Insulin Receptor of Human Cerebral Gliomas

Structure and Function

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Abstract

The insulin receptor from human brain tumors of glial origin was examined for the first time using intact cells (from an established cultured human glioblastoma cell line) and partially purified solubilized membranes (from cultured cells and freshly isolated human brain tumors). The structure of the glial insulin receptor subunits was assessed by affinity cross-linking of ^{125}I-insulin with the α-subunit of the receptor, neuraminidase treatment of the cross-linked receptor, behavior of the receptor on lectin columns, and electrophoretic mobility of the phosphorylated β-subunit. The functions of the insulin receptor were examined by measuring specific ^{125}I-insulin binding (receptor concentration, affinity, specificity, pH-, time-, and temperature dependence), insulin-induced down-regulation of the receptor, insulin-stimulated autophosphorylation of the β-subunit, and phosphorylation of exogenous substrates as well as insulin-stimulated glucose uptake in glioblastoma cells. All of these properties were typical for the insulin receptor from target tissues for insulin action. The insulin receptor of the normal human brain showed the altered electrophoretic mobility and lack of neuraminidase sensitivity of its α-subunit previously reported for the rat brain receptor. There was no difference, however, in the functions of the receptor subunits (binding, phosphorylation) from the normal brain tissue and the eight human gliomal tumors. Since the glial elements compose a majority of the brain cells, the "normal" structure and function of their insulin receptor might provide a key to understanding the role of insulin in the carbohydrate metabolism of the human central nervous system.

Introduction

Traditionally, the central nervous system has not been considered a target tissue for insulin action. A major difficulty with most of the previous studies has been the use of preparations from brain containing heterogeneous cell populations. Since the various components of the whole brain homogenates, brain slices, or membranes (neuronal, glial, and vascular) possess different metabolic and functional properties, it is difficult to dissect the role of insulin in the glucose metabolism of any specific central nervous component. A regulatory role of insulin during fetal brain growth and development (1, 2) as well as its role in neurotransmission has been postulated (3). Despite the presence of the blood–brain barrier, insulin has been identified within the central nervous system (4). This finding, coupled with the presence of specific insulin receptors in the various components of the rat, rabbit, mouse, and neonatal human brain (1, 2, 5, 6), suggests a function of this hormone in the mammalian central nervous system.

Extensive studies have compared the properties of "peripheral" (classic target tissues for insulin action) and "central" (brain) insulin receptors. Functional properties of the α-subunit of the insulin receptor, such as the binding kinetic parameters, pH optima, time, and temperature dependence, and specificity of insulin binding, are indistinguishable from the peripheral receptors (1, 2, 5, 7). In contrast, other properties of adult rodent brain receptor, such as its apparent inability to down-regulate after exposure to high insulin concentration (7, 8), differ from those of peripheral insulin receptors. The structure of the receptor's α-subunit in rat brain differs from that of other tissues: the electrophoretic mobility of the α-subunit covalently labeled with ^{125}I-insulin is consistently more rapid ($M_r \sim 115{,}000$ vs. 130,000–135,000 for the peripheral receptor) (9–12). In addition, neuraminidase treatment, removing sialic acid residues, failed to alter the electrophoretic properties of the α-subunit of the brain receptor while significantly decreasing the apparent molecular weight of the α-subunit of the peripheral receptors (10, 11), suggesting a difference of glycosylation of the two types of insulin receptors. The other, β-subunit of the insulin receptor has also been studied in the rat brain and found to possess the usual functional (insulin-stimulated autophosphorylation and tyrosine kinase activity) properties (9, 13). Structural studies of the β-subunit of the brain receptor revealed either a slightly lower (10, 13) or identical (9) apparent molecular weight (M_r) when compared with nonneural tissues.

Heterogeneity of tissues studied makes it difficult to identify the precise role of any particular component of the central nervous system in insulin action. In an attempt to address this question, Clarke et al. (14) demonstrated specific insulin receptors and insulin-stimulated 2-deoxy-D-glucose uptake by cultured neonatal glial cells from rat brain. Since insulin did not stimulate this uptake in cultures of neuronal cells, a functional difference between the glial and neuronal insulin receptors was suggested.

Several growth factor and hormone receptors possessing tyrosine-specific protein kinase activity are thought to play a role in cell transformation or differentiation. Indeed, Libermann et al. (15) reported that in 20 samples of human brain tumors, expression of the epidermal growth factor (EGF)[1] was increased compared with control specimens. In a follow-up study (16),

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1. *Abbreviations used in this paper:* DSS, disuccinimidyl suberate; EGF, epidermal growth factor; hGH, human growth hormone; IGF-1, insulin-like growth factor-1; KRP, Krebs-Ringer phosphate buffer; PMSF, phenylmethyl-sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

they found that 4 out of 10 primary human brain tumors of glial origin with increased expression of EGF receptors also showed amplification of the EGF receptor gene.

In the present study, we analyzed for the first time the structural and functional characteristics of the insulin receptors from cultured human glioblastoma cells as well as from freshly isolated human brain tumors of glial origin. Our results show that all properties of the human nonneuronal insulin receptors mimic those of peripheral tissues. All gliomal tumors also express the "peripheral" insulin receptors; in this small group of patients we could not correlate the receptor activity with the tumor grade. In addition, the unique features of the insulin receptor from the normal human brain, predicted from the rodent model, were identified.

Methods

Cells

Cells of the cultured human glioblastoma cell line (S., established March 1981, from a 62-yr-old male with left paraoccipital glioblastoma) were obtained from the Surgical Neurology Branch, National Institute of Neurological and Communicative Disorders and Strokes, National Institutes of Health. Samples of freshly isolated human glial cerebral tumors were obtained from the operating room and processed immediately. Tissue was homogenized in a 0.25 M sucrose buffer in presence of protease inhibitors (17). The 550 *g* supernatant was centrifuged at 190,000 *g* for 90 min. The pellet was resuspended in the binding buffer and used as "crude membranes" as described below. Aliquots of the preparations were solubilized by 1% Triton X-100 (New England Nuclear, Boston, MA) and spun at 120,000 *g* for 45 min. Portions of the solubilized membranes were used for some studies, and the remainder was then processed by wheat-germ agglutinin coupled to agarose chromatography (18). The solubilized, lectin-purified samples were used for both the binding and phosphorylation experiments. Protein content of the preparations was determined by the Bio-Rad method (Bio-Rad Laboratories, Richmond, CA).

^{125}I-insulin binding studies

Cells of the cultured human glioblastoma cell line were harvested, washed three times, and resuspended in the binding buffer (NIH Media Unit) at a concentration of 1×10^7 cells/ml. 400 μl of the suspension was incubated with 0.1 ng/ml ^{125}I-insulin (monoiodinated, tyrosine A-14, sp act 370 mCi/mg; New England Nuclear, Boston, MA) in the absence or presence of various concentrations of unlabeled insulin (Eli Lilly & Co., Indianapolis, IN) at 15°C for 3 h (19). Nonspecific insulin binding, assessed in the presence of excess insulin (100 μg/ml), represented <1% of the total tracer binding. Cell viability, assessed by exclusion of trypan blue dye, was always at least 95%.

^{125}I-insulin binding studies with crude membranes from the freshly isolated tumors were performed in an analogous fashion except that 50 μl of the resuspended membranes (100–500 μg protein) was used. Insulin binding was then performed with the Triton X-100 solubilized membranes as well as solubilized, lectin-purified preparations to account for the insulin receptors at each step of preparation. 20 μl of the preparations (~1,800 μg protein/ml) was used for the 4°C, 16-h binding assays (18). Briefly, lectin column eluates were incubated with tracer amount of ^{125}I-insulin and increasing amounts of unlabeled insulin (0–10 μg/ml) in a total volume of 200 μl in 50 mM Hepes, 10 mM Mg_2SO_4, and 0.1% bovine serum albumin, pH 7.5. The final concentration of Triton X-100 was 0.1%. Receptor-bound insulin was separated from free insulin by precipitation with polyethylene glycol (final concentration, 12.5%) in carrier human γ-globulin (final concentration, 0.05%). On average, nonspecific binding represented 41% (range, 32–49%) of total binding. When we compared the insulin binding results with the crude, solubilized, and wheat-germ agglutinin–purified preparations, we could account for practically all specific insulin binding sites (range, 69–116%). Specific activities of insulin binding (expressed per microgram protein) of the various brain tumor membrane preparations revealed, on average, 52-fold purification (range, 38–77-fold) when the lectin-purified and crude membranes were compared.

^{125}I-EGF (Collaborative Research, Lexington, MA; sp act 212 μCi/μg) binding studies were carried out for 3 h at 15°C in the absence and presence of excess unlabeled EGF (10^{-7} M). Specific ^{125}I-labeled human growth hormone (hGH) (sp act 26 μCi/μg; gift of Dr. K. Asakawa, Diabetes Branch, National Institutes of Health) binding at 30°C for 90 min was measured with and without 1 μg/ml unlabeled hGH. Down-regulation studies were carried out as described in (20). Briefly, cells were preincubated with unlabeled insulin at 37°C for 3 h. After extensive washing employing the optimal procedure (21), ^{125}I-insulin binding studies were done at 15°C for 3 h.

Phosphorylation studies

Endogenous substrates. The lectin-purified, solubilized extracts from either the cultured glioblastoma cell lines or freshly isolated human cerebral gliomas were preincubated in the absence or presence of insulin for 30 min at 22°C. Phosphorylation reaction was carried out for 10 min at 22°C as previously described (17). The phosphorylated β-subunit of the insulin receptor was identified by immunoprecipitation with human anti–receptor antibody (B-10, 1:100) and protein A (Calbiochem-Behring Corp., La Jolla, CA), followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (17, 22, 23) and autoradiography (X-Omat AR Film; Eastman Kodak Co., Rochester, NY).

Artificial substrates. Aliquots of the partially purified insulin receptor were preincubated without or with insulin for 30 min at 22°C. The phosphorylation of casein or poly ($GluNa^{80}Tyr^{20}$) (both from Sigma Chemical Co., St. Louis, MO) in the presence of $MgCl_2$, ATP (Sigma Chemical Co.), and (γ-^{32}P) ATP (New England Nuclear) was carried out exactly as described in (17). Endogenous phosphorylation (measured in the absence of artificial substrates) was always <10% and was subtracted from total to assess phosphorylation of the exogenous substrates. When either the crude membranes or Triton X-100 solubilized membranes were used for the phosphorylation studies, incorporation of ^{32}P into the exogenous substrates in the basal state was too high to allow reliable quantification of the insulin effect.

Cross-linking of ^{125}I-insulin to insulin receptors

Cross-linking was performed using a modification of the method of Pilch and Czech (24). Human brain and glioblastoma membranes were prepared as described above. Membranes (final concentration 2.5 mg/ml) and ^{125}I-insulin (final concentration 10 ng/ml) were incubated overnight at 4°C. Incubations were terminated by centrifugation at 20,000 *g* at 4°C. Membrane pellets were resuspended and washed twice in bovine serum albumin-free Krebs-Ringer phosphate buffer (KRP), pH 7.8. Crosslinking was performed in KRP, pH 7.8, in the presence of 0.1 mM disuccinimidyl suberate (DSS) (Pierce Chemical Co., Rockford, IL) for 16 min on ice and was terminated by 100 mM Tris with 10 mM EDTA, pH 7.4. Pellets were resuspended in 50 mM Hepes, pH 7.6, and solubilized by stirring on ice for 3 h with 1% Triton X-100 with 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were ultracentrifuged at 180,000 *g* for 30 min at 4°C. Anti–insulin receptor antibody (B-10; 1:100) was used for immunoprecipitation as described previously (17). The cross-linked receptors were subjected to SDS-PAGE under reducing conditions according to Laemmli (23). The acrylamide concentration of the resolving gel was 7.5%, while that of the stacking gel was 5%.

Neuraminidase digestion of cross-linked receptors

Receptors from normal human brain, glioblastoma, and human erythrocytes were cross-linked to ^{125}I-insulin as described above. Neuraminidase digestion was performed using a modification of the method of Hendricks et al. (11). Before solubilization, the pellets were resuspended in 1 ml 5 mM 2(*N*-morpholino) ethanesulfonic acid and 1 mM $CaCl_2$ in the presence or absence of 2.5 units of neuraminidase (type VII) from *Clostridium perfringens* (Sigma Chemical Co.). Digestion was carried out at 37°C for 30 min in the absence of protease inhibitors for the brain

preparation but in the presence of 2 mM PMSF, 10 μg/ml leupeptin, 0.5 U/ml α_2-macroglobulin, 10 μg/ml pepstatin A, and 1 mM *N*-ethylmaleimide (Sigma Chemical Co.) for the erythrocyte and glioblastoma preparations. The pellets were washed once in KRP, pH 7.8, and solubilized, immunoprecipitated, and subjected to SDS-PAGE.

Cross-linking of ^{125}I-insulin to the partially purified insulin receptor

Solubilized, wheat-germ agglutinin-purified membranes were prepared as above. About 100 μg of protein was used in each tube. Samples were preincubated with ^{125}I-insulin in the absence or presence of excess unlabeled insulin (100 μg/ml) at 4°C for 16 h. To separate the bound from free insulin, samples were run on G-25M Sephadex columns (Pharmacia Fine Chemicals, Uppsala, Sweden) and 1-ml fractions collected. Fractions with the highest radioactivity (usually No. 5) were used for the cross-linking studies in presence of 0.1 mM DSS as described above. After immunoprecipitation with anti-receptor antibody, SDS-PAGE and autoradiography were used to estimate the apparent molecular weight of the cross-linked α-subunit of the insulin receptor.

Degradation of ^{125}I-insulin by the human glioblastoma cells

Degradation of ^{125}I-insulin by the cultured glioblastoma cells was assessed as described previously (19). Briefly, tracer degradation by both the incubation medium and by the cells was measured by TCA precipitability after a 3-h incubation at 15°C. Less than 5% of ^{125}I-insulin was degraded by the medium after the 3-h incubation. About 7% of the tracer was degraded by the cells after the 3-h incubation at 15°C.

Insulin-stimulated glucose uptake

The uptake of D-glucose was studied using cultured human glioblastoma cells (cell line S.). Cells, at 2×10^5/well, were suspended in Dulbecco's minimal essential medium containing 10% fetal bovine serum and glutamine, plated, and grown for 5 d at 37°C in humidified atmosphere (5% CO_2). The cells were washed twice with Eagle's minimal essential medium and incubated with it for 1 h at 37°C. This was followed by two washes with phosphate buffered saline (PBS), pH 7.4, at 37°C, and incubation with or without insulin for 15 min. 0.5 mM D-glucose and 1 μCi of D-[^{14}C(U)]-glucose (New England Nuclear; sp act 341.2 mCi/mmol) were added for 5 min at 37°C. The reaction was terminated by 2 ml of ice cold PBS, pH 7.4. Cells were washed three more times with PBS and digested with 0.5 ml of 0.1% SDS. The digest was aliquoted for protein determination and liquid scintillation counting. All assays were done in quadruplicates.

Results

Characterization of the structure and function of the insulin receptor from the human glial tumors was carried out using two approaches: first, cells of established high-grade cultured glioblastoma lines were used; and second, tissue from freshly resected human cerebral glial tumors was utilized.

Specific ^{125}I-insulin binding. Studies of ^{125}I-insulin binding to intact cells of the cultured human glioblastoma line revealed characteristics typical of those obtained with classic target tissues with regard to time-, temperature-, and pH-dependence. Results of the insulin binding studies are shown in Fig. 1 where competition-inhibition studies were carried out for 3 h at 15°C with various concentrations of unlabeled insulin in the presence of 0.1 ng/ml ^{125}I-insulin. 2.3 ng/ml of insulin inhibited ^{125}I-insulin binding by 50% (ID_{50}). Scatchard analysis (25) of the binding data revealed a curvilinear plot, typical of the insulin receptor from other tissues (Fig. 1). Specificity of the insulin receptor binding to intact cells or crude membranes from the cultured glioblastoma cells was assessed measuring inhibition of ^{125}I-insulin binding by various related or unrelated peptides. Hormones

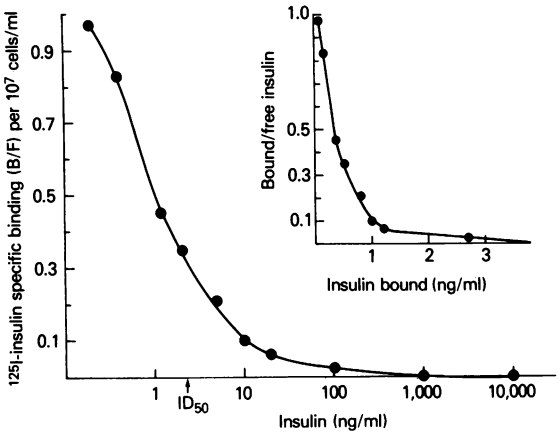

Figure 1. Specific ^{125}I-insulin binding to cultured human glioblastoma cells. Cells of an established human glioblastoma-derived line were harvested and specific ^{125}I-insulin binding studies done as outlined in Methods. Shown is a competition-inhibition curve for ^{125}I-insulin binding at 15°C for 180 min (steady state). ID_{50} (2.3 ng/ml) refers to concentration of unlabeled insulin inhibiting ^{125}I-insulin binding by 50%. Scatchard analysis of the binding data is shown in the insert. Results plotted are means of three separate studies.

related to insulin (e.g., proinsulin or insulin-like growth factor-1 [IGF-1]) did compete with ^{125}I-insulin binding, while unrelated peptides (e.g., hGH or EGF) were ineffective.

Insulin binding studies were also done with crude membranes and solubilized, wheat-germ agglutinin-purified membranes from the glioblastoma cells (Fig. 2). In contrast with the report of Heidenreich et al. (10), all specific insulin binding activity was recovered in the lectin-column eluate; no significant insulin binding was measured in the flow-through of the wheat germ agglutinin column. Mean insulin concentration required to inhibit 50% of ^{125}I-insulin binding was 3.7 ng/ml for the solubilized, lectin-purified preparations from the cultured glioblastoma cells.

We next examined insulin binding to the insulin receptor from freshly obtained cerebral gliomas from eight male patients.

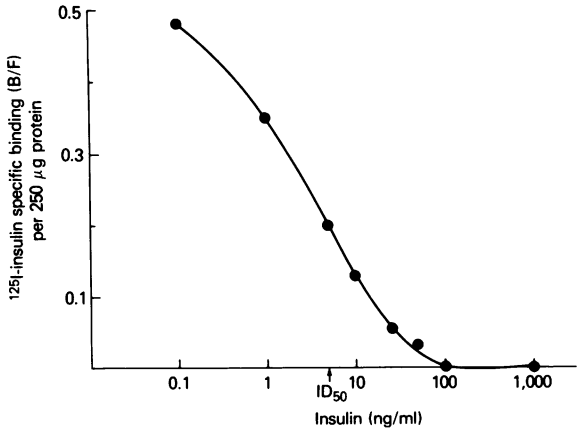

Figure 2. Specific ^{125}I-insulin binding to solubilized, lectin-purified membranes of the cultured human glioblastoma cell line. Results are expressed as specific ^{125}I-insulin binding per 250 μg protein as a function of insulin concentration. Binding studies were carried out at 4°C for 16 h. Nonspecific binding, as assessed in the presence of 1 μg/ml insulin, represented 20% of the total insulin binding. Results shown are means of three separate studies.

Clinical parameters of the subjects are shown in Table I. One of the patients (No. 4) underwent a frontal lobectomy during the brain surgery; the histologically normal tissue served as a source of "normal brain." Crude membranes were prepared from the gliomal tissues; no attempt was made to separate microvessels from the glia. Specific ^{125}I-insulin binding studies were carried out with these membranes. No clear correlation could be established between the amount of insulin bound per milligram of the membrane protein and the tumor grade, location, or patient's age. Concentration of insulin required for the 50% inhibition of ^{125}I-insulin binding ranged from 1.2 to 9 ng/ml. Results obtained with the normal brain fell within the range obtained with the tumor tissue.

The malignant and normal brain tissue was further processed. Specific ^{125}I-insulin binding was demonstrated with Triton X-100 (1%) solubilized membranes and partially purified (by wheat germ agglutinin coupled to agarose chromatography) receptor preparations (not shown). As in the case of the cultured glioblastoma cells, all specific insulin binding activity was recovered in the lectin column eluates. When expressed per standard amount of protein, the specific ^{125}I-insulin binding to the partially purified insulin receptors from the various tumors was remarkably similar and did not differ from the normal brain. ID_{50} range (1.7 to 5.5 ng/ml of insulin) obtained in the ^{125}I-insulin binding experiments with the partially purified receptors did not differ from the results obtained with the crude membranes.

^{125}I-EGF and ^{125}I-hGH binding. Specific binding of other peptide growth factors to the crude membranes from the gliomal tumors was assessed. ^{125}I-EGF binding was measured in five tumors and the normal brain. Except for patient no. 5, whose tumor had no significant EGF binding, all tissues showed higher levels of EGF than insulin binding. However, there was no correlation between specific ^{125}I-EGF binding capacity and the tumor grade. No specific binding of ^{125}I-hGH was detected in any of the crude membranes.

Regulation of cerebral glial insulin receptors. Studies in the past showed failure of insulin to down-regulate its receptors in rat neuron–enriched cultured brain tissue (7, 8). When cells of the cultured human glioblastoma line were preincubated with various concentrations of insulin at 37°C, and ^{125}I-insulin binding was carried out after extensive washing of the cells, the glial insulin receptors were down-regulated by the ambient insulin concentrations (Fig. 3). Increasing insulin levels during preincubation inhibited subsequent specific ^{125}I-insulin binding. 50% down-regulation occurred after preincubation with ~46 ng/ml insulin. In comparison, the cells of the human U-937 line, which is known for the rapid and sensitive regulation of the insulin receptor (20), was about fivefold more sensitive to insulin regulation (ID_{50} ~ 10 ng/ml).

Table I. Clinical Characteristics of Patients with Brain Gliomas

Patient	Age	Tumor type	Location
1	35	Gliosarcoma	L Frontal
2	50	Glioma	R Temporal
3	60	Glioma	L Frontal
4	75	Glioblastoma	R Frontal
5	5	Juvenile astrocytoma	Hypothalamus
6	61	Glioblastoma	R Frontal
7	15	Glioblastoma	L Frontoparietal
8	37	Glioblastoma	L Temporal

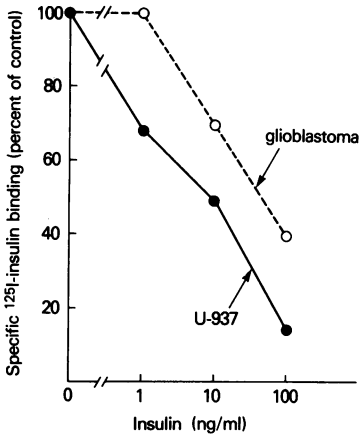

Figure 3. Down-regulation of the insulin receptors of the cultured human glioblastoma cell line. The cells of the glioblastoma and human monocyte-like cell line (U-937) were preincubated in the presence of various concentrations of porcine insulin at 37°C for 3 h. After extensive washing (21), ^{125}I-insulin binding studies were done at 15°C for 3 h. The results are plotted as percentage of ^{125}I-insulin binding to cells preincubated with the culture medium alone. The glioblastoma insulin receptor is down-regulated by the previous exposure to insulin, with 50% inhibition of binding by ~46 ng/ml insulin; ID_{50} for the U-937 cell line was ~10 ng/ml. Data shown are averages of two experiments.

α-Subunit structure. Insulin binding is the presumed function of the α-subunit of the insulin receptor. The rat brain α-subunit, which was identified either by photo-labeling or covalent cross-linking with ^{125}I-insulin, was shown to migrate with an apparently lower molecular weight by electrophoretic criteria than the α-subunits of peripheral insulin receptors (9–12). When we covalently cross-linked, in presence of 0.1 mM DSS, ^{125}I-insulin to the receptor from the cultured human glioblastoma cells or crude membranes derived from these cells, the α-subunit of the glioblastoma receptor had an M_r of 135,000–138,000 in our studies. Excess concentration of unlabeled insulin completely abolished labeling of the insulin receptor on the autoradiographs (results not shown). The α-subunit of the IM-9 insulin receptor, representative of peripheral tissues, showed identical electrophoretic mobility on the same gels.

All of the insulin receptors from the freshly isolated glioma tumors yielded α-subunit with M_r ~ 135,000–140,000 (Fig. 4). Significantly, when membranes from normal brain tissue and from the glioblastoma derived from the brain of the same individual were studied, there was a marked difference in the mobility of the α-subunit of the insulin receptor. The α-subunit of the glioblastoma receptor moved with an M_r ~ 140,000, while that of the normal brain had an M_r ~ 118,000 (Fig. 4), similar to that previously reported with the whole rat brain as well as several regions from the rat brain (9–12). In addition, the electrophoretic mobility of the rat brain insulin receptor subunits was not changed by neuraminidase treatment (10, 11). Therefore, we next assessed the response of normal human brain as well as human glioblastoma receptor to neuraminidase digestion. As shown in Fig. 5, neuraminidase (2.5 U/ml) treatment resulted in a small (from an M_r 136,000 to 132,000) but significant decrease in the M_r of the α-subunit of the glioblastoma receptor. The insulin receptor of a peripheral cell, the erythrocyte, showed identical response to neuraminidase (Fig. 5, *C* and *D*). In contrast, the cross-linked insulin receptor of the normal human brain migrated more rapidly and did not respond to neuraminidase (Fig. 5, *E* and *F*).

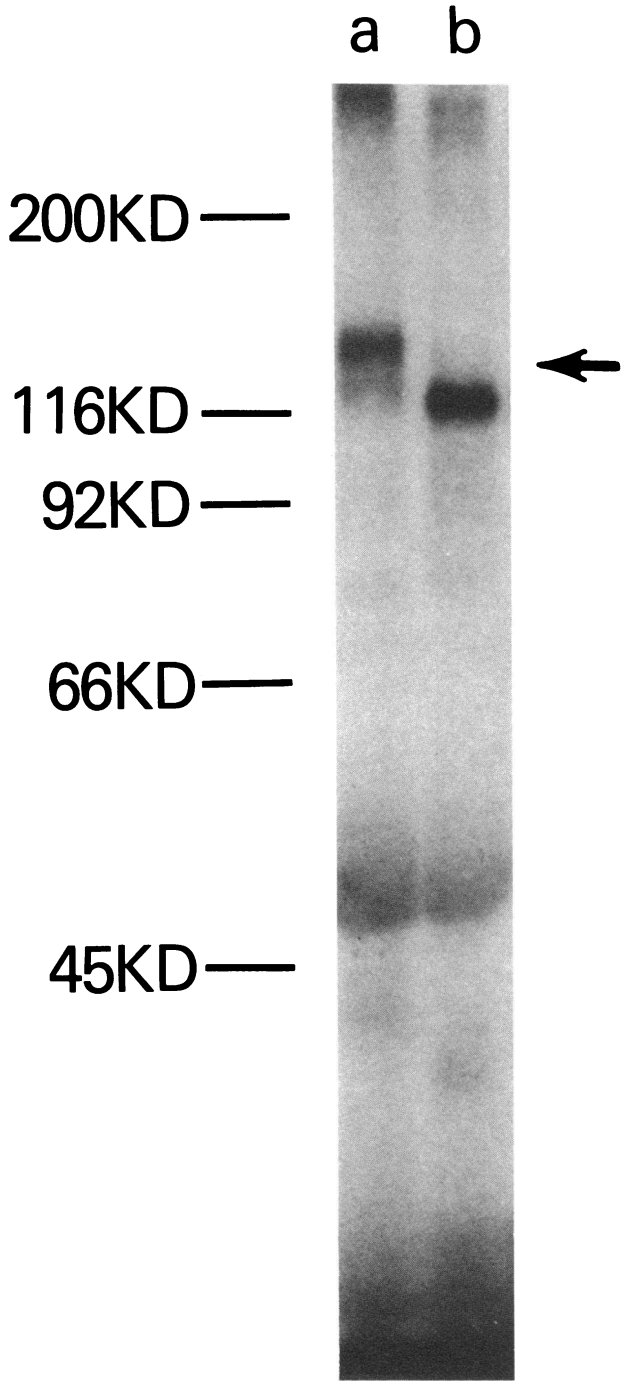

Figure 4. Electrophoretic mobility of the α-subunit of the insulin receptor of the normal human brain and glioblastoma. Crude membranes from freshly resected glioblastoma (case no. 4, lane *a*) and normal brain tissue (case no. 4, lane *b*), both at 3.75 mg/ml protein, were preincubated with ^{125}I-insulin at 4°C for 16 h. Covalent cross-linking of the ligand to the receptor was done in the presence of 0.1 mM DSS. The cross-linked α-subunit of the insulin receptor was identified after immunoprecipitation with anti-receptor antibody (B-10, 1:100), SDS-PAGE, and autoradiography. The arrow points to the region of the α-subunit migration; M_r of the glioblastoma receptor was 140,000, while that of the normal brain was ~118,000. Preincubation of the membranes with excess of unlabeled insulin abolished the labeling of the α-subunit. Immunoprecipitation of the samples with normal human serum did not result in labeling of any bands in the region of the receptor's α-subunit (not shown).

Structure and function of the β-subunit. β-subunit of the insulin receptors from numerous tissues contains tyrosine kinase activity which can be stimulated by insulin (26, 27). In our study insulin significantly stimulated autophosphorylation of the cultured glioblastoma β-subunit (Fig. 6). The time-, temperature-, and insulin concentration-dependence as well as ionic requirements for the optimal activity of the receptor kinase were identical for the glioblastoma preparation to those previously reported with other tissues (17, 22, 28). The apparent molecular weight of the β-subunit of the glioblastoma insulin receptor, at ~95,000, was typical of the receptors of peripheral cells. Insulin-activated receptor kinase is capable of phosphorylating exogenous substrates (27–29). The partially purified glioblastoma receptor also contained insulin-sensitive kinase activity toward artificial substrates. Insulin at 10^{-8} M stimulated phosphorylation of casein ~2.5-fold by this preparation (Fig. 7). In addition, insulin (10^{-8} M) also stimulated phosphorylation of a substrate containing tyrosine as the only phosphorylatable amino acid residue, poly (GluNa^{80}Tyr^{20}), ~3.75-fold in the presence of (γ-^{32}P) ATP and 20 mM Mg^{2+} (Fig. 7). Thus, the human glioblastoma insulin receptor contained functionally and structurally normal β-subunit.

Analogous results were obtained with the partially purified insulin receptor from the cerebral gliomas and normal human brain. Insulin significantly increased autophosphorylation of the receptor's β-subunit as identified by immunoprecipitation with anti-receptor antibody, SDS-PAGE, and autoradiography (Fig. 8). The optimal insulin effect occurred at a concentration of 1 μg/ml for all freshly isolated tissues examined and was approximately equal in magnitude with all receptor preparations. The electrophoretic mobility of the receptor's β-subunit from all tumor or normal brain tissues was identical (M_r ~ 95,000). Insulin-stimulated phosphorylation of exogenous substrates by the partially purified human brain receptor preparations was next assessed. The insulin dose-response was similar to that seen in the autophosphorylation experiments; the optimal stimulation of the receptor kinase activity occurred with 1 μg/ml insulin (Fig. 9). The receptor kinases from the normal brain and the tumors showed the same insulin sensitivity. No insulin-stimulated kinase activity was detected in the lectin column flow-through collection.

The insulin-stimulated tyrosine kinase activities of the partially purified insulin receptors from the normal brain and the patients' gliomas were compared. As we previously discussed (17), the optimal expression of the insulin-stimulated receptor kinase activity involves the insulin-stimulated increment in the enzyme activity expressed per unit of the receptor binding. No significant difference in the receptor tyrosine kinase activity was detected between the normal brain, high- or low-grade tumors from the small number of patients examined [range, 4.4–26.1 insulin-stimulated kinase activity (= picomoles ATP per milligram substrate per 10 min) per B/F].

Insulin-stimulated glucose uptake. Preincubation of cultured human glioblastoma cells with insulin resulted in an increase in [^{14}C]glucose uptake. Insulin at 10^{-7} M stimulated glucose uptake maximally by 45.7±8.2% over the control value after a 15-min preincubation period.

Discussion

Rat brain insulin receptors have been found to possess unique features (9–12). The covalently labeled α-subunit–insulin com-

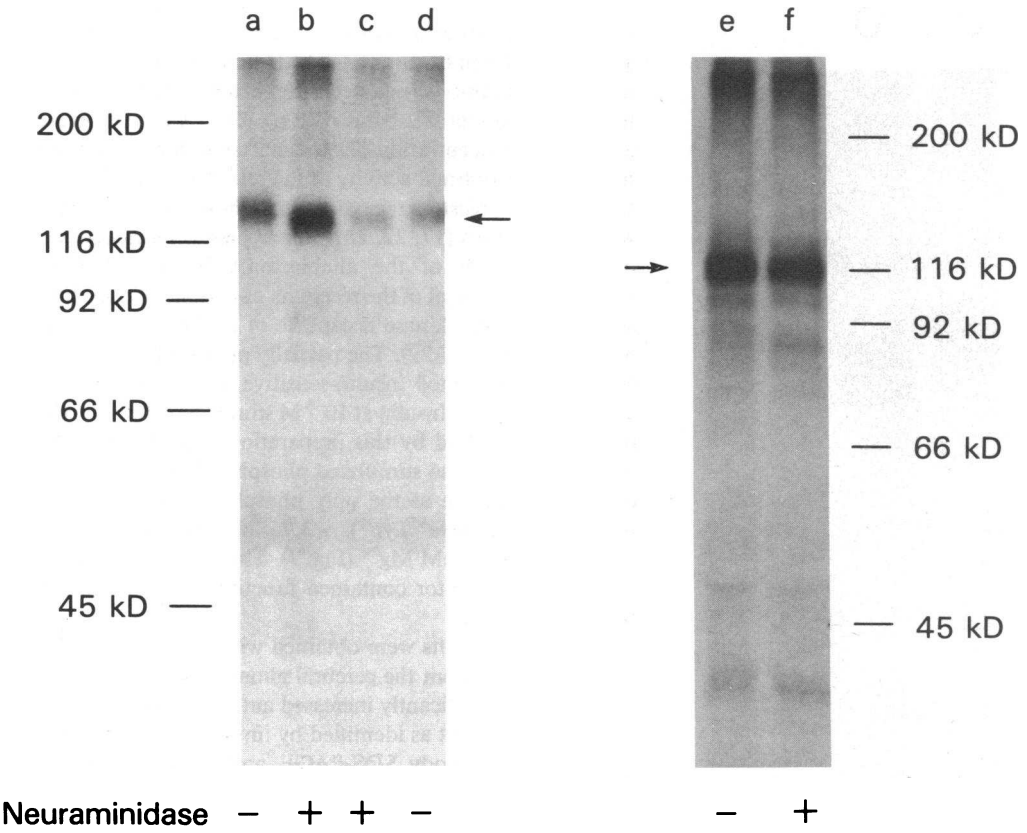

Figure 5. Effect of neuraminidase on the electrophoretic mobility of the insulin receptor of normal human brain, glioblastoma, and erythrocytes. Crude membranes (3.5 mg/ml protein) were preincubated with ^{125}I-insulin and covalent cross-linking done in the presence of 0.1 mM DSS. Neuraminidase (2.5 U/ml) treatment was done in the presence of protease inhibitors (see Methods). After solubilization, immunoprecipitation with anti-receptor antibody (B-10, 1:100) was carried out, followed by SDS-PAGE and autoradiography. Arrows point to the position of α-subunit of the insulin receptor. Lanes *A* and *B*, human glioblastoma; *C* and *D*, human erythrocytes; *E* and *F*, normal human brain. Apparent molecular weights were reduced by the neuraminidase treatment of the glioblastoma (from an M_r of 137,000 to 132,000) and erythrocyte (from an M_r of 136,000 to 132,000) receptor, but not whole brain (unchanged at an M_r of ~118,000) insulin receptor.

plex possessed a more rapid electrophoretic mobility; exposure to neuraminidase failed to alter the electrophoretic properties of the α-subunit of the brain receptors, and whole brain and neuronal insulin receptors did not down-regulate after a chronic exposure to insulin. We tested, with human tissue, the predictions from the rat brain model and extended the studies to the insulin receptors from normal human brain and cerebral gliomas. To avoid the problems inherent in dealing with mixed cell populations, we first studied properties of the insulin receptors from cultured human cells of purely glial origin. The glial elements compose up to 85% of the brain cells (14). We found that all properties of the human glioblastoma insulin receptors were indistinguishable from those of peripheral target tissues. We identified specific insulin receptors with binding parameters (receptor affinity constants, shape of the Scatchard plot, pH-, time-, and temperature-dependence, as well as specificity) typical of peripheral target tissues for insulin. The α-subunit of the glioblastoma insulin receptor (from intact cells, crude membranes, and partially purified receptor) cross-linked with ^{125}I-insulin showed electrophoretic mobility identical to that of peripheral tissues. In addition, the insulin receptor of the glioblastoma clearly down-regulated after a 3-h exposure to as little as 10 ng/ml insulin. The β-subunit of the cultured glioblastoma insulin receptor showed the same apparent molecular weight, insulin-stimulated autophosphorylation, and tyrosine kinase activity towards exogenous substrates as that of peripheral receptors.

Further, we characterized for the first time insulin receptors from human brain glial tumors immediately upon their removal to rule out any effects of the cell culturing per se. Even though no specific method was used to remove the vascular elements from the glial tumor tissue, it is unlikely that the presence of microvessel insulin receptors (30, 31) would affect our conclusions since the results with the cultured and freshly isolated glial tissues were analogous.

We found that all the human glial tumors possessed specific insulin receptors with the typical binding activities and characteristics of the peripheral receptors. The cross-linked α-subunit migrated identically with a peripheral receptor on SDS-PAGE. Carbohydrate moieties of the glial and other peripheral receptors are identical or closely related since all the glial preparations bound to the wheat germ agglutinin columns and were specifically eluted with *N*-acetyl-D-glucosamine. The eluates contained the full binding and insulin-stimulated kinase activities. Neuraminidase treatment decreased the apparent molecular weight

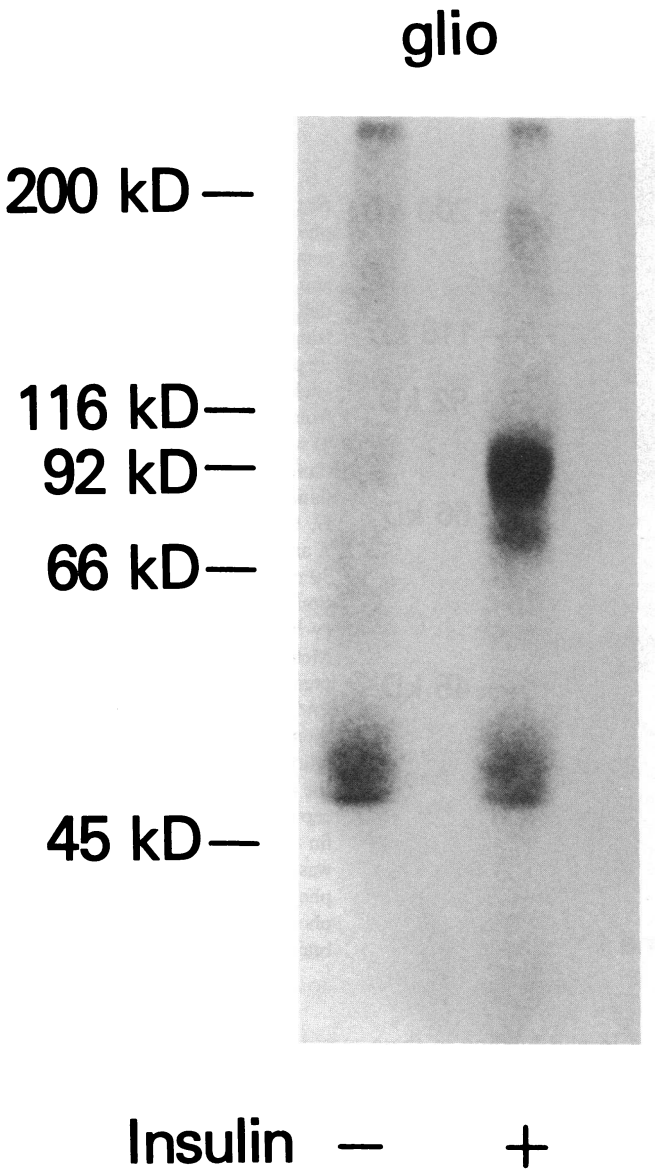

Figure 6. Insulin-stimulated phosphorylation of the cultured glioblastoma (*glio*) insulin receptor. Partially purified insulin receptor from the cultured glioblastoma cell line was preincubated in the absence and presence of 1 μg/ml insulin at 22°C for 30 min. Autophosphorylation experiments were done in the presence of (γ-^{32}P)ATP and Mn^{2+} at 22°C for 10 min. The phosphoproteins were identified by immunoprecipitation with anti-receptor antibody (B-10, 1:100), SDS-PAGE, and autoradiography.

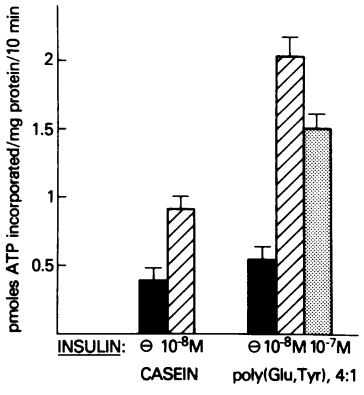

Figure 7. Insulin-stimulated phosphorylation of exogenous substrates by the cultured human glioblastoma insulin receptor. The partially purified glioblastoma insulin receptor was preincubated without or with insulin at 22°C for 30 min. Phosphorylation of exogenous substrates [casein or poly ($GluNa^{80}Tyr^{20}$) = poly (Glu,Tyr), 4:1] was carried out in the presence of (γ-^{32}P) ATP and Mg^{2+} (see Methods) at 22°C. Results are shown as mean of three separate experiments ±1 SD; four individual values of the kinase activity were determined for each condition in every experiment.

of the glial receptor's α-subunit in the same fashion as that of a peripheral receptor.

Significantly, when preparations of the malignant and normal portions of the same lobe from the brain of the same patient were examined, we showed major differences between the two types of tissue in the apparent molecular weight of the α-subunits and their response to neuraminidase. The "whole brain" preparation behaved as predicted by the rat brain model (10, 11): markedly faster electrophoretic mobility and failure to respond to neuraminidase. In contrast, there were no differences seen between the normal human brain and glial tumor tissue in the level of specific insulin binding, receptor affinity, structure of the β-subunit, or its insulin-stimulated kinase activity.

The finding of identical insulin binding activity per unit of protein of the normal and malignant brain tissue despite the marked difference in both the structure of the binding (α) subunit of the insulin receptor deserves a comment. It has been assumed that the lower apparent molecular weight of the brain receptor's α-subunit is due to its decreased or absent neuraminidase-sensitive sialic acid content (10–12). In rat adipocytes removal of sialic acid preserves insulin binding but impairs insulin action (32, 33). We have reported that in the cultured human monocyte-like cell (U-937) and freshly isolated human monocytes the altered apparent molecular weight of the α-subunit did not change insulin binding (34). If the structural differences are due to altered posttranslational glycosylation of the brain and peripheral receptors, these changes might not take place in the binding domain of the α-subunit of the insulin receptor. Alternatively, our methods of assaying the specific insulin binding might not be sensitive enough to detect subtle changes in binding.

One puzzling aspect of the structural studies of the α-subunit remains to be solved. Since the glial elements of the brain comprise the majority of the cells in the whole brain, why do we detect only one, the "central," ^{125}I-insulin–labeled species on the autoradiographs of the cross-linking studies of the receptor from the normal brain? Is it possible that this reflects an inhibitory influence of the neuronal elements on the glial receptors in the in vivo setting or during the preparation of the samples? It is unlikely, from our results, that a major discrepancy in the affinity of the neuronal and glial receptors for insulin could explain the predominance of the "central" type of the receptor's α-subunit.

To investigate metabolic consequences of the presence of the typical insulin receptors on the human glial cells, we measured the effect of insulin on glucose uptake. As demonstrated with the rat neonatal cultured glial cells by Clarke et al. (14), we show here that insulin stimulated glucose uptake by cultured human glioblastoma cells.

We can thus extend the results previously obtained with the rodent insulin receptors to human brain receptors. The predominant cellular component of the brain, the glia, possesses specific insulin receptors with structural and functional properties typical of the target tissues for insulin. Importantly, a metabolic effect

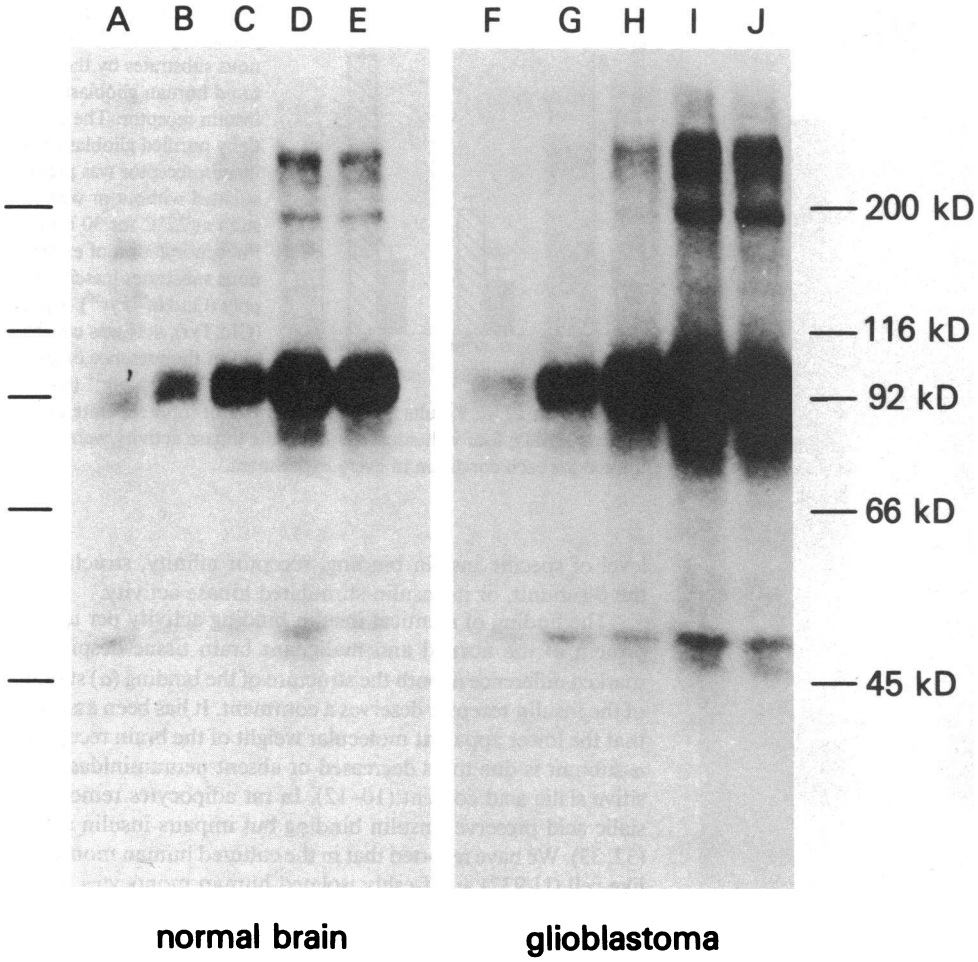

Figure 8. Insulin-stimulated phosphorylation of the insulin receptor from normal human brain and cerebral glioblastoma. Partially purified insulin receptors from normal human brain (27.4 μg protein) and freshly resected glioblastoma (25 μg protein) from the same patient (case no. 4) were preincubated without (lanes *A* and *F*) or with insulin (lanes *B* and *G*, 10 ng/ml; *C* and *H*, 100 ng/ml; *D* and *I*, 1 μg/ml; *E* and *J*, 10 μg/ml) at 22°C for 30 min. Phosphorylation reaction was done in the presence of (γ-^{32}P) ATP and Mn^{2+} (see Methods) for 10 min. Immunoprecipitation (B-10, 1:100), SDS-PAGE and autoradiography were then carried out. Optimal stimulation of autophosphorylation of the β-subunit of the insulin receptor occurred at 1 μg/ml insulin for both preparations. There was no difference in the electrophoretic mobility of the phosphorylated β-subunit of normal brain or tumor insulin receptor.

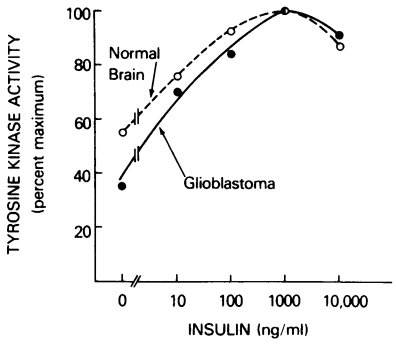

Figure 9. Insulin-stimulated tyrosine kinase activity of the insulin receptor from normal human brain and cerebral glioblastoma. Partially purified insulin receptor preparations of normal brain tissue and glioblastoma from the same subject (case no. 4) were preincubated without or with insulin at 22°C for 30 min (see Methods). Phosphorylation of poly (GluNa^{80}Tyr^{20}) in the presence of (γ-^{32}P)ATP and Mg^{2+} (20 mM) was carried out for various lengths of time up to 20 min at 22°C (reaction remains linear for the initial 30 min). The tyrosine kinase activity in picomoles ATP incorporated per milligram substrate per 10 min was plotted as percent of the maximal values (achieved with 1 μg/ml insulin) for each preparation. There was no significant difference in the shapes of the insulin dose-response curves for receptor preparations from the brain tumors of other patients. Similar qualitative results were obtained with other exogenous substrates, such as casein (not shown).

of insulin binding to these receptors can be measured in the glioblastoma cells.

Crude membrane preparations from glial tumors of our patients contained high specific EGF binding activity, as also reported by Libermann et al. (15). It is possible that expression of tyrosine-kinase–containing growth factor receptors is related to the transformation of brain glial cells. However, we did not find any correlation between the insulin binding (α-subunit function) or tyrosine-kinase activities (β-subunit function) and grade of malignancy of the brain tumors in our small group of patients. No specific hGH binding to the tumor membranes could be measured. hGH, unlike insulin and EGF, does not stimulate autophosphorylation of its receptor or tyrosine kinase activity (35) towards exogenous substrates.

In summary, the insulin receptors of the cultured human glioblastoma cell line and freshly isolated human brain tumors of glial origin are indistinguishable from the typical peripheral receptors of target tissues for insulin. The insulin receptor of the normal human brain differs from the peripheral receptor in the electrophoretic mobility of its α-subunit as well as its failure to respond to neuraminidase treatment, as was predicted by the rodent brain model.

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