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Tyrosine-Kinase Defect of the Insulin Receptor in Cultured Fibroblasts from Patients with Lipoatropic Diabetes*

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ABSTRACT. Postbinding defects in insulin action were described previously in cultured fibroblasts from six patients with lipoatropic diabetes. To define the contribution of the insulin receptor tyrosine kinase in these defects, we studied autophosphorylation and kinase activity of lectin purified receptors from these six patients and six normal cell lines. The patients' insulin receptors, prepared by precipitation with polyethylene glycol, had normal insulin binding characteristics and autophosphorylation properties, but a 56% decrease in the tyrosine kinase activity toward an exogenous substrate. To identify more subtle qualitative defects in autophosphorylation, insulin receptors were sequentially immunoprecipitated and analyzed for their phosphoaminoacid content. The phosphorylated receptors precipitated with an antiphosphotyrosine antibody contained labeled phosphotyrosine, whereas those in the supernatant, when further precipitated with an antireceptor antibody, contained only phosphoserine. Under these conditions, the insulin-stimulated autophosphorylation of tyrosine was significantly decreased by 54% in the patient receptors compared to normal subjects' receptors. In addition, insulin-like growth factor-I stimulation of autophosphorylation of its receptor was reduced by 59% in the patients' cells compared to those from normal subjects. We conclude that fibroblasts from patients with lipoatropic diabetes have defects in the tyrosine kinase activity of their insulin and their insulin-like growth factor-I receptors that might give rise to the *in vitro* hormone resistance and be related to the *in vivo* hormone resistance that occurs in these patients. (*J Clin Endocrinol Metab* **69:** 142, 1989)

LIPOATROPIC diabetes is a rare human disorder characterized by complete or partial lack of adipose tissue, hepatosplenomegaly, hyperlipidemia, and severe insulin resistance (1, 2). This syndrome, the type A insulin resistance syndrome, and leprechaunism belong to a family of apparently inherited diseases associated with severe insulin resistance and acanthosis nigricans (2). The pathogenesis of the insulin resistance in lipoatropic diabetes is unknown. The presence of abnormalities in glucose metabolism which are maintained in cultured fibroblasts from patients with lipoatropic diabetes strongly suggests a primary defect in insulin action located at the cellular level (2–4). Most studies of lipoatropic diabetes have focused on the measurement of insulin binding to identify alterations in receptor number or affinity as possible mechanisms of insulin resistance. However, in contrast to leprechaunism or the type A syndrome, in which the insulin resistance can often be explained by a major decrease in insulin receptor number (2, 5–7), in most patients with lipoatropic diabetes, insulin binding to both circulating and cultured cells is normal (3, 4, 8, 9) or moderately decreased (10). Thus, the cellular defect is presumably a postbinding defect.

The insulin receptor contains tyrosine-specific protein kinase activity, which is required for the transmembrane signaling induced by the hormone (11–18) and which may be abnormal in diabetic syndromes. The binding of insulin to the α-subunit of the receptor (mol wt, 135 K) is followed rapidly by the activation of a tyrosine-kinase located in the β-subunit (mol wt, 95K). This activation results in phosphorylation of the β-subunit, primarily on tyrosine residues (autophosphorylation), as well as transfer of phosphate from the receptor to exogenous substrates (phosphotransferase activity). Furthermore, in intact cells, serine residues of the insulin receptor are

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also phosphorylated; this serine phosphorylation may inhibit the tyrosine kinase activity of the insulin receptor (11–14, 18, 19).

In several genetic syndromes of severe insulin resistance and acanthosis nigricans, such as the type A syndrome and leprechaunism, there are specific tyrosine kinase abnormalities (20–22). However, for lipoatropic diabetic patients a defect in insulin receptor autophosphorylation has been reported only once (23).

We previously demonstrated the presence of postbinding defects in insulin action in cultured fibroblasts from six patients with lipoatropic diabetes (4). In this study we attempted to detect, in these six patients, an abnormality in receptor autophosphorylation and tyrosine kinase activity. Then, using antiphosphotyrosine antibodies, which allowed us to discriminate between the tyrosine- and serine-labeled receptor β-subunits, we tried to define the nature of the alteration. Simultaneously, the tyrosine kinase activity of the insulin-like growth factor-I (IGF-I) receptor was studied in lectin-purified receptors from these patients compared to those from normal subjects. We found that both insulin and IGF-I receptor tyrosine kinase activities were altered in lipoatropic diabetes. This alteration may be located at a common step involved in the phosphorylation of serine *vs.* tyrosine residues of the receptor β-subunits.

Materials and Methods

Materials

Porcine insulin was purchased from Novo Research Institute (Copenhagen, Denmark); [^{125}I-Tyr^{A14}]monoiodoinsulin (73 TBq/mmol), IGF-I, and [γ-^{32}P]ATP (110 TBq/mmol) from Amersham (Buckinghamshire, United Kingdom); Dulbecco's Modified Eagle's Medium (H16) and fetal calf serum from Gibco (Grand Island, NY); ATP, poly-Glu:Tyr (4:1 ratio), phenylmethylsulfonylfluoride, *N*-acetyl-D-glucosamine, and cellulose thin layer plates from Sigma Chemical Co. (St. Louis, MO); Triton X-100 and polyethylene glycol (PEG; mol wt, 8000) from Merck (Darmstadt, West Germany); bovine γ-globulins (fraction II) and protein-A (Pansorbin) from Calbiochem-Behring Corp. (La Jolla, CA); and aprotinin (Iniprol) from Choay Laboratories (Paris, France). All compounds for electrophoresis were obtained from Bio-Rad Chemical Division (Richmond, CA). Wheat germ agglutinin-agarose was obtained from Pharmacia PL Biochemical, Inc. (Uppsala, Sweden). All other reagents used were of the best analytical grade. The antiphosphotyrosine antibody (αPY) was prepared as previously described (19). The antibody against the human insulin receptor (serum B-8) was a gift from Dr. C. R. Kahn (Boston, MA).

Cells for study

Fibroblasts were obtained by forearm skin biopsy in six female patients (age range, 1–32 yr; designated D1–D6) with lipoatropic diabetes. Each patient had lipoatrophy, acanthosis nigricans, and hepatomegaly (except D5), and severe insulin resistance as estimated by the euglycemic clamp technique or the inefficacy of insulin therapy. The clinical and laboratory features of these patients were described previously in detail (4). The control group consisted of six normal subjects, four women and two men (age range, 20–23 yr), who had no personal or family history of diabetes mellitus.

The fibroblasts were grown in monolayers in Dulbecco's Modified Eagle's Medium containing glucose (1 mg/mL) and supplemented with 10% fetal calf serum. The cells were subcultured at a 1:3 ratio by mild digestion with trypsin (0.05% trypsin and 0.02% EDTA). Confluent cell monolayers were used between the 5th and 12th passages. Before the experiments, fibroblasts were incubated for 18 h in serum-free culture medium.

Purification of insulin receptors by lectin affinity chromatography

Fibroblast monolayers (75-cm^2 flasks of confluent cells) were washed with cold Dulbecco's phosphate-buffered saline and scraped from flasks at 4 C in a solution of 50 mmol/L HEPES (pH 7.6), 1% Triton X-100, 1,200 PIU/mL aprotinin, and 2 mmol/L phenylmethylsulfonylfluoride as previously described (21, 24). The cell extract then was stirred for 1 h at 4 C and centrifuged (100,000 × *g*; 2 h; 4 C) in a Beckman ultracentrifuge (Palo Alto, CA). The supernatant containing the soluble receptor was applied to a 1-mL column of wheat germ agglutinin coupled to agarose and recycled three times. The column was washed with 400 mL of a buffer containing 50 mmol/L HEPES (pH 7.4) and 0.1% Triton X-100 (25). The partially purified receptors were eluted with 0.3 mmol/L *N*-acetyl-D-glucosamine, and the eluates were collected in 500-μL fractions. The protein concentration of an aliquot of each fraction was measured by the dye-binding method (Bio-Rad) using BSA as standard. The fraction containing the maximal protein concentration (~70 μg protein/mL) was used for subsequent assays.

Insulin binding assay

Lectin-purified receptors (~3 μg protein) were incubated in duplicate with 50 pg/mL [^{125}I]monoiodoinsulin and increasing concentrations of unlabeled insulin (from 0–10,000 ng/mL) for 15 h at 4 C in a final volume of 50 μL. The incubation buffer contained 50 mmol/L HEPES (pH 7.6), 150 mmol/L NaCl, 0.08% Triton X-100, and 0.1% BSA (buffer A). Bound and free hormone were separated by precipitation of the former with PEG at a final concentration of 12.5%, using 0.1 mg/mL bovine γ-globulin as carrier (24). After 15 min at 4 C, the samples were centrifuged at 12,000 × *g* for 5 min in a Beckman microfuge, and the pellet obtained was washed with 12.5% PEG and counted for radioactivity in a Kontron γ-counter.

Autophosphorylation assay

Lectin-purified receptors (~3 μg protein) were preincubated in duplicate at 4 C for 15 h with increasing concentrations of insulin or IGF-I in a final volume of 50 μL buffer A. The

phosphorylation reaction was initiated by the addition of $MnCl_2$ (4 mmol/L) and 30 μCi/tube [γ-^{32}P]ATP (50 μmol/L) and allowed to proceed for 15 min at 20 C. The reaction then was stopped by addition of 50 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, and 5 mM ATP (24).

Two procedures were used to measure insulin-stimulated receptor autophosphorylation. In one set of experiments, the phosphorylated lectin-purified proteins were precipitated (15 min; 4 C) with PEG (12.5%) in the presence of 0.1 mg/mL bovine γ-globulin. The precipitated proteins were solubilized in Laemmli sample buffer containing 100 mmol/L dithiothreitol and boiled for 3 min, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide (7.5%) gel electrophoresis (11, 25). The phosphorylated proteins were detected by autoradiography of the stained and dried gels on Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) with an intensifying screen. The incorporation of ^{32}P into the 95K band was quantified by scanning densitometry of autoradiograms of the same time exposure (dual-wavelength TLC scanner CS-930, Shimadzu Co. Tokyo, Japan) and expressed as arbitrary units (au), which represented the integrated area of the peak and corresponded to the scanning result × 10^{-6}. In some instances, the labeled band was cut off and counted; the ^{32}P radioactivity determinations were very similar to the scanning results.

In a second set of experiments the phosphorylation of phosphotyrosine-containing proteins was determined by specific immunoprecipitation with an antiphosphotyrosine antibody (αPY, at a final dilution of 1:50) (19). After incubation (6 h; 4 C), the immune complexes were precipitated with protein-A, separated by electrophoresis, and autoradiographed as described above (11, 25). To analyze the remaining receptors, the supernatant obtained after the precipitation with αPY was submitted to a second immunoprecipitation using an antiinsulin receptor antibody (serum B-8, at a final dilution of 1:100).

Tyrosine kinase assay

Lectin-purified receptors (~1.5 μg protein) were incubated in duplicate with or without insulin (10,000 ng/mL) for 30 min at 20 C in a final volume of 50 μL buffer A. The synthetic copolymer Glu:Tyr at a final concentration of 2.5 mg/mL, $MnCl_2$ (4 mmol/L), $MgCl_2$ (12 mmol/L), unlabeled ATP (50 μmol/L), and 10 μCi/tube [γ-^{32}P]ATP were added at time zero (20). After incubation (20 min; 20 C), 25-μL aliquots were spotted on filter papers (Whatman 3 MM), which were dried and placed in 10% trichloroacetic acid containing 10 mmol/L sodium pyrophosphate. After extensive washing with the same buffer for 24 h, the filter papers were counted for radioactivity in a liquid scintillation counter.

Phosphoamino acid analysis

Phosphoamino acids were analyzed after trypsin digestion and hydrolysis of the protein bands in polyacrylamide gel fragments as previously described (13, 26). The position of the insulin-stimulated phosphorylated proteins on the polyacrylamide gel was located by autoradiography. The corresponding bands were excised, rehydrated in 20% methanol (12 h at 37 C), dried at 110 C, and digested for 24 h at 37 C in 50 mmol/L NH_4HCO_3, pH 8, containing 50 μg/mL trypsin. The tryptic peptides were lyophilized and hydrolyzed in 6 mol/L HCl (2 h; 110 C). The phosphoamino acids were separated by high voltage electrophoresis on cellulose thin layer plates (250 μm) in H_2O-acetic acid-pyridine (89:10:1). Standards of phosphoamino acids were identified by reaction with ninhydrin, and the radioactivity of the samples was evaluated by autoradiography.

Statistical analysis

Individual values obtained by scanning densitometry of the 95K protein band, expressed in arbitrary units, were corrected for protein concentration. The results in the patient and the normal groups were compared by Student's t test. The results are expressed as the mean ± SEM. To compare individual patient values with the normal group, the 95% confidence interval of the normal group was calculated. $P < 0.05$ was considered significant.

Results

Insulin binding to soluble receptors

Previous studies of insulin binding to intact fibroblasts obtained from the six patients with lipoatropic diabetes revealed normal binding properties compared to normal cell lines (4). To quantify the amount of receptor from each cell line and to compare the level of hormone binding to that of hormone-induced protein phosphorylation, [^{125}I]insulin binding to partially purified soluble receptors was determined. The mean values for insulin binding to soluble receptors were similar in the patients and the normal group (Fig. 1); the levels of tracer binding were 11.3 ± 1.8% and 11.5 ± 2.2% (±SEM), and half-maximal inhibition of tracer insulin binding (ED_{50}) occurred at 3.7 ± 1.2 and 3.9 ± 1.0 nmol/L insulin, respectively. Five patient cell lines yielded binding curves within the 95% confidence interval of the normal group (5.4–17.6%), and only one cell line had a slightly increased value (patient D_6; 19.1%). Scatchard analysis of the insulin binding data for patient D_6 revealed an increase in the insulin receptor number without an alteration in affinity constant (data not shown).

Insulin-stimulated phosphorylation of the lectin-purified receptors

Lectin-purified receptors were incubated with increasing concentrations of insulin, phosphorylated in the presence of [γ-^{32}P]ATP, and precipitated with PEG. By this procedure all proteins over the molecular mass of 120K were precipitated. The autoradiograms of the gels obtained under reducing conditions revealed only one major protein band (with an apparent mol wt of 95K), the phosphorylation of which was markedly enhanced in the presence of insulin. Autoradiograms obtained from a

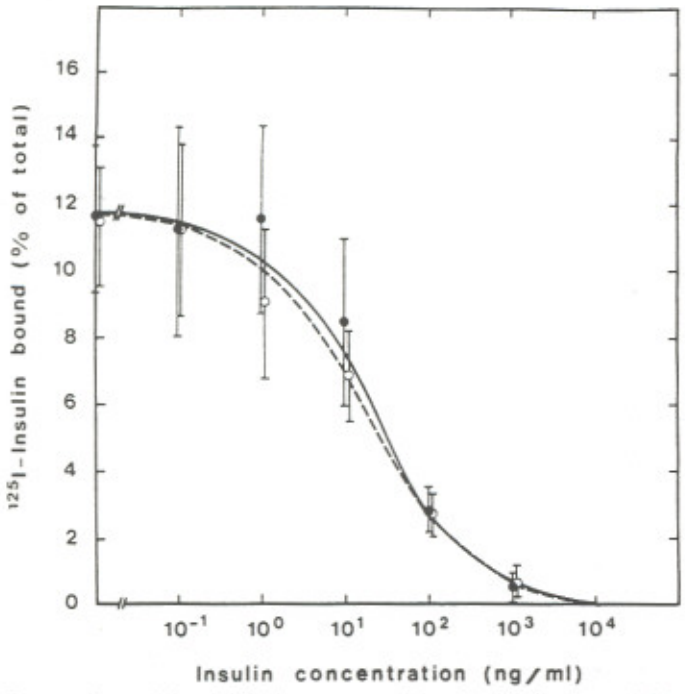

FIG. 1. Competition-inhibition curves of insulin binding to soluble insulin receptors from fibroblasts of normal subjects and lipoatropic diabetic patients. [^{125}I]Insulin binding to lectin-purified receptors from the normal (●) and patient (○) groups was performed by incubation for 15 h at 4 C with 50 pg/mL [^{125}I-Tyr^{A14}]monoiodoinsulin and various concentrations of unlabeled insulin. The results are expressed as the mean (± SEM) percentage of [^{125}I]insulin bound per 4 μg protein.

patient and a normal subject are shown in Fig. 2 (*left panel*). The presence in this fraction of the insulin receptor β-subunit was verified. When phosphorylated lectin-purified receptors were immunoprecipitated with an antiinsulin receptor antibody (serum B-8), among the 95K phosphorylated proteins in the supernatant was a minor band whose labeling was not modified by insulin. Conversely, labeling of the insulin receptor β-subunit, recovered in the precipitate, was markedly insulin dependent (data not shown).

The quantification by scanning densitometry of autoradiograms revealed that the mean dose-response curves from the patient and the normal groups were similar (Fig. 3). The basal levels of phosphorylation were similar, representing 0.67 ± 0.12 and 0.68 ± 0.17 au for the patient and the control group, respectively. Insulin was active at a concentration as low as 1 ng/mL, and insulin activity was maximal at 5,000–10,000 ng/mL. For the patient group the ED_{50} was 14.8 ± 2.2 nmol/L (normal group, 11.5 ± 3.0 nmol/L), and the maximal insulin-stimulated autophosphorylation was 1.96 ± 0.41 au (normal group, 2.08 ± 0.48 au). The insulin stimulation relative to the basal activity in the normal subjects is shown in Table 1. When analyzed as individual values, the dose-response curves from five patient cell lines were situated within the 95% confidence interval of the normal group, and a decreased level of phosphorylation was found in only one patient (D3). These data indicate that insulin stimulates the autophosphorylation of the receptor β-subunit to the same extent in normal subjects and patients with lipoatropic diabetes.

As shown in Fig. 4B (*left panel*), the phosphoamino acid analysis of the PEG-precipitable 95K proteins revealed that under the conditions used, phosphorylation occurred on both phosphoserine and phosphotyrosine residues.

Protein kinase assay of the lectin-purified receptors

The receptor kinase activity was measured using the exogenous substrate poly-Glu:Tyr as a phosphoacceptor. In the absence of insulin, the basal activity was 66.5 ± 10.0 × 10^3 cpm/mg protein in soluble receptors of patients and 108.8 ± 11.6 × 10^3 cpm/mg protein in soluble receptors from normal subjects (Fig. 5). The maximal effect of insulin (at 10,000 ng/ml) was reduced by 56% in the patient group compared to that in the normal

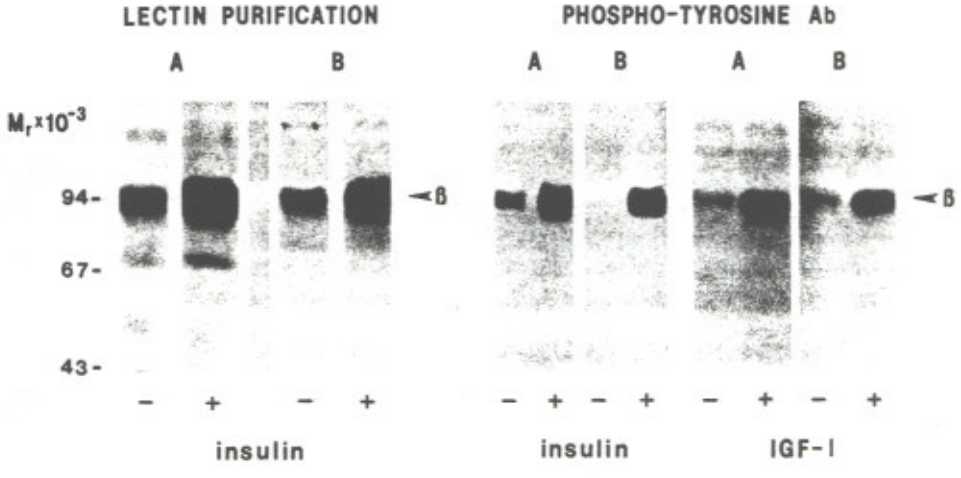

FIG. 2. Autoradiograms of insulin-stimulated phosphorylation of the 95K proteins from a lipoatropic diabetic patient and a normal subject. Lectin-purified receptors from the normal subject (lane A) and patient fibroblasts (lane B) were preincubated with (+) or without (−) insulin (10,000 ng/mL) or IGF-I (1,000 ng/mL), and phosphorylation was carried out as described in *Materials and Methods*. The phosphorylated glycoproteins were analyzed by electrophoresis after precipitation with PEG (lectin purification; *left panel*) or with an anti-phosphotyrosine antibody (*right panel*). M_r, Mol wt.

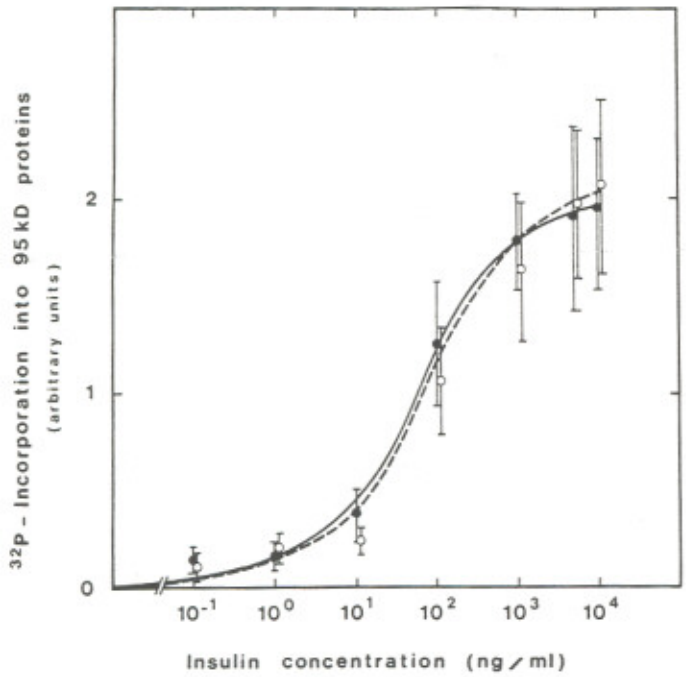

FIG. 3. Dose response of autophosphorylation of insulin receptors from normal subjects and lipoatropic diabetic patients. Lectin-purified receptors from lipoatropic diabetic patients (○) and normal subjects (●) were preincubated with increasing concentrations of insulin for 15 h at 4 C and phosphorylated in the presence of [γ-^{32}P]ATP for 15 h at 20 C. The phosphorylated proteins were analyzed after precipitation with PEG (12.5%) using 0.1 mg/mL bovine γ-globulin as carrier. The basal activity was subtracted from each value, and the results were expressed in arbitrary units (mean ± SEM).

group (95.2 ± 17.9 and 218.8 ± 35.5 × 10^3 cpm/mg protein; $P < 0.05$). This stimulation represented 143 ± 26% of the basal level in the patients and was reduced compared to the stimulation in the normal receptors (202 ± 32% of the basal value; $P < 0.01$).

Thus, receptors from patient fibroblasts appeared less able to tyrosine-phosphorylate exogenous substrate despite the equal level of insulin binding and the equal degree of autophosphorylation. These results prompted us to analyze more specifically receptor tyrosine autophosphorylation.

Immunoprecipitation of the autophosphorylated receptor with the antiphosphotyrosine antibodies

To estimate the level of tyrosine phosphorylation in patient and normal insulin receptors, the phosphorylated proteins were analyzed after specific immunoprecipitation with an αPY antibody directed against phosphotyrosine-containing proteins (19).

The antibody αPY selectively immunoprecipitated one protein (mol wt, 95K) which displayed increased labeling upon hormone stimulation and, thus, could be identified as the β-subunit of the insulin receptor (Fig. 2). The phosphoamino acid analysis of this band revealed the presence of only radiolabeled phosphotyrosine residues, thus confirming the specificity of this antibody (Fig. 4B). The immunoprecipitation was complete because a second immunoprecipitation of the supernatant with αPY did not reveal any phosphorylated band at the 95K level (Fig. 4A, *right panel*). Interestingly, when the same supernatant was further immunoprecipitated with an antiinsulin receptor antibody (serum B-8), one insulin-stimulated 95K band was identified on the autoradiogram (Fig. 4A, *right panel*). The phosphoamino acid analysis revealed only the presence of labeled phosphoserine (Fig. 4B, *right panel*). We concluded from these experiments that under the conditions used, at least two pools of insulin receptors were present in the soluble fraction, and specific precipitation with αPY allowed the identification of the receptors phosphorylated on tyrosine, whereas the antiinsulin receptor antibody precipitated the remaining receptors phosphorylated on serine.

To investigate further the insulin receptor in the patients with lipoatropic diabetes, the level of receptor autophosphorylated on tyrosine residues was specifically quantified using αPY. An autoradiogram of the phosphotyrosine-containing proteins in the absence or presence of insulin from a patient and a normal cell line is shown in Fig. 2 (*right panel*). The quantification by scanning densitometry of autoradiograms obtained from the patients and the normal group is shown in Table 1. In the basal state, tyrosine phosphorylation of the 95K proteins was 0.18 ± 0.04 and 0.44 ± 0.10 au for the patient and

TABLE 1. ^{32}P incorporation into the 95K proteins from fibroblasts of normal subjects and lipoatropic diabetic patients

Cells	Receptor precipitated with PEG (au)		Receptor precipitated with antiphosphotyrosine antibody (au)		
	No treatment	+ Insulin	No treatment	+ Insulin	+ IGF-I
Normal subjects (n = 6)	0.68 ± 0.17 (100)	2.08 ± 0.48 (457)	0.44 ± 0.10 (100)	1.57 ± 0.27 (358)	1.54 ± 0.29 (350)
Patients (n = 6)	0.67 ± 0.12 (99)	1.96 ± 0.41 (446)	0.18 ± 0.04 (40)	0.72 ± 0.18 (164)	0.63 ± 0.20 (143)

The glycoproteins phosphorylated in the absence or presence of insulin (10,000 ng/mL) or IGF-I (1,000 ng/mL) were analyzed after precipitation with PEG or antiphosphotyrosine antibody. The results are expressed in arbitrary units (mean ± SEM). The numbers in parentheses represent the phosphorylation relative to the basal activity in normal subjects (100%).

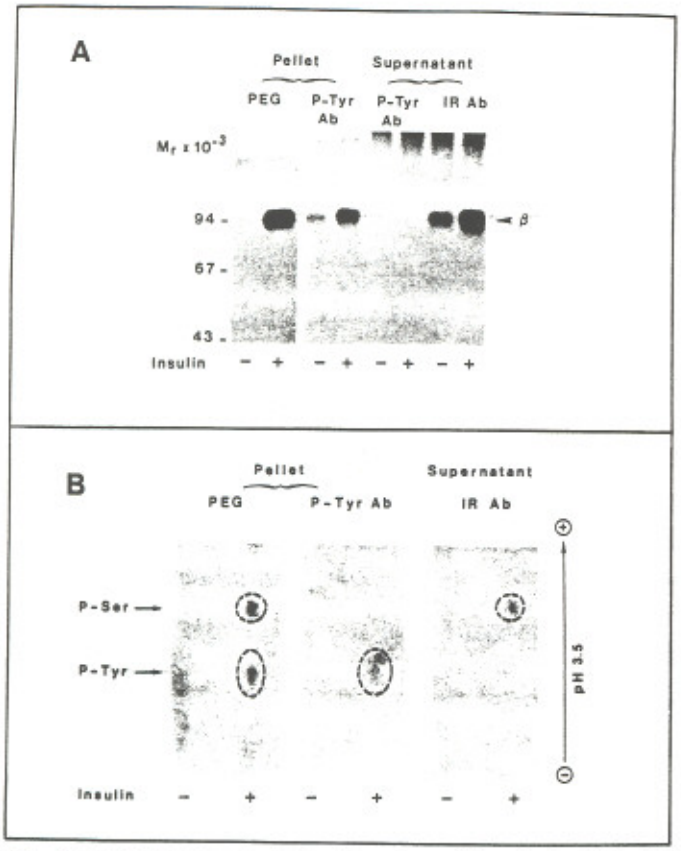

FIG. 4. Autoradiograms and phosphoamino acid analysis of the 95K phosphorylated proteins from a normal subject. A, The phosphorylated glycoproteins in the absence (−) or presence (+) of insulin (10,000 ng/mL) were analyzed after precipitation with PEG or antiphosphotyrosine antibody (P-Tyr Ab; *left panel*). The supernatant obtained after initial reaction with the antiphosphotyrosine antibody was further immunoprecipitated with either P-Tyr Ab or antiinsulin receptor antibody (IR Ab; *right panel*). B, Phosphoamino acid content of the 95K protein bands obtained under the conditions described in A. M_r, Mol wt.

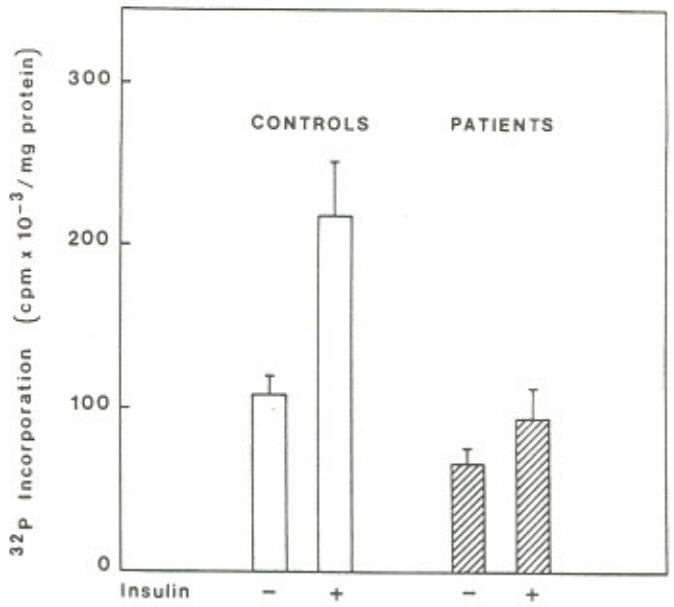

FIG. 5. Tyrosine-specific kinase activity of the insulin receptor from normal subjects and lipoatropic diabetic patients toward the synthetic copolymer poly-glu:Tyr (4:1). Lectin-purified receptors from the normal subjects (□) and the patient group (▩) were incubated with (+) or without (−) insulin (10,000 ng/mL) and phosphorylated in the presence of poly-Glu:Tyr (2.5 mg/mL) as indicated in *Materials and Methods*. The data are expressed as the mean ± SEM (counts per min × 10^{-3}/mg protein).

normal groups, respectively ($P < 0.05$). In the presence of insulin, tyrosine-specific phosphorylation of the 95K proteins reached 0.72 ± 0.18 au in the patient group, which was 46% of the value in the normal group (1.57 ± 0.27 au; $P < 0.05$). Moreover, the increment in autophosphorylation stimulated by insulin (*i.e.* stimulated minus basal) also was reduced to a similar extent in the patient group (0.54 au) compared to that in the normal group (1.13 au). These data indicate that lipoatropic diabetes is characterized by an impaired ability of insulin to stimulate the tyrosine-containing receptor subpopulation.

Immunoprecipitation of phosphorylated phosphotyrosine-containing proteins stimulated by IGF-I

To determine whether the defect in tyrosine phosphorylation was selective for the insulin receptor, we studied the phosphotyrosine-containing proteins stimulated by IGF-I using specific immunoprecipitation with the αPY antibody.

On the autoradiogram (Fig. 2, *right panel*) only one labeled band appeared to be stimulated by IGF-I. It had an apparent mol wt of 95K, which corresponded to the β-subunit of the IGF-I receptor (27). The quantification by scanning densitometry of autoradiograms is shown in Table 1. In the presence of a maximally effective concentration of IGF-I (1000 ng/mL), the tyrosine phosphorylation in the patient receptors reached 0.63 ± 0.20 au, 41% of the value in the normal group (1.54 ± 0.29 au; $P < 0.05$). The increment in phosphorylation stimulated by IGF-I also was decreased to a similar extent in the patient group (0.45 au) compared to that in the normal group (1.10 au).

Discussion

Among the genetic syndromes of severe insulin resistance and acanthosis nigricans, the type A syndrome and leprechaunism have been extensively studied in order to understand the pathogenesis of insulin resistance (2, 5, 7). Decreased insulin binding to freshly isolated or cultured cells from these patients has been proposed as a common mechanism of insulin resistance. However, there are patients whose cells bind insulin normally but respond poorly to insulin, suggesting defects located at a

postbinding level. In a few patients the defect was located at the level of the β-subunit of the receptor, *e.g.* tyrosine-specific kinase activity was decreased (20–22), or it resulted from a low mol wt circulating factor that uncoupled insulin action from receptor binding and phosphorylation (28, 29).

In lipoatropic diabetes, insulin binding to cultured cells usually is normal (3, 4, 8, 9) or moderately decreased (10), and the contribution of insulin receptor kinase defects to the insulin resistance has not been studied extensively. Whittaker *et al.* (9) found insulin receptor phosphorylation to be normal in Epstein-Barr virus-transformed lymphocytes of three lipoatropic diabetic patients, while Kriauciunas *et al.* (10), studying three related patients with lipoatropic diabetes, found that insulin binding to fibroblasts and insulin-stimulated receptor autophosphorylation were decreased in parallel in two cases, while in the third this latter parameter was normal. Thus, in these studies, a specific defect at the level of receptor autophosphorylation was not revealed. Such a defect was only found in erythrocytes from one patient (23).

In an attempt to investigate further insulin receptor kinase function in lipoatropic diabetes, we studied receptor autophosphorylation and kinase activity in skin fibroblasts from six such patients (4). Cultured fibroblasts were used because they can be grown for several generations after removal from the *in vivo* milieu, thus allowing the detection of cellular abnormalities due to a primary defect. We previously reported the presence of a normal level of insulin receptors together with heterogenous alterations in glucose metabolism in these cell lines (4). As expected from our previous experiments using intact cells, insulin binding to lectin-purified receptors from patient fibroblasts was not altered compared to those from normal subjects. Moreover, insulin-induced autophosphorylation of the soluble receptor was normal, and the phosphorylation dose-response curves were similar in both groups. Thus, measurements of insulin binding and receptor autophosphorylation in lectin-purified material revealed no specific defect at the level of insulin receptor kinase, in accordance with the results of previous studies of lipoatropic diabetic patients (9, 10) or other insulin-resistant patients (6, 9).

We then studied the ability of insulin receptors to phosphorylate tyrosine contained in an exogenous substrate (poly-Glu:Tyr). Both basal and hormone-activated phosphorylation of poly-Gly:Tyr were decreased. Such a discrepancy between endogenous and exogenous receptor kinase activities is rather uncommon, since in most studies of insulin-resistant patients, both activities were affected in parallel (6, 20, 21). Nevertheless, similar discrepancies have been reported recently in patients with noninsulin-dependent diabetes (30) and leprechaunism (22). Another study (31) dealt with cells transfected with a plasmid coding for a human insulin receptor mutated on the twin tyrosines in positions 1162 and 1163 (15), which behave as a major site of receptor autophosphorylation (32, 33); even though receptor autophosphorylation was minimally modified, exogenous kinase activity and insulin metabolic responses were markedly reduced.

To further investigate endogenous receptor kinase activity, sequential immunoprecipitations were conducted in parallel with analysis of labeled phosphoamino acids. When the phosphorylated receptors were immunoprecipitated with an antiphosphotyrosine antibody (αPY), insulin-stimulated phosphate incorporation was reduced in the patient group. Under these conditions, phosphoamino acid analysis revealed only phosphotyrosine, while both phosphoserine and phosphotyrosine were present in the receptor fraction before immunoprecipitation. The extent of this alteration correlated well with the defect in exogenous kinase activity. Thus, 1) the striking discrepancy between the normal level of autophosphorylation when the receptor was precipitated with PEG and the defect when αPY was used along with 2) the detection of receptors labeled on both amino acids strongly suggest the presence of a subpopulation of serine-phosphorylated insulin receptors. We were able to document this suggestion in the supernatant obtained after immunoprecipitation with αPY. Phosphorylation of serine residues, in addition to tyrosine residues, on the insulin receptor has been reported previously in most studies using intact cells and less often in cell-free systems (11–14, 33, 34). Two recent studies (35, 36) analyzed more precisely the conditions that allowed the copurification of the insulin receptor; with serine kinase activity responsible for insulin-dependent phosphorylation of the insulin receptor; some of these conditions, such as the absence of NaCl during the purification procedure or the performance of the kinase assays at 22 C, were fulfilled in our experiments. Thus, it is likely that our partially purified receptor fractions contain an insulin-responsive serine kinase. As previously found *in vivo*, serine phosphorylation of the insulin receptor β-subunit appears to antagonize tyrosine phosphorylation and, thus, reduce receptor signalling (12, 18, 19). Similarly, the presence of a population of insulin receptors phosphorylated on either serine or tyrosine residues was recently reported in noninsulin-dependent diabetic patients (37).

To investigate further whether the defect in kinase activity in patient cells was due to an intrinsic receptor defect rather than increased serine kinase activity in the lectin-purified fraction, we performed several additional experiments. Insulin receptors were separated from the other glycoproteins by immunoprecipitation with an antiinsulin receptor antibody (serum B-8) before the phos-

phorylation assay. The defect in autophosphorylation was still found in the patient receptors (data not shown), thus suggesting an intrinsic alteration of the receptor.

The abnormal tyrosine kinase activity in the insulin receptor from the lipoatropic diabetic patients correlated well with the *in vitro* insulin resistance in each cell line (4). However, just as for postbinding abnormalities concerning glucose metabolism, some heterogeneity was found in the defects of receptor kinase activities, the most important alterations being observed in the D3 and D4 cell lines. Nevertheless, as the moment a clear correlation between alterations at the kinase and postbinding levels was not found in the cells from these six patients. Interestingly, two recent studies using cells transfected with plasmids coding for human insulin receptors either truncated by the C-terminal 43 amino acids (38) or mutated on the twin tyrosines at the major site of autophosphorylation (31) revealed that these modifications induced insulin resistance for some biological responses while others were conserved. These data led the researchers to conclude that different actions of insulin may be mediated by different domains of the insulin receptor and, in particular, by distinct and specific receptor phosphorylation sites. Similarly, in our patient cell lines, the heterogeneous alterations in insulin-responsive pathways could result from alterations in the receptor serine or threonine *vs.* tyrosine phosphorylation state. In addition, the heterogeneity of the defects among the different patients could be easily related to the well known heterogeneity in clinical and biological features that was previously reported in the different syndromes of insulin resistance (2, 6, 22) and in lipoatropic diabetes in particular (4, 8, 10), suggesting that the defects in the different patients could result from different mutations in the same gene. It is obvious that the assays concerning endogenous and exogenous kinase activities that were carried out in this study could not document the precise nature of such subtle alterations.

As the β-subunits of both insulin and IGF-I receptors have important structural homologies (39), we also investigated the extent of tyrosine autophosphorylation of the IGF-I receptor in response to IGF-I; marked defects were found in the patient group, the extent of which were similar to those of the insulin receptor. This alteration could be ascribed chiefly to an alteration of the IGF-I receptor β-subunit; indeed, since IGF-I was 400-fold less potent than insulin as an inhibitor of insulin binding to its receptor (data not shown), in accordance with previous studies (40), the IGF-I concentration used would have been too low to fully activate the insulin receptor. Moreover, binding and metabolic assays performed with some of the patient cell lines revealed a normal level of IGF-I binding to cultured fibroblasts together with postbinding alterations similar to those found with insulin (4) (our unpublished data). These data further argue for an actual alteration of the IGF-I receptor tyrosine kinase. Thus, although insulin at the concentrations used could cross-react with IGF-I receptors, the fact that similar alterations were found with both hormones clearly indicates that the two kinds of receptors were affected in the same way.

In addition to the defects in hormone-activated receptor endogenous and exogenous tyrosine kinase activities in the patient group, the basal activities also were decreased. Even though similar results have been reported previously for insulin-resistant patients (6, 20, 21), the meaning of such a defect is unclear. It may result from an altered structural conformation of the receptor β-subunit, a more generalized defect such as a chronic lack of insulin, an inhibitor, or another factor.

These results indicate the presence of a primary defect in the tyrosine kinase activity of both insulin and IGF-I receptors from fibroblasts obtained from lipoatropic diabetic patients. These alterations might be located at a common step or implicate a common cellular component, such as lipid environment, which would be involved in both receptor phosphorylation processes and thus could induce subtle structural modifications of the receptor β-subunits. As a result, increased serine and/ or decreased tyrosine autophosphorylation of the receptors could lead to reduced exogenous kinase activity and receptor signalling, and these defects might be involved in the pathogenesis of the insulin resistance in this syndrome.

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