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Structural Analysis of Normal and Mutant Insulin Receptors in Fibroblasts Cultured from Families with Leprechaunism

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SUMMARY

Leprechaunism is an inherited disorder characterized by insulin resistance and intrauterine growth restriction. In this study we analyze insulin binding and subunit structure of the insulin receptor in dermal fibroblasts cultured from three unrelated families whose probands (Ark-1, Atl, and Minn) were affected by leprechaunism. Cells cultured from all three probands had markedly reduced insulin binding at equilibrium. Fibroblasts cultured from the parents of Ark-1 and Atl had partial and differing degrees of impairment in insulin binding. The structure of the *alpha* subunit of insulin receptors was analyzed by cross-linking ^{125}I-insulin to plasma membranes. A major band of 350 kilodaltons (kD) (corresponding to the heterotetrameric insulin receptor $alpha_2$ $beta_2$) was observed in control and leprechaun fibroblasts. The relative amount of radioactivity cross-linked to plasma membranes reflected the genetic variations seen in insulin binding to intact cells. In reducing gels, ^{125}I-insulin was cross-linked equally to a 250-kD (*alpha-alpha* dimer) and a 125-kD (*alpha* monomer) protein in cells from controls, the parents of Ark-1 and Atl, and probands Atl and Minn. By contrast, cells from the Ark-1 proband had diminished cross-linking of *alpha-alpha* dimers. The ratio of dimer to monomer in cells from controls was 0.93 ± 0.06, and that in cells from Ark-1 was 0.31 ± 0.19 ($P < .01$). *Beta*-subunit structure and function was analyzed by studying insulin-enhanced autophosphorylation. Although maximal stimulation of *beta*-subunit phosphorylation was reduced to 30% in proband Ark-1 fibroblasts, this reduction was quantitatively

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related to reduced insulin binding. These results indicate that mutations causing severe insulin resistance and defective insulin binding are transmitted with autosomal recessive patterns of inheritance and that heterogeneity exists for these mutations. The mutation in pedigree Ark-1 most likely produces conformational changes in *alpha*-subunit interaction.

INTRODUCTION

Families expressing inherited resistance to specific hormones have contributed to our understanding of hormone action and cellular signaling (Verhoeven and Wilson 1979; Elsas et al. 1985*a*). Leprechaunism is a syndrome of extreme insulin resistance associated with intrauterine growth restriction and defective insulin binding (Donohue 1948; Uchida and Donohue 1954; Kobayashi et al. 1978; D'Ercole et al. 1979; Schilling et al. 1979; Podskalny and Kahn 1982*a*, 1982*b*; Rechler 1982; Craig et al. 1984; Elsas et al. 1985*b*). Although many individual patients have been reported, only a few have been investigated at a cellular level to correlate biological responses with the kinetics of insulin binding (Kobayashi et al. 1978; D'Ercole et al. 1979; Schilling et al. 1979; Rechler 1982; Elsas et al. 1985*b*). The most thoroughly studied patient, Ark-1, had absent high-affinity but retained low-affinity insulin binding to her fibroblasts and erythrocytes (Rechler 1982; Elders et al. 1983; Craig et al. 1984; Elsas et al. 1985*b*). Fibroblasts cultured from her skin had decreased insulin stimulation of glucose utilization, 2-deoxy-D-glucose transport, and glycogen synthase activity (Kobayashi et al. 1978; Craig et al. 1984; Elsas et al. 1985*b*). In studies of her parents' cells, we found different degrees of reduction in the number of high-affinity insulin-binding sites, suggesting that the affected proband was a compound heterozygote for two mutant alleles (Elsas et al. 1985*a*, 1985*b*). This conclusion is also supported by recent experiments with transformed lymphocytes (Taylor et al. 1986).

Several radiolabeling methods are available for evaluating insulin-receptor structure and subunit interaction in diploid cells. One method chemically cross-links ^{125}I-insulin to membrane receptor proteins using the noncleavable, bifunctional cross-linker disuccinimidyl suberate (DSS) (Hill et al. 1979; Pilch and Czech 1979; Czech and Massague 1982). When applied to either membrane-bound or soluble insulin receptors, this procedure identifies a subunit, *alpha*, as the primary binding site for insulin. A second radiolabeling procedure uses insulin to potentiate phosphorylation of its own receptor from ^{32}P-γ-adenosine 5′ triphosphate (ATP) (Avruch et al. 1982*b*; Kasuga et al. 1982). In several cell and cell-free systems insulin promotes tyrosine-specific autophosphorylation of its receptor with preferential labeling of the *beta* subunit (Kasuga et al. 1983; Zick et al. 1983; Nemenoff et al. 1984). This characterization of the mature insulin-receptor structure was recently confirmed by analysis of the insulin receptor purified from human placenta and by deduction of its

cDNA sequence (Fujita-Yamaguchi 1984; Ebina et al. 1985; Ullrich et al. 1985). The purified holoreceptor is a glycoprotein of 350 kD comprising two *alpha* and two *beta* subunits bound by disulfide bonds (Fujita-Yamaguchi 1984). Both subunits are coded for by a single gene located on chromosome 19 (bands p13.2→p13.3) (Ebina et al. 1985; Yang-Feng et al. 1985). The portion of the cDNA coding for the *beta* subunit has considerable sequence homology with other growth-promoting receptors and *src* oncogenes and contains the transmembrane and tyrosine-kinase domains (Ebina et al. 1985; Ullrich et al. 1985).

In the present study we describe insulin binding and insulin-receptor structure of fibroblasts cultured from the families of two patients affected by leprechaunism—Ark-1 (Kobayashi et al. 1978; Elders et al. 1983; Craig et al. 1984; Elsas et al. 1985*b*) and Atl (Longo et al. 1986)—and from a third patient, Minn (Podskalny and Kahn 1982*a*, 1982*b*). Using affinity cross-linking, we found a difference in *alpha*-subunit relationships of insulin receptors from the Ark-1 cell line only. Insulin-enhanced autophosphorylation in the *beta* subunit of these cells is in direct proportion to the number of binding sites.

MATERIAL AND METHODS

Materials

^{125}I Na was purchased from New England Nuclear, ^{32}P-γ-ATP from Amersham. Bovine serum albumin (fatty acid free) was obtained from Sigma Chemical (St. Louis). DSS was from Pierce; *N*-ethylmaleimide (NEM) from Aldrich; and dithiothreitol (DTT) from Calbiochem. Nitrocellulose was from Sartorius. Porcine insulin was a gift from Dr. R. E. Chance of Eli Lilly (Indianapolis). ^{125}I-insulin was prepared using a chloramine T method, as described elsewhere (Santora et al. 1979).

Cell Culture

Fibroblasts were cultured from skin biopsies of the Ark-1 family (provided by Dr. Joycelyn Elders) and the Atl family (provided by Dr. Elspeth McPherson). Minn fibroblasts were from the human-genetic-mutant-cell-repository number GM5241. Cells were grown in Dulbecco and Vogt's modified Eagle's medium with 15% fetal bovine serum in Corning 75-cm^2 or 150-cm^2 polystyrene tissue-culture flasks. For insulin-binding studies, cells were cultured in either sterile scintillation vials or plastic Costar 24-well culture dishes, allowed to grow to confluency, and analyzed in monolayers as described elsewhere (Elsas et al. 1985*b;* Longo et al. 1986).

Biochemical Techniques

Plasma membranes were isolated from human fibroblasts, affinity labeled with ^{125}I-insulin, and resolved by electrophoresis as described elsewhere (Laemmli 1970; Endo and Elsas 1984; Elsas et al. 1985*b*). The following protein standards were used: thyroglobulin (669,000), fibronectin (440,000), catalase (232,000), myosin (205,000), β-galactosidase (116,000), phosphorylase B (94,000), bovine serum albumin (67,000), and ovalbumin (43,000). Autoradio-

grams were analyzed with an E.C. Apparatus densitometer, and radiodense peaks were integrated by computer.

Two-way gel electrophoresis was performed using 10 mM NEM in the first dimension. The lane was then excised and soaked in 62.5 mM Tris-HCl buffer, pH 6.8, 2% sodium dodecyl sulfate (SDS), and 50 mM DTT for 2 h at 24 C. The lane was then placed on a 5% acrylamide stacking gel and a 5%–15% gradient of acrylamide:bis acrylamide (75:1). Electrophoresis in the second dimension was conducted in the presence of 50 mM DTT.

Phosphorylation of Partially Purified Insulin Receptor

Approximately 200 mg of cell protein was obtained from 50 150-cm^2 Falcon flasks. Ten milligrams of membranous protein was obtained after solubilization with 2% Triton X-100 in 50 mM Tris-HCl, pH 7.4, containing 1 mg aprotinin/ml, and 0.2 mM phenylmethylsulfonylfluoride. The solubilized protein was diluted three times with 50 mM Tris-HCl, pH 7.4, 10 mM MgCl. Glycosylated proteins were adsorbed to wheat-germ agglutinin Sepharose prepared according to the method of Porath et al. (1967). Adsorbed proteins were eluted with 0.3 M *N*-acetylglucosamine. This procedure produced a fivefold increase in specific insulin binding.

Soluble, partially purified receptor (200–600 μg) was diluted to 250 μl and incubated with or without insulin at various concentrations for 4 h at 24 C. Phosphorylation was initiated by adding 50 μl of ATP solution (20 μCi of ^{32}P-γ-ATP). The final concentration of ATP was 50 μM. After 15 min incubation at 24 C, the reaction was terminated with iced NaF and 10 mM ATP. Insulin receptors were precipitated either with human serum B-2 containing antibody against insulin receptor (Kahn et al. 1977) or with rabbit anti-insulin-receptor IgG (Nagata et al. 1986). For experiments outlined in figures 4 and 5, human serum B-2 at a 1:200 dilution was used with addition of staphyloccocal protein A. Serum B-2, containing anti-insulin-receptor antibodies from patients with type B insulin-resistant diabetes mellitus, was the gift of Dr. Philip Gorden. In rabbits, we raised polyclonal antibodies against insulin receptors purified from human placenta (Fujita-Yamaguchi 1984). This antibody recognized nanogram quantities of purified receptor and proteins of predicted insulin-receptor size from fibroblast membrane (fig. 6). For the studies indicated in figures 7 and 8, insulin-receptor protein was immunoprecipitated with 25 μg of our rabbit anti-insulin-receptor IgG in protein A. Immunoprecipitates were resuspended in SDS sample buffer containing either 10 mM NEM or 0.1 M DTT. The entire suspension of resolubilized precipitate was then separated by gel electrophoresis and autoradiographed.

All data are expressed as means ± SD unless otherwise stated.

RESULTS

Insulin Binding to Intact Cells

Figure 1 describes attenuated pedigrees indicating family members from whom cells were cultured. Insulin (1 ng/ml) binding to fibroblasts is reported

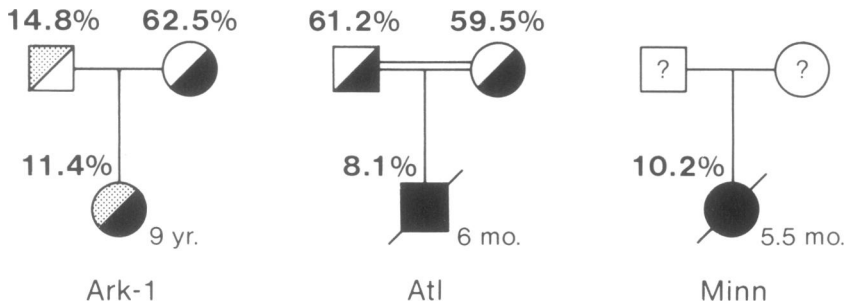

FIG. 1.—Binding of ^{125}I-insulin by fibroblasts cultured from three families with leprechaunism. By means of previously described methods (Elsas et al. 1985*b*; Longo et al. 1986), monolayers of cultured dermal fibroblasts were analyzed for ^{125}I-insulin (1 ng/ml) binding at 24 C. Superscripts represent the mean percent of specific insulin binding in four experiments comparing mutant and four control-cell lines matched for population doublings. Subscripts represent age at which the skin biopsy was obtained. Families Ark-1 (Elsas et al. 1985*b*), Atl (Longo et al. 1986), and Minn (Podskalny and Kahn 1982*a*) have been reported in part elsewhere. Parental cells from Minn were not available for study. Pedigrees show Mendelian inheritance when high-affinity insulin binding is used as the genetic discriminant. Compound heterozygosity in the Ark-1 proband is schematized by differing allele symbols.

above each symbol, expressed as percent of control. At equilibrium, specific insulin binding to four different control-cell lines ranged between 1.98 ± 0.09 and 2.52 ± 0.12, with a mean value of 2.24 ± 0.11 fmol/mg of cell protein in 20 observations. Insulin binding by fibroblasts cultured from probands Ark-1, Atl, and Minn was ~10% of the normal value. Fibroblasts cultured from the parents of Ark-1 had different degrees of impairment of insulin binding, accounting for the 14.8% and 62.5% of control values for father's and mother's cells, respectively. In both parents of Atl insulin binding by fibroblasts was significantly reduced compared with that in controls but was greater than that in their affected offspring ($P < .01$, by analysis of variance). Cells from the parents of Minn were not available for this study. Recently, it was reported that the mother of leprechaun Minn had a 50% reduction of insulin binding to her circulating monocytes, a finding further supporting the Mendelian-inheritance pattern shown in figure 1 (Taylor 1985). Of considerable interest is the reduced insulin binding by cells cultured from the father of the Ark-1 proband. Despite evidencing a binding defect similar to that of his daughter, he had no growth restriction or stigmata of leprechaunism; but he did have an insulin-resistant glucose-tolerance test (Elsas et al. 1985*b*).

Insulin-Receptor Structure

By means of chemical cross-linking of ^{125}I-insulin to plasma membranes prepared from the cells of Ark-1 and her parents, the structural characteristics of their insulin receptors were compared. Affinity-labeled insulin receptors were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. NEM was added to prevent spontaneous dissociation of the native receptor complex (Endo and Elsas 1984). Compared with the mother's cells, decreased specific labeling of insulin-receptor complexes was found in

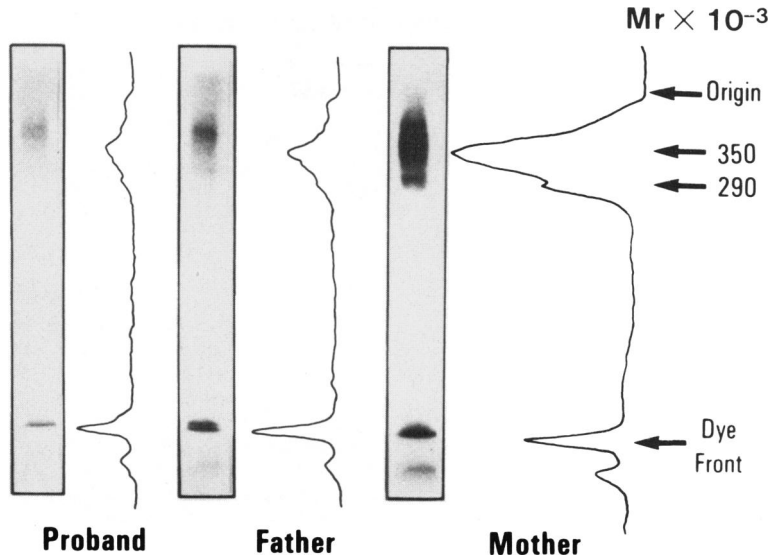

FIG. 2.—Autoradiograms and their densitometric profiles of membrane proteins cross-linked to ^{125}I-insulin and resolved in nonreducing SDS-PAGE. Membranes of fibroblasts cultured from the Ark-1 proband and both parents were equilibrated with ^{125}I-insulin (30 ng/ml) for 90 min at 24 C and cross-linked with 10 mM DSS. Extracted proteins were placed in 10 mM NEM and electrophoresed in a 5%–15% gradient of polyacrylamide. Equal amounts (200 μg) of cross-linked protein from each cell line were applied and analyzed in parallel.

plasma-membrane protein from the proband's and the father's cells (fig. 2). Per unit of solubilized membrane protein, chemical radiolabeling of the insulin receptor from cells of the proband was 15% of her mother's value and 56% of her father's value. In separate experiments insulin receptors from both the Atl and Minn probands' cells demonstrated a decreased labeling compared with control cells. Thus, affinity cross-linking of ^{125}I-insulin at higher insulin concentrations reflected qualitatively the variations of insulin binding observed in intact cells. The molecular weight of the insulin receptor was estimated at 350,000 for cells cultured from Ark-1, her parents, Atl, Minn, and controls. The specifically labeled band was broad and contained several higher- and lower-sized labeled proteins, including a 290-kD minor band seen as a shoulder on the densitometric profile (fig. 2).

In reducing conditions (DTT 10 mM), two specific ^{125}I-insulin-binding proteins with estimated molecular weights of 125,000 (*alpha* subunit) and 250,000 (fig. 3) were seen in control membrane. In control cells, the relative intensity of the two bands was related to the concentration of DSS used to cross-link ^{125}I-insulin to its receptor. In control membranes, increasing concentrations of DSS augmented the relative intensity of the 250-kD vis-à-vis the 125-kD band. The two bands had the same intensity (ratio 1:1) with 10 mM of cross-linking agent. In insulin receptors obtained from Ark-1 cells, DSS was less effective in promoting these changes (fig. 3). At 10 mM DSS (the concentration that saturates aqueous buffers), in membranes derived from Ark-1 cells, the 125-kD band was

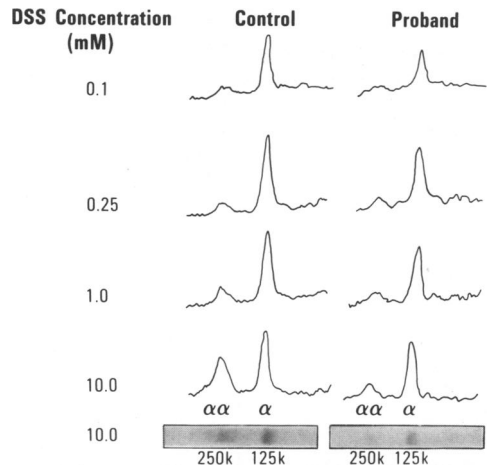

FIG. 3.—Densitometric profiles of monomer (*alpha*) and dimer (*alpha-alpha*) subunits formed by cross-linking ^{125}I-insulin to cell-membrane receptors from control and Ark-1 fibroblasts. Membrane proteins were cross-linked to ^{125}I-insulin at increasing concentrations of DSS and separated in 5%–15% PAGE in reducing conditions (DTT 10 mM). *Alpha* peaks were normalized to compare the dimer:monomer ratios in both cell lines.

three times more intense than the 250-kD band. The 350-kD band was no longer present in these reducing gels (see figs. 2, 3). Two-way gel analysis confirmed that both the 125-kD and 250-kD bands derived from the 350-kD insulin receptor. The 250-kD band contained insulin-receptor epitopes, since it reacted with rabbit anti-placental insulin-receptor antibodies on immunoblotting. Since this 250-kD band was derived from the fibroblast holoreceptor ($alpha_2$ $beta_2$), reacted with anti-insulin-receptor antibodies, and, in other purified-receptor preparations was identified as composed of *alpha* subunits (Endo and Elsas 1984; Fujita-Yamaguchi 1984), we assume that it is an *alpha-alpha* dimer.

The dimer:monomer ratio formed by 10 mM DSS in insulin receptors from the different cells is presented in table 1. In five different experiments, insulin receptors from Ark-1 fibroblasts had a mean dimer:monomer ratio of 0.31 ± 0.19, a value significantly different ($P < .01$) from the control-cell value of 0.93 ± 0.06. Insulin receptors from two other homozygous affected probands (Atl and Minn) and from four obligate heterozygotes had dimer:monomer ratios similar to that in controls. Although fibroblasts from Ark-1's father expressed decreased high-affinity insulin binding (fig. 1), his insulin receptor produced normal dimer:monomer ratios.

Insulin-Receptor Phosphorylation

The autophosphorylating activity of the insulin receptor of cultured human fibroblasts was investigated by measuring the incorporation of ^{32}P into the receptor protein following hormonal exposure. Insulin (600 ng/ml), added for 4 h at 24 C, promoted the phosphorylation of a 350-kD membrane protein of control (200 μg protein/lane) and Ark-1 cells (600 μg protein/lane) (fig. 4). A

TABLE 1

αα:α-SUBUNIT RATIOS FORMED BY AFFINITY LABELING (with ^{125}I-Insulin) NORMAL AND MUTANT INSULIN RECEPTORS FROM CULTURED HUMAN FIBROBLASTS

Source	Mean ± SD αα:α Ratio[a]
Controls[b]	0.93 ± 0.06 (6)
Ark-1:	
Proband	0.31 ± 0.19[c] (5)
Father	0.98 (1)
Mother	0.99 (1)
Atl:	
Proband	1.05 ± 0.06 (2)
Father	0.91 (1)
Mother	0.92 (1)
Minn	1.01 (1)

[a] ^{125}I-insulin was affinity cross-linked (by 10 mM DSS) to fibroblast membranes, extracted, and 20,000-cpm electrophoresed in 5%–15% SDS-PAGE, 10 mM DTT. Radiodense bands at 125 kD (α) and 250 kD (αα) were identified, as was a geometrically identical radiofree "background" band. After subtracting this background, αα:α ratios were calculated from specific counts per minute in the 250-kD and 125-kD areas. The number of gels analyzed is given in parentheses.

[b] Four different control cell lines were used.

[c] $P < .01$ (by Student's two-tailed t-test) when compared with controls.

greater quantity of membrane proteins from Ark-1 fibroblasts (600 μg/lane) was used to correct for the lower amount of binding sites present on their plasma membranes (Elsas et al. 1985*b*). Insulin enhanced ^{32}P incorporation in receptors purified from cells cultured from both Ark-1 parents in a manner similar to that shown for control cells. To determine which subunit of the insulin receptor was phosphorylated, two-way gels were analyzed. Figure 5 shows the effect of insulin on the phosphorylation of its receptor partially purified from Ark-1 fibroblasts. Insulin increased ^{32}P incorporation into a 350-kD protein in the horizontal dimension. The horizontal dimension was characterized by nonreducing conditions with 10 mM NEM. The holoreceptor was reduced into its subunits in the vertical dimension by means of 10 mM DTT. Enhanced labeling of a 90-kD band indicated that insulin augmented phosphorylation of the *beta* subunit of the receptor. Other proteins, particularly one of 160 kD, were present in the immunoprecipitate and were phosphorylated. However, their phosphorylation was independent of insulin, indicating that antiserum B-2 was not strictly specific for the insulin receptor. To determine the dose-response curve for insulin-stimulated phosphorylation of the *beta* subunit of its receptor, we needed an antibody more specific for the insulin receptor. We developed polyclonal anti-insulin-receptor antibodies in a rabbit. The purified IgG fraction recognized nanogram quantities of purified insulin receptor (fig. 6, lane A). When 250 μg of Triton X-100–solubilized fibroblast-membrane proteins were separated by electrophoresis under nonreducing conditions and blotted to nitrocellulose, this antibody recognized a 350-kD protein (fig. 6, lane B). It also

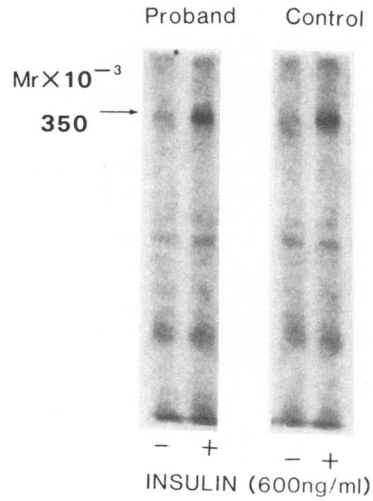

FIG. 4.—Autoradiography of ^{32}P-labeled insulin receptors resolved by SDS-PAGE. Plasma-membrane fractions, prepared from Ark-1 and control fibroblasts, were partially purified by lectin-column chromatography. Fractions eluted by *N*-acetyl-D-glucosamine and containing insulin-binding activity were pooled. Partially purified insulin receptors (200 μg for control cells, 600 μg for Ark-1 cells) were incubated for 4 h with or without insulin (600 ng/ml). After a further incubation for 15 min with ^{32}P-γ-ATP, insulin receptors were immunoprecipitated by human autoantibody B-2, resolved by SDS-PAGE in the presence of 10 mM NEM, and identified by autoradiography. A threefold-higher amount of proteins was used for Ark-1 fibroblasts, since they have only 30% of normal insulin binding at these insulin concentrations (Elsas et al. 1985*b*).

detected higher aggregates of 430 kD and 520 kD. Immunoblots of reducing gels identified only 125-kD and 90-kD proteins, indicating high specificity toward the insulin receptor and its subunits by this polyclonal antibody. This more specific antibody was used in subsequent experiments to determine whether a quantitative defect in insulin-enhanced autophosphorylation was present in cells from the Ark-1 proband (figs. 7, 8). Experimental conditions for insulin stimulation of its receptor phosphorylation were identical to those previously used for binding, chemical cross-linking of ^{125}I-insulin to the *alpha* subunit, and functional responsivity (Elsas et al. 1985*b*). Insulin increased ^{32}P incorporation from ^{32}P-γ-ATP into the *beta* subunit of both control and mutant cells in a dose-dependent manner (figs. 7, 8). Maximum stimulation occurred in both cell lines at 1,000–10,000 ng insulin/ml. Of interest was the reduced gross radiolabeling of the 200-μg aliquots of membrane proteins solubilized from the Ark-1 cells. At maximum enhanced autophosphorylation this represented 30% of the insulin-stimulated counts incorporated into control-cell proteins (fig. 8, left panel). Reduced phosphorylation was expected, when normalized to total membrane protein, since the Ark-1 cell line had only one-third of insulin binding to control cells at an insulin concentration of 100–10,000 ng/ml (Elsas et al. 1985*b*). This same ratio would be expected for ^{32}P incorporation if it were not normalized to number of insulin receptors. Therefore, the dose response was expressed as percent of maximum (as displayed in fig. 8, right panel). The ED_{50} for insulin

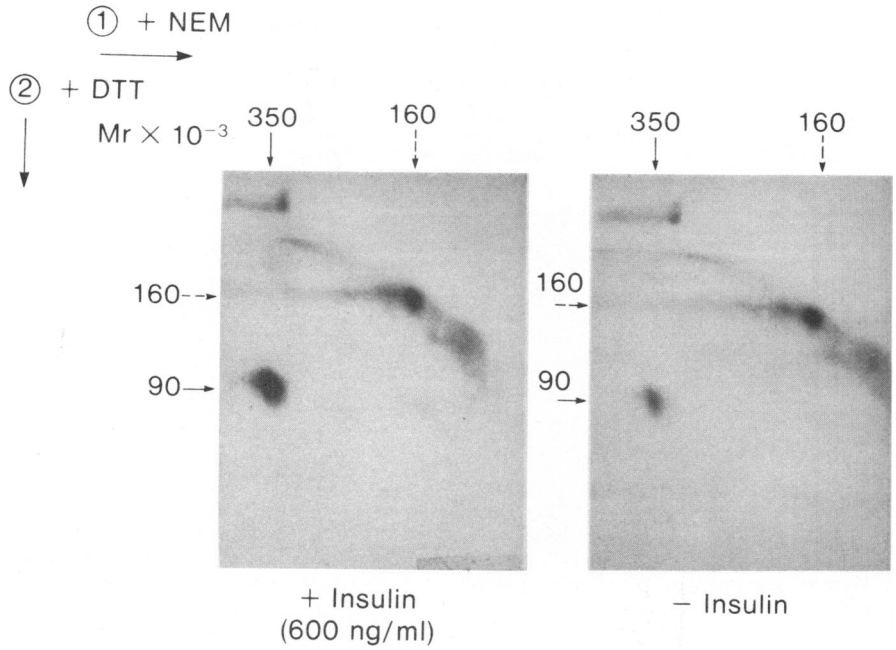

FIG. 5.—Autoradiography of two-way gel electrophoresis. Insulin receptors of Ark-1 were partially purified by lectin-column chromatography, phosphorylated with ^{32}-γ-ATP in the presence (+) and absence (−) of 600 ng insulin/ml, and immunoprecipitated with serum B-2 as described in fig. 4. After electrophoresis in the horizontal dimension in 10 mM NEM, the lane was cut out, soaked in 50 mM DTT, and electrophoresed in the vertical dimension.

stimulation of autophosphorylation was 100 ng/ml at 24 C for both control and Ark-1 receptors.

DISCUSSION

Insulin receptors of membranes from a variety of cultured cells and species have been studied at a structural level by use of several different approaches (Czech and Massague 1982). Covalent cross-linking of ^{125}I-insulin to cell membranes consistently defined the *alpha* subunit of the insulin receptor as the principal binding site for insulin (Pilch and Czech 1979; Czech and Massague 1982). Therefore, we used this chemical approach on membranes from cultured cells with genetically impaired insulin binding to evaluate subunit composition and interaction in the insulin receptor (Elsas et al. 1985*a*, 1985*b*). We found that DSS not only covalently bound radiolabeled insulin to *alpha* subunits of the insulin receptor but also that, with increasing concentrations, it cross-linked juxtaposed subunits (fig. 3) (Endo and Elsas 1984). This effect was markedly reduced in Ark-1 insulin receptors (table 1). Since the chemical cross-linker has a length of 11 Å (Hill et al. 1979), these data suggest an alteration in the physical proximity of the two *alpha* subunits of the mature insulin receptor in Ark-1 cells. Several observations support this interpretation. First, high-affinity binding of ^{125}I-insulin to intact cells was absent (Elsas et al. 1985*b*). This impaired

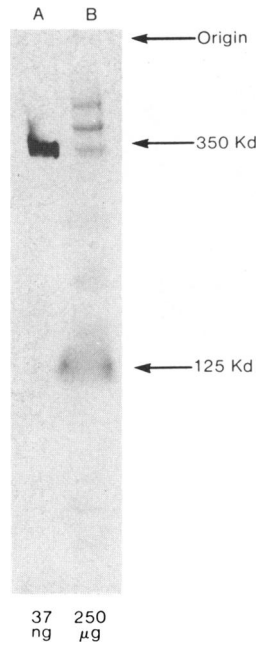

FIG. 6.—Immunoblots of human insulin receptors from placenta and cultured fibroblasts. Insulin receptors (37 ng) purified from human placenta (lane A) and 250 μg of Triton X-100 extract of normal fibroblast membranes (lane B) were separated in 5%–15% SDS-PAGE in the presence of 5mM NEM. After electrophoresis, proteins were transferred to nitrocellulose. Proteins reacting with the polyclonal rabbit antibody were stained using goat anti-rabbit antibodies conjugated with peroxidase and developed with diaminobenzidine.

binding to intact cells correlated with decreased insulin cross-linking to holoreceptor in affinity-labeling experiments (fig. 2). Furthermore, *alpha-alpha* dimer formation was consistently low when ^{125}I-insulin was cross-linked to membrane-bound receptor from the Ark-1 proband (fig. 3, table 1). This latter observation was unique to the Ark-1 proband's cell line and was not related to the degree of impaired insulin binding, since other cells with similarly decreased insulin binding had normal dimer cross-linking (table 1).

The phosphorylation of insulin receptors obtained from Ark-1 fibroblasts was enhanced by insulin (figs. 5, 7). Insulin-stimulated phosphorylation was decreased in Ark-1 cells in direct proportion to the reduced number of low-affinity binding sites present on their membrane (fig. 8) (Elsas et al. 1985*b*). Others have demonstrated—in intact cells, in cell-free systems, and in the purified insulin receptor—that insulin potentiates phosphorylation of the *beta* subunit of its receptor on both serine and tyrosine residues (Avruch et al. 1982*b;* Kasuga et al. 1982; Rosen et al. 1983; Roth and Cassell 1983; Shia et al. 1983; Von Obberghen et al. 1983; Zick et al. 1983; Nemenoff et al. 1984). The *beta* subunit of the insulin receptor also acts as a protein kinase toward certain exogenous substrates (Rosen et al. 1983; Roth and Cassell 1983). It is recognized that insulin alters the phosphorylation of a number of important proteins

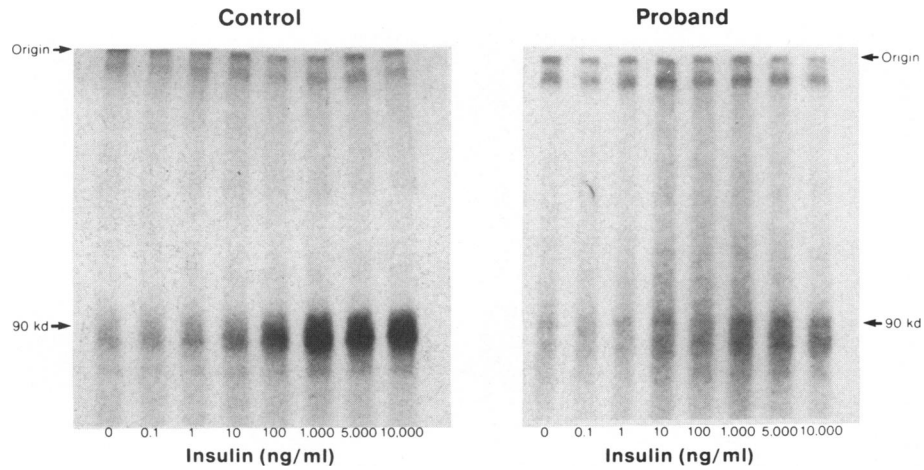

FIG. 7.—Autoradiograms of insulin-enhanced phosphorylation of the *beta* subunit from normal and mutant human fibroblasts. Membrane proteins (200 μg) from cells of the Ark-1 proband and controls were phosphorylated as described in fig. 4. However, phosphorylated insulin receptors were immunoprecipitated by monospecific, polyclonal rabbit anti-insulin receptor IgG, resolved on SDS-PAGE in the presence of 50 mM DTT, and identified by autoradiography.

in target cells, leading to phosphorylation of some and dephosphorylation of others through mechanisms involving plasma-membrane interaction with insulin (Avruch et al. 1982*a;* Czech 1985). Two laboratories have questioned the postulate that acute cellular signaling by insulin is secondary to receptor autophosphorylation (Simpson and Hedo 1984; Zick et al. 1984). Antisera B-2 and B-10, which are polyclonal and inhibit insulin binding to its receptor, elicit several high-affinity insulin-receptor responses and down-regulation. Neither of these antibodies potentiates insulin-receptor phosphorylation. However, *beta*-subunit phosphorylation and activation of a kinase probably is involved in signal transduction and has clinical significance (Grigorescu et al. 1984, 1986; Grunberger et al. 1984). Impairment of insulin-enhanced phosphorylation and consequent phosphorylation of other substrates has been associated with insulin-resistant syndromes other than leprechaunism. In at least one patient with Type A insulin resistance and acanthosis nigricans, insulin binding was not impaired—but defective insulin-stimulated tyrosine kinase was found, suggesting a defective coupling mechanism between binding and signaling (Grunberger et al. 1984). In three other patients with Type A insulin-resistant syndromes, heterogeneous binding defects and impairment of *beta*-subunit autophosphorylation were described (Grigorescu et al. 1984, 1986). The phenotype of this insulin-resistant syndrome shares some features with leprechaunism, with respect to (1) cystic changes in membranes of some internal organs and (2) acanthosis nigricans. However, in these other syndromes of insulin resistance there is no evidence for heritability and there is no congenital growth restriction.

It should be emphasized that in our experiments receptor binding, affinity

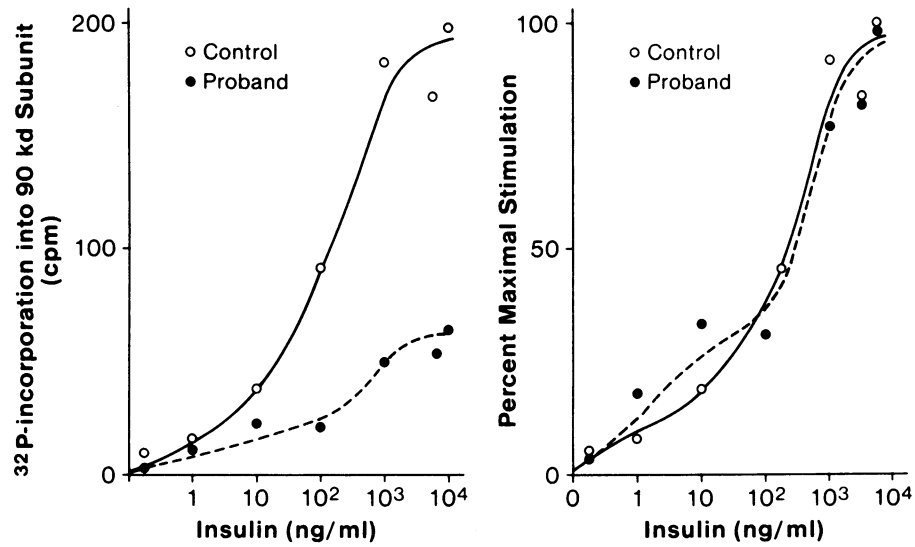

FIG. 8.—Dose-response curves for insulin-enhanced autophosphorylation of its receptor's *beta* subunit. Cells cultured from Ark-1 and control were used. Radiodense areas seen in fig. 7 at 90 kD were cut out from the gel and counted for ^{32}P levels. Background counts from nonradiodense areas were subtracted, and specific counts were calculated. The left panel displays the specific counts obtained. Since Ark-1 cells had 33% of control cells' binding sites at insulin concentrations >10 ng/ml (Elsas et al. 1985*b*), the data were displayed in the right panel as percent of maximal stimulation. Phosphorylation at 10,000 ng insulin/ml was considered as 100%.

cross-linking, autophosphorylation, and cellular responsivity to insulin were conducted at the same temperature (24 C) and under identical experimental conditions. Thus we could compare binding, structure, and functional response. In Ark-1 fibroblasts the number of binding sites was reduced (Elsas et al. 1985*b*) and *alpha*-subunit interaction was different. There was also a reduction in total cellular receptor phosphorylation (figs. 7, 8). However, when corrected for reduced number of binding sites, insulin-enhanced autophosphorylation was similar to that seen in control cells (fig. 8). This finding is in contrast to that for some patients with Type A insulin resistance, in whom impaired phosphorylation was not related to decreased binding (Grunberger et al. 1984).

It is clear that there are many different mutations in man that produce insulin resistance. Even within the leprechaun syndrome, which includes defective insulin binding, *alpha*-subunit interaction may or may not be altered (table 1). No studies of subunit interaction or conformational change in receptor following occupancy by insulin have been published previously. Thus, whether this physical change links insulin binding to some but not all signaling mechanisms remains problematic.

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