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Leprechaunism: An Inherited Defect in a High-Affinity Insulin Receptor

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SUMMARY

We examined in vivo oral glucose tolerance tests and in vitro insulin binding, cellular response, and insulin-receptor structure of fibroblasts cultured from the skin of a patient with leprechaun syndrome and her parents. In response to oral glucose, the proband exhibited marked hyperinsulinism (maximum plasma insulin = 4,120 μU/ml), the father had mild hyperinsulinism (maximum plasma insulin = 240 μU/ml), and the mother was normal. [^{125}I]insulin binding to monolayers of intact fibroblasts demonstrated complex kinetics that were interpreted using a two-receptor model. Normal high-affinity binding had an apparent K_A of 1.6×10^{10}/molar with 1,100 sites/cell. The proposed low-affinity state receptor had an apparent K_A of 6.8×10^7/molar with approximately 30,000 sites/cell. Insulin binding to the proband's cells had no high-affinity binding but had normal low-affinity binding. Cells from the mother had 60%, and cells from the father, 2%, of control insulin binding to the high-affinity receptor, but normal, low-affinity site binding. Two different, insulin-stimulable responses were evaluated under experimental conditions identical with those used for insulin binding. Insulin stimulation of 2-methylaminoisobutyric acid uptake occurred with half-maximal responses between 25 and 50 ng/ml insulin. This response was similar in cells from controls and the patient. By contrast, the uptake and phosphorylation of 2-deoxy-D-glucose was stimulated at half-maximal insulin concentrations between 1 and 10 ng/ml in control cells but was not significantly increased in the proband's cells until 1,000 ng/ml concentrations of insulin were attained. In affinity crosslinking experiments, [^{125}I]insulin was covalently bound to insulin receptors of fibroblast membranes using disuccinimidylsuberate. [^{125}I]insulin specifically bound to 125,000 dalton monomeric subunits and 250,000 dalton dimers. In

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control cells, the ratio of monomer to dimer was approximately one, but significantly fewer dimers were crosslinked in insulin receptors from the patient's cells.

We conclude that in this family two different recessive mutations impair high-affinity insulin-receptor binding and that the proband with leprechaunism is a compound heterozygote for these mutations. The two mutations produced structural changes in the receptor that altered subunit interactions and loss of high-affinity binding and cellular responsivity.

INTRODUCTION

Binding to specific receptors located on plasma membrane surfaces is thought to initiate the process by which many peptide hormones produce intracellular signaling [1–4]. Studies of families with probands expressing "hormone resistance" have advanced our knowledge of the mechanisms of peptide hormone action and focused attention on peptide hormone receptors as functional proteins under genetic control. Examples of heritable disorders resulting from impaired membrane receptors for peptide hormones include: ACTH unresponsiveness [5, 6], Alstrom syndrome [7, 8], diabetes insipidus [9, 10], Laron dwarfism [11], familial hypercholesterolemia [12], pseudohypoparathyroidism [13], familial insulin-resistant diabetes [14–16], and leprechaunism [17, 18].

Thirty-one patients with leprechaunism have been reported since Donahue's original description in 1948 [19–44]. A common phenotype is characterized by: severe intrauterine growth restriction; small elfin-like face with protruberant ears; distended abdomen; relatively large hands, feet, and genitalia; and abnormal skin with hypertrichosis, acanthosis nigricans, and decreased subcutaneous fat. At autopsy, patients have had cystic changes in membranes of gonads and hyperplasia of pancreatic islet cells. Biochemical evidence for insulin resistance is found including: markedly elevated plasma insulin, impaired glucose homeostasis, absence of anti-insulin receptor antibodies, and altered insulin-receptor binding and/or response to insulin by patients' cells.

Several other human disorders have insulin-receptor or post-receptor resistance to insulin, but their phenotypes are quite different from "leprechaunism" and they have considerable variation in phenotypic expression. They include: insulin-resistant diabetes types A, C, and "Rudiger," with associated acanthosis nigricans, virilization, and cystic ovaries [14–16]; lipoatrophic diabetes [45]; myotonic dystrophy [46]; and some patients with ataxia telangiectasia [47]. In patients with leprechaunism, there are only five reports that include formal evaluation of insulin binding to monocytes or cultured skin fibroblasts [21, 22, 24, 44, 48]. Despite a postulated genetic causation for abnormal insulin-receptor function, little evaluation is reported of first-degree relatives to clarify what, if any, genetic mechanisms apply.

Here we define "insulin resistance" in vivo and insulin-binding kinetics and response by monolayers of skin fibroblasts cultured from the skin of a proband with leprechaunism, her parents, and six control cell lines. Replicate plates of fibroblast monolayers cultured in scintillation vials are used with identical experimental conditions for insulin binding and responses. Using affinity crosslinking of [^{125}I]insulin to fibroblast membranes, we identify a structural variation in the insulin receptor and postulate a genetic mechanism for aberrant insulin-receptor response.

MATERIALS AND METHODS

Case Report

The proband in this family is a black female who is 8 years old at present. She has been studied and reported as "Arkansas 1" [22, 48–54]. Her survival is unusual in that most other patients with this syndrome have died by 10 months of age. The mechanism of impaired insulin-receptor interaction and intracellular signaling is controversial in this patient in that one group of investigators found normal insulin binding to peripheral monocytes and fibroblasts and reported "post-receptor" impairments in insulin-stimulated responses [22]. These insulin-binding studies were performed at low temperature on trypsinized fibroblast suspensions. Three other laboratories found abnormal insulin binding to monolayers of cultured fibroblasts, B lymphocytes, and erythrocytes from this patient [48–53]. We reported our initial family studies in abstract form [49]. There is no evidence of parental consanguinity, and both parents are phenotypically normal without any stigmata of leprechaunsim.

Tissue Culture

Human fibroblast cell lines were developed from explants of skin biopsies from the proband (Ark 1), her two unaffected parents, and six age- and sex-matched controls. Control cell lines were matched with respect to doublings for each experiment. Initial cultures were in Dulbecco and Vogt's medium with 15% fetal bovine serum and added nonessential amino acids using Corning 75-cm^2 polystyrene tissue culture flasks. Stock cultures were harvested at early mean population doublings and frozen for storage at −70°C. Cultures were routinely monitored for mycoplasma by the standard, Hoechst-stain, fluorescence method. We harvested cells that had undergone between nine and 20 mean population doublings with 0.25% trypsin, diluted with culture medium to 2.5 × 10^4 cells per ml, and seeded replicate, sterile, scintillation vials with 100,000 cells. From 30 to 50 vials were prepared per cell line (a control and study line were always compared in parallel) for each experiment. Vials were covered with sterile foil, placed in 5% CO_2-95% air, and incubated at 37°C. Experiments were performed at confluence, which was 5–8 days after seeding depending on a given cell line's division rate. Replicate vials for each cell line when assayed for protein or DNA content had a range of variation of 3%–6% after performing the experiments. Culture media was removed, and monolayers were washed with phosphate-buffered saline containing 116 mM NaCl, 2.6 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.13 mM KCl, 0.9 mM NaH_2PO_4, 0.8 mM $MgSO_4$, and 5.5 mM D-glucose, pH 7.4.

Insulin-binding and Transport Studies

Materials. 2-*D*eoxy [1-^{14}C]-D-glucose (57 mCi/mmol), [^{125}I]Na (17 Ci/mg), [^{3}H]inulin (0.61–2.5 Ci/mmol), [^{3}H] and [^{14}C]urea, 2 amino-3-methyl [1-^{14}C]-isobutyric acid (19 mCi/mmol), Liquifluor, and Aquasol were from New England Nuclear, Boston, Mass., 2-Methylaminoisobutyric acid (MeAIB) 2-deoxy-D-glucose and HEPES were obtained

from Sigma, St. Louis, Mo. Fraction V bovine serum albumin (fatty acid poor, lot 27, containing 0.1 mol fatty acid per mol albumin) and ovalbumin (lot 6) were from Miles, Kankakee, Ill., and the Phadebas insulin immunoassay test was from Pharmacia, Uppsala, Sweden. Sodium bovine insulin (lot IDG 04-94-195, 25 U/mg) was a gift from Dr. R. L. Jackson of Eli Lilly, Indianapolis, Ind. Multiplication stimulating activity (MSA) was a gift from Dr. M. M. Rechler, NIADDK, Bethesda, Md.

The Transport Assay

Transport-assay buffers were the same as used previously [55] with the exception that HEPES replaced $NaHCO_3$. The assay buffer (290 mOsm) contained the following: 25 mM HEPES, 2.5 mM KCl, 0.81 mM $MgSO_4$, 0.94 mM NaH_2PO_4, 2.38 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 5.55 mM D-glucose, NaOH to adjust pH, and NaCl to bring final Na^+ concentration to 143.1 mM. For all assays of transport and insulin binding, the pH was adjusted to 7.8 and the temperature was 24°C.

Assays for MeAIB transport and intracellular fluid volume were quantitated as described using [^{14}C]urea (0.2 μc) as a marker of total fluid volume and [^{3}H]inulin (1.3 μc) as a marker for extracellular volume [56]. Distribution ratios were corrected for retained counts in the extracellar fluid and represented the substrate concentration in the intracellular space per microliter of intracellular fluid volume, divided by the media substrate concentration per microliter of extracellular fluid volume.

The model sugar 2-deoxy-[U-^{14}C]-D-glucose was used as a substrate for sugar uptake and phosphorylation. Since 2-deoxy-D-glucose is phosphorylated after or during entry into the cell, cellular fractions were analyzed for free and phosphorylated 2-deoxy-D-glucose by radiochromatography using techniques described [55].

The Student's one-tailed *t*-test statistic was computed to compare values obtained under experimental conditions with control values. The probabililty (*P*) that the computed values of *t* would be exceeded by chance was used to determine significance.

Insulin Iodination

Bovine, porcine, or chicken insulins were iodinated stoichiometrically to specific activities of 259–270 μCi/μg with chloramine T and metabisulfite [57]. Iodination mixtures were purified using Sephadex G-10 swollen in 100 mM HEPES receptor-assay buffer containing 20 mg/ml bovine serum albumin. The Sephadex slurry was poured into a 5-ml plastic syringe containing a 1.3-μ millipore filter and packed at 100 g for 5 min. The iodination reaction mixture was then applied to the packed Sephadex and centrifuged at 1,000 *g* for 5 min. [^{125}I]Na, unreacted chloramine-T, and metabisulfite were retained in the Sephadex, while [^{125}I]monoiodoinsulins and unlabeled insulin were eluted. [^{125}I]monoiodoinsulins were stored at −20°C and repurified by the same gel filtration-centrifugation technique before each experiment. Trichloracetic acid precipitability of iodinated insulins prepared in this manner was 96%–98%. By reverse phase HPLC, the tyrosines at either A-14 or A-19 were found iodinated on porcine insulin.

Insulin Binding

Insulin-binding assays used [^{125}I]-labeled chicken insulin at specific activities of 210 μc/mg and concentrations of 0.8 ng/ml because of its high affinity [57]. Cells in monolayer were incubated for varying times or with varying insulin concentrations. To terminate an assay, cells were rinsed three times with 1 ml cold 0.9 M NaCl and the cell layer counted in situ. Nonspecfic background counts were defined as counts remaining in the presence of 10,000 ng/ml of cold insulin. Analysis of insulin receptors used a Scatchard graphic display and iterative computer program for least-squares analysis assuming a two-receptor model as defined [57, 58]. Specific insulin bound was normalized to protein content for each vial, and results were expressed as specific picograms of insulin bound per 100 μg of cell protein.

In this method, the bound insulin/free insulin ratio (bound/unbound) was computed as a function of the concentration of bound insulin (bound) at each experimental point and compared to observed bound-to-free ratios for each bound value by a least-squares regression analysis to determine the goodness of fit of the equilibrium kinetic parameters. These parameters were then reestimated, and the calculation was repeated to obtain the "best-fit" estimates for a two-receptor model [58].

Affinity Labeling, Solubilization, and Autoradiography of Insulin Receptor

Preparation of plasma membranes required monolayers of fibroblasts from about six 150-cm^2 Falcon flasks at confluence for each lane (about 6×10^6 cells). Membranes were prepared by differential centrifugation in EDTA-containing Tris buffers with added Aprotinin and phenylmethylsulfonylfluoride to prevent proteolysis. Final membranes were frozen at −70°C to accumulate sufficient quantities of plasma membrane for crosslinking. Frozen pellets were resuspended at 24°C in 30 ng/ml [^{125}I]insulin at 0.75–1 mg/ml membrane protein for 1 hr, diluted with 10 vol of ice-cold Krebs-Ringer phosphate, and centrifuged at 32,000 *g* for 30 min at 4°C. Crosslinking of [^{125}I]insulin to membrane proteins was accomplished at pH 7.4 on 1.5–2 mg/ml of membrane protein using freshly prepared disuccinimidylsuberate (0.1 M in dimethylsulfoxide) at 1:100 dilution for 30 min. Membrane pellets were centrifuged, washed, and radioactively crosslinked proteins extracted with 1% Triton X-100. In parallel experiments, "nonspecific" [^{125}I]insulin crosslinking and membrane extraction were carried out in insulin concentrations of 10,000 ng/ml. About 50% of radioactivity was extracted into the supernatant. The equivalent of 300 μg of extracted plasma membrane protein was used for each lane. The extracts were analyzed using a discontinuous buffer system with 3% stacking gels and 5%–15% gradients of sodium dodecylsulfate polyacrylamide gels (acrylamide to bis-acrylamide, 100:1). After electrophoresis, gels were stained, destained, dried, and autoradiographed on Kodak X-Omat film for 168 hrs using an intensified screen.

RESULTS

Insulin resistance was defined in the proband and evaluated in both parents using standard oral glucose tolerance tests measuring plasma glucose and insulin at timed intervals (table 1). Following oral glucose administration (1.75 gm/kg), the proband had fasting hypoglycemia, but hyperglycemia at 2 hrs. She also demonstrated marked and sustained hyperinsulinemia as high as 4,420 μU/ml. The mother's glucose tolerance test was normal, but the father at age 32 had mild fasting hyperinsulinism and mild carbohydrate intolerance. He has no physical signs or symptoms of leprechaunism. Neither the proband nor her father have

TABLE 1

GLUCOSE AND INSULIN RESPONSES TO ORAL GLUCOSE IN ARK-1 FAMILY WITH LEPRECHAUNISM

	CONTROLS		PROBAND		MOTHER		FATHER	
TIME (MIN)	Glucose (mg%)	Insulin (μU/ml)	Glucose (mg%)	Insulin (μU/ml)	Glucose (mg%)	Insulin (μU/ml)	Glucose (mg%)	Insulin (μU/ml)
0	81	4	38	130	101	7	77	38
30	122	82	170	1700	150	90	165	197
60	105	122	320	2820	114	87	168	199
90	87	71	310	4420	70	58	130	278
120	90	32	210	4120	70	51	127	240
180	73	26	58	3900	97	62	98	149

circulating antibodies to insulin or the insulin receptor. In vitro studies of [^{125}I]insulin binding at low concentrations to fresh erythrocytes at various temperatures and pH demonstrated qualitative changes that correlated with these abnormal glucose tolerance curves. Binding of 0.2 ng/ml [^{125}I]insulin to the mother's red blood cells was about 70% of control cell binding, while erythrocytes from the father and proband bound approximately 10% of control cells [51]. Since these cells had been exposed in vivo to high concentrations of plasma insulin and since "down regulation" is a well-described phenomenon by which insulin reduces its own surface receptors, we could not be sure whether insulin binding to these freshly obtained peripheral cells was reduced because of "down regulation" or because of a primary heritable defect in the insulin receptor [59]. Since monolayer cultures of dermal skin fibroblasts are exposed in culture to small (less than 0.1 nM insulin), we could obviate this in vivo problem of "down regulation" from high circulating concentrations of insulin greater than 10 nM by evaluating heritability of insulin binding and cellular response to insulin in dermal cells cultured from this family.

Insulin Binding to Monolayers of Fibroblasts

The mutant and control cell lines were studied for specific insulin binding. The time course is shown for insulin binding and displacement by control's and proband's cell lines (fig. 1). Cell lines obtained from the patient with leprechaunism bound approximately 10% of control. Equilibrium for insulin-receptor interaction was reached by 2 hrs for both cell lines under these conditions at 24°C. When excessive amounts (10,000 ng/ml) of unlabeled insulin were added at this time point, specific insulin was displaced completely from the cell layer. The rate to equilibrium for association and the rate to equilibrium during the dissociation phases were approximately equal whether insulin receptors were partially or fully occupied. Thus, negative cooperative events are unlikely in producing complex

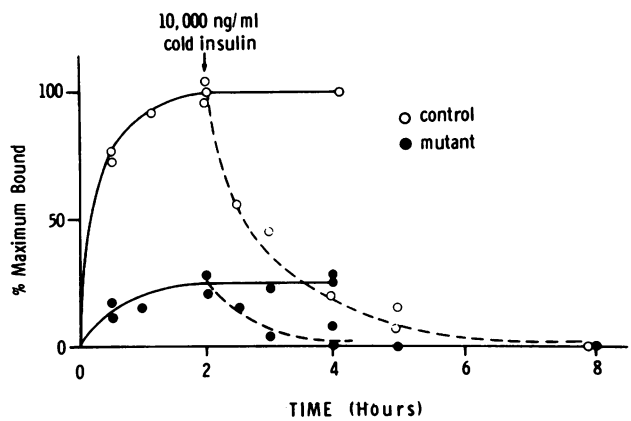

FIG. 1.—Time course of specific [^{125}I]insulin binding and displacement by monolayers of control and mutant dermal fibroblasts. Specific binding of 0.8 ng/ml of [^{125}I]chicken insulin (230 μCi/μg) was determined in replicate scintillation vials at 24°C in insulin-free, HEPES buffer, pH 7.8. Nonspecific binding was subtracted. Data are the average of duplicate observations at each time point in two overlapping experiments.

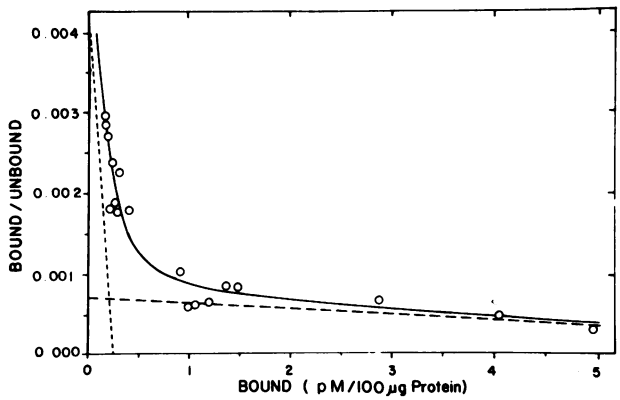

FIG. 2.—Scatchard analysis of insulin binding by control fibroblasts. *Solid curvilinear line* represents a best-fit curve for the observed control data (*open circles*), which are the mean of six observations in three overlapping experiments. *Broken lines* are derived linear functions from a weighted iterative program assuming a two-receptor model.

insulin binding seen over a wide concentration range of ligand (*vide infra*) [58]. In data not shown here, cellular protein was proportional to specific binding. Thus, measurement of equilibrium-binding parameters using Michaelis-Menten kinetics would pertain over saturating concentrations of ligand.

Insulin binding to control fibroblasts when analyzed by the method of Scatchard produced a curvilinear function. (fig. 2). Because negative cooperative events were an unlikely mechanism, we analyzed these results using a two-receptor state model. A high-affinity, low-capacity, insulin-binding state, represented by the dashed line to the left in figure 2, had an apparent K_A of 1.6×10^{10}/molar with 1,100 sites/cell (type I receptor). The proposed low-affinity, high-capacity insulin-receptor state had an apparent K_A of 6.8×10^7/molar with approximately 30,000 sites/cell (type II receptor) as represented by the dashed line below in figure 2. When cells from the mutant cell line were analyzed by this method, a single linear binding function was present (fig. 3). This linear function had a

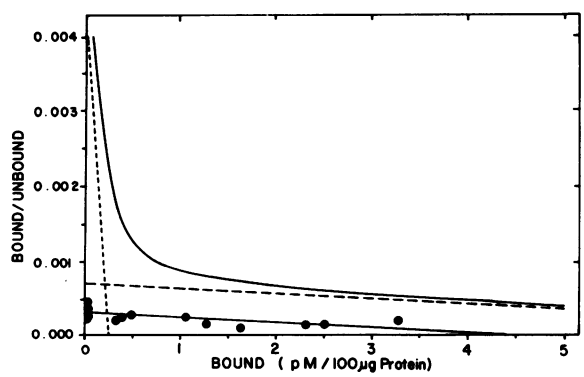

FIG. 3.—Scatchard analysis of insulin binding by mutant fibroblasts (proband). *Closed circles* are the mean of quadruplicate values observed in the proband's cells, and the *solid line* is derived by a best-fit least-squares analysis. *Broken lines* are from control studies (cf., fig. 2).

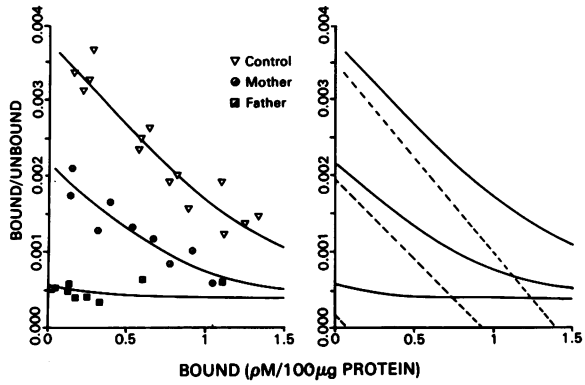

FIG. 4.—Scatchard analysis of high-affinity insulin binding to parental fibroblasts. Specific insulin binding was analyzed over insulin concentrations between 0.08 and 1.0 ng/ml. Data were treated as described in figures 2 and 3. *Dashed lines in graph to the right* are the high-affinity binding sites derived by iterative computer analysis assuming a two-receptor model for each cell line.

slope similar to that of the low-affinity, high-capacity, type II receptor in control cells with no evidence for high-affinity, insulin binding. Since this interpretation of the insulin-binding data suggested that a high-affinity, insulin-binding site was absent or impaired as a result of genetic variation, we evaluated binding at low insulin concentrations in cells cultured from the parents and control. Over the concentration range 0.01–0.1 ng/ml of [^{125}I]insulin, 92% was preferentially bound to receptors in their high-affinity binding state [58, 59]. At these concentrations, the proband's cells bound less than 10% of control (figs. 1 and 3) and the mother's and father's cells had 50% and 15% of control, respectively. This high-affinity binding was then analyzed over 0.08–1.0 ng/ml concentration range (fig. 4). As in the previous Scatchard plots, figures 2 and 3, the solid line represents the computed best-fit function for the observed binding data. In the plot to the right, indicated by dashed lines, are computer-derived functions for the postulated

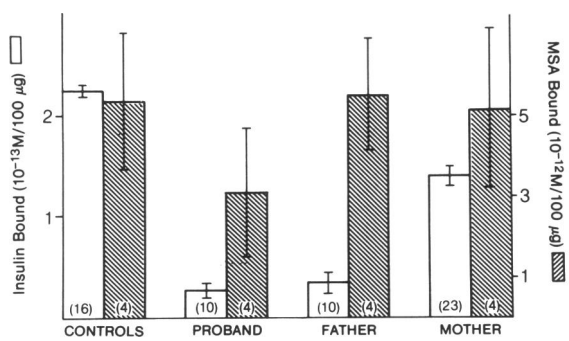

FIG. 5.—Insulin and multiplication stimulating factor (MSA) binding to monolayers of fibroblasts cultured from controls and family members. Specific binding of [^{125}I]chicken insulin (0.8 ng/ml) and [^{125}I]MSA (0.5 ng/ml) was compared among cell lines in four overlapping experiments. The mean specific binding is given by *bar heights bracketed by 2 SD with the no. observations in parentheses.*

high-affinity receptor (type I). Note that the slopes of all three lines are approximately parallel, indicating that the equililbrium association constants are similar (about 1.2×10^{10}/molar). However, there are marked differences in the abscissal intercepts that approximate the number of type I receptors. The mother has 60% and the father has only 2% of control type I receptors per cell, respectively.

The specificity of this defect for insulin binding was tested using radiolabeled MSA. Binding experiments were conducted under identical conditions to insulin except that [^{125}I]MSA II, fraction 25 (from Dr. M. M. Rechler) was used as ligand at low concentrations of 0.5 ng/ml (fig. 5). We found similar binding of MSA to the parental cells and to those of the proband in simultaneous experiments when binding of insulin was impaired. As can be seen from the brackets in figure 5, there was considerable variation for MSA binding among the quadruplicate values. However, no significant difference in MSA binding was found among cells from controls, proband, or the parents.

Insulin Response by Cultured Fibroblasts

It appeared that high-affinity insulin binding (type I) was impaired in cells from the proband. Both parents' cells had partial but quantitatively different impaired high-affinity insulin binding (figs. 4 and 5). Conversely, low-affinity insulin binding (type II) was intact as evidenced both by kinetic analysis of [^{125}I]insulin binding (figs. 3 and 4) and by normal [^{125}I]MSA binding (fig. 5). This hypothesis was further tested by evaluating insulin responses in these cultured cells.

Insulin stimulation of 2-deoxy-D-glucose uptake and phosphorylation occurred at low insulin concentrations and correlated with occupancy of the putative high-affinity or type I insulin receptor (table 2). In control cells, 1 ng/ml of insulin resulted in 12% stimulation, and maximum stimulation occurred between 10 ng/ml (1.67 nM) and 100 ng/ml (46.7 nM). The approximate K_D for high-affinity insulin binding was 1.7 nM. By contrast, insulin failed to stimulate any 2-deoxy-D-glucose uptake by the mutant cells until 100 ng/ml was present, and at 1,000

TABLE 2

INSULIN STIMULATION OF 2-DEOXY-D-GLUCOSE UPTAKE AND PHOSPHORYLATION BY MONOLAYERS OF FIBROBLAST FROM CONTROL AND THE PROBAND

INSULIN CONCENTRATION (ng/ml)	PERCENT STIMULATION		% MAXIMUM STIMULATION	
	Controls	Proband	Controls	Proband
0	0 (4)	0 (4)	0	0
1	12.3 ± 2.1 (4)	0 (4)	22.0	0
10	40.3 ± 3.7 (4)	0 (4)	72.0	0
100	56.0 ± 2.9 (4)	5.6 ± 0.5 (4)	100.0	...
1,000	47.9 ± 10.1 (4)	13.1 ± 1.0 (3)	100.0	...

NOTE: 3 μM 2-deoxyglucose-U-^{14}C was incubated with monolayers of cultured fibroblasts at 24°C for 3 hrs under identical conditions used for insulin-binding studies. Update was normalized by intracellular fluid volume and expressed as percent control and percent maximum stimulation. Data are the mean of two experiments ± 1 SD with the no. observations in parentheses.

ng/ml, only 13% stimulation occurred. No further increase in hexose uptake by either cell line was seen at 10,000 ng/ml insulin. The father's cell lines had similar responsivity at 1 and 10 ng/ml as control cells. When the cell-contained counts were studied by ion-exchange chromatography using BioRad gels, 78% of the [^{14}C] was present as 2-deoxy-D-glucose-6-phosphate. This degree of phosphorylation occurred at the earliest time points studied following insulin exposure (10 min). Insulin stimulation of uptake and phosphorylation continued without reaching equilibrium over 5 hrs.

MeAIB is an amino acid analog transported by an insulin-responsive, membrane-transporter system [58]. Transport of this neutral compound was accomplished under experimental conditions by methods identical with those used for insulin binding and 2-deoxyglucose uptake and phosphorylation except that D-glucose was present in the medium. Intracellular fluid spaces for these fibroblast monolayers were 90 ± 1.2%, and extracellular fluid spaces were 10 ± 0.1% of total water spaces. Maximum stimulation by insulin occurred between 100 ng/ml and 250 ng/ml with half maximum at between 25 and 50 ng/ml (3.5×10^{-9}M) (table 3). This dose-response relationship correlated with the predicted occupancy of the putative low-affinity (type II) insulin receptor and were clearly higher concentrations than those required to stimulate hexose transport and phosphorylation (cf., table 2). As shown in table 3, parallel observations were made of insulin-responsive, MeAIB transport by cells from controls and the proband. Absolute values for transport in distribution ratios were: by control cells without insulin, 3.10 ± 0.08, and with 1,000 ng/ml insulin, 4.17 ± 0.10; by the proband's cells without insulin, 1.95 ± 0.02, and at maximum insulin stimulation, 2.67 ± 0.12. It can be seen from table 3 that half-maximal stimulation of the proband's cells also occurred at insulin concentrations of approximately 50 ng/ml and that maximum stimulation occurred between 100 and 250 ng/ml. Thus, for this cellular response, both control and mutant cell lines had similar insulin concentration requirements.

TABLE 3

INSULIN STIMULATION OF [^{14}C]METHYLAMINOISOBUTYRIC ACID (ME-AIB) TRANSPORT BY MONOLAYERS OF FIBROBLASTS FROM CONTROL AND THE PROBAND

Insulin (ng/ml)	Control (% maximum stimulation)	Proband (% maximum stimulation)
0	0	0
0.3	0	0
2.5	18	13
10	20	10
25	40	33
50	64	68
100	85	90
250	100	100
1,000	100	100

NOTE: Cells were exposed to porcine insulin at the indicated concentrations for 2 hrs before washing and adding 3 μM[^{14}C]MeAIB for a subsequent 30-min transport study. Data are mean values of percent maximal stimulation from four separate experiments and at least quadruplicate observations.

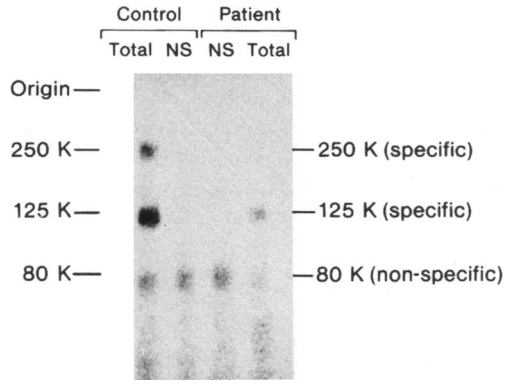

FIG. 6.—Autoradiograms of fibroblast insulin-receptor subunits after crosslinking [^{125}I]insulin, solubilizing, and electrophoresing in sodium dodecylsulfate-polyacrylamide gels. [^{125}I]insulin was chemically crosslinked to plasma membranes in low (25 ng/ml) and high (10,000 ng/ml) insulin concentrations. Membrane proteins were extracted in Triton X-100 and reduced in dithiothreitol (10 mM) and SDS, then electrophoresed in a 5%–15% polyacrylamide-sodium dodecylsulfate gradient gel. Gels were dried, fixed, and autoradiographed. "*Total*" represents low and "*nonspecific*" high unlabeled insulin concentrations used in the initial crosslinking procedure. The 80,000-dalton band was identified under both conditions and was considered nonspecific-binding.

This observation suggests that cellular signaling through the low-affinity, type II, insulin receptor is not impaired by the mutation in this patient.

Chemical Characterization of the Mutant Insulin Receptor

Chemical characterization of the insulin-receptor structure was attempted to better understand the fundamental mechanisms involved in both normal and abnormal receptor proteins. We chemically crosslinked [^{125}I]insulin to surface membranes. Quite high concentrations (10–25 ng/ml) of [^{125}I]insulin were required in crosslinking experiments to accomplish enough membrane-receptor labeling for visualization by autoradiography of the femtomolar concentrations expected in these cell membranes. Calculations from our two-receptor model suggested that 25 ng/ml of insulin would occupy about 1,100 high-affinity (type I) and 22,500 low-affinity (type II) insulin receptors per control fibroblast at 24°C. We were able to visualize specific insulin binding on SDS-PAGE and could characterize specific subunits of the insulin receptor that bound insulin (fig. 6). In control cells, most specific insulin binding was to a subunit of 125,000 daltons (in 10 mM dithiothreitol) with some additional binding to a larger, 250,000 dalton band. This latter band generated 125,000 dalton bands by two-dimensional PAGE (data not shown) and therefore represented partially crosslinked dimers of the 125,000 dalton protein. In membranes from the patients' cells, less total binding was seen (fig. 6). However, when the same specific counts were applied, only a single band was seen at 125,000 daltons. No 250,000 dalton dimer was covalently crosslinked. In subsequent experiments using prolonged film exposure (7 days) and integrating peaks from densitometric profiles of these autoradiograms, 250,000 to 125,000 daltons ratios were calculated. They were 0.50, 0.98, 0.99, and 0.99 for receptors from the proband, father, mother, and control, respectively.

DISCUSSION

It is probable from these data that an impaired high-affinity (type I) insulin receptor was inherited and caused the phenotype, leprechaunism, in this family. This genetic postulate included a kinetic interpretation of the nonlinearity of insulin binding to cultured skin fibroblasts using a two-receptor model. Such a two-receptor model assumes either two separate receptors or two conformational states of the same receptor with fixed affinity and capacity [57–61]. An alternate kinetic proposal to explain nonlinear Scatchard plots for insulin binding is that insulin-receptor molecules undergo decreased affinity as their neighboring receptors are occupied [62]. Although this mechanism is not excluded by these studies, the low-affinity state or type II receptor is intact, while the type I, high-affinity state receptor is impaired in these mutant cells. Additionally, when two responses with widely different dose requirements were compared, the low-affinity functional response was intact while high-affinity response was not. Insulin maximally stimulated 2-deoxyglucose uptake and phosphorylation at 10 ng/ml, a functional response conforming to occupancy of the insulin receptor in a high-affinity state. Cells from the proband had no type I insulin binding and marked impairment of insulin-stimulated, 2-deoxyglucose uptake. By contrast, the low-affinity, type II receptor was present in cells from the proband, and her cells responded to insulin, by increasing MeAIB transport. Using a two-receptor model and high-affinity binding as a genetic discriminant, both phenotypically normal parents of the proband were found to have impaired high-affinity, type I insulin binding. The more pronounced decrease in insulin binding by the father's cells as compared with those from the mother suggested two different recessive mutations. The degree of impaired binding expressed by cells from the father was almost as low as that of the proband's cells. He did, in fact, have abnormal glucose tolerance with hyperinsulinism, but did not express leprechaunism. From the kinetic analysis of insulin binding, from the normal binding of MSA, and from the diverse functional responses to insulin, we formulated the following genetic hypothesis: Each parent is heterozygous for a different mutation affecting the high-affinity, type I state of the insulin receptor, and the proband is a compound heterozygote having inherited two noncomplementing mutant genes for insulin binding.

There are probably many normal variations in the structure and function of human insulin receptors, and closer scrutiny of the paternal cell line provides several implications for milder disorders of insulin resistance. The father's cells had only 2% of high-affinity insulin binding, but normal MSA and low-affinity insulin binding. We employed methods of affinity crosslinking and autoradiography to further explore the structure and subunit interactions of his cells' insulin receptor [60, 61, 63, 64]. In data not shown here, his insulin receptor demonstrated normal 250,000 dalton dimer to 125,000 dalton monomer ratios [65]. He did not have growth restriction or other phenotypic changes of leprechaunism. But he did evidence in vivo insulin resistance and decreased insulin binding to cultured cells and is therefore most likely heterozygous for a more severely altered insulin receptor than is his wife. Mild, insulin-independent diabetes mellitus and some less severe forms of insulin resistance are caused by relatively common heritable

defects in the insulin receptor [48, 66]. In one family study, mild insulin-receptor impairment conformed to a dominant pattern of inheritance [14]. This pedigree probably reflects heterozygotes for mutations in the insulin receptor, while the relatively rare, but more severe leprechaunism syndrome probably reflects the inheritance of two recessive mutations of the insulin receptor. Although leprechaunism is a relatively rare syndrome, other more common genetic variations in the insulin receptor may result in a variety of adult or childhood-onset, insulin-resistant disorders.

The importance of high-affinity insulin binding on embryonic growth has been emphasized by the phenotype of intrauterine growth restriction in the absent pancreatic beta cell syndrome [68]. Additional experimental evidence in fat cells suggests that when insulin-receptor sites are occupied, MSA binding is enhanced as is the binding of other growth factors [63, 64, 67]. This may explain the growth-stimulating effects of low concentrations of insulin in rat liver cells [69, 70]. If insulin-receptor signaling or insulin itself were absent during embryogenesis, growth restriction might occur as evidenced by the leprechaun syndrome and the syndrome of agenesis of pancreatic cells [68]. The father, who has barely detectable high-affinity insulin-binding sites, does not have somatic growth restriction. Perhaps he has enough type I receptors to preserve insulin's growth-receptor potentiation and thereby was protected from the severe, dysmorphic features expressed by his daughter who has no functioning type I receptors.

These genetic studies suggest that the complex binding of insulin to its receptor is mediated by intrinsic subunit interactions. The proband's cells had no high-affinity insulin binding, no high-affinity cellular signaling, and decreased crosslinking of α subunits. We theorize that the mutations affect α subunit interaction producing spatial differences between these 125,000 dalton subunits greater than 11Å, the length of the crosslinking agent. This hypothesis predicts the need for subunit interaction by the insulin receptor upon occupancy by insulin complex to signal intracellular response. It also offers an explanation as to why the insulin receptor did not express genetically impaired binding when another laboratory examined fibroblasts from this patient [22]. In these studies, the cells had been trypsinized and suspended before binding was analyzed. Furthermore, insulin binding was measured at 4°C after 90 min. Under these experimental conditions, we measured only 10% of total binding at equilibrium and could not differentiate normal from abnormal. We suggest that the physiological relationships of insulin-subunit interactions under normal membranal conditions were disrupted by trypsin, cold, and nonequilibrium kinetics.

REFERENCES

1. VERHOEVEN GFM, WILSON JD: The syndromes of primary hormone resistance. *Metabolism* 28:253–289, 1979
2. BAR RS, HARRISON LC, MUGGEO M, GORDEN P, KAHN CR, ROTH J: Regulation of insulin receptors in normal and abnormal physiology in humans. *Adv Intern Med* 24:23–52, 1979
3. KAHN CR: Membrane receptors for hormones and neurotransmitters. *J Cell Biol* 70:(2 part 1):261–286, 1976

4. CZECH MP: Cellular dynamics of insulin action. *Fed Proc* 41:2717–2718, 1982
5. MIGEON CJ, KENNY FM, KOWARSKI A, ET AL.: The syndrome of congenital adrenocortical unresponsiveness to ACTH. Report of six cases. *Pediatr Res* 2:501–513, 1968
6. FRANKS RC, NANCE WE: Hereditary adrenocortical unresponsiveness to ACTH. *Pediatrics* 45:43–48, 1970
7. GOLDSTEIN JL, FIALKOW PJ: The Alstrom syndrome. Report of three cases with further delineation of the clinical, pathophysiological and genetic aspects of the disorder. *Medicine* 52:53–71, 1973
8. EDWARDS JA, SETHI PK, SCOMA AJ, BANNERMAN RM, FROHMAN LA: A new famililal syndrome characterized by pigmentary retinopathy, hypogonadism, mental retardation, nerve deafness and glucose intolerance. *Am J Med* 60:23–32, 1976
9. FORSSMAN H: On hereditary diabetes insipidus with special regard to a sex-linked form. *Acta Med Scand [Suppl]* 159:1–196, 1945
10. BODE HH, CRAWFORD JD: Nephrogenic diabetes insipidus in North America—the Hopewell Hypotheses. *N Engl J Med* 280:750–754, 1969
11. LARON Z: The syndrome of familial dwarfism and high plasma immunoreactivated human growth hormone. *Birth Defects: Orig Art Ser* 10(4):231–238, 1974
12. BROWN MS, GOLDSTEIN JF: Familial hypercholesterolemia: a genetic defect in the low-density lipoprotein receptor. *N Engl J Med* 294:1386–1390, 1976
13. FITCH N: Albright's hereditary osteodystrophy: a review. *Am J Med Genet* 11:11–29, 1982
14. SCARLETT JA, KOLTERMAN OG, MOORE P, ET AL.: Insulin resistance and diabetes due to a genetic defect in insulin receptors. *J Clin Endocrinol Metab* 55:123–131, 1982
15. RUDIGER HW, DREYER M, KUHNAU J, BARTELHEIMER H: Familial insulin-resistant diabetes secondary to an affinity defect of the insulin receptor. *Hum Genet* 64:407–411, 1983
16. KAHN CR, FLIER JS, BAR RS, ET AL.: The syndromes of insulin resistance and acanthosis nigricans. Insulin-receptor disorders in man. *N Engl J Med* 294:739–745, 1976
17. DONOHUE WL: Dysendocrinism. *J Pediatr* 32:739–748, 1948
18. DONOHUE WL, UCHIDA I: Leprechaunism: a euphemism for a rare familial disorder. *J Pediatr* 45:505–519, 1954
19. DAVID R, GOODMAN RM: Leprechaunism in 4 siblings: new pathologic and electron microscopic observations. *Hum Hered* 27:172A, 1977
20. GILLEROT Y, BUEZ A, HUSTIN J: Leprechaunism (Donohue syndrome). A case with growth hormone deficiency. *Acta Paediatr Belg* 32:279–282, 1979
21. SCHILLING EE, RECHLER MM, GRUNFELD C, ROSENBERG AM: Primary defect of insulin receptors in skin fibroblasts cultured from an infant with leprechaunism and insulin resistance. *Proc Natl Acad Sci USA* 76:5877–5881, 1979
22. KOBAYASHI M, OLEFSKY JM, ELDERS J, ET AL.: Insulin resistance due to a defect distal to the insulin receptor: demonstration in a patient with leprechaunism. *Proc Natl Acad Sci USA* 75:3469–3473, 1978
23. ROSENBERG AM, HAWORTH JC, DEGROOT GW, TREVENEN CL, RECHLER MM: A case of leprechaunism with severe hyperinsulinemia. *Am J Dis Child* 134:170–175, 1980
24. D'ERCOLE AJ, UNDERWOOD LE, GROELKE J, PLET A: Leprechaunism: studies of the relationship among hyperinsulinism, insulin resistance and growth retardation. *J Clin Endocrinol Metab* 48:495–502, 1979
25. EVANS PR: Leprechaunism. *Arch Dis Child* 30:479–483, 1955
26. PATTERSON JH, WATKINS WL: Leprechaunism in a male infant. *J Pediatr* 60:730–739, 1962
27. SALMON MA, WEBB JN: Dystrophic changes associated with leprechaunism in a male infant. *Arch Dis Child* 38:530–535, 1963
28. DEKABAN A: Metabolic and chromosomal studies in leprechaunism. *Arch Dis Child* 40:632–636, 1965

29. KALLO A, LAKATOS I, SZIJARTO L: Leprechaunism (Donohue's sydrome). *J Pediatr* 66:372–379, 1965
30. ROGERS DR: Leprechaunism (Donohue's syndrome). *Am J Clin Pathol* 45:614–619, 1966
31. FERGUSON-SMITH MA, HAMILTON W, FERGUSON IC, ET AL.: An abnormal metacentric chromosome in an infant with leprechaunism. *Ann Genet (Paris)* 11:195–200, 1968
32. SUMMITT RL, FAVARA BE: Leprechaunism (Donohue's syndrome): a case report. *J Pediatr* 74:601–610, 1969
33. DER KALOUSTIAN VM, KRONFOL NM, TAKLA R, HABASH A, KHAZIN A, NAJJAR SS: Leprechaunism: a report of two new cases. *Am J Dis Child* 122:442–445, 1971
34. PORADOVSKA W, JAWORSKA M, HANC I: Donohue's syndrome (leprechaunism) in a female infant. *Acta Chir Plast (Praha)* 14:4–12, 1972
35. GROSS-KIESELSTEIN E, BEN-GALIM E, AMIR N, ABRAHAMOV A: Leprechaunism (Donohue syndrome). *Am J Dis Child* 126:500–503, 1973
36. ORDWAY NK, STOUT LC: Intrauterine growth retardation, jaundice and hypoglycemia in a neonate. *J Pediatr* 83:867–874, 1973
37. IWASAKI H, ABE M, NAWATE G, KATO H, SHINOHAR T: Case of chromosome abnormality (46, XX, gq+) with congenital heart disease and leprechaunism. *Jpn J Hum Genet* 19:82–83, 1974
38. TSUJINO G, YOSHINAGA T: A case of leprechaunism and an analysis of some clinical manifestations of this syndrome. *Z Kinderheilkd* 118:347–360, 1975
39. HARTDEGEN RG, DOGLIOTTI M, RABINOWITZ L, BARTLETT RG: Leprechaunism: case report in a black African child. *Br J Dermatol* 93:587–591, 1975
40. RAMAAT LG, SHAFI KM, DAJANI FF: Donohue's syndrome (report of a case). *Indian Pediatr* 14:325–327, 1977
41. ADAMS JM, GORDON LP, DUTTON RV, ROSENBERG HS, RUDOLPH AJ: Leprechaunism (Donohue's syndrome) in a low birth weight infant. *South Med J* 70:998–1001, 1977
42. SACCHI F, MAGGIORE G, BIANCHI E, ET AL.: Staphylocidal defect of polymorphonuclear leukocytes in a patient with leprechaunism. *Eur J Pediatr* 137:89–90, 1981
43. MENON PS, SACHDEV H, VERMA IC: Diagnostic criteria in leprechaunism (Donohue syndrome). *Indian J Pediatr* 47:161–164, 1980
44. KAPLOWITZ PB, D'ERCOLE AJ: Fibroblasts from a patient with leprechaunism are resistant to insulin, epidermal growth factor, and somatomedin-C. *J Clin Endocrinol Metab* 55:741–748, 1982
45. DUNNIGAN MG, COCHRANE MA, KELLY A, SCOTT JW: Familial lipoatrophic diabetes with dominant transmission. A new syndrome. *Q J Med* 43:33–48, 1974
46. GORDEN P, GRIGGS RC, NISSLEY SP, ROTH J, ENGEL WK: Studies of plasma insulin in myotonic dystrophy. *J Clin Endocrinol Metab* 29:684–690, 1969
47. BAR RS, LEVIS WR, RECHLER MM, ET AL.: Extreme insulin resistance in ataxia telangiectasia. *N Engl J Med* 298:1164–1171, 1978
48. RECHLER MM: Leprechaunism and related syndromes with primary insulin resistance: heterogeneity of molecular defects, in *Membranes and Genetic Diseases,* New York, Alan R. Liss, 1982, pp 245–281
49. ELSAS LJ, STRUMLAUF E, GOODRUM G: Inherited defects in high affinity insulin binding by cultured skin fibroblasts. *Am J Hum Genet* 34:51A, 1982
50. TAYLOR SI, SAMUELS B, ROTH J, ET AL.: Decreased insulin binding in cultured lymphocytes from two patients with extreme insulin resistance. *J Clin Endocrinol Metab* 54:919–930, 1982
51. ELDERS MJ, HILL DE, SCHEDEWIE HK, HERZBERG V, BOUGHTER M, TAYLOR SI: Characterization of the erythrocyte insulin receptor in leprechaunism. *Clin Res* 30:897A, 1983
52. TAYLOR SI, ROTH J, BLIZZARD RM, ELDERS MJ: Qualitative abnormalities in insulin binding in a patient with extreme insulin resistance: decreased sensitivity to alterations in temperature and pH. *Proc Natl Acad Sci USA* 78:7157–7161, 1981

53. TAYLOR SI, HEDO JA, UNDERHILL LH, KASUGA M, ELDERS MJ, ROTH J: Extreme insulin resistance in association with abnormally high binding affinity of insulin receptors from a patient with leprechaunism: evidence for a defect intrinsic to the receptor. *J Clin Endocrinol Metab* 55:1108–1113, 1982
54. BIER DM, SCHEDEWIE H, LARNER J, ET AL.: Glucose kinetics in leprechaunism: accelerated fasting due to insulin resistance. *J Clin Endocrinol Metab* 51:988–994, 1980
55. ELSAS LJ, WHEELER FB, DANNER DJ, DEHAAN RL: Amino acid transport by aggregates of cultured chicken heart cells: effect of insulin. *J Biol Chem* 250:9381–9390, 1975
56. ELSAS LJ, MACDONELL RC, ROSENBERG LE: Influence of age on insulin stimulation of amino acid uptake in rat diaphragm. *J Biol Chem* 246:6452–6459, 1971
57. SANTORA AC, WHEELER FB, DEHAAN RL, ELSAS LJ: Relationship of insulin binding to amino acid transport by cultured 14-day embryonic chick heart cells. *Endocrinology* 104:1059–1068, 1979
58. WHEELER FB, SANTORA AC, ELSAS LJ: Evidence supporting a two-receptor model for insulin binding by cultured embryonic heart cells. *Endocrinology* 107:195–207, 1980
59. SERRAVEZZA JC, ENDO F, DEHAAN RL, ELSAS LJ: Insulin-induced receptor loss reduces responsiveness of chick heart cells to insulin. *Endocrinology* 113:497–508, 1983
60. CZECH MP, OPPENHEIMER CL, MASSAGUE J: Interrelationships among receptor structures for insulin and peptide growth factors. *Fed Proc* 42:2598–2601, 1983
61. CZECH MP, MASSAGUE J: Subunit structure and dynamics of the insulin receptor. *Fed Proc* 41:2719–2723, 1982
62. DEMEYTS P, BIANCO AR, ROTH J: Site-site interactions among insulin receptors: characterization of the negative cooperativity. *J Biol Chem* 251:1877–1888, 1976
63. MASSAGUE J, CZECH MP: The subunit structures of two distinct receptors for insulin-like growth factors I and II and their relationship to the insulin receptor. *J Biol Chem* 257:5038–5045, 1982
64. CZECH MP: Structural and functional aspects of the receptors for insulin and the insulin-like growth factors. *Physiologist* 26:190–193, 1983
65. ENDO F, ELSAS LJ: Human mutation of subunit interaction in the insulin receptor. *Clin Res* 32:519A, 1984
66. OLEFSKY JM: Insulin resistance and insulin action: an in vitro and in vivo perspective. *Diabetes* 30:148–162, 1981
67. KING GL, RECHLER MM, KAHN CR: Interactions between the receptors for insulin and the insulin-like growth factors on adipocytes. *J Biol Chem* 257:10001–10006, 1982
68. HILL DE: Effect of insulin on fetal growth. *Semin Perinatol* 2:319–328, 1978
69. MASSAGUE J, BLINDERMAN LA, CZECH MP: The high affinity insulin receptor mediates growth stimulation in rat hepatoma cells. *J Biol Chem* 257:13958–13963, 1982
70. KOONTZ JW, IWAHASHI M: Insulin as a potent, specific growth factor in a rat hepatoma cell line. *Science* 211:947–949, 1981