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Expression of Epidermal Growth Factor Receptor and Associated Glycoprotein on Cultured Human Brain Tumor Cells

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The expression of epidermal growth factor (EGF-R) in normal glial and glioma cells grown in culture was examined by using several independent assays. Immunoprecipitation with the monoclonal antibody R1 of extracts from metabolically labeled glial and glioma cells revealed a protein of $M_r \sim 170{,}000$, with a migration in sodium dodecyl sulfate-polyacrylamide gels identical to the EGR-R of A431 epidermal carcinoma cells. Furthermore, in the majority of glioma extracts, a protein of $M_r \sim 190{,}000$ was specifically immunoprecipitated by this antibody. Similar results were obtained by immunoblotting with a second antibody directed against a synthetic peptide in the sequence of the v-erb-B oncogene. In cell lines expressing both proteins, each was specifically phosphorylated on tyrosine in immune complex kinase assays. The majority of glioma cells bound between 40,000 to 80,000 ^{125}I-labeled epidermal growth factor molecules per cell. These results suggest that the expression of EGF-R is common in cultured human glioma cells. In addition, a structurally related protein, is expressed in some of these cells.

Key words: epidermal growth factor, brain tumors, cell surface glycoproteins

Epidermal growth factor (EGF) is a mitogenic polypeptide for a variety of cells in vitro and in vivo and has been extensively studied in terms of its biochemistry and

Abbreviations used: EGF-R, epidermal growth factor receptor(s); EGF, epidermal growth factor; DME/F12, Dulbecco's modified minimal essential medium/Ham's F12 medium; PBS, phosphate-buffered saline; DPBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE; polyacrylamide gel electrophoresis; FBS, fetal bovine serum; Hepes, N-Z-hydroxyethylpiperazine-N′-Z-ethanesulfanic acid; BSA, bovine serum albumin. The single-letter amino acid nomenclature follows the rules as stated in J Biol Chem 250:14–42, 1985.

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mode of action [1–4]. The binding of EGF to its target cells involves a specific membrane glycoprotein, epidermal growth factor receptor (EGF-R), and initiates a cascade of events resulting in cellular proliferation. An early response to the binding of EGF is the phosphorylation of a number of endogenous proteins [5,6]. The protein kinase activity has been shown to be associated with the EGF-R glycoprotein itself and to be a cyclic nucleotide-independent tyrosine protein kinase [7–9]. A major substrate of the in vitro phosphorylation appears to be EGF-R as identified by biochemical and immunological procedures. Furthermore, Downward et al [10] have shown sequence homology between EGF-R and the oncogene product v-erb B.

The overexpression of EGF-R has been reported for most squamous cell carcinomas [11], the A431 carcinoma cell line [5], and recently for some primary brain tumors [12]. Libermann et al [12] showed that the expression of EGF-R kinase activity was increased significantly in a number of non-neural brain tumor tissues over that in normal brain specimen without EGF stimulation. Furthermore, a few human malignant glioblastomas were observed to have an amplified and possibly rearranged EGF-R gene [13]. However, previous reports have demonstrated the similar binding of EGF to both human glioma and normal glial cells in culture [14]. To examine the difference in the binding of EGF to cells and the enzymatic activities of EGF-R, we investigated the expression of EGF-R on a number of human glial and glioma cells in culture with several independent assays. We observed the expression of the EGF-R (M_r ~ 170,000) on cells of glial origin. Furthermore, another protein of M_r ~ 190,000 cross-reacted with both antibody preparations. The possible relationship between EGF-R and the M_r 190,000 protein is discussed.

MATERIALS AND METHODS

Cells and Culture Conditions

Cell lines were derived from surgical specimens of primary brain tumors or from brain tissue specimens from patients with unrelated trauma. The glial origin of the cells was assessed by the presence of glial acidic fibrillary protein, DNA content, and morphologic histologic criteria as described [15]. The glioma cell lines (EFC-2, KE, LG, AO_2, MB, CT-3, CT-1, and PL-1) and normal glial cell lines (GB and CDG) were initiated from surgical specimens of human gliomas of different histological grades and of non-neoplastic brain tissues as previously described [15]. Human epidermoid carcinoma $A431_8$ cells were obtained from T. Hunter (Salk Institute, La Jolla, CA). Cells were routinely grown in a mixture of DME/F12 medium (Grand Island Biological Co., Grand Island, NY) containing 10% FBS (Hyclone Lab., Logan, UT) and no antibiotics. Cells were metabolically radiolabeled by incubating the cultures at 37° for 30 min with 1 mCi/ml of [^{3}H]leucine or 100 μCi/ml [^{35}S]methionine in DME/F12 medium, devoid of either leucine or methionine, respectively. Alternatively, the cells were labeled 24 hr with the radioactive precursors at 100 μCi/ml in DME/F12 medium at one tenth the usual concentration of the appropriate amino acid plus 10% FBS.

Immunoprecipitation of EGF-R

The metabolically radiolabeled cells were washed twice with cold PBS, lysed, and homogenized in a detergent-containing buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 100 KIU /ml aprotinin in 20 mM sodium phosphte, pH 7.5). The

lysates were cleared by centrifugation for 10 min at 8,000g, and 5 μl (1 mg/ml) of purified R1 monoclonal antibody R1 [16] was added. After 3 hr 60 μl of formalin-inactivated *Staphylococcus aureus* (Cowan strain) was added, and then precipitates were washed as described by Kessler [17]. The immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [18] by using a 5–15% linear acrylamide gradient running gel. For gels containing ^{3}H-labeled or ^{35}S-labeled samples, the gels were processed by fluorography by treatment with Enhance (New England Nuclear, Boston, MA) and then were dried and exposed to x-ray film.

Autophosphorylation of Immunoprecipitated EGF-R Complexes

The cell extracts were prepared as previously described, except that the lysates were clarified by centrifugation in a Beckman Ti 50 rotor (Beckman Instruments, Fullerton, CA) at 100,000g for 60 min at 4°C. R1 antibody (5 μl) was added to the lysate and the mixture was incubated for 1 hr at 4°C, followed by another 15-min incubation after addition of 10 μl of pansorbin. The immune complex was washed twice with 3 ml of a mixture of 0.1% Triton X-100 and 150 mM NaCl in 10 mM sodium phosphate, pH 7.5, and was collected by centrifugation (8,000g, 10 min). The phosphorylation reaction was initiated by addition of 50 μl of 0.1% Triton X-100, 5 μCi of γ-[^{32}P]ATP (adenosine triphosphate, New England Nuclear, 2,000–3,000 Ci/mmol) and 6 mM $MnCl_2$ in 20 mM Hepes, pH 7.0, to the immunoprecipitated pellet. After 10 min on ice, the reaction was terminated by the addition of 3 ml of lysis buffer. The precipitates were washed again and then subjected to SDS-PAGE analysis as already described.

Western Blotting

Immunoblotting of the proteins was performed by a modification of the procedure as described by Towbin et al [19]. Briefly, equal aliquots (100 μg) of cell extracts were subjected to SDS-PAGE on a 5–15% polyacrylamide gradient gel, after which the proteins were electroblotted from the gel onto 0.1 μm nitrocellulose paper for 12–18 hr at 40 V in 20% ethyl alcohol, 5 mM sodium acetate, and 2 mM Hepes pH 8.2. The nitrocellulose paper was then incubated for 3 hr at 37°C in TNE/NP-40 (0.1% NP-40, 150 mM NaCl, 2 mM EDTA, in 50 mM Tris HCl, pH 7.5) containing 3% BSA. The blocking solution was removed and replaced with new buffer that contained antiserum (1:100 dilution) directed against a synthetic v-erb-B polypeptide (described below). Incubation proceeded for 3 hr at room temperature, followed by five washes with TNE/NP-40. Control immunoblots contained 100 μg synthetic polypeptide along with the antiserum. The filters were then incubated for 3 hr with 0.01 μCi/ml of ^{125}I-goat antirabbit IgG (40 μCi/μg; Amersham, Chicago, IL) in TNE/NP-40 plus 3% BSA. After the incubation, the filters were washed five times with TNE/NP-40 and then dried and exposed to x-ray film with intensifying screens. Densitometric scans of the exposed x-ray film were performed by using a Beckman DU-8 spectrophotometer with gel scan accessory.

The antiserum against a synthetic polypeptide of v-erb-B sequence was generated in a manner similar to the method previously described [20]. A hydrophobic domain of the v-erb-B gene product sequence with sequence homology of EGF-R [10] was chosen by using a computer program (Intelligenetics, Inc.) The amino acid sequence of v-erb B 421-437 (LMEEEDMEDIVDADEYL) was synthesized in a Vega

solid-phase amino acid synthesizer (Vega Biotechnologies, Inc., Tucson, AZ) using standard procedures. An additional cysteine was added to the C-terminus of the peptide and was used to crosslink it to keyhole-limpet hemocyanin [21]. Antiserum was generated in rabbits by using an initial subcutaneous injection of 200 μg of the conjugate in Freund's complete adjuvant given at multiple sites. Two additional injections followed at 3-wk intervals—200 μg of conjugate in Freund's incomplete adjuvant.

Binding of ^{125}I-Labeled EGF to Glioma Cells

Receptor-grade EGF was purchased from Collaborative Research (Boston, MA) and was radiolabeled with ^{125}I (carrier free; ICN, Irvine, CA) as previously described [3]. Specific activity was determined to be 4.92×10^4 cpm/μg.

Binding assays followed the procedures previously described [3]. Briefly, cells were plated into multiwell plates ($0.5–1 \times 10^5$ cells/well) and cultured for 2 days. The medium was then aspirated, and the cells were washed once with DME/F12 followed by incubation an additional 24 hr in DME/F12 and no serum. The dishes were then washed twice in cold DPBS containing 0.2% BSA. To each well was added 1 ml of ^{125}I-labeled EGF at various concentrations in the DPBS/BSA solution, and then the plates were incubated for 1 hr at 4°C. The cultures were then washed five times with DPBS/BSA, the cells were lysed with 1% SDS in 100 mM NaOH, and radioactivity was determined with a Beckman 8000 gamma counter. Nonspecific binding was determined by using a 100-fold excess of unlabeled EGF.

RESULTS

Detection of EGF-R in Cultured Glioma Cells

Initially, to determine whether glioma cells expressed EGF-R, the ability of a monoclonal antibody, R1, to immunoprecipitated this protein was examined. Cells were metabolically radiolabeled with [^{3}H]leucine or [^{35}S]methionine for 30 min to minimize degradation; then EGF-R was immunoprecipitated as described in Materials and Methods. Figure 1 displays the SDS-PAGE profile from several cultured glioma cells. In most of the glial and glioma cells, a protein of M_r 170,000 was observed (Fig. 1, lowest arrow). These bands migrated in the same position as did an immunoprecipitated protein from A431 cells, which had been demonstrated previously to be EGF-R. In addition to the 170,000 protein (p170), a band with a slower migration M_r 190,000 (p190; Fig. 1, top arrow) was observed in most cells of glial origin. The p190 was not observed to be immunoprecipitated in cultured human fibroblasts, colon, breast, or head and neck carcinoma cells (unpublished observation). A more slowly migrating band (M_r 220,000), observed in CT-3 cells was identified in other experiments as fibronectin, which had been previously observed to bind nonspecifically to *S aureus* protein A. In two cell lines, AO_2 and NG-1, no bands were observed at either the 170,000 or the 190,000-dalton molecular weight range. To determine whether this result was due to a slow metabolism rate in these cells, the metabolic labeling period was increased to 24 hr. Under these conditions, a p170 and p190 were observed in AO_2 cells. However, these proteins were not observed in NG-1 cells. Two immunoprecipitated bands were consistently seen in the NG-1 cells (with relative molecular weights of 150,000 and 95,000); these bands migrated with the same relative mobility as did immunoprecipitated bands from A431 cells. Preliminary

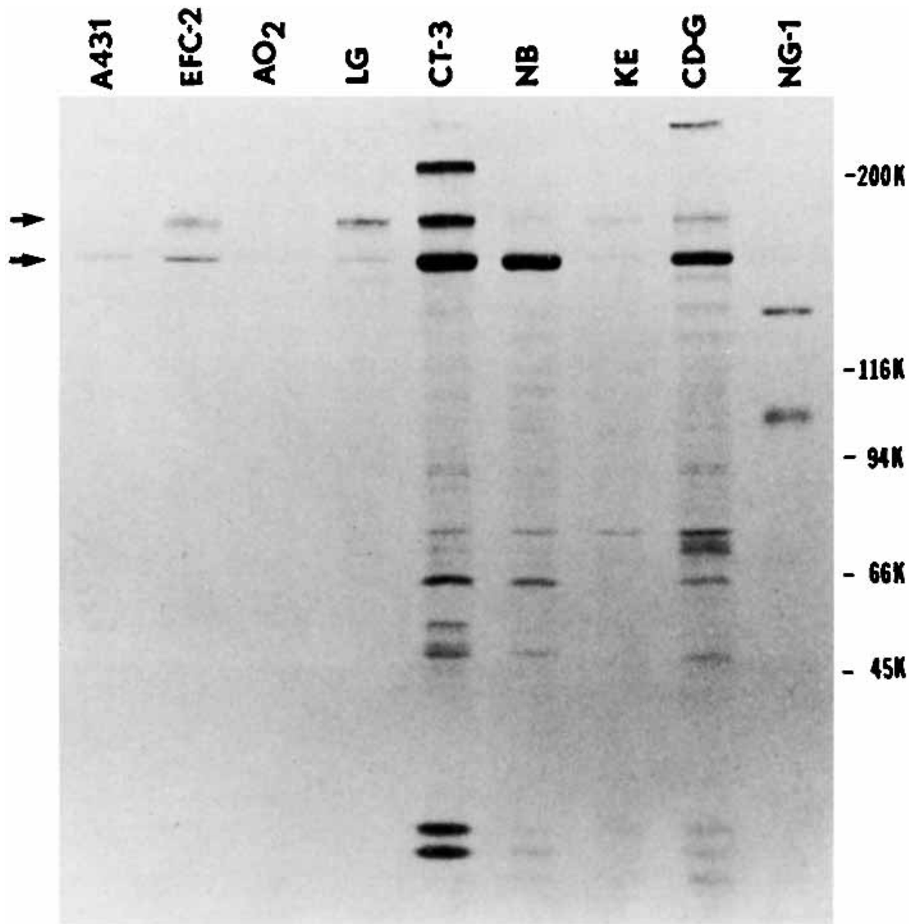

Fig 1. Immunoprecipitations of glioma, glial, and A431 cell extracts of established cultured cells with a monoclonal antibody directed against EGF-R, followed by SDS-PAGE. Immunoprecipitation was performed as described in Materials and Methods. The top arrow is for an observed band at M_r 190,000, and the bottom arrow is directed toward a band at 170,000 (EGF-R). The protein standards include myosin, galactosidase, phosphorylase b, bovine serum albumin, and ovalbumin, with M_rs ($\times 10^3$) of 200, 116, 94, 66, and 45, respectively.

Southern analysis has suggested that NG-1 cells may contain a rearranged EGF-receptor gene, which may account for this result (unpublished observation).

Immunoblotting

Several explanations might account for the presence of the p190 in R1 monoclonal antibody immunoprecipitates of glial and glioma cell extracts. To determine whether the p190 was a coprecipitating band or whether it shared other similar antigenic determinants with EGF-R, proteins from glial and glioma cell extracts were separated by SDS-PAGE and then were electroblotted onto nitrocellulose paper. Proteins were reacted with an antiserum generated against a synthetic peptide with sequence homology to v-erb B and then with ^{125}I-labeled *S aureus* protein A as described in Materials and Methods. An example of the reactivity with KE cell

extracts is shown in Figure 2. Both a p170 and p190 are observed. In contrast, when the specific reactivity of the antiserum is blocked by prior addition of excess free peptide, little or no reactivity is observed in the M_r 170,000–190,000 region (Fig. 2b, blocked). A densitometric scan of the difference in intensities in this region is shown in Figure 2a and indicates that both the p170 and p190 react specifically with this serum. Thus, the ability of a v-erb-B antiserum to react independently with the p170 and p190 suggests that these proteins share antigenic determinants.

Phosphorylation of p170 and p190 in Immune Complex Kinase Assays

To further characterize the p170 and p190 peptides, their ability to undergo posttranslational modifications was examined. In metabolic labeling experiments followed by immunoprecipitation with the R1 monclonal antibody, both p170 and p190 were found to incorporate $[^{32}P]O_4$ and $[^3H]$mannose. Incorporation of mannose

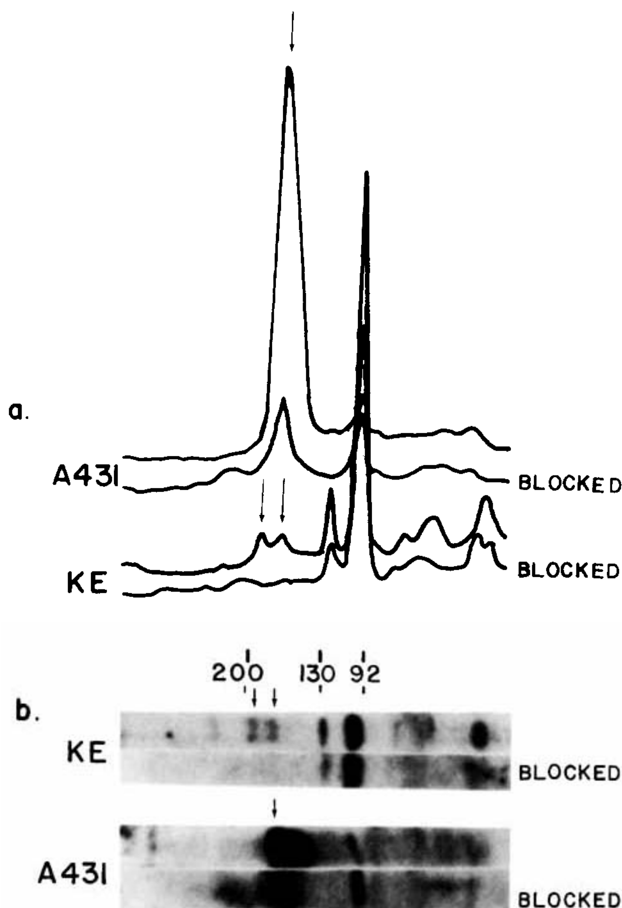

Fig. 2. Immunoblots of A431 and glioma cell KE cell extracts with an antisera generated against a synthetic polypeptide with homology to v-erb-B (see Materials and Methods) after SDS-PAGE. a) The densitometric tracings of the immunoblots shown in (b). The arrows (left to right) show p190 and p170, respectively in (a) and (b). The blocked lanes show that the immunoblot performed in the presence of the synthetic polypeptide. The molecular weight standards are myosin, β-galactosidase, and phosphorylase b.

into p170 was increased approximately fourfold over that of p190 by similar metabolic labeling (data not shown). Because p170 has been demonstrated to be a tyrosine protein kinase, it was of considerable interest to determine whether the immunoprecipitate proteins had associated kinase activity. Immunoprecipitates containing these proteins were incubated with [^{32}P]ATP as described in Materials and Methods for immunocomplex-kinase assay. The results of the proteins after SDS-PAGE analysis are shown in Figure 3. In each of the glial and glioma cells in which a p170 and p190 were present in immunoprecipitation, these proteins were also found phosphorylated in immune complex kinase assays. Whether p190 is itself also a kinase or is phosphorylated by the kinase associated with EGF-R remains to be determined. Furthermore, both proteins were found to be phosphorylated on tyrosine residues.

Quantitation of Cell-Surface EGF-R

Although direct immunoblotting techniques are not a good quantitative measured of an antigen, both immunoblotting and immunoprecipitation experiments suggested that EGF-R was not significantly overexpressed in normal glial and glioma cells compared with EGF-R expression on A431 cells. To directly measure the number of EGF-R on the cell surface, the binding of ^{125}I-labeled EGF to cells in culture was performed at 4°C. Scatchard plots of the binding data were performed for each of the cell lines, and the calculated number of EGF-R is shown in Table I. For A431 cells, 1.5×10^6 molecules of EGF per cell were bound, in agreement with the published number of receptor sites for these cells [7]. Furthermore, two affinity

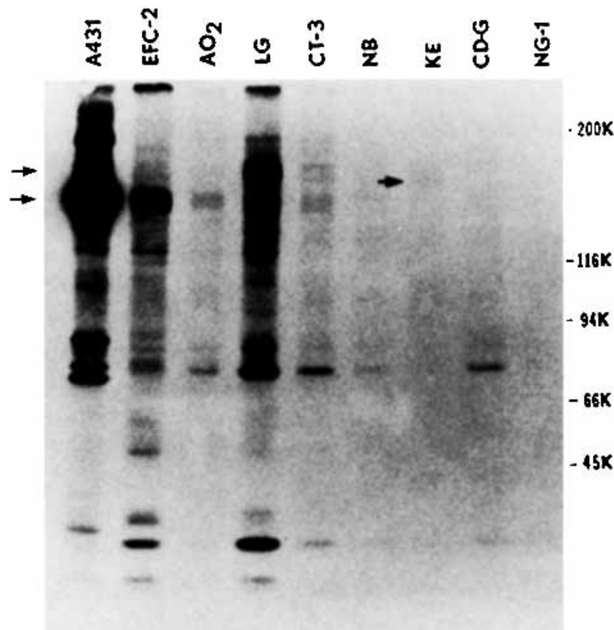

Fig. 3. Gel electrophoresis of autophosphorylated EGF-R immunoprecipitated with R1 monoclonal antibody from the various cultured glial/glioma cells. Immunoprecipitation of EGF-R-complex kinases were performed as described in Materials and Methods. The top arrow shows phosphorylation of a band of M_r 190,000, and the bottom arrow shows the EGF-R, also demonstrated by A431 cells. The protein standards are the same as those described in Figure 1.

TABLE I. Epidermal Growth Factor Receptor Expression on Cultured Human Glioma Cells

Cell line	Immuno-precipitation[a]	Immunocomplex kinase[a]	Immuno-blot[a]	Binding sites/cell[b]	K_d[b] ($\times 10^{-10}$)
A431	170K	170K	170K	1.3×10^6	900
				2.4×10^5	2
EFC-2	170K	170K	170K	4.5×10^4	17
	190K	190K	190K		
KE	170K	170K	170K	7.2×10^4	44
	190K	190K	190K		
LG	170K	170K	170K	14.1×10^4	10
	190K	190K	190K		
AO_2	170K	170K	170K	5.4×10^4	6
	190K	190K	190K		
NG-1	120K	—	ND[c]	0	—
	80K				
MB	190K	170K	—	2×10^4	500
	170K				
PL-1	190K	190K	170K	12.2×10^4	6
	170K	170K	150K		
CT-3	170K	170K	—	3×10^4	700
	190K	190K			
CDG	190K	170K	170	5×10^4	2

[a]The immunoprecipitation, immunocomplex kinase and immunoblot was performed as described in Materials and Methods and Figures 1–3. The 170K or 190K refers to the presence of either a p170 or a p190 band.
[b]The binding sites per cell and apparent dissociation constants (K_d) were determined by Scatchard analysis of the binding of ^{125}I-labeled EGF to the various cells as described in Materials and Methods. The analysis was performed by using linear regression by computer.
[c]Not detectable. Due to the high background of nonspecific bands at less than $M_r \sim 130$, specific binding could not be determined.

classes of binding sites were observed for the A431 cells as represented by a biphasic Scatchard plot. The majority of glial and glioma cells were calculated to express between 40,000 and 80,000 EGF-R. Scatchard analysis suggested only one class of binding site (linear relationship), in cells which expressed mainly p170, as well as in cells which expressed both p170 and p190. Whether the single observed class of EGF binding sites is due to experimental limitations or to the interaction of EGF-R with a specific component, particularly p190, cannot be ascertained presently. Studies are underway to examine these possibilities. However, no significant increase in binding of EGF was observed between cells that express approximately equal amounts of p190 and p170 (EFC-Z, KE, LG) and those expressing more p170 and p190 (CDG), suggesting p190 does not bind EGF.

DISCUSSION

Aberrant expressions of growth factors and their receptors are receiving considerable study to determine their possible roles in tumorigenesis and progression. The EGF-R has been implicated with several types of tumors due to its structural similarity to the v-erb-B oncogene and because of the frequency of aberrant expression in many types of human tumors. Several explanations have been proposed for why aberrant expression of these gene products may be involved in tumorigenesis. An altered gene (eg, the v-erb-B gene) may encode a protein in which the kinase is activated indepen-

dently of EGF binding. Overexpression of the gene products may result in an increase in the mitogenic response induced by EGF. An autocrine mechanism, such as production of transforming growth factor α (a growth-stimulatory factor related to EGF and which binds EGF-R) might also trigger aberrant cell division [22]. In glial cells, which are differentiated, cell division may be relatively slow. Our studies have indicated that these cells nonetheless produce levels of EGF-R that are comparable with levels observed in dividing fibroblasts. Preliminary data have indicated that EGF receptor maybe differentially expressed on actively dividing glial cells compared to their quiescent counterparts (unpublished observation). This would agree with the cell-cycle variation of EGF-R previously shown [23]. Thus, expression of EGF receptor in these cell lines may correlate with active division of cells and may be a result rather than a cause of tumorigenicity. Amplification and rearrangement of EGF receptor, which has been observed in other cells [11,12], may confer further selective advantage.

An additional protein, designated p190, was also observed in most glial and glioma cells. Several types of evidence suggest that this protein might be the product of, or similar to, the human c-neu proto-oncogene. The neu proto-oncogene has been reported to share structural homology with the EGF-R, but is a distinct gene, located on human chromosome 17 [24], in contrast to the EGF-R gene, which is located on human chromosome 7. Our studies have indicated that the p190 is recognized in immunoprecipitates with the R1 monoclonal antibody to EGF-R as well as in immunoblots with an antiserum to a synthetic v-erb-B peptide. Thus, p190 shares some antigenic determinants with EGF-R. Pulse-chase studies (to be presented elsewhere) have indicated that p190 is not a precursor to p170. In addition, p190 appears to incorporate less carbohydrate by metabolic labeling than does p170, an observation that has been reported for the rat c-neu product. Scatchard analysis, which suggests only one class of EGF binding site for the glioma cells, supports the published observations that the neu gene product does not bind EGF, but may be intimately involved with its activity. Thus, while the possibility that p190 is an aberrant form of EGF-R cannot be eliminated, our current evidence suggests that the former represents a distinct gene product.

The possibility that neu is expressed in glioma cells is intriguing, especially as transfection studies have determined that this gene is activated in glioblastomas as well as in neuroblastomas [24]. Whether the expression of p190 that we observe represents normal expression of the c-neu product, or aberrant expression of the "activated" c-neu gene, or, less likely, the expression of an aberrant form of EGF-R, remains to be determined. Future studies should focus on the significance of p190 to growth regulation in glial and glioma cells.

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