

Abnormality of Insulin Binding and Receptor Phosphorylation in an Insulin-resistant Melanoma Cell Line

HANS U. HARING, MORRIS F. WHITE, C. RONALD KAHN, MASATO KASUGA, VILMA LAURIS, ROBERT FLEISCHMANN,* MARILYN MURRAY,* and JOHN PAWELEK*
*E. P. Joslin Research Laboratory, Joslin Diabetes Center, and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02215; and *Department of Dermatology, Yale Medical School, New Haven, Connecticut 06510*

ABSTRACT The insulin receptor possesses an insulin-stimulated tyrosine-kinase activity; however, the significance of receptor phosphorylation in terms of the binding and signaling function of the receptor is unclear. To help clarify this problem, we have studied insulin binding and receptor phosphorylation in a Cloudman S91 melanoma cell line and two of its variants: the wild type (1A) in which insulin inhibits cell growth, an insulin-resistant variant (111) in which insulin neither stimulates or inhibits growth, and a variant (46) in which insulin stimulates cell growth.

¹²⁵I-insulin binding to intact cells was similar for the wild-type 1A and insulin-stimulated variant 46. The insulin-resistant variant 111, in contrast, showed ~30% decrease in insulin binding. This was due to a decrease of receptor affinity with no major difference in receptor number. When the melanoma cells were solubilized in 1% Triton X-100 and the insulin receptor was partially purified by chromatography on wheat germ agglutinin-agarose, a similar pattern of binding was observed.

Phosphorylation was studied by incubation of the partially purified receptor with insulin and [γ -³²P]ATP, and the receptor was identified by immunoprecipitation and NaDodSO₄ PAGE. Insulin stimulated phosphorylation of the 95,000-mol-wt β -subunit of the receptor in all three cell types with similar kinetics. The amount of ³²P incorporated into the β -subunit in the insulin-resistant cell line 111 was ~50% of that observed with the two other cell lines. This difference was reflected throughout the entire dose-response curve (10⁻⁹ M to 10⁻⁶ M). Qualitatively similar results were obtained when phosphorylation was studied in the intact cell. Peptide mapping of the β -subunit using tryptic digestion and reverse-phase high-performance liquid chromatography column separation indicated three sites of phosphorylation in receptor from the wild type and variant 46, but only two major sites of phosphorylation of variant 111. These data suggest that the insulin-resistant variant melanoma 111 possesses a specific defect in the insulin receptor which alters both its binding and autophosphorylation properties, and also suggests a possible role of receptor phosphorylation in both the binding and the signaling function of the insulin receptor.

Insulin stimulates phosphorylation of the 95,000-mol-wt (β) subunit of the insulin receptor. This phenomenon, first observed in hepatoma cells and lymphocytes (1), has been shown to occur in all cell types containing insulin receptors (2-12). Insulin receptor phosphorylation can be demonstrated in intact cells (1, 2, 6, 7) and in cell-free systems using both partially purified receptor (2-11) and highly purified receptor preparations (5). In broken cell systems, the insulin receptor

kinase is tyrosine specific, whereas in whole cells, insulin stimulates phosphorylation at both tyrosine and serine residues (2, 3). Evidence has been presented using ATP-affinity labeling that the protein kinase activity that catalyzes the phosphorylation is present in the β -subunit of the receptor itself (12).

The role of receptor autophosphorylation in the binding and signaling function of the receptor is not known. One

approach to understanding the role of this process is to study receptor phosphorylation in cells with altered insulin response. Cloudman S91 melanoma cells and its variants are a good system for such studies. Pawelek and co-workers have shown that insulin inhibits growth of the wild-type melanoma cell (13). Thus, it has been possible to select several variants from this cell line with altered insulin response. In this paper, we have characterized insulin binding and receptor phosphorylation in the wild-type Cloudman S91 melanoma (IA) and two variants: one (III) in which insulin has no effect on growth and one (46) in which insulin has a growth-stimulating effect (14, 15). We find that receptor from the insulin-resistant cell type III shows decreased autophosphorylation and decreased receptor affinity. By peptide mapping, the decreased phosphorylation appears to be due to a loss of one of three sites of phosphorylation. These data suggest that receptor phosphorylation plays a role in both the binding and the signaling function of the insulin receptor and that the mutant III possesses a defect which alters these functions.

MATERIALS AND METHODS

Materials

Porcine insulin (lot 1JM95AN) was purchased from Elanco Products Co. (Indianapolis, IN); [32 P]orthophosphate, [γ - 32 P]ATP, and Triton X-100 were purchased from New England Nuclear (Boston, MA). Aprotinin, phenylmethylsulfonyl fluoride, and *N*-acetyl-D-glucosamine were obtained from Sigma Chemical Co. (St. Louis, MO); wheat germ agglutinin coupled to agarose was from Vector Laboratories, Inc. (Burlingame, CA); and TPCK-treated trypsin was from Worthington Biochemical Corp. (Freehold, NJ). All reagents for NaDodSO₄ PAGE were from Bio-Rad Laboratories (Richmond, CA).

Biologically active 125 I-insulin was prepared by the lactoperoxidase method and the A₁₄[Tyr]monoiodoinsulin was isolated by high performance liquid chromatography (HPLC)¹, resulting in a specific activity of ~360 μ Ci/ μ g (16).

Cell Culture

Melanoma cells were cultured in modified Ham's F10 medium with 5% fetal calf serum in monolayer in plastic dishes in a humidified atmosphere of 5% CO₂/95% O₂ at 37°C (13). Medium was changed every 3rd d and cells were split when confluence was reached. EDTA (1 mM) was used for cell detachment. Confluent cells were used for binding and phosphorylation experiments. Because insulin inhibits growth of the wild-type IA cells (13), the insulin-resistant III and insulin-dependent 46 variants could be selected from cells treated with ethylmethanesulfonate then cultured in the presence of insulin (10⁻⁷ to 10⁻⁶ M). The variant colonies were isolated and cloned in medium containing soft agar as previously described (15). The wild-type and variant cells have maintained their characteristic insulin responses for over 2 hr in culture.

Type 46 was cultivated in the presence of insulin 10⁻⁸ M. Unless otherwise stated, however, insulin was removed from the medium 24–36 h before cells were used for insulin binding or phosphorylation experiments.

Determination of Rates of Proliferation

To determine rates of proliferation under various culture conditions, (2 × 10⁴) cells were inoculated in 25-cm² Corning tissue culture flasks (Corning Glass Works, Corning, NY) in 4 ml medium, and medium was changed three times per week. Triplicate cultures were harvested and counted in a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) every 2 or 3 d over a 2-wk period. Variation within triplicate samples was \pm 15%. The generation time was determined from the rate of division in log phase from the following equation: generation time = [0.69 (H)]/[ln (C₂/C₁)], where H is the time in hours between counting the initial cell number (C₁) and the final cell number (C₂). Generation time was determined using only the linear portion of a growth curve.

¹ Abbreviation used in this paper: HPLC, high-performance liquid chromatography.

Receptor Phosphorylation in Intact Cells

Cells were plated in 10-cm tissue culture dishes. After reaching confluence, cells were washed twice with phosphate-free Krebs–Ringer bicarbonate buffer containing 0.1% BSA and 25 mM HEPES, pH 7.4, and incubated at 37°C in this buffer with 0.125 mCi/ml of [32 P]orthophosphate in a humidified atmosphere containing 7% CO₂. After 120 min, insulin (final concentration 5 × 10⁻⁷ M) was added to half of the flasks and incubation was continued for 20 min at 37°C. The reaction was stopped by addition of cold Krebs–Ringer bicarbonate buffer containing 10 mM sodium pyrophosphate, 100 mM NaF, and 4 mM EDTA, and the cells were washed with this "stopping buffer."

The [32 P]phosphate-labeled cells were solubilized in 25 mM HEPES buffer (pH 7.4) containing 1% Triton X-100, phenylmethylsulfonyl fluoride (2 mM), Aprotinin (1 TIU/ml), 10 mM sodium pyrophosphate, 100 mM NaF, and 4 mM EDTA at 4°C for 30 min. Insoluble material was removed by centrifugation at 200,000 g for 60 min, and the supernatants were applied to columns containing wheat germ agglutinin coupled to agarose. After extensive washing with 25 mM HEPES buffer (pH 7.4) containing 0.1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM NaF, and 4 mM EDTA, the bound material was eluted with buffer supplemented with 0.3 M *N*-acetylglucosamine. With other cell types, this has been shown to result in a 20-fold purification and a 100% recovery of the insulin receptor (17). The eluted fractions were precipitated with sera from patients which contain autoantibodies against the insulin receptor (B-8, 1:300 dilution) or normal pooled serum as previously described (1). Alternatively, a 1:100 dilution of serum from rabbits containing receptor antibody (kindly provided by Dr. S. Jacobs, Burroughs-Wellcome) was used.

Immunoprecipitates were solubilized by boiling for 3 min in a solution of 2% NaDodSO₄, 0.1 M dithiothreitol, 0.01% bromophenol blue, 10% glycerol, and 10 mM sodium phosphate (pH 7.0). Electrophoresis was performed according to Laemmli (18) in 4% stacking and 7.5% resolving gels. After electrophoresis, the slab gels were stained, dried, and autoradiographed with Kodak X-Omat film (Eastman Kodak Co., Rochester, NY) for 10–14 d. Molecular weights were calculated by using standard proteins (5).

Phosphorylation of Solubilized Wheat Germ Purified Receptor In Vivo

Preparation of solubilized receptor and purification over wheat germ was performed as described above except that the cells were grown in normal medium until extraction of the receptors. NaF, sodium pyrophosphate, EDTA, and insulin were omitted from the solubilization buffer. For phosphorylation studies, aliquots of the eluted fractions from the lectin column were incubated with or without insulin at room temperature for 1 h followed by incubation with [γ - 32 P]ATP 50–100 μ M in 100 mM HEPES buffer, pH 7.4, containing 10 mM MgCl₂ and 2 mM MnCl₂ at 4°C or 22°C for 5–60 min. The phosphorylation reaction was stopped at 4°C by addition of NaF (100 mM), sodium pyrophosphate (10 mM), EDTA (5 mM), and ATP (5 mM). The insulin receptor subunits were identified by immunoprecipitation with antibodies against insulin receptor followed by NaDodSO₄ PAGE under reducing conditions and autoradiography as described above.

Insulin Binding

INTACT CELLS: 36–48 h before an experiment, cells were detached in a saline solution containing EDTA (0.2 mg/ml) and transferred to six-well tissue culture dishes with 1.2–1.4 × 10⁶ cells per well. For the binding experiments, cell incubations were carried out in a modified F10 medium without glucose containing 20 mM HEPES at pH 7.8 and 1% bovine serum albumin (buffered medium). Confluent monolayer cultures of cells (~2 × 10⁶ cells/35-mm plate) were incubated with 1 ml buffered medium (pH 7.8) containing 1–3 × 10⁻¹¹ M 125 I-insulin and various concentrations of unlabeled insulin for 3 h at 15°C. At the end of the incubation, the monolayers were washed three times with 2 ml of cold PBS, solubilized in 1 ml of 0.01% SDS and the radioactivity was quantitated in a Tracer 1190 autogamma counter (TM Analytic, Elk Grove Village, IL).

BINDING TO SOLUBILIZED RECEPTOR: Solubilized and wheat germ-purified receptor was prepared as described above. Aliquots of wheat germ eluate containing between 10 and 15 μ g protein were incubated with 125 I-insulin (~10⁻¹¹ M) and various concentrations of unlabeled insulin overnight at 4°C in a medium of 150 mM NaCl and HEPES 25 mM at pH 7.4. Separation of the free and receptor-bound insulin was then performed by precipitation of the solubilized receptor with polyethylene glycol (12.5% final concentration) as described earlier (19), using human γ -globulin (0.1 mg) as carrier protein. The protein pellets were washed once with polyethylene glycol and counted in a Tracer 1190 autogamma counter.

Tryptic Peptide Mapping by HPLC

Fixed and dried polyacrylamide gel fragments containing the phosphorylated receptor (located by autoradiography) were washed for 12 h at 37°C with 20 ml of 10% methanol. The absorbent paper was removed from the gel fragment. The gel was dried at 70°C for 60 min and rehydrated in 2 ml of 50 mM NH_4HCO_3 containing 100 μg of TPCK-treated trypsin (Worthington Biochemical Corp.). This mixture was incubated for 24 h at 37°C, the gel fragment was removed and the supernatant was clarified by centrifugation and lyophilized. The residue was dissolved in 25–50 μl of 0.1% trifluoroacetic acid. The phosphopeptides were separated using a Waters HPLC system (Waters Instruments, Inc., Rochester, MN) equipped with a $\mu\text{Bondapak C}_{18}$ reversed-phase column (20). Phosphopeptides applied to the column were eluted at a flow rate of 1 ml/min. The column was washed for 5 min with 0.05% trifluoroacetic acid in water and then the peptides were eluted with a gradient of acetonitrile also containing 0.05% trifluoroacetic acid increasing at a rate of 1% per min. Radioactivity in 0.5–1.0-ml fractions eluted from the reversed-phase column was measured in 5 ml of scintillation mixture.

RESULTS

Insulin Effect on Cell Growth

The effect of insulin on growth rates of the wild type Cloudman S91 melanoma cells and the two variants is shown in Fig. 1. Insulin ($\sim 7 \times 10^{-7}$ M) inhibited the growth of the wild-type cells; the generation time for these cells increased from 35 to 86 h in the presence of insulin. This effect of insulin has been previously shown to be mediated via the insulin receptor based on dose-response curves for insulin analogues and the ability of antibody to the insulin receptor to mimic this effect (13–15). By contrast, insulin stimulated growth of the variant cell line 46; and the generation time decreased from 103 to 47 h. A similar but smaller effect was also observed with physiological concentrations of insulin (15). No effect of insulin on generation time was detectable in the cell line 111. On this basis, we have referred to variant 111 as an insulin-resistant cell line.

Insulin Binding

Insulin binding was studied in the three melanoma cell lines both in intact cells and with solubilized receptor partially

purified by wheat germ affinity chromatography (Fig. 2). At tracer concentrations, ^{125}I -insulin binding to the insulin resistant variant line 111 was 30% lower than that of the wild type, whereas binding of insulin to the insulin-stimulated line 46 was $\sim 25\%$ higher than the wild type. In both cases, the change was due to a change in specific binding with no change in nonspecific binding. Unlabeled insulin competed for labeled-insulin binding with a similar dose dependence, and at maximal insulin concentrations there was no difference of binding between the three cell lines. When insulin binding was studied in solubilized wheat germ-purified material from each of the three cell lines, a similar picture was observed (Fig. 2B), i.e., binding was slightly lower in the extract of 111 and slightly higher in the extract of 46 than in the wild type when expressed per microgram of protein. Thus, there was no apparent difference in the yield of receptor after solubilization and wheat germ purification among the three cell types, and the differences in the binding present in the three cell lines was retained after solubilization and partial purification.

Analysis of insulin binding for all three cell types yielded curvilinear Scatchard plots (Fig. 3). Quantitative analysis of the Scatchard plots were performed using a negative cooperative model (21) and an M-lab computer modeling program. Using this model, the decrease in binding in the resistant cell line was due to a decrease in affinity with no change in receptor concentration (84.5 vs. 95 fmol/mg of protein). Receptor concentration was also similar in the insulin-dependent cell line 46. Thus, all three melanoma lines exhibit similar receptor numbers, but the insulin-resistant line 111 exhibits decreased receptor affinity, and the insulin-dependent line 46 exhibits a slightly increased receptor affinity.

Receptor Phosphorylation in Variant and Wild-Type Cells

In vitro autophosphorylation of the 95,000-*M*, subunit of the insulin receptor was studied after wheat germ purification of the receptor. As in other cell lines, when equal amounts of the partially purified receptor isolated from each of the cell

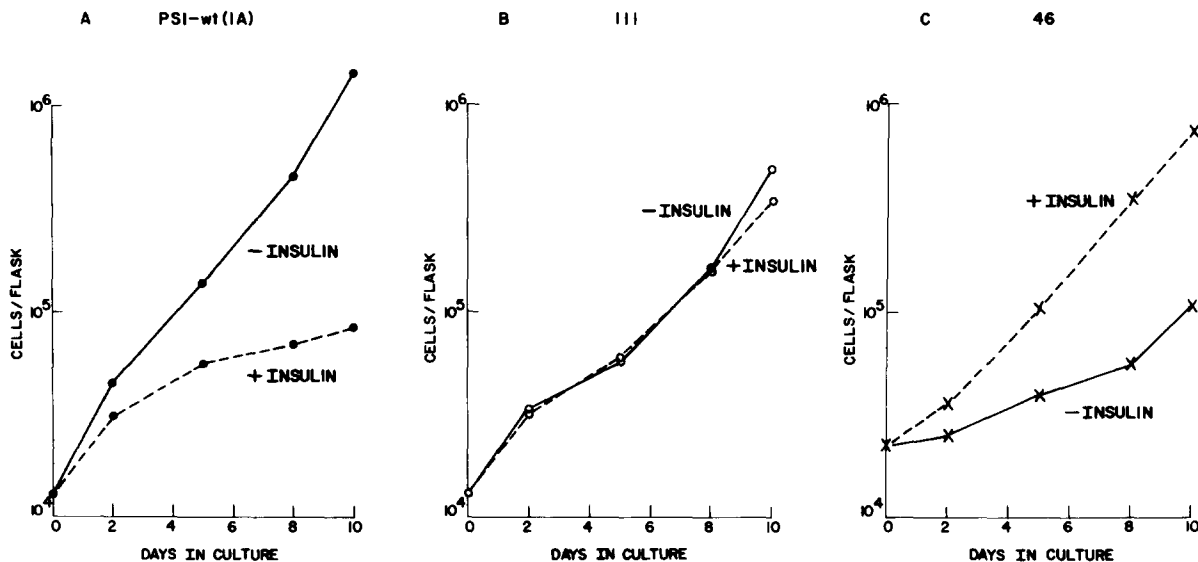


FIGURE 1 Growth of Cloudman melanoma cell lines. Cells were cultured and cell number was determined as described in Materials and Methods. A contains data on the wild-type cell (1A); C is data on the insulin-dependent variant (46); and B is data on the insulin-resistant variant (111). The data in the solid line are in the absence of insulin, and with the broken line in the presence of 7×10^{-7} M insulin.

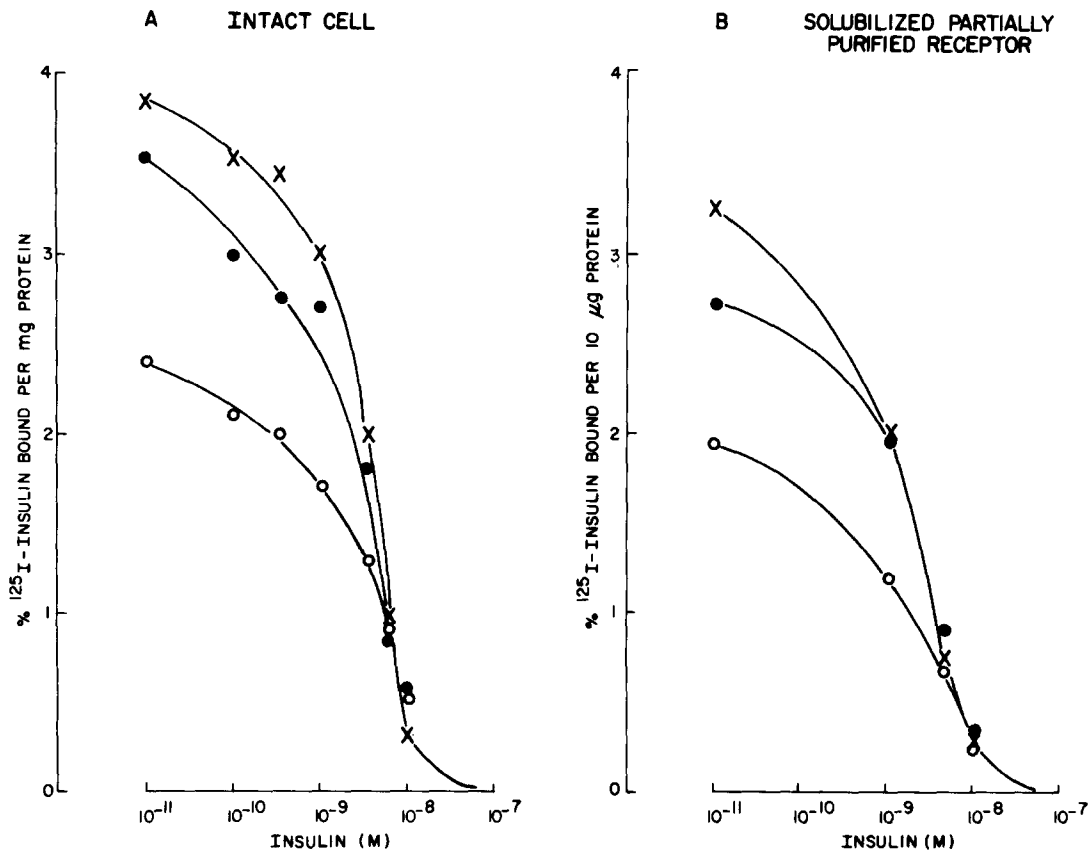


FIGURE 2 Competition for ^{125}I -insulin binding by unlabeled insulin in intact melanoma cells (A) and solubilized receptors partially purified by wheat germ affinity chromatography (B). Data on the wild type 1A are shown by ●, variant 111 by ○, and variant 46 by x.

lines were incubated *in vitro* with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, Mn^{++} , and insulin, and the proteins analyzed by immunoprecipitation and NaDodSO_4 gel electrophoresis, autophosphorylation of the receptor was observed (Fig. 4). In all three cell lines, the major phosphorylated protein had a $M_r = 95,000$, the position previously shown to contain the β -subunit of the insulin receptor. Further evidence that this was the β -subunit of the receptor was the finding that the protein was specifically precipitated by antireceptor antibody (Fig. 5, lanes A, C, E, G, I, K vs. B, D, F, H, J, L) and that the phosphorylation was stimulated by insulin (Fig. 5, lanes C, G, K vs. A, E, I). Quantitatively, insulin receptor from the insulin-resistant cell line 111 was less heavily phosphorylated than the receptor from the two insulin-sensitive cell lines when studied at equal protein concentration (Figs. 4 and Fig. 5, lane G vs. C and K).

Two kinds of evidence indicate that the lower phosphorylation of the 95,000- M_r band in 111 cells was not caused by a less efficient immunoprecipitation of the receptor in this cell line. First, when antibodies to the insulin receptor raised in rabbits were used for immunoprecipitation instead of human autoantibodies, a similar decrease in phosphorylation was seen. Secondly, in experiments in which the partially purified receptor was not immunoprecipitated, but subjected directly to electrophoresis after phosphorylation, the insulin-dependent phosphorylation of the 95,000- M_r band was decreased in line 111 when compared with the other two cell lines (data not shown).

As shown in Fig. 3, when insulin was removed for the culture medium 24 h before study, type 46 cells had a similar

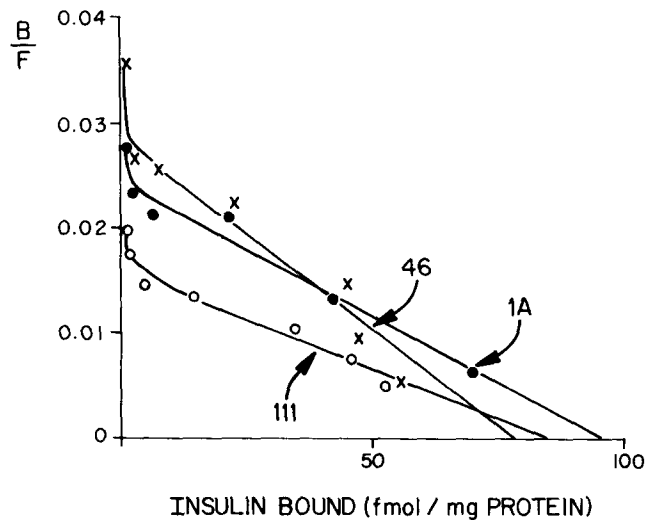


FIGURE 3 Scatchard plot of insulin binding to intact melanoma cells. The data are those in Fig. 2, and the code is the same. The Scatchard plots were fit using a negative cooperative model (21) and an M-lab computer program.

number of insulin receptors as the wild-type melanoma, and under these conditions, this cell line showed the same intensity of autophosphorylation as the wild-type cell. If insulin was not removed from the culture media, type 46 cells exhibited ~50% loss of receptors due to down-regulation (data not shown) and also a less intense phosphorylation of the β -subunit than the wild-type 1A melanoma (Fig. 5, lane C vs.

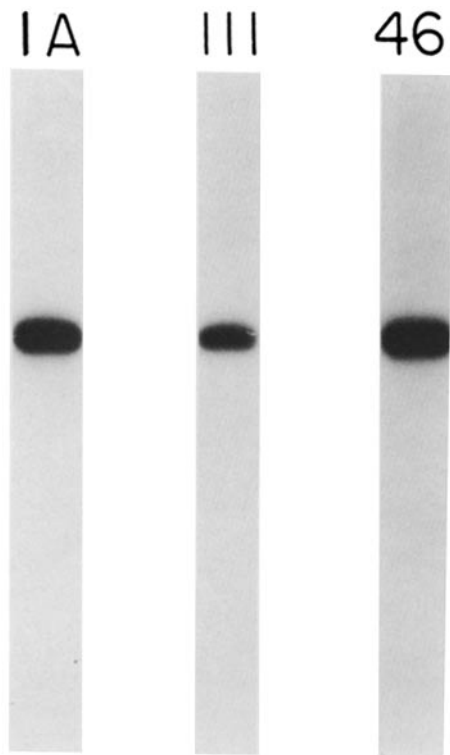


FIGURE 4 Insulin-receptor autophosphorylation in vitro. Insulin receptors were partially purified from the wild-type melanoma 1A and variants 111 and 46 as described in Materials and Methods. The partially purified receptor (24 μg in 500 μl) was then phosphorylated at 22°C by incubation for 10 min with 10^{-7} M insulin, 10 μM [γ - ^{32}P]ATP, and 5 mM Mn^{2+} . The reaction was stopped, the receptor was immunoprecipitated, and the β -subunit was separated by NaDodSO₄ PAGE under reducing conditions. The figure shows autoradiograms of the gel lanes.

K). Even after down-regulation, however, receptor phosphorylation in type 46 cells was greater than in variant 111.

Characterization of the Autophosphorylation

Fig. 6 shows the time course of receptor phosphorylation in all three cell types. In this in vitro system, all cell types exhibited sigmoidal kinetics of phosphorylation with an initial lag period and a half time of ~ 20 min. The decreased phosphorylation of receptor from line 111 was evident at all time points studied. The exact significance of the sigmoidal kinetics is unknown at present, but may suggest a positive cooperativity of the phosphorylation process in the solubilized system due to activation of the receptor kinase by the autophosphorylation, as suggested by Rosen et al. (22).

Quantitation of phosphorylation was performed by densitometric scanning of gels. An effect of insulin was detectable at 10^{-9} M in all three cell types and half-maximal stimulation was observed between 10^{-9} and 10^{-8} M (Fig. 7). This is similar to the dose-response in other cell lines and is similar to the displacement curves of insulin binding in Fig. 2. A decrease in the phosphorylation of the receptor in type 111 was observed in six separate experiments at all insulin concentrations. At concentrations of insulin which saturate all receptors, this decrease averaged 50% (Table I). In general, receptors from type 46 cells exhibited slightly higher levels of insulin-stimulated phosphorylation than receptor from wild type (Table I).

Phosphopeptide Analysis

In an effort to determine if the decreased phosphorylation in the 111 insulin-resistant variant was due to a general or specific modification in this receptor, the sites of phosphorylation were analyzed by digesting the phosphorylated receptor

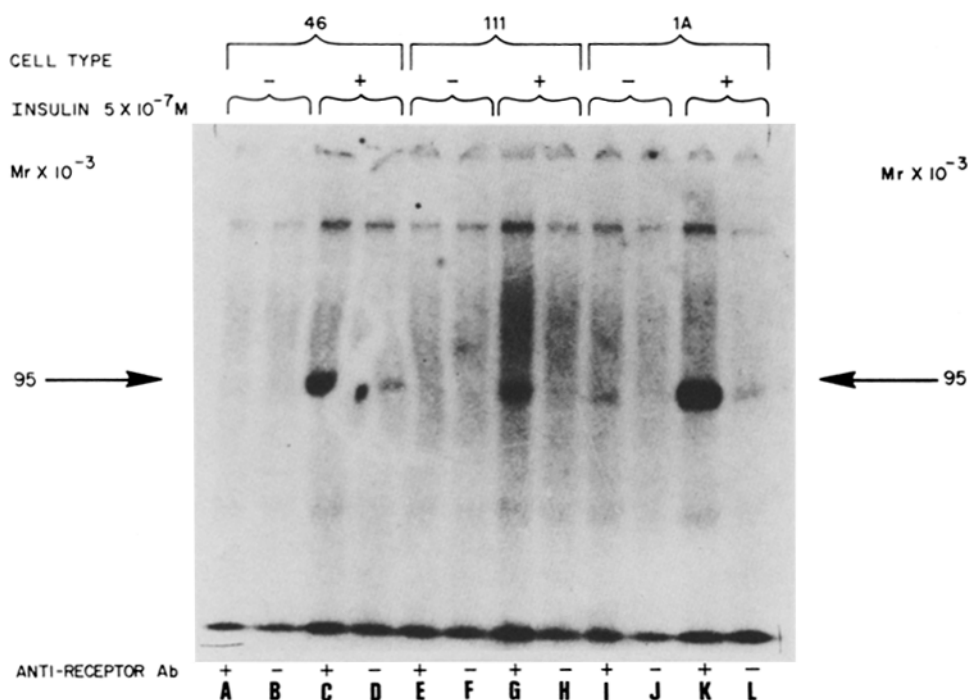


FIGURE 5 Autoradiogram showing the incorporation of ^{32}P from [γ - ^{32}P]ATP into the β -subunit of the insulin receptor in a cell-free system. The insulin receptors were partially purified from wild-type melanoma 1A (four right lanes), variant 111 (four middle lanes), and variant 46 (four left lanes), and the phosphorylation assay was performed as described in the Materials and Methods section. For each lane, ~ 20 μg of receptor protein and 0.1 mCi [γ - ^{32}P]ATP was used. Duplicate samples were preincubated with insulin (5×10^{-7} M) for 1 h at room temperature and phosphorylation was performed for 60 min at 4°C. The receptor was immunoprecipitated by serum containing antibody against the receptor (first and third lanes for each cell type) or control-pooled serum (second and fourth lanes). The immunoprecipitates were reduced with 0.1 M dithiothreitol, analyzed in NaDodSO₄-polyacrylamide (7.5%), and autoradiographed.

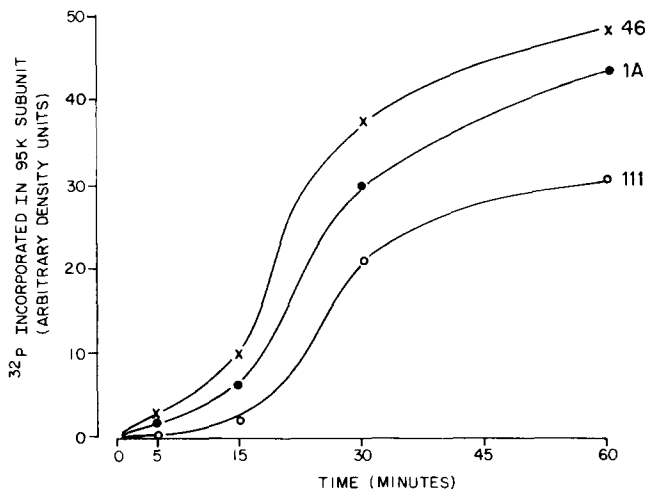


FIGURE 6 Time course of incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into the β -subunit of the insulin receptor in the cell-free system. The insulin receptors were partially purified from wild-type melanoma 1A cells (●), type 46 cells (X), and type 111 (○). Receptors from all three cell types ($\sim 20\ \mu\text{g}$) were preincubated with insulin $5 \times 10^{-7}\ \text{M}$ for 1 h at room temperature. The phosphorylation assay was carried out for the time intervals shown in the figure at 4°C as described in the Materials and Methods section. The receptor was immunoprecipitated with serum containing antibody against the receptor. The immunoprecipitates were reduced with 0.1 M dithiothreitol, analyzed in NaDodSO₄ polyacrylamide (7.5%), and autoradiographed. Phosphorylation of the 95,000- M_r (95K subunit) receptor subunit was then determined by scanning densitometry and is expressed in relative values.

with trypsin and subjecting the resultant phosphopeptides to analysis by HPLC on a reversed-phase column. In the wild-type 1A cells, three phosphopeptides were identified (Fig. 8, top), consistent with our previous observation that in vitro there are multiple sites of autophosphorylation in the insulin receptor (20). A similar pattern was observed in the insulin-sensitive variant 46 (Fig. 8, bottom). By contrast, the insulin-resistant variant 111 appeared to be lacking a discrete peak at position 2, whereas peaks 1 and 3 were unchanged (Fig. 8, center). Due to the low amount of radioactivity in each peak, statistical evaluation was impossible; however, similar HPLC elution patterns were observed in two separate experiments.

Receptor Phosphorylation in Intact Cells

Insulin receptor phosphorylation was also studied in the intact cell. To reduce the background of other phosphoproteins in these experiments, a rabbit serum containing antibodies to the insulin receptor was used for precipitation or the wheat germ-purified labeled material was first precipitated with control serum and the supernatant was then precipitated with serum containing antireceptor antibody. An autoradiogram of such an experiment is shown in Fig. 9. Although the labeling of the receptor was low in intact cells as compared with the in vitro assays, in both the wild-type 1A cell and type 46 phosphorylation of the β -subunit of the insulin receptor was detectable after insulin stimulation. By contrast, little or no phosphorylation of the 95,000- M_r subunit of the insulin receptor could be detected in type 111 cells.

DISCUSSION

Although the exact mechanism of insulin action remains unknown, insulin has been shown to affect the state of phos-

phorylation of a variety of cellular proteins and enzymes (23–26). This protein modification has been suggested to play a role in the post receptor transmission of the insulin signal. Recently, we have presented evidence that the insulin receptor is a protein kinase, and have proposed that receptor-induced phosphorylation may be an early step in the signal transmission process (1–5). These findings have been confirmed and extended using receptors from a wide variety of tissues (5–12).

Insulin-receptor phosphorylation occurs at multiple sites (2). The receptor itself is tyrosine-specific protein kinase which will autophosphorylate, as well as catalyze the incorporation of phosphate into the tyrosine residues of artificial substrates in in vitro systems (5, 22, 27). In the intact cell, there is also phosphorylation of the β -subunit of the receptor on serine and threonine residues (2) suggesting that in vivo the receptor kinase changes in specificity or, more likely, that other (serine) kinases which are not part of the receptor may act upon the receptor in an insulin-sensitive way. The function of the receptor kinase and autophosphorylation is unclear.

In the present study, we have shown that an insulin-resistant melanoma cell line possesses an altered insulin receptor, which is defective both in its affinity for insulin and its autophosphorylation properties. In the cell-free system, phosphorylation of partially purified receptor from insulin-resistant melanoma cells is $\sim 50\%$ lower than the phosphorylation of receptor from the insulin-sensitive cell lines at maximal insulin concentration. This difference cannot be explained by differences in recovery, differences in immunoprecipitation or the different binding characteristics of the receptor from this cell line. Although insulin-receptor affinity is decreased by $\sim 30\%$, decreased binding is seen only at low insulin concentrations; at saturating insulin concentrations the number of occupied receptors per milligram of protein is equal in all three cell types. Even at saturating insulin concentrations, however, phosphorylation is reduced $\sim 50\%$.

The view that the reduced phosphorylation is not caused by a lower receptor number is also supported by two other types of experimental evidence. In the experiment shown in Fig. 5, receptors from down-regulated type 46 cells, which show $\sim 50\%$ reduction in number were used. Phosphorylation of the insulin receptor of the down-regulated 46 cells was lower than in the 95,000- M_r band of 1A, but was still higher than that of the β -subunit of type 111 cells. Thus, it seems likely that the decreased phosphorylation observed with the insulin-resistant cell 111 is a reflection of a defect in the receptor itself.

Peptide mapping of the phosphorylated receptor reveals three sites of autophosphorylation in the wild-type and insulin-sensitive cells, whereas in the insulin-resistant mutant one is missing. This would suggest a defect in the receptor as a substrate for autophosphorylation rather than as a kinase. This is supported by a preliminary observation that the insulin receptor from 111 cells is as active as receptor from the wild type in catalyzing phosphorylation of an exogenous substrate such as histone (White, M. F., and C. R. Kahn; unpublished observation). Whether there is actually a modification in sequence of the receptor or in some other factor that alters accessibility of site 2 remains to be determined. It is also possible that there is microheterogeneity of receptors, and that the lack of site 2 reflects a loss of a particular receptor form.

Recent studies with epidermal growth factor (EGF) (28–30), platelet-derived growth factor (PDGF) (31, 32) and RNA-

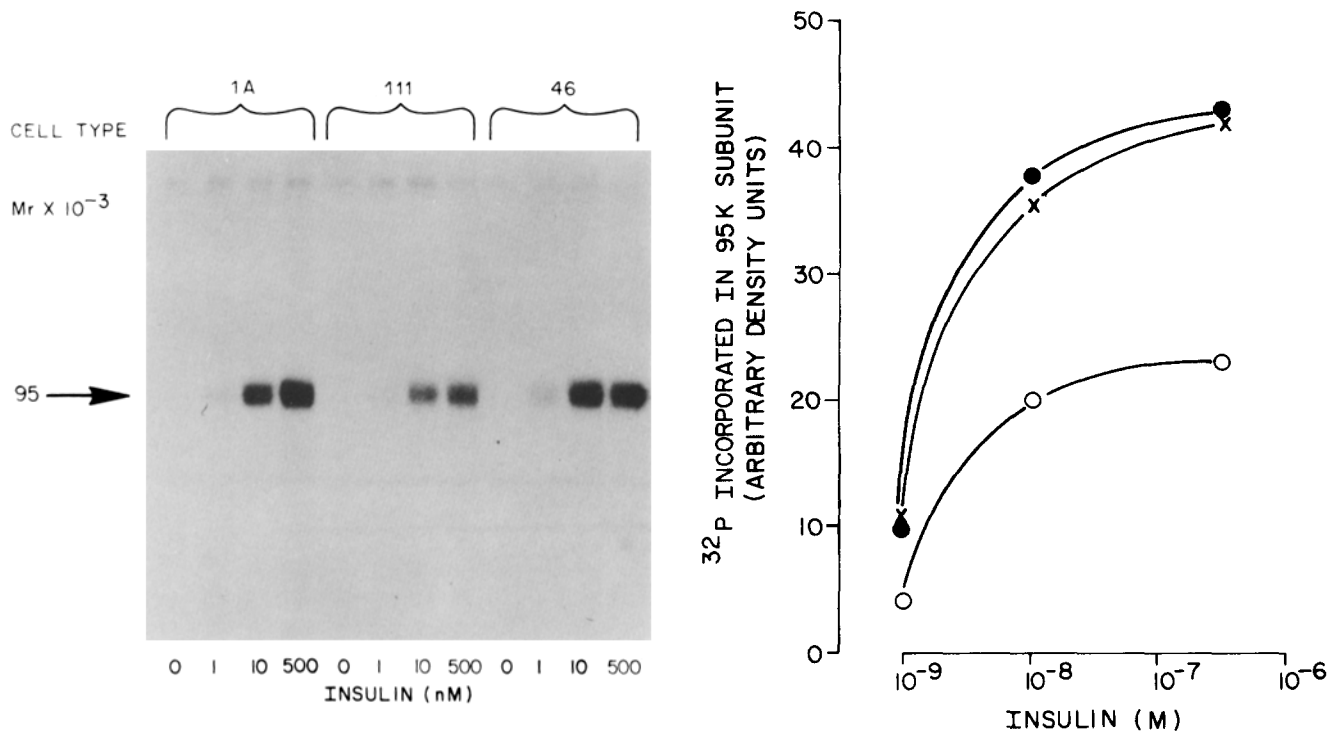


FIGURE 7 (left) Autoradiogram showing the dose response of the insulin effect on incorporation of ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ into the β -subunit of the insulin receptor in a cell free system. Insulin receptors were partially purified from wild-type melanoma 1A cells (four left lanes), from cell type 111 (four middle lanes), and from cell type 46 (four right lanes). Receptors ($\sim 20 \mu\text{g}$) were preincubated for 1 h at room temperature with insulin in the concentrations indicated for each lane in the figure. The phosphorylation assay was performed as described in Materials and Methods with $\sim 15\text{--}20 \mu\text{g}$ protein and 0.1 mCi $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ per lane at 4°C for 60 min. Immunoprecipitation was performed with antireceptor antibody containing serum (1:300). The immunoprecipitates were reduced with 0.1 M dithiothreitol and analyzed in $\text{NaDodSO}_4\text{-7.5\% PAGE}$. (right) Dose response of insulin action on phosphorylation of the insulin receptor 95,000-M_r subunit from wild-type 1A (●), type 111 (○), and type 46 (×) as determined by densitometry scanning in arbitrary units.

TABLE I
Comparison of Binding and Autophosphorylation
Characteristics in the Three Melanoma Cell Lines

Cell line	Tracer binding	Receptor affinity	Receptor capacity	Receptor phosphorylation
	%	%	%	%
1A	100	100	100	100
111	63	79	90	49 ± 7
46	125	156	84	137 ± 18

The binding values are derived from the data shown in Figs. 2 and 3 and represent the mean of three experiments. The phosphorylation data are the mean of seven *in vitro* experiments using four separate receptor preparations. All data were expressed as the percent of control observed in the parental cell line (1A) which was taken as 100%. The values of phosphorylation are expressed as the mean \pm SEM.

tumor viruses (33–38) suggest that phosphorylation of proteins on tyrosine is associated with the regulation of cell proliferation. Both EGF and PDGF stimulate tyrosine-directed phosphorylation of their own plasma membrane receptors. Both growth factors have also been shown to stimulate the phosphorylation of exogenous substrates via their receptor-associated kinases. In the case of EGF, a series of mutants of the A431 cells in which the effect of EGF on growth is altered have been studied to determine if there were also altered hormone effect on phosphorylation of the receptor (39). Unlike our study, however, all of the variants that showed decreased phosphorylation also exhibited a comparable decrease in receptor number.

Insulin has been shown to be a growth-regulating hormone

in a number of cell types (40–43). The observation that insulin, like EGF and PDGF, acts on tyrosine phosphorylation is consistent with the concept that tyrosine-specific kinases may be important in regulation of cell growth. The coincidence of a defect in insulin-receptor phosphorylation with a loss of the effect of insulin on growth in the 111 variant further supports this concept.

Another result of the present study is the finding that the reduced receptor phosphorylation present in 111 cells is associated with a reduced receptor affinity. Whether this is a result of the same modification of the receptor that results in altered phosphorylation activity is not clear. Certainly, it is also possible that autophosphorylation may play a role in modulation of the binding activity of the receptor affinity. The data in the melanoma cells would suggest that receptors in a high autophosphorylation state also possess a high affinity for hormone. This hypothesis is also supported indirectly from studies in adipocytes and hepatocytes (43–46). In these cells, the rate of insulin dissociation decreases within seconds after insulin is bound to the cell. This rapid change appears to be independent of internalization and results from a change in the receptor from a low to a high affinity state. This change of affinity does not occur in ATP-depleted cells (46), and it is possible that insulin-induced autophosphorylation is the underlying process of this insulin-induced change from a low to a high affinity state of the receptor.

Another point worth noting is the difference between the *in vitro* and *in vivo* studies. The phosphorylation defect of the receptor of type 111 can be clearly demonstrated in the

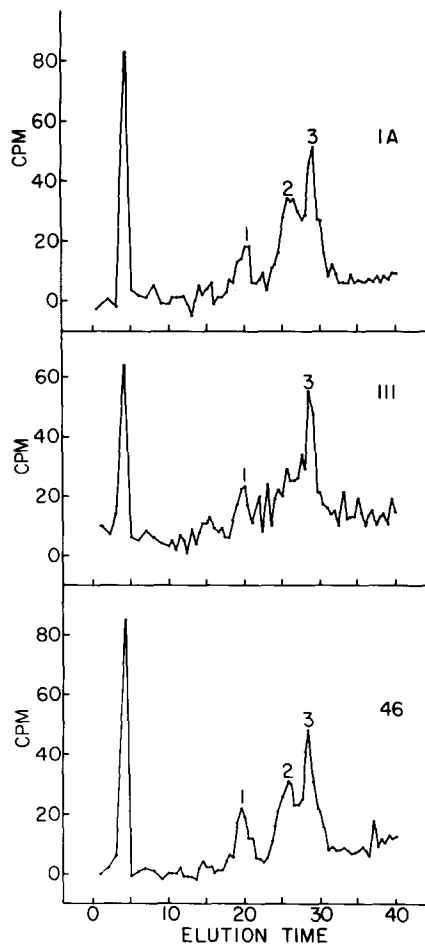


FIGURE 8 Trypsin digestion of the β -subunit of the insulin receptor obtained from three melanoma cell lines. Partially purified insulin receptor (24 μ g) was phosphorylated at 22°C during a 10-min interval in a mixture (500 μ l) containing 100 nM insulin, 10 μ M [γ - 32 P]ATP, and 5 mM Mn^{2+} . The reaction was stopped, the receptor was immunoprecipitated, and the β -subunit was separated by NaDodSO₄ PAGE under reducing conditions. The polyacrylamide gel fragments containing the β -subunit were identified by autoradiography, excised, and incubated with trypsin for 24 h as described in Materials and Methods. The phosphopeptides obtained from each cell type (1A top; 111 middle; 46 bottom) were separated by reversed-phase HPLC on a μ Bondapak C₁₈ column. The experiment shown is one of two, both of which showed a decrease of material in the peak 2 region.

in vitro system, whereas its demonstration in the intact cell was more difficult to interpret because of the low intensity of the 95,000- M_r band. In two experiments with the intact 111 cells in which a double immunoprecipitation was performed to reduce background, there was no detectable insulin effect consistent with an abnormality of receptor phosphorylation in this cell line. Phosphorylation of the receptor in intact cells involves both a tyrosine kinase (the receptor) and an unknown serine kinase (2). The latter is not present on the in vitro system (3) and, therefore, a defect in the intrinsic insulin receptor kinase could be more or less apparent in the intact cell than in the partially purified system depending on the activity of this serine kinase.

In summary, the insulin-resistant melanoma 111 contains a mutant insulin receptor, which is altered in both its auto-phosphorylation and insulin binding activity. The data of this study support the concept that receptor phosphorylation is

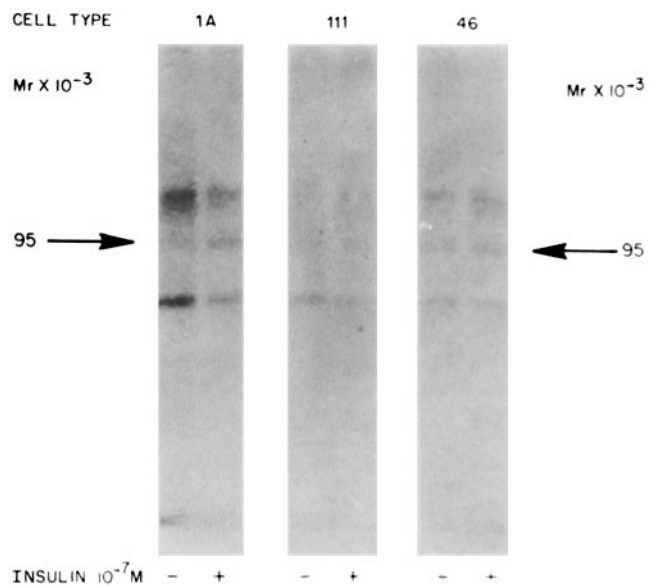


FIGURE 9 Autoradiogram showing the ^{32}P labeling of the β -subunit of the insulin receptor in intact melanoma cells. Melanoma cells labeled with [32 P]orthophosphate for 7 h and half of the dishes were incubated with 5×10^{-7} M insulin for another 3 min at 37°C. Cells were solubilized and the insulin receptor were partially purified on wheat germ agglutinin-agarose. Immunoprecipitation was performed first with pooled human control serum followed by immunoprecipitation of the supernatant with serum containing antireceptor antibody in a 1:300 final dilution. The immunoprecipitates were reduced with 0.1 M dithiothreitol and analyzed in NaDodSO₄ PAGE. Autoradiography was performed for 10 d. The two left lanes show the data of the melanoma wild-type 1A, the two middle lanes show the data of the insulin-resistant variant 111, and the two right lanes show the insulin-stimulated variant 46. The second lane of each panel shows the effect of insulin.

involved in insulin signal transmission. Furthermore, the data suggest the possibility that receptor phosphorylation also may influence the binding affinity of the insulin receptor.

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