including marked insulin resistance, acanthosis nigricans, and polycystic ovaries with hyperandrogenism. Her oral glucose tolerance test achieved the National Diabetes Data Group criteria (19) for diabetes mellitus (Table I). The patient's two sisters, S1 and S2 (Fig. 1), presented at age 10 and 11, respectively, and are

* This work was supported by National Institutes of Health Grant NIDDK 28082-09 and a grant from the Juvenile Diabetes Foundation (to J. S. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: PCR, polymerase chain reaction; CHO, Chinese hamster ovary; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); WGA, wheat germ agglutinin; α PY, anti-phosphotyrosine antibodies; α IR, anti-insulin receptor antibodies; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

² The nucleotide and amino acid numbering system of Ebina *et al.* (33) is used hereafter.

TABLE I

Clinical characteristics of insulin-resistant family, BI-1

Glucose tolerance tests were performed using a standard (75 g) oral glucose load. Plasma glucose and serum insulin concentrations were measured after an overnight (12-h) fast and after glucose ingestion.

Member ^a	Age	Glucose		Insulin	
		Fasting	1 h/2 h	Fasting	1 h/2 h
		mg/dl		microunits/ml	
BI-1	20	89	200/220	76.6	760/710
S1	16	107		98.5	
S2	17	96		134	
Father	47	94	200/103	57	682/521
Mother	46	111	/126	17.9	/57
Normal range		<115	<200/<140	<20	

^a See pedigree (Fig. 1).

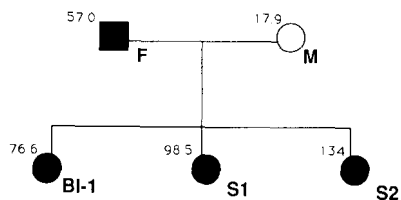


FIG. 1. Pedigree of insulin-resistant family BI-1. Squares, male; circles, female. Closed symbols indicate individuals affected with insulin resistance including the proband (BI-1), her two sisters (S1, S2), and their father (F). The mother (M) has no evidence of insulin resistance. Fasting serum insulin levels (microunits/ml) for each subject are shown next to each symbol.

both similarly affected with the mild Type A phenotype and insulin resistance (Table I). The father is insulin-resistant as evidenced by endogenous hyperinsulinemia to a degree similar to the proband but lacks acanthosis nigricans or other signs of insulin resistance. His oral glucose tolerance test only partially achieves criteria for impaired glucose tolerance (Table I) (19). The mother is phenotypically normal and is without biochemical evidence of insulin resistance. Peripheral blood leukocytes or surgically obtained (provided by Jose Caro, East Carolina University, Greenville, NC) liver and muscle samples from 30 normal subjects were used as sources of control human genomic DNA.

Isolation of Cellular DNA and RNA cDNA Synthesis—Genomic DNA was isolated from peripheral blood leukocytes, human skin fibroblasts, and tissue samples using standard techniques (20). Total cellular RNA was isolated using the guanidinium thiocyanate-CsCl technique (21) or by the method of Chomczynski and Sacchi (22). First strand cDNA was prepared using each of seven insulin receptor cDNA-specific oligonucleotide primers (see below) with 1–10 μ g of total cellular RNA, 0.5 μ M oligonucleotide primer, and 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories as previously described (12, 23).

Enzymatic Amplification of Genomic DNA and cDNA—Insulin receptor cDNA was subjected to 40 cycles of PCR amplification using seven overlapping 25-base oligonucleotide primer sets complementary to the insulin receptor cDNA (see Table II) with 0.25 μ M each of two primers and 1.5 units of *Thermus aquaticus* DNA polymerase in an automated DNA thermal cycler (Perkin-Elmer Cetus Instruments) as described previously (12, 23). Each primer contains additional noncomplementary bases at the 5' end, creating restriction enzyme recognition sites for use in subsequent subcloning (12). Genomic DNA (0.1–1.0 μ g) was amplified in an identical way except that the DNA was initially denatured for 6 min at 94 °C at the beginning of the first cycle. For PCR from genomic DNA, two 30-base oligonucleotide primers corresponding to the sequence of intronic DNA flanking exon 19 were constructed using the genomic sequence data of Seino et al. (24, 25).

Sequencing of PCR-amplified cDNA and Genomic DNA—Amplified cDNA fragments were treated with RNase A as described previously (12), purified by agarose gel electrophoresis, electroeluted or eluted from agarose with Gene Clean (Bio 101, San Diego, CA), digested with the appropriate restriction enzymes, and ligated into M13 replicative form DNA (mp 18 and mp 19) (20). Single-stranded M13 phage DNA template was sequenced with ³⁵S-dATP using the dideoxy

chain termination method (26). Several independent clones for each insulin receptor PCR primer set were isolated and sequenced in this way.

Alternatively, PCR-amplified cDNA and genomic DNA were sequenced directly. Amplified genomic DNA and cDNA fragments were purified as noted above (RNase A was used only with cDNA). One hundred to 200 ng of PCR product was alkaline-denatured, neutralized, and ethanol-precipitated. This denatured double-stranded DNA template was sequenced with modified T7 DNA polymerase (Sequenase, United States Biochemical Corp., Cleveland, OH) using a [γ -³²P] ATP end-labeled sequencing primer (either of two PCR primers used to amplify the cDNA/DNA fragment or internal sequencing primer(s)) as we have described previously (27).

Allele-specific Oligonucleotide Hybridization—Samples of amplified genomic DNA which included exon 19 of the insulin receptor were quantitated by agarose minigel electrophoresis. Approximately 50–100 ng of each sample was alkaline-denatured and applied to nitrocellulose filters using a slot-blot manifold (Bethesda Research Laboratories). Slot-blots were prehybridized (1 h)/hybridized (overnight) according to a modification of the method of Myerowitz (28) in 5 × Denhardt's solution, 0.9 M NaCl, 5.0 mM EDTA, 50 mM NaH₂PO₄/Na₂HPO₄, pH 8.4, and 333 μ g/ml salmon sperm DNA at 37 °C. A single 19-base oligonucleotide (5'-GGACCTGACAGCGAGAAAC-3') complementary to the antisense strand of the mutant allele was end-labeled with [α -³²P]dCTP and terminal deoxyribonucleotidyltransferase (International Biotechnologies, Inc., New Haven, CT) and hybridized to filters. Filters were washed in 6 × SSC, 0.1% SDS twice at ambient temperature (10 min), twice at 48 °C for 10 min ("low stringency"), and finally at 57 °C for 10 min ("high stringency").

Expression of Insulin Receptor cDNA in CHO Cells—A normal human insulin receptor plasmid, pCVSHIRC (6), was used to express the wild-type insulin receptor and for reconstruction of a mutant cDNA expression vector. Using primers 11 and 15 (Table II), a 1418-bp cDNA fragment containing the mutation was amplified from patient BI-1. This fragment was digested with *Xho*I and *Spe*I, generating a 1267-bp fragment, and substituted for the corresponding region (insulin receptor cDNA nucleotides 3068–4334) of pCVSHIRC which had been digested with *Spe*I and partially digested with *Xho*I. Prior to transfection, the entire PCR-derived region of the mutant plasmid was sequenced to confirm that only the desired mutation was present.

Normal and mutant cDNA vectors (10 μ g) were cotransfected with a neomycin-resistant gene, pSVNeo (1 μ g) into CHO cells using the calcium phosphate precipitation method as previously described (9). Following selection of neomycin-resistant cells with G418 (800 μ g/ml), cells expressing high levels of surface insulin receptors were selected by fluorescence-activated cell sorting (29) using an insulin receptor monoclonal antibody (MA-51) provided by Ira Goldfine (University of California, San Francisco, CA). Clonal cell lines expressing wild-type (CHO/HIRC and CHO/HIRC/2) and mutant (CHO/BI-1/A5 and CHO/BI-1/A12) receptors were prepared by plating cells at limiting dilution.

Insulin Binding—Transfected CHO cells were grown to confluence in 24-well dishes and incubated with 0.2 ng/ml ¹²⁵I-insulin (2200 Ci/mmol, Du Pont-New England Nuclear) and variable concentrations of cold insulin as previously described (9). Insulin binding was analyzed using the "Ligand" program (30). Insulin binding to solubilized receptors was measured as previously described (31) using wheat germ agglutinin (WGA)-agarose purified receptors (32).

[³⁵S]Methionine and [³²P]Phosphate Labeling of CHO/HIRC and CHO/BI-1 Cells—Confluent CHO cells were labeled overnight with 0.1 mCi/ml [³⁵S]methionine (Amersham Corp.) or for 2 h with 0.2 mCi/ml [³²P]phosphate (Du Pont-New England Nuclear) followed by a 2-min incubation with or without 100 nM insulin and subsequent rapid freezing with liquid nitrogen (9). Frozen cells were solubilized and centrifuged as previously described (9). In experiments using [³⁵S]methionine, insulin receptors were sequentially immunoprecipitated from the cell extract with antiphosphotyrosine antibodies (α PY) followed by anti-insulin receptor antibody. This antibody (α IR) is a polyclonal IgG which recognizes both transfected human and endogenous hamster insulin receptors and was derived from a patient with anti-insulin receptor antibodies. In experiments using [³²P]phosphate, insulin receptors were separately immunoprecipitated with α IR and α PY. Proteins were eluted from washed precipitates with Laemmli sample buffer, reduced with dithiothreitol, and analyzed by SDS-PAGE (9). Labeled proteins were identified by autoradiography and quantitated by scintillation counting (³⁵S) or Cerenkov counting (³²P) of gel fragments.

Autophosphorylation and Kinase Activity of WGA-purified Receptors—Equal amounts of WGA-purified insulin receptor, as assessed by ¹²⁵I-insulin binding, were incubated for 20 min at 22 °C in 50 μl of 50 mM HEPES (pH 7.6) and 5.0 mM MnCl₂ with variable concentrations of insulin (0–100 nM). Autophosphorylation was initiated by adding 25 μM ATP (cold plus 25 μCi of [γ -³²P]ATP) followed by 5 min of further incubation at 22 °C. The reaction mixture was then added to Laemmli sample buffer with dithiothreitol, and phosphoproteins were separated by SDS-PAGE and analyzed by autoradiography and Cerenkov counting.

Insulin receptor kinase activity was assessed as previously described (9) using a synthetic peptide (Thr-12-Lys) corresponding to amino acid residues 1154–1165 of the human insulin receptor (33). Equal amounts of WGA-purified receptor as assessed by binding (approximately 1.0 μg each) were assayed with or without insulin (100 nM) with 1.0 mM Thr-12-Lys. Kinase activity without prior autophosphorylation was determined after a 20-min preincubation with or without insulin by adding [γ -³²P]ATP and Thr-12-Lys simultaneously followed by a 5-min incubation before stopping the reaction. Kinase activity after receptor autoactivation was assessed by incubating with [γ -³²P]ATP for 15 min after the 20-min insulin preincubation. This was then followed by the addition of Thr-12-Lys and a further 5-min incubation.

RESULTS

Identification of a Missense Mutation in Codon 1134—Patient BI-1 was strongly suspected of having an insulin receptor β -subunit defect on the basis of prior studies with the patient's cells demonstrating normal insulin binding with diminished autophosphorylation and kinase activity (17) (patient A3, Ref. 34). Total cellular RNA was isolated from BI-1's fibroblasts and PCR-amplified following synthesis of first strand cDNA. The PCR products were subcloned into M13 prior to dideoxy sequencing. Using PCR primer set 11, 12 (Table II), which spans a central region of the intracellular β -subunit, three of seven independent clones that were sequenced contained a single base substitution in codon 1134 (GCA^{Ala} → ACA^{Thr}). This finding is consistent with heterozygosity for this defect. The remainder of the insulin receptor cDNA sequence was evaluated by sequencing individual cDNA clones obtained by PCR amplification with primers 1–14 (Table II). For each of the seven overlapping amplified regions, a minimum of five clones was completely sequenced with the exception of a 681-bp span between codon 648 and codon 875 where only three clones were sequenced. The sequence data corresponded to the published normal insulin receptor sequence (33, 35) with the following exceptions: a

silent change (TCC → TGT) in codon Cys³¹² was found in 1 of 5 clones; two additional regions contained nucleotide substitutions in single clones which were subsequently confirmed to be PCR misincorporation artifacts (27, 36) by direct sequencing of PCR products and by sequencing of large numbers (10–14) of clones. Thus it is extremely likely that the Ala¹¹³⁴ mutation is the only amino acid substitution encoded by the insulin receptor gene of BI-1.

Direct sequencing of PCR product DNA allows simultaneous detection of two or more alleles on a single sequencing gel ladder. This technique was applied to two differently derived PCR products containing the region of Ala¹¹³⁴. In patient BI-1, PCR amplification of leukocyte-derived genomic DNA (exon 19) and fibroblast-derived cDNA (primers 11, 12) followed by direct sequencing (Fig. 2), confirmed the presence of the Ala¹¹³⁴ mutation in 1 of 2 alleles. Direct sequencing of exon 19 revealed both normal and abnormal alleles in the two affected sisters and the affected father. However, both of the mother's alleles were normal in this region. Furthermore, the autosomal dominant pattern of inheritance in this family implicates the existence of a single dominant disease-causing allele.

As shown in Fig. 3, PCR amplification of exon 19 followed by slot-blotting and hybridization to an oligonucleotide specific for the mutant allele confirmed the presence of the mutation in the above affected individuals and excluded it in the mother and 30 normals (60 normal alleles). Thus all the affected members of this family are heterozygotes for the Ala¹¹³⁴ mutation which does not appear to be a common polymorphism in the normal population.

Two lines of evidence support the view that both normal and mutant alleles are equally expressed as mRNA; nearly 50% of independent M13 clones were of each type; the appearance of direct sequencing gels from genomic DNA where both alleles are equally present was similar to the appearance of direct sequencing gels from cDNA (not shown). Thus, the apparent dominant inheritance of insulin resistance is not a consequence of a disproportionate accumulation of mRNA encoding the mutant receptor.

The Ala¹¹³⁴ Mutant Insulin Receptor Is Normally Expressed and Displays Normal Insulin Binding—In order to study the functional significance of the amino acid substitution in codon 1134, we substituted a 1267-bp portion of BI-1's insulin recep-

TABLE II
Sequence of oligonucleotides used for synthesis of first strand insulin receptor cDNA and subsequent PCR amplification

Oligonucleotides were synthesized with phosphoramidite chemistry using an Applied Biosystems 381A DNA synthesizer. The location of each oligonucleotide within the insulin receptor cDNA is reported according to the system of Ebina *et al.* (33). Underlined letters are noncomplementary nucleotides (nt) substituted within or added to the 5' end to create restriction enzyme recognition sites for potential subcloning.

Primer	Sequence	Location
1	5'-CGCTGCAGGTGGCGGTGGCCGCGCT-3'	nt 175→200
2	5'-GTTGCTGGAATTCATCGTGTACCCG-3'	nt 1113→1088
3	5'-CCTGATGAACATCACCCGGGGTTCT-3'	nt 543→568
4	5'-GCGAAGCTTGGTTCTCGAACTTCT-3'	nt 1571→1546
5	5'-AACTGCAGCAACTGCTGTGCACCC-3'	nt 1101→1126
6	5'-GTGAATTCGGGTCGTTGGACCTCAG-3'	nt 1830→1855
7	5'-TCCTGCAAGTTGGACGGTGGTAGACA-3'	nt 1794→1819
8	5'-CCGAATTCGTGGGCACGCTGGTCTGA-3'	nt 2516→2491
9	5'-GGAAGCTTGGCGATGTTGGGAATGT-3'	nt 2422→2447
10	5'-AAGAATTCAGTGGCACTGAGATACT-3'	nt 3155→3130
11	5'-ATCTGCAGCCAGATGGGCCCGCTGGG-3'	nt 3084→3109
12	5'-GCGAATTCGGTGGCACTCCACCGTAC-3'	nt 3756→3736
13	5'-ATCTGCAGAGACATATGAAACGG-3'	nt 3675→3700
14	5'-AGGAATTCAAACCAGAGGAAAGCGA-3'	nt 4352→4327
15	5'-GCCCTCCAGGTTACAGTT-3'	nt 4498→4479

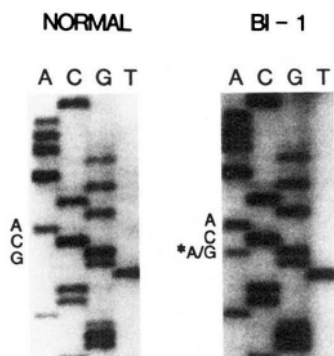


FIG. 2. Partial nucleotide sequence of insulin receptor exon 19. Genomic DNA from patient BI-1 and a normal subject was PCR-amplified using primers which flank exon 19. The double-stranded PCR product DNA fragments were then directly sequenced. For BI-1, the sequencing ladder reveals a heterozygous change (*) in codon 1134 (GCA^{Ala}/ACA^{Thr}); the normal subject is homozygous for the GCA^{Ala} wild-type codon.

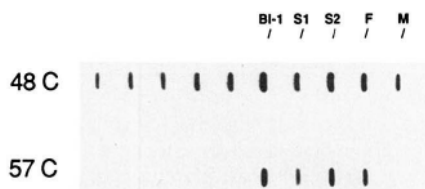


FIG. 3. Allele-specific hybridization to detect the Ala¹¹³⁴ insulin receptor mutation. A synthetic oligonucleotide containing the codon 1134 mutation (ACA^{Thr} rather than GCA^{Ala}) was end-labeled with [α -³²P]dCTP and hybridized to slot-blots containing PCR-amplified genomic DNA from insulin receptor exon 19. This example shows results for five normal subjects (first five slots) and members of the BI-1 family including the proband (BI-1), her two affected sisters (S1, S2), the affected father (F), and the unaffected mother (M). When washed at low stringency (48 °C) the mutant probe hybridizes to all DNA samples. At high stringency (57 °C) washing, the mutant probe remains hybridized only to DNA samples from affected subjects who have the mutant allele.

tor cDNA which contained the mutation for the corresponding portion of a normal insulin receptor cDNA expression vector. The expression vector bearing the Ala¹¹³⁴ mutation was then transfected into CHO cells, and two clones, CHO/BI-1/A5 and CHO/BI-1/A12, that expressed high levels of the mutant insulin receptor were selected for further studies in comparison with two wild-type clones, (CHO/HIRC and CHO/HIRC/2).

Insulin binding to intact transfected CHO cells was performed both at 22 and 4 °C. An example of insulin binding/accumulation performed at 22 °C is shown in Fig. 4. The concentration of added cold insulin to half-maximally displaced bound tracer ¹²⁵I-insulin (ED₅₀) was \approx 0.9 nM for CHO/HIRC cells, \approx 0.4 nM for CHO/HIRC/2 cells, and \approx 0.4–0.6 nM for both mutant clones.

Analysis of Scatchard plots with the Ligand program (30) (data not shown) employed 1-binding site and 2-binding site models. When studies at 4 °C, affinity binding constants (K_d) for wild-type and mutant receptors were 0.48–2.2 and 0.55 nM, respectively, for a 1-site model and 0.32–2.2 and 0.36 nM, respectively, for a 2-site model (high affinity site). Thus the insulin binding affinity of transfected mutant receptors was similar to the transfected wild-type receptors.

Tyrosyl Phosphorylation of Insulin Receptors in [³⁵S]Methionine-labeled CHO Cells—Biosynthetic labeling of transfected cells with [³⁵S]methionine followed by sequential immunoprecipitation with α PY and α IR was performed to assess

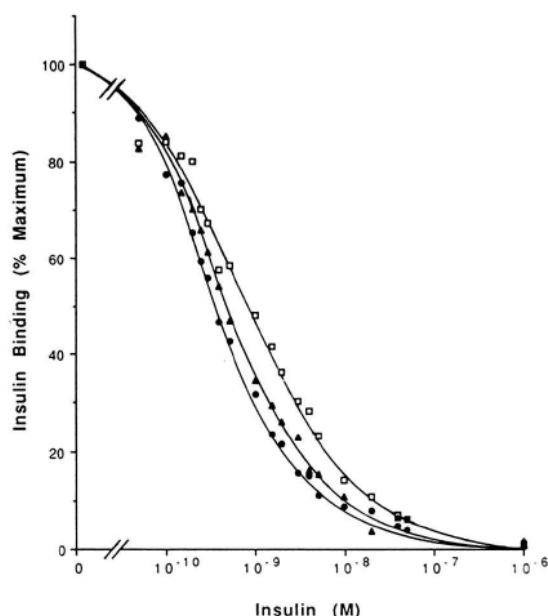


FIG. 4. ¹²⁵I-insulin binding/accumulation in CHO cells expressing wild-type and mutant insulin receptors. The total binding/accumulation of ¹²⁵I-insulin in CHO/HIRC (□) and two clones expressing the Ala¹¹³⁴ mutation, CHO/BI-1/A5 (●) and CHO/BI-1/A12 (▲), was measured in the presence of the indicated concentrations of unlabeled insulin after 3 h at 22 °C. Results are expressed as a percentage of ¹²⁵I-insulin bound/accumulated in the absence of unlabeled insulin. Experiments conducted at 4 °C overnight reveal similar results.

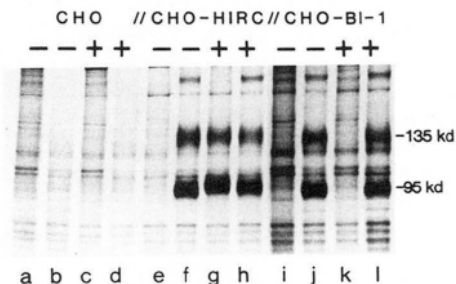


FIG. 5. Insulin-stimulated autophosphorylation of intact transfected CHO cells assessed with [³⁵S]methionine labeling. Cells were labeled overnight with [³⁵S]methionine and then stimulated with insulin (100 nM) for 2 min (+ lanes). Phosphotyrosine-containing proteins were immunoprecipitated from each cell line lysate with α PY (lanes a, c, e, g, i, k). Labeled proteins that were not tyrosyl-phosphorylated were subsequently immunoprecipitated with α IR (lanes b, d, f, h, j, l). Samples were analyzed by SDS-PAGE and subsequent autoradiography. Insulin receptor α - (135 kDa) and β - (95 kDa) subunit bands are indicated.

receptor processing and determine the stoichiometry of autophosphorylation. Similar numbers of [³⁵S]methionine-labeled receptors were precipitated from wild-type or mutant transfected CHO cells with α IR in the absence of insulin (compare lanes f and j, Fig. 5). No receptors were immunoprecipitated from native CHO cells (Fig. 5, a–d), reflecting the very low level of endogenous rodent receptors in CHO cells. Both wild-type and mutant receptors displayed normally processed α - and β -subunits of equivalent size (Fig. 5). The β -subunits of receptors precipitated from CHO/HIRC and CHO/BI-1 cells contained twice as much radioactivity as their corresponding α -subunits (as predicted by the relative number of methionine residues in each subunit). Following exposure of [³⁵S]methionine-labeled cells to 100 nM insulin, \approx 60% of total wild-type α - and β -subunits (the sum of lanes g and h,

Fig. 5) were precipitated with α PY (Fig. 5, lane g). Migration of the tyrosyl-phosphorylated β -subunits was slightly retarded during SDS-PAGE (compare lanes f and g, Fig. 5). However, about \approx 40% of the insulin-stimulated wild-type receptor molecules did not react with α PY and were only precipitated with α IR (Fig. 5, lane h). This subset migrated to the position of unphosphorylated receptors, suggesting that insulin-stimulated CHO/HIRC cells contain both tyrosyl-phosphorylated and unphosphorylated receptors as described previously in Fao cells (37). In contrast, and most importantly, insulin stimulation of CHO/BI-1 cells with the Ala¹¹³⁴ mutation did not result in tyrosyl phosphorylation of [³⁵S]methionine-labeled receptors as assessed by α PY immunoprecipitation (Fig. 5, lane k). Even after insulin stimulation, these receptors were precipitated only with α IR (Fig. 5, lane l). Quantitation of these autophosphorylation experiments (Fig. 6) shows that tyrosyl phosphorylation of receptors is stimulated 11-fold by insulin in CHO/HIRC cells, but there is no insulin stimulation of tyrosyl phosphorylation in the 2 CHO/BI-1 clones.

Autophosphorylation and Kinase Activity in Intact CHO Cells—Immunoprecipitation with α IR following [³²P]phosphate labeling of intact cells (Fig. 7A) revealed detectable levels of basal phosphorylation of the β -subunit. The extent of this phosphorylation was similar in CHO/HIRC AND CHO/BI-1 cells and was increased relative to that seen in native CHO cells. Whereas insulin stimulated receptor autophosphorylation \approx 5-fold in CHO/HIRC cells, it completely failed to stimulate receptor autophosphorylation in CHO/BI-1 cells.

Immunoprecipitation with α PY following [³²P]phosphate labeling of intact cells (Fig. 7B) revealed barely detectable basal tyrosyl phosphorylation of the β -subunit from native CHO, CHO/HIRC, and CHO/BI-1 cells. Insulin markedly stimulated the tyrosyl phosphorylation of the β -subunit in CHO/HIRC cells; in contrast, autophosphorylation of the mutant β -subunit in CHO/BI-1 cells in response to insulin was barely detectable. This was consistent with the results using the [³⁵S]methionine-labeled cells. Thus, substitution of Ala¹¹³⁴ with Thr has no effect on basal Ser/Thr phosphorylation

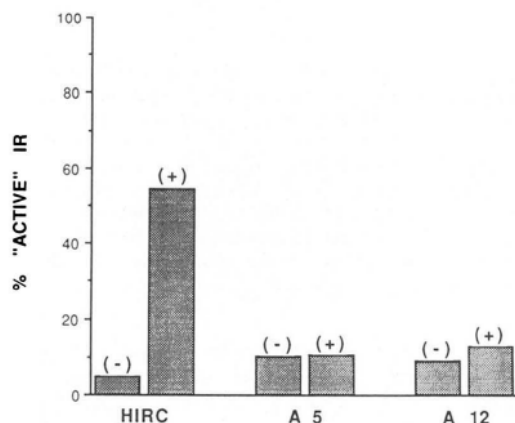


FIG. 6. Quantitation of insulin-stimulated autophosphorylation in transfected CHO cells. In experiments with [³⁵S]methionine labeling of transfected CHO cells such as that shown in Fig. 5, labeled insulin receptor β -subunit bands were identified by autoradiography, excised, and quantitated (relative to background cpm) by scintillation counting. For each cell line, results are expressed as the β -subunit cpm immunoprecipitated with α PY divided by the sum of β -subunit cpm immunoprecipitated with both α PY and α IR \times 100 (the percent "active insulin receptor") with (+) or without (-) insulin stimulation. For cells expressing wild-type receptor (HIRC) there is an 11-fold stimulation of autophosphorylation by insulin. For two clonal cell lines expressing the Ala¹¹³⁴ mutant receptor (A5, A12) there is no apparent insulin stimulation.

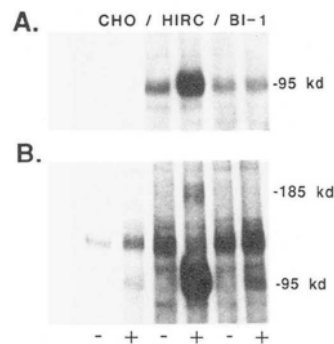


FIG. 7. Insulin-stimulated autophosphorylation and phosphorylation of endogenous substrate (pp185) in [³²P]phosphate-labeled CHO cells. Control CHO cells (CHO), wild type CHO/HIRC cells (HIRC), and mutant CHO/BI-1 (BI-1) cells were labeled for 2 h with [³²P]phosphate and then incubated with (+) or without (-) 100 nM insulin for 2 min. Cell lysates were then immunoprecipitated with α IR (panel A) or with α PY (panel B). The bands corresponding to the insulin receptor β -subunit (95 kDa) and pp185 (185 kDa) are shown.

ation but blocks insulin-stimulated tyrosyl phosphorylation of the β -subunit.

An endogenous substrate of the insulin receptor pp185 is found in nearly all cell types immediately following insulin stimulation (2). After 2 min of acute insulin stimulation, this phosphoprotein was detected in CHO/HIRC cells but not in native CHO or mutant CHO/BI-1 cells (Fig. 7). Thus mutation of codon Ala¹¹³⁴ appears to impair the receptor's ability to catalyze the phosphorylation of this protein.

Autophosphorylation and Kinase Activity of the Partially Purified Insulin Receptor—The insulin dose response of autophosphorylation with equal amounts of wild-type and mutant WGA-purified insulin receptors was assayed *in vitro*. The half-maximal concentration for insulin-stimulated autophosphorylation was similar (\approx 5.0 nM) for both wild-type and mutant receptors, but the basal and maximal incorporation of [γ -³²P]ATP into the receptor β -subunit was \approx 10-fold higher in wild-type receptors (data not shown). Thus autophosphorylation of the Ala¹¹³⁴ mutant receptor was also largely blocked *in vitro*.

The *in vitro* phosphotransferase activity of WGA-purified insulin receptors was assessed with a synthetic 12-amino acid peptide fragment of insulin receptor β -subunit (Thr-12-Lys). Using equal amounts of unphosphorylated receptor, insulin stimulated the phosphorylation of Thr-12-Lys by both wild-type and mutant receptors; however, the absolute activity was \approx 5.5-fold higher with wild-type than with mutant receptors. When autophosphorylation of the receptors was carried out before adding the Thr-12-Lys, absolute kinase activity was \approx 8.5-fold higher for the wild-type receptors (Fig. 8). Also, the basal phosphotransferase activity of receptors after prior autophosphorylation was only modestly reduced in the mutant, but the -fold response to insulin was deficient in the mutant versus wild-type receptors (4.5- versus 21-fold insulin stimulation, respectively) (Fig. 8). In subsequent experiments the kinase activity of WGA-purified cell lysate derived from control cells transfected only with psVeneo (NEO) was compared with an equivalent protein amount of mutant cell WGA-purified lysate and wild-type WGA-purified lysate. Stimulation of phosphotransferase activity by insulin was 35.4-fold for wild-type receptors, 5.1-fold for mutant receptors, and 5.3-fold for NEO lysate. Thus, the slight kinase activity seen with mutant receptor WGA lysate could be accounted for by the presence of a small number of normally functioning endogenous CHO cell receptors.

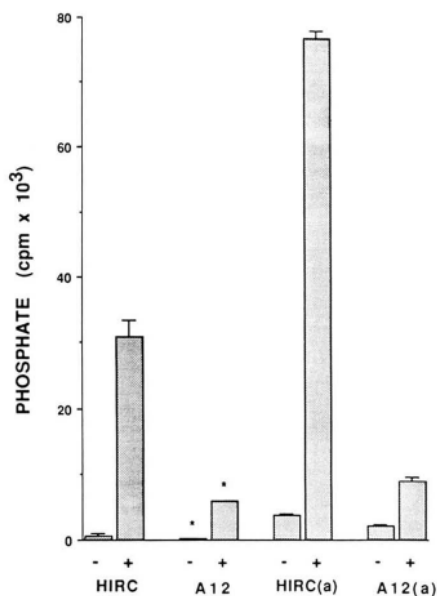


FIG. 8. Phosphotransferase activity of partially purified insulin receptors. Equal amounts of wild-type (HIRC) and mutant (A12) WGA-purified insulin receptors were incubated in the presence (+) or absence (-) or 100 nM insulin for 20 min followed by the simultaneous addition of [γ -³²P]ATP and 1 mM substrate (Thr-12-Lys). The effect of prior autophosphorylation on kinase activity (*a lanes*) was measured by incubating insulin-stimulated receptors with [γ -³²P]ATP for 15 min before adding Thr-12-Lys. Each point represents the mean of 3 (\pm S.D.) determinations of phosphate cpm incorporated into the substrate (*bars* indicated by * are single determinations only).

DISCUSSION

In this report, we have identified a previously undescribed genetic variant of the insulin receptor (Ala¹¹³⁴ → Thr¹¹³⁴) that appears to account for the severe insulin resistance in a patient (BI-1) with the Type A syndrome and her affected family members. We reported previously that BI-1 has normal insulin binding to her erythrocytes (34, patient A3) and cultured fibroblasts (17) but has a 40–50% decrease in maximal insulin-stimulated receptor autophosphorylation and kinase activity toward an exogenous substrate (17, 34). We have also directly determined the functional significance of this mutation. When the mutant Thr¹¹³⁴ receptor was expressed in CHO cells it was deficient in insulin-stimulated autophosphorylation, kinase activity toward an exogenous substrate, and the ability to phosphorylate the endogenous substrate, pp185 (2). The marked impairment of these insulin receptor functions occurred despite normal processing of receptor molecules that displayed normal insulin binding affinity.

The tyrosyl kinase activity of the insulin receptor is critical for insulin receptor signal transmission (1). Site-directed point mutations in the consensus sequence encoding the ATP binding site of the insulin receptor completely inhibit both kinase activity and transmission of biological signals (6–8). A naturally occurring ATP binding site mutation also abolishes kinase activity and was reported in a patient with severe insulin resistance (15). However, other site-directed mutants of the receptor β -subunit which delete potential sites of autophosphorylation have variable and unpredictable effects on the biological activity of the receptor (9, 38). Thus the relation between autophosphorylation, substrate phosphorylation, and signal transmission may vary depending on the biological activity observed. Further evaluation of the impact of the Ala¹¹³⁴ mutation on insulin-mediated processes may provide additional insights into the hypothesis that different domains

of the receptor direct discordant biological responses (38, 39).

Ala¹¹³⁴ and the immediate region surrounding it are part of a consensus sequence found in many protein tyrosine kinases. The alanine residue in this position is present in the mouse (40) and rat (41) insulin receptor, the IGF-1 receptor (42), a newly described insulin receptor-related peptide (43), and several viral protein tyrosine kinases including the *abl*, *fps*, *fes*, *ros*, and *erb-B* tyrosyl kinases (44). In fact, insulin receptor Ala¹¹³⁴ resides within a short region of nearly 100% homology among known protein tyrosine kinases and is located between 2 residues (Leu¹¹³³, Ala¹¹³⁵) which are absolutely conserved in all known tyrosine kinases (44). Therefore, it seems likely that Ala¹¹³⁴ and adjacent residues are important for normal function of the kinase domain of the entire family of membrane tyrosine kinases.

Further studies will be required to evaluate the molecular basis for dominantly inherited insulin resistance caused by the Ala¹¹³⁴ mutant allele, which could have a number of possible explanations. If steady state levels of receptor α/β monomers encoded by each allele are equal in number, consistent with evidence that the two mRNAs are equally expressed, the severe insulin resistance *in vivo* appears to exceed that predicted for impairment of one-half of the normal receptor complement. This would suggest that the mutant receptor protein must in some way interfere with the function of the normal protein. Assuming random sorting of α/β monomers, 25% of mature $\alpha_2\beta_2$ dimers would be mutant/mutant, 25% normal/normal, and 50% mutant/normal hybrids in a heterozygote. Given the evidence that autophosphorylation may involve transphosphorylation of one α/β half-receptor by the other (45, 46) or that there may be intermolecular transphosphorylation between adjacent receptor molecules (47), a dominant effect could therefore be exerted at the level of autophosphorylation. Alternatively, a dominant effect on one or more signaling pathways could occur as has been suggested with site-directed mutants of the ATP binding site (6, 8) or mutation of Tyr¹¹⁵⁸ (38) where there is apparent inhibition of some functions of native rodent receptors in transfected cells. We have also recently shown that severe insulin resistance may be caused by another mutation (Trp¹²⁰⁰ → Ser¹²⁰⁰) in one allele of the insulin receptor gene (12, 48).

The three affected sisters in the BI-1 family all displayed features of the Type A syndrome of insulin resistance, which include the skin lesion of acanthosis nigricans, hyperandrogenism with polycystic ovaries, and insulin resistance (18). This syndrome is thought to be uncommon and is usually only recognized in females due to the pathologic elevation in androgen levels. High androgen levels are thought to be a consequence of chronic stimulation of insulin-like growth factor-1 receptors on the ovaries (49) by elevated insulin levels. The insulin-resistant father in this family, who is also a heterozygote for the Ala¹¹³⁴ mutation, has no physical features of the Type A syndrome. The present study illustrates that severe insulin resistance with dominant inheritance may be caused by a mutation in one allele of the insulin receptor gene and can be clinically silent in a male. Although the impaired receptor kinase function described in non-insulin-dependent diabetes mellitus (50, 51) is thought to be mostly an acquired defect (52), it is plausible that a subpopulation of individuals have insulin receptor mutations that cause relatively mild glucose intolerance or, in combination with other defects, render them at risk for non-insulin-dependent diabetes mellitus.

Acknowledgments—We would like to thank Drs. Peter A. Wilden and Jonathan M. Backer (Joslin Diabetes Center, Boston, MA) for helpful discussions and technical advice.

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