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Tandem duplication of the epidermal growth factor receptor tyrosine kinase and calcium internalization domains in A-172 glioma cells

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Amplification and rearrangement of the epidermal growth factor receptor (EGFR) gene occur frequently in malignant gliomas. Rearrangement may also lead to the expression of potentially oncogenic EGFR deletion mutants. Data presented here indicate the existence of a 190 kDa mutant form of the EGFR in A-172 glioma cells that is substantially different from the deletion mutants characterized previously. The EGFR-like protein is expressed along with the 170 kDa wild type EGFR. It is detectable with antibodies to both extracellular and intracellular regions of the EGFR, but is not crossreactive with other HER-family members. The wild type and mutant receptors undergo phosphorylation in response to treatment with TGFα and are associated with expression of both 10.5 kb and 11.5 kb EGFR-related transcripts. Combined reverse transcription-polymerase chain reaction (RT–PCR) identifies a unique transcript in A-172 cells that encodes an in-frame, tandem duplication of both tyrosine kinase and calcium internalization (TK/CAIN) domains (exons 18 through 26). The duplication of these domains is associated with a specific genomic rearrangement between potential v-*myb* and c-*myb* consensus binding sites within introns 26 and 17 of the EGFR gene resulting in the formation of a chimeric intron.

Keywords: epidermal growth factor receptor; mutation; genetic recombination; glioma; intron; oncogene

Introduction

Malignant gliomas are the most common primary brain tumors. The prognosis for patients with these tumors remains extremely poor (Mahaley, 1991). As a result of multistep tumor progression, malignant gliomas are genetically heterogeneous tumors (Louis and Gusella, 1995). The most consistent genetic alterations identified to date include p53 abnormalities (Bogler *et al*., 1995; Rasheed *et al*., 1994), alterations in the p16/CDKN2/pRb pathway (Ueki *et al*., 1996) and EGFR gene amplification and rearrangement (Libermann *et al*., 1985; Wong *et al*., 1987; Humphrey *et al*., 1988; Bigner *et al*., 1990). Levels of EGFR in gliomas are proportional to tumor invasiveness (Lund-Johansen *et al*., 1990) and overexpression of wild type EGFR has been shown to induce ligand-dependent cellular transformation of astrocytes, indicating a direct role for the receptor in oncogenesis (Frisa *et al*., 1996; O'Rourke *et al*., 1997).

The full-length (wild type) EGFR requires binding of ligand for activation (Carpenter and Cohen, 1979). Presumably, transcription of multiple gene copies per cell produces higher EGFR expression in malignant glioma cells than in normal astrocytes, with increased sensitivity of the cell to available ligand. Hence, ectodomains of the other HER-family proteins have been shown to act as dominant negative factors by forming inactive heterodimers with EGFR. This leads to inhibition of anchorage-independent growth of human U87 glioma cells (O'Rourke *et al*., 1997). Similarly, differential splicing of the EGFR transcript in normal rat liver produced a secreted 95–100 kDa form of EGFR consisting of the extracellular portion of the molecule which may also act as a negative regulator (Basu *et al*., 1989; Petch *et al*., 1990). Hence, there is abundant evidence implicating EGFR in tumorigenesis of malignant gliomas, particularly glioblastoma multiforme.

EGFR amplification occurs in up to 50% of malignant gliomas with gene rearrangement in approximately one-half of those tumors leading to frequent co-expression of both wild type and mutant receptors (Libermann *et al*., 1985; Wong *et al*., 1987; Humphrey *et al*., 1988; Bigner *et al*., 1990; Ekstrand *et al*., 1991). Expression of these EGFR mutants has been reported to be associated with a poor prognosis (Schlegel *et al*., 1994). In addition, such mutants are capable of oncogenic transformation in the absence of ligand (Batra *et al*., 1995; Moscatello *et al*., 1996).

The EGFR (c-*erb*B-1) gene encodes a cell surface receptor that is structurally similar to the viral *erb*B-1 gene product (Downward *et al*., 1984). Proviral insertion within intron 14 results in separation of the ligand-binding domain of this receptor from its transmembrane and cytoplasmic regions (Raines *et al*., 1985). The resulting N-terminally truncated molecule produces tissue-specific tumorigenesis (i.e. erythroleukemia). In contrast, certain mutations of *erb*B-1 occurring in the C-terminal region produce sarcomas (Yamamoto *et al*., 1983). Such mutations include C-terminal truncation, point mutations and internal deletions within the tyrosine kinase domain (Pelley *et al*., 1988). Therefore, the *erb*B-1 gene can become oncogenic by way of a number of different structural alterations.

Tumorigenic activation of the EGFR in human gliomas appears to arise primarily as a result of deletion mutation. In addition to the 170 kDa EGFR present in many gliomas, certain mutant EGFR arise from specific gene arrangements and internal deletions

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(Humphrey *et al*., 1991; Wong *et al*., 1992). Such mutants may contribute to tumor progression via constitutive receptor activation. Most contain a deletion of specific groups of exons encoding part of the extracellular portion of the EGFR molecule. Although uncommon in gliomas, one extensive N-terminal truncation has been reported (EGFRvI). This mutation creates a molecule similar to the viral *erb*B-1 gene product which induces malignant transformation and potent constitutive receptor activation (Halley *et al*., 1991; Wong *et al*., 1992). The EGFRvII mutation consists of an in-frame deletion of 83 amino acids in domain IV of the extracellular region of the molecule (amino acids 520–603; exons 14 and 15). This mutant receptor remains capable of binding ligand and has enhanced tyrosine kinase activity (Humphrey *et al*., 1991). EGFRvIII is the most common variant seen in gliomas occurring in at least 17% of them (Humphrey *et al*., 1990). It contains an in-frame loss of a large portion of the extracellular region of the molecule (amino acids 6–273; exons 2–7). This leads to the expression of a 140 kDa receptor with formation of unique epitopes that are of interest for receptor-targeted antitumor strategies (Humphrey *et al*., 1990. Each of the EGFR mutants appears to result from loss of specific exons from the genes which encode them (Ekstrand *et al*., 1991; Wong *et al*., 1992). Thus, the 190 kDa EGFR-like molecule, which is the subject of this report, represents a new and distinctly different type of EGFR mutant.

Results

Expression of a functional 190 kDa EGFR-like protein in A-172 human glioma cells and its relationship to other HER family proteins

EGFR-related 190–200 kDa proteins have been observed previously in A-172, KE and A-1235 human glioma cell lines (Steck *et al*., 1988; Panneerselvam *et al*., 1995). We have detected the EGFR-like species in A-172 cells using antibodies to both intracellular (Figure 1) and extracellular (data not shown) regions of the EGFR. Hence, the p190 appears to be an EGFR-like molecule with similarities to both intracellular and extracellular regions of the wild type p170 EGFR found in A-431 cells. In addition to the p190, A-172 cells express a 170 kDa form of EGFR that is indistinguishable from that produced by A-431 carcinoma cells.

The EGFR-like p190 present in A-172 cells is not detectable by antibodies to HER2/neu, HER3 or HER4 proteins (Figure 2). Wild type HER1, HER2 and HER4 proteins are all expressed in A-172 cells, although HER3 is not. HER3 is detectable in A-431 cells, however. Consequently, based on size differences of the various immunoreactive proteins, the EGFR-like p190 is unlikely to be either a wild type or mutant form of HER2, HER3 or HER4. Instead, the p190 appears to be most closely related to the EGFR. In addition, treatment of serum-starved A-172 cells with TGFα induces phosphorylation of both 170 kDa and 190 kDa species (Figure 3; lanes 5 and 6). Neither of the two EGFR forms is constitutively autophosphorylated, however.

A-172 cells produce a unique 11.5 kb EGFR-like transcript

Experiments with glycosylation inhibitors suggest that the protein cores of the 190–200 kDa EGFR-like protein in A-172 and A-1235 glioma cells are glycosylated to a similar extent as the wild type EGFR (Panneerselvam *et al*., 1995). Thus, the difference in molecular weight between wild type EGFR and the p190 is not due to differential glycosylation. The difference in molecular weight between deglycosylated protein cores suggests that, if the p190 were encoded by a mutant EGFR transcript, approximately one kilobase of additional sequence would be required beyond that present in the full-length 10.5 kb EGFR transcript (Panneerselvam *et al*., 1995).

At least three separate EGFR-related transcripts have been noted in A-431 carcinoma and in normal syncytiotrophoblast cells including 10.0–10.5 kb, 5.6–5.8 kb and 2.6–2.8 kb forms (Ullrich *et al*., 1984; Ekstrand *et al*., 1991). In addition to the 10.5 kb species present in A-431 and A-172 cells, we have identified an 11.5 kb EGFR-like mRNA species in the latter (Figure 4). A transcript of similar size has also been reported in KE glioma cells which have been shown to express an EGFR-reactive 190 kDa protein (Steck *et al*., 1988). We have been unable to detect the larger transcript in A-431 carcinoma cells (Figure 4) or in any other cell lines that do not express the p190.

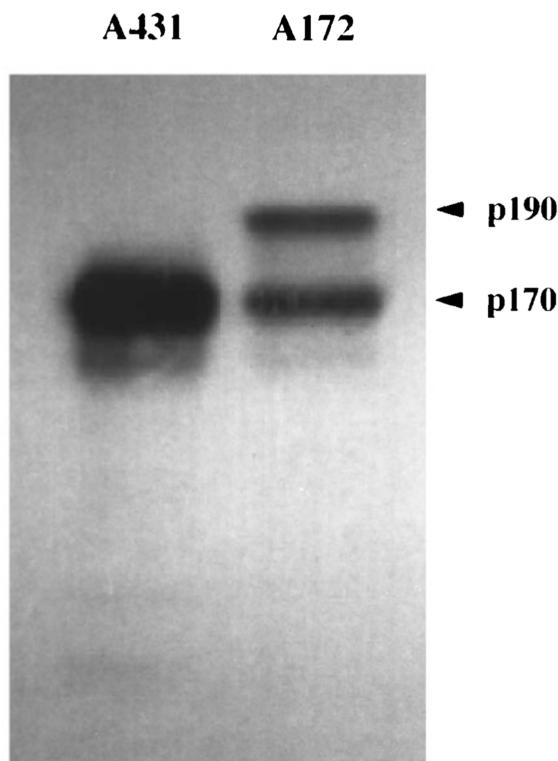

Figure 1 Western blot of lysate from A-431 carcinoma and A-172 glioma cell lines. Anti-EGFR antibody 1005 (Santa Cruz) was used to detect the intracellular region of the molecule. Similar results were obtained with anti-EGFR antibody R-1 recognizing the extracellular region (data not shown). Proteins were resolved on 5% SDS–PAGE, transferred to nylon membranes, and detected as described

Thus, expression of the larger transcript corresponds closely to that of the p190. The ~1 kb difference in the two transcripts is also compatible with RT–PCR results indicating duplication of a 1101 bp sequence within an EGFR-like transcript of A-172 cells as described.

Characterization of EGFR transcript expression in A-172 cells by RT–PCR using multiple oligodeoxyribonucleotide (ODN) pairs

To characterize EGFR-related transcripts present in A-172 cells, we used several different sets of EGFR-

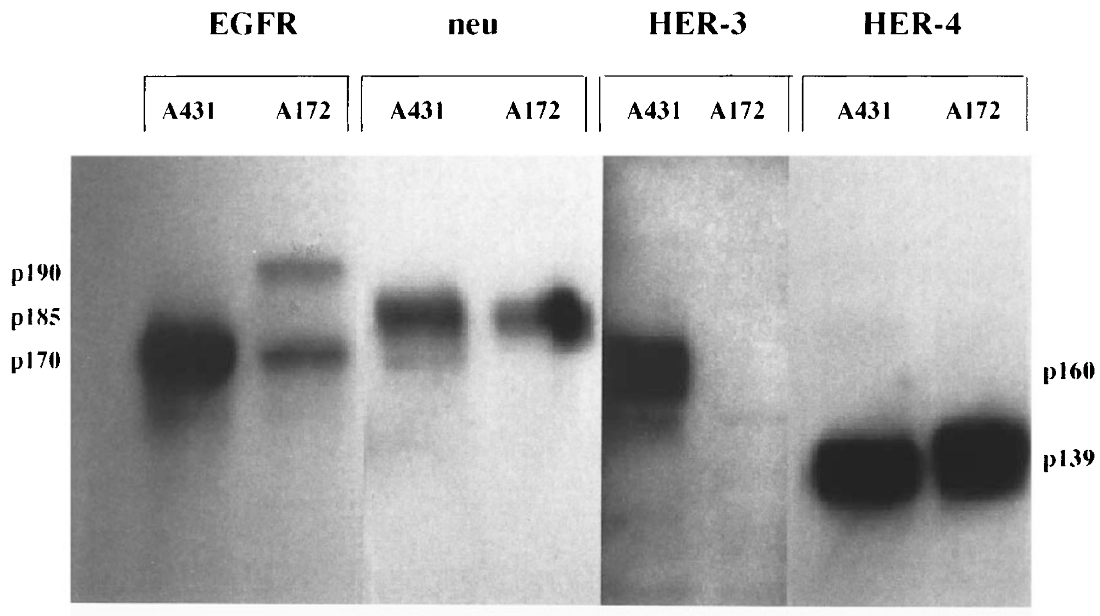

Figure 2 Western blots of cellular proteins from A-431 carcinoma and A-172 glioma cells probed with antibodies to the indicated HER-family members and detected with a chemiluminescent system

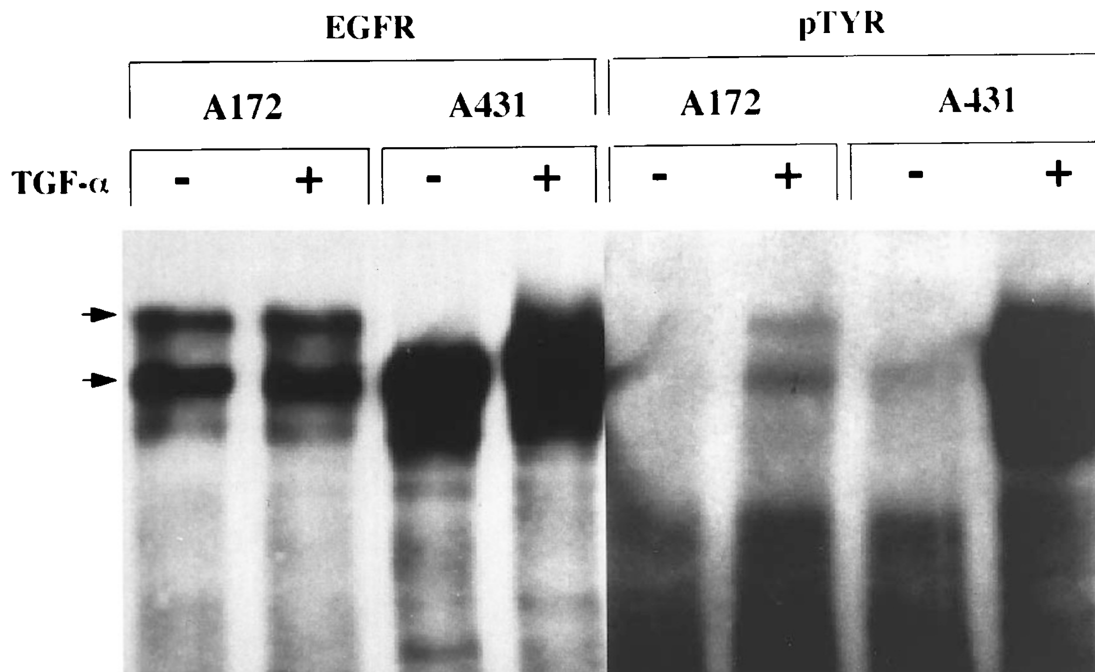

Figure 3 Duplicate Western blots of EGFR proteins from A-172 and A-431 cells. Cells were maintained in serum-free medium for 24 h and treated for 10 min (+) with or (−) without TGFα (40 ng/ml) with lysates prepared as described. Anti-EGFR antibody 1005 (Santa Cruz; lanes 1–4) and anti-phosphotyrosine antibody PY69 (Santa Cruz; lanes 5–8) were used for immunoprecipitation. Detection was with anti-EGFR antibody and a chemiluminescent system

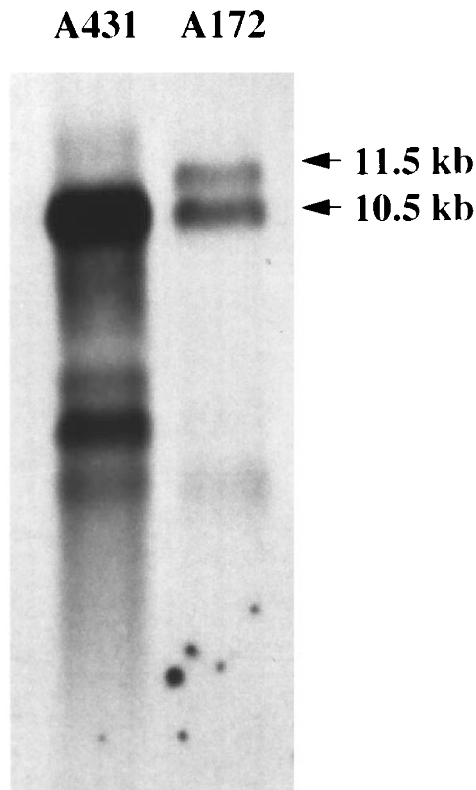

Figure 4 Northern blot of A-431 and A-172 poly(A)$^+$RNA probed with a DIG-labeled riboprobe spanning nucleotides +1348 to +1804 of the human EGFR cDNA (Ullrich *et al.*, 1984). Detection was performed with a chemiluminescent system as described

specific ODN pairs in reverse transcription-polymerase chain reaction (RT–PCR) reactions. All ODN pairs produced fragments of identical size with A-172 and A-431 templates (Figure 5a–d) corresponding to the length of the wild type sequence (Ullrich *et al.*, 1984). ODN pairs binding at the 5′ and 3′ ends of the EGFR coding region (+187 and +3819; Figure 5b) also produced a second major band in A-172 cells that was ~1 kb larger than the wild type sequence. Similarly, ODN spanning the region from +3016 to +3819 produced one PCR fragment (804 bp) in A-431 cells and two PCR fragments (804 bp and 1905 bp) in A-172 cells (Figure 5c). Consistent results were obtained with two other ODN combinations (+2101 to +2292; Figure 5d; and +3116 to +3379, data not shown). In contrast, no second band was detectable in either cell line using ODN to the extracellular region of the EGFR molecule (+187 to +2221; Figure 5a). These findings are consistent with the presence of two separate species of EGFR mRNA in A-172 cells (Figure 4). RT–PCR reactions using independently isolated RNA samples gave identical results. Only the smaller of the two species was ever detected in A-431 cells, regardless of the ODN combination used.

Nucleotide sequence of the mutant EGFR mRNA

The DNA sequence of the 804 bp PCR fragment derived from A-431 cells (Figure 5c) precisely matched the published EGFR cDNA sequence extending from nucleotide +3016 in the tyrosine kinase domain to +3819 corresponding to the third nucleotide of the terminal codon. In contrast, the larger (1905 bp)

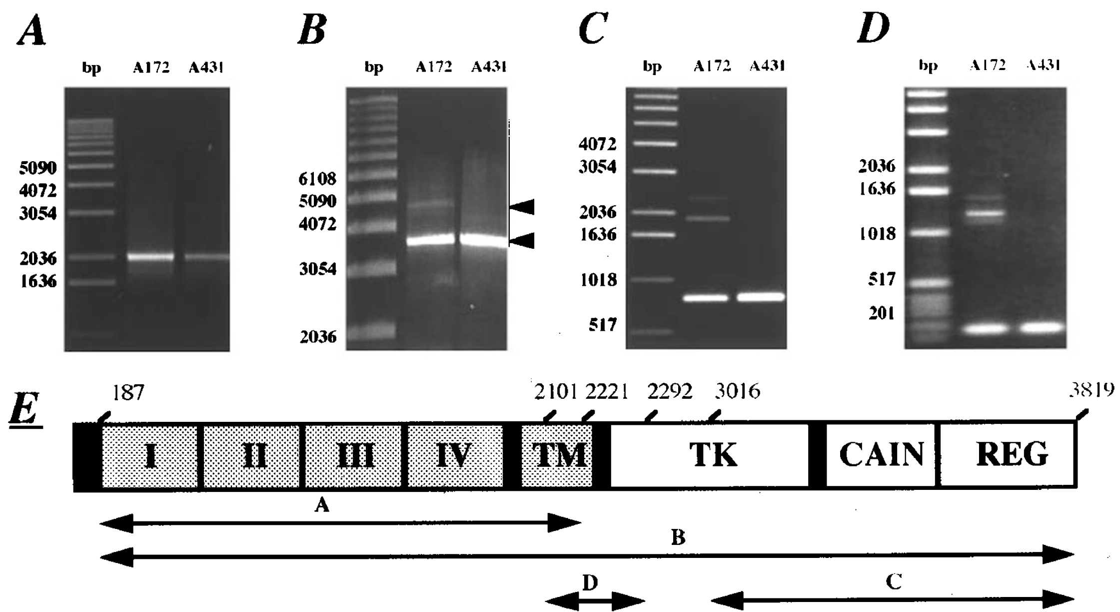

Figure 5 PCR reactions performed using four different pairs of EGFR-specific ODN on A-172 and A-431 cDNA template mixes. ODN pairs and resulting PCR products correspond to: (**a**), the extracellular region of EGFR (nucleotides +187 to +2221); (**b**), the full-length coding sequence (nucleotides +187 to +3819); (**c**) part of the intracellular region (nucleotides +3016 to +3819), and (**d**) part of the intracellular region (nucleotides +2101 to +2292). (**e**) The numbering system for ODN is derived from Ullrich *et al.* with numbers corresponding to the 5′-end of each ODN relative to the human EGFR coding strand. Solid lines indicate regions of the molecule amplified by each ODN pair as depicted in **a–d**

fragment from A-172 cells (Figure 5c) contains a tandem repeat of exons 18 through 26 as derived from the exon-intron structure of the chicken EGFR gene (Callaghan *et al*., 1993). This produces an in-frame duplication of the tyrosine kinase (TK) and calcium-mediated receptor internalization (CAIN) functional domains (Figure 6). In addition to tandem duplication of the TK/CAIN region, five nucleotide substitutions are present within the sequenced region. Four of the substitutions occur within the third nucleotide of each representative codon leading to conservation of amino acid sequence in each case. In the fifth example, a nucleotide substitution at +4080 (A→C) in the TK/CAIN-2 region encodes aspartate rather than glutamine. Analysis of two separate plasmids containing the 1905 bp PCR fragment derived from a single PCR reaction and RT–PCR fragments from a different isolate of A-172 RNA gave identical results.

Detection and sequence of intron rearrangement in A-172 genomic DNA with ODN from exons 26 and 18

Using an ODN homologous to the EGFR coding strand near the 3′-end of exon 26 and an ODN homologous to the non-coding strand near the 5′-end of exon 18, we amplified a unique fragment from A-172 genomic DNA (Figure 7a, lane 7). No such fragment could be detected in genomic DNA from either A-431 cells or normal human kidney (Figure 7a, lanes 8 and 9). In order to determine the significance of this finding, we isolated wild type introns 17 and 26 from human renal genomic DNA using PCR (Figure 7a, lanes 3 and 6). Sequencing of the A-172-specific PCR fragment and comparison to wild type introns 17 and 26 from human renal genomic DNA reveals elements of both wild type introns incorporated into a 615 bp chimeric intron (Figure 7c). The mutant intron contains 432 bp of intron 26 (wild type intron 26=737 bp) and 183 bp of intron 17 (wild type intron 17=982 bp). Analysis of the sites of recombination of the three introns using a mutation matrix derived from systematic binding affinity measurements (Deng *et al*., 1996) revealed potential c-*myb* and v-*myb* consensus binding sequences in the non-coding strands at sites of rearrangement in introns 17 and 26 respectively (Figure 7b). In both introns, recombination occurs within the core regions of the putative binding sites. Moreover, a potential c-*myb* binding site is reconstituted in the chimeric 26/17 intron.

Discussion

We and others have observed the presence of 190–200 kDa EGFR-related proteins in A-172 human glioma cells and in two other glioma cell lines (Steck *et al*., 1988; Panneerselvam *et al*., 1995). Certain characteristics of these proteins such as glycosylation status have been described previously (Panneerselvam *et al*., 1995). Until the current study, however, genetic sequence alterations that might encode the p190 have not been reported. RT–PCR studies described here indicate the presence of a mutant EGFR transcript in A-172 human glioma cells which encodes two copies of the TK/CAIN region (exons 18 through 26) in tandem array. It is likely that this arises from a genomic alteration in which EGFR introns 26 and 17 have undergone recombination. Together with identification of the TK/CAIN duplication amplified from separate mRNA isolates using several different ODN pairs, the 26/17 chimeric intron strongly suggests that genomic rearrangement between introns 17 and 26 creates a mutant gene which encodes the TK/CAIN transcript. Moreover, our results may help to explain why previous studies involving phosphopeptide mapping of the p190 in KE cells failed to reveal significant differences between it and the wild type receptor (Steck *et al*., 1988).

A detailed analysis of the chicken EGFR exon-intron junction structure reveals that exon duplication probably occurred within the ligand binding region of the EGFR gene at some time during its history (Callaghan *et al*., 1993). Thus, as one step in the evolution of the EGFR gene, certain susceptible introns may have undergone genetic recombination giving rise to exon duplication. The multi-domain organization of EGFR is present in the other HER-family receptors as well. Studies in which various functional domains of EGFR and HER-2 (neu) are exchanged reveal that the ligand-binding, transmembrane, and tyrosine kinase domains are capable of functioning as independent modules (Lehvaslaiho *et al*., 1989). Thus, in malignant gliomas, where genetic instability is common, EGFR rearrangements that

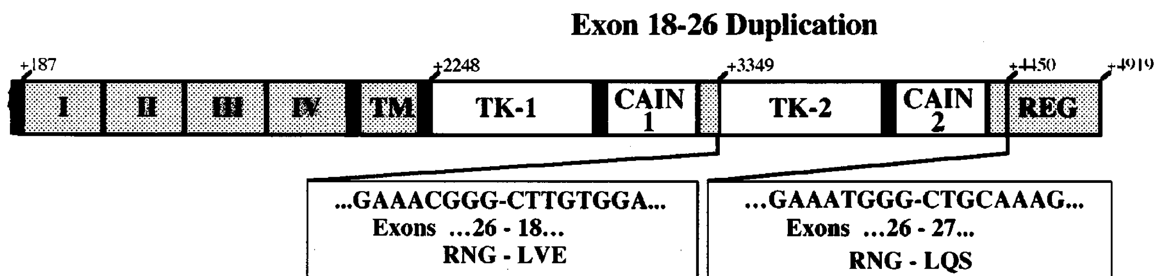

Figure 6 Structure of the TK/CAIN tandem duplication mutant cDNA with partial nucleotide sequence at boundaries of the TK/CAIN-2 region. Numbers at the top indicate nucleotide positions relative to the ATG start codon beginning at +187 (Ullrich *et al*., 1984) and are modified to account for the duplicated TK/CAIN sequence. Conservative substitutions occur at nucleotides +3168 (C→T) and +3345 (T→C) in the TK/CAIN-1 region and at +3648 (A→G) and +4269 (C→T) in the TK/CAIN-2 region. An additional substitution at +4080 (A→C) in TK/CAIN-2 leads to a glutamine to aspartate change.

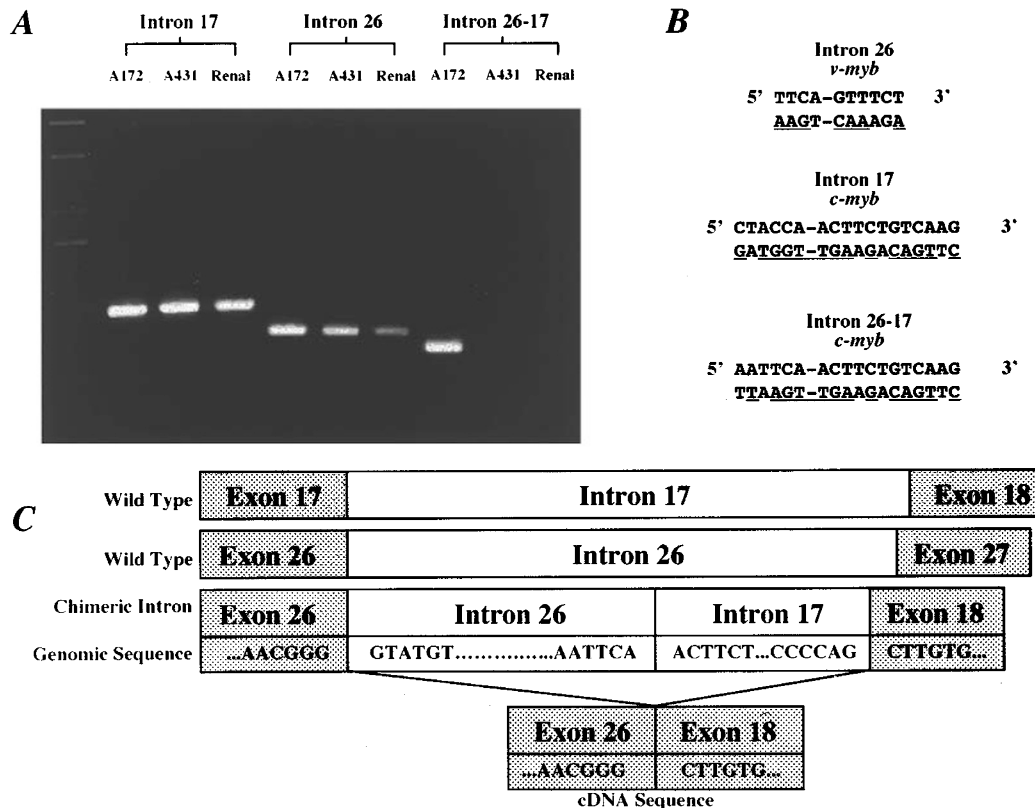

Figure 7 Isolation and partial structure of wild type and mutant introns. (**a**) PCR with genomic DNA from A-172 cells, A-431 cells, and human kidney. ODN used are from exons 17 and 18 (intron 17), exons 26 and 27 (intron 26) and exons 26 and 18 (chimeric intron 26/17). (**b**) The homology of sequences flanking each breakpoint to c-*myb* and v-*myb* binding sites is shown. Underlined nucleotides have strong homology to *myb* consensus sequences. (**c**) Structure of wild type introns 26 and 17 from renal genomic DNA (**a**, lanes 3 and 6) and the 26/17 chimera present in A-172 cells (**a**, lane 7). The genomic sequences at the intron-exon boundaries and at intron breakpoints are indicated along with the corresponding nucleotide sequence derived from the mutant cDNA

involve either loss or duplication of modular groups of exons is not entirely surprising. In addition to duplication of the TK/CAIN functional domains, other regions of the EGFR, including those that are the site of deletion mutation, can undergo tandem duplication (our unpublished observations). If such mutants are oncogenic, tumor cells expressing them may have a distinct survival advantage.

The site of rearrangement in intron 17 consists of a potential c-*myb* binding site (87.4% homology by mutation matrix) with 100% conservation of the core binding motif (Deng *et al*., 1996). In addition, a v-*myb* consensus sequence (89.2% homology by matrix) is present at the site of rearrangement in intron 26 with 87.6% homology in the core. In both introns, rearrangement results from recombination within the core regions leading to reconstitution of a potential c-*myb* binding sequence in the chimeric 26/17 intron (87.2% homology by matrix) with 100% homology in the core. Although primarily a transcriptional regulator, the c-*myb* protein has been implicated in VDJ recombination at the T cell receptor delta locus where an intact c-*myb* binding site is necessary for efficient rearrangement of the minilocus substrate (Hernandez-Munain *et al*., 1996). Moreover, the c-*myb* gene itself is amplified and highly expressed in two of four malignant glioma cell lines examined in one study (Welter *et al*., 1990). Additional studies will be necessary, however, to determine whether or not the c-*myb* protein is actually involved in EGFR rearrangement in human glioma cells.

The subclass I growth factor receptors such as EGFR have a single tyrosine kinase catalytic domain. In contrast, certain members of the subclass III receptor family, such as FLT3, c-kit, c-fms and members of the Janus (JAK) family of receptors have two separate tyrosine kinase domains separated by a KI or 'kinase insert' region (Agnes *et al*., 1994). Small internal tandem duplication of nucleotide sequences within the juxtamembrane and TK-1 regions of the FLT3 gene have been reported in the cells of patients with acute myeloid leukemia (Yokota *et al*., 1997). While these mutations may lead to constitutive activation and are associated with a poor prognosis for patients, they have not yet been shown to involve further duplication of either the TK-1 or TK-2 domains in their entirety (Horiike *et al*., 1997). Similarly, an *in vitro* mutation of the trk receptor has been described which contains an inverted duplication of the tyrosine kinase domain in head-to-tail arrangement (Coulier *et al*., 1990). Nevertheless, tandem duplication of the entire tyrosine kinase and calcium-

dependent receptor internalization domains appears to be distinctly unusual among tumors expressing subclass I receptors.

Overexpression of TGFα, which occurs in a large percentage of malignant gliomas, may lead to autocrine-stimulated tumor cell proliferation and cellular transformation (Maruno *et al*., 1991; Blasband *et al*., 1990). More specifically, ligand-dependent cellular transformation has been demonstrated in immortalized murine glia in which the wild type EGFR has been expressed at high levels (Frisa *et al*., 1996). Hence, in the presence of other critical genetic alterations, ligand-dependent glial cell transformation may occur via overexpression of a functional EGFR.

Treatment of A-172 cells with TGFα induces tyrosine phosphorylation of wild type p170 and tandem mutant p190. Although this suggests that both wild type and tandem mutant receptors bind ligand and undergo receptor autophosphorylation, it does not exclude the possibility of transphosphorylation of the p190 by ligand-activated wild type receptors. Moreover, although the TK/CAIN mutant may be activated directly by ligand, it does not appear to be constitutively autophosphorylated. Similarly, the EGFRvIII does not exhibit enhanced basal autophosphorylation even though it is oncogenic (Chu *et al*., 1997; Huang *et al*., 1997). As with EGFRvIII, the tandem mutant could cause ligand-independent transformation despite the absence of high-level constitutive autophosphorylation. Either or both TK domains could phosphorylate substrates resulting in activation of a number of different signal cascades. In the tandem mutant, the spatial relationship of TK-2 to its regulatory domain is unchanged; however, TK-1 is separated from the regulatory domain by 367 amino acid residues. Thus, it is possible that TK-1 could be unregulated and hence oncogenic due to its spatial separation from the regulatory domain, or as a result of secondary conformational alterations. In addition to the tandem duplication, a single point mutation is present at nucleotide +4080 located within the TK-2 region of the mutant. This results in an amino acid substitution (glutamine to aspartate) that could potentially alter the function of that tyrosine kinase domain, although the similarity of charge makes this unlikely.

Although EGFRvIII is oncogenic, the precise means by which the aberrant signal is transduced remains a subject of investigation. In comparison to the wild type EGFR, EGFRvIII constitutively complexes with the adapter proteins Grb2 and Shc (Montgomery *et al*., 1995; Moscatello *et al*., 1996; Chu *et al*., 1997). EGFRvIII also activates MAP kinase in some studies (Moscatello *et al*., 1996), but not in others (Chu *et al*., 1997). In addition, EGFRvIII activates extracellular signal-regulated kinase (MEK) (Moscatello *et al*., 1996). Therefore, among other differences, the EGFRvIII activates a different complement of substrates than the wild type receptor. Thus, co-expression of wild type EGFR and one or more of the EGFR mutants, could help to maintain a transformed state by simultaneous activation of parallel signaling pathways.

There is evidence that EGFRvIII may be constitutively active as a result of inefficient receptor down-regulation, rather than by way of enhanced autophosphorylation (Chu *et al*., 1997; Huang *et al*., 1997). Thus, unlike wild type receptors, EGFRvIII may be unable to attenuate mitogenic signaling by receptor internalization (Huang *et al*., 1997). Within the C-terminal region of the EGFR there is a domain designated CAIN which modulates receptor internalization (Chen *et al*., 1989). Deletion of this region leads to increased transforming ability presumably via prolongation of cell-surface receptor half-life (Wells *et al*., 1990). Thus, there are a number of possible mechanisms by which deletion mutations in the EGFR could lead to propagation of an oncogenic signal in the absence of ligand. It will be of interest to determine the functional effects that duplication of the CAIN region might have on this process, as well as the effects of tyrosine kinase duplication on glial transformation and signal transduction

Materials and methods

Cell culture

A-172 astrocytoma and A-431 carcinoma cell lines were grown on 100 mm tissue culture plates in Dulbecco's Modified Eagle Medium (DMEM) with 1500 mg/L glucose, 2 mM L-glutamine, and 110 mg/L sodium pyruvate, supplemented with 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were grown at 37°C in 5% CO_2 with media changes 2–3 times per week.

RNA isolation and Northern blotting

Poly(A)$^+$ RNA was isolated from A-172 and A-431 cells using a batch oligo dT method (Invitrogen). RNA samples were resolved on formaldehyde-agarose gels and transferred to positively charged nylon membranes (MSI) using a turboblotting apparatus as recommended by the manufacturer (Schleicher and Schuell). Blots were hybridized with a DIG-UTP-labeled riboprobe generated from pBluescript (KS$^-$; Stratagene) containing a 456 bp *Bam*HI–*Hind*III restriction fragment from the human EGFR cDNA (nucleotides +1348 to +1804; Ullrich *et al*., 1984). Hybridization of the EGFR riboprobe was detected using alkaline-phosphatase-conjugated, anti-DIG antibodies and the CDP-STAR chemiluminescent substrate (Bio-Rad).

Reverse transcription (RT) and PCR analysis of EGFR cDNA

RT reactions were performed with poly(A)$^+$ RNA and using oligo dT and MMLV reverse transcriptase (Perkin-Elmer). RT reactions were examined for EGFR cDNAs by PCR using multiple oligodeoxyribonucleotide (ODN) pairs. ODN were prepared on an Applied Biosystems DNA synthesizer in the Biopolymer Facility at Roswell Park Cancer Institute. All ODN used for PCR were designed with a T_M of 66°C. PCR fragments were generated using a mixture of 1.25 units *Taq* polymerase (Life Technologies) and 0.075 units *Pfu* polymerase (Stratagene). Reactions were incubated in a thermal cycler (Perkin Elmer) at 94°C for 5 min, then 94°C for 30 s alternating with 72°C for 6 min ×5 cycles; then 94°C for 30 s alternating with 70°C for 6 min × five cycles; then 94°C for 30 s alternating with 68°C for 6 min × 25 cycles. Reaction products were resolved on 1% agarose gels and eluted using Geno-bind reagent (Clontech). Isolated fragments were tailed with *Taq* polymerase and dATP and introduced into the pCRII-Topo vector (Invitrogen). Sequencing was performed using automated methods and homology searches were performed using MatInspector (Quandt *et al*., 1995). Each plasmid insert was sequenced at least twice.

Western blotting and immunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH = 7.5, 0.25% sodium deoxycholate, 1% NP-40, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EGTA, 1 μg/ml each of pepstatin, leupeptin and aprotinin). Lysates were passed through a 20 guage needle 15 times to shear DNA and centrifuged to remove debris. Protein content was measured by the bicinchoninic acid method (Sigma). Cellular extracts (50 μg) were boiled for 5 min and applied to 5% SDS-polyacrylamide gels. Protein was electroblotted onto positively charged nylon membranes (MSI) and then probed with anti-EGFR, neu, HER3, or HER4 antibodies (Santa Cruz). Specific binding was detected by secondary antibodies conjugated to alkaline phosphatase which was then exposed to CDP-STAR chemiluminescent substrate (Bio-Rad). Immunoprecipitations were performed with equal amounts of protein extract from A-172 and A-431 cells using either anti-EGFR (EGFR 1005; Santa Cruz) or anti-phosphotyrosine (PY69; Santa Cruz) antibodies (1 μg/ml) followed by incubation with protein-A agarose beads. Immunoprecipitates were washed three times with RIPA buffer, boiled in 2 × Laemmli buffer, run on 5% SDS-polyacrylamide gels and detected by Western blotting with anti-EGFR antibody as described above.

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