The Enhanced Tumorigenic Activity of a Mutant Epidermal Growth Factor Receptor Common in Human Cancers Is Mediated by Threshold Levels of Constitutive Tyrosine Phosphorylation and Unattenuated Signaling*

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H.-J. Su Huang‡§§, Motoo Nagane‡, Candice K. Klingbeil†, Hong Lin‡, Ryo Nishikawa†‡‡, Xiang-Dong Ji‡‡, Chun-Ming Huang‡‡, Gordon N. Gill§§, H. Steven Wiley¶¶, and Webster K. Cavenee‡§§

From the ‡Ludwig Institute for Cancer Research, §Department of Medicine, ¶Department of Chemistry and Biochemistry and §§Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92093-0660, ‡‡PharMingen, Inc., San Diego, California 92121, and the ¶¶Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84132

Deregulation of signaling by the epidermal growth factor receptor (EGFR) is common in human malignancy progression. One mutant EGFR (variously named ΔEGFR, dc2-7 EGFR, or EGFRVIII), which occurs frequently in human cancers, lacks a portion of the extracellular ligand-binding domain due to genomic deletions that eliminate exons 2 to 7 and confers a dramatic enhancement of brain tumor cell tumorigenicity in vivo. In order to dissect the molecular mechanisms of this activity, we analyzed location, autophosphorylation, and attenuation of the mutant receptors. The mutant receptors were expressed on the cell surface and constitutively autophosphorylated at a significantly decreased level compared with wild-type EGFR activated by ligand treatment. Unlike wild-type EGFR, the constitutively active ΔEGFR were not down-regulated, suggesting that the altered conformation of the mutant did not result in exposure of receptor sequence motifs required for endocytosis and lysosomal sorting. Mutational analysis showed that the enhanced tumorigenicity was dependent on intrinsic tyrosine kinase activity and was mediated through the carboxyl terminus. In contrast with wild-type receptor, mutation of any major tyrosine autophosphorylation site abolished these activities suggesting that the biological functions of ΔEGFR are due to low constitutive activation with mitogenic effects amplified by failure to attenuate signaling by receptor down-regulation.

Ligand binding to wild-type epidermal growth factor receptor (wt EGFR) results in receptor dimerization, kinase activation, and autophosphorylation that provides both docking sites for proteins involved in signal transduction and exposure of endocytic and lysosomal targeting sequence codes required for receptor internalization and down-regulation. The biochemical and biological roles of each autophosphorylation site in wt EGFR have been explored by mutational analysis, and mutation of any single autophosphorylation site does not significantly abrogate binding of the activated receptor to specific SH2-containing proteins associated with distinct signaling pathways. Likewise, such single mutations are generally incapable of reducing the biological functions of the receptor in in vitro models. Correspondingly, the mitogenic and transforming activities of wild-type receptor were diminished only when combinations of favorable autophosphorylation sites (i.e., Tyr-1068, Tyr-1148, and Tyr-1173) were mutated, suggesting that the autophosphorylation sites of wt EGFR may have less specificity for signaling proteins and can compensate for each other. Sites of tyrosine phosphorylation may be provided via heterodimerization with other members of the erb B family of receptors. Point mutations that inactivated the tyrosine kinase activity of wt EGFR eliminated occupancy-induced receptor internalization, whereas mutant receptors lacking multiple autophosphorylation sites also lacked the ability to undergo ligand-induced endocytosis, suggesting that kinase-regulated receptor internalization is mediated by the phosphotyrosine residues. The role of the kinase and phosphotyrosine residues in EGFR down-regulation may be more indirect. Evidence suggests that autophosphorylation of EGFR results in a more open conformation to exposure the otherwise cryptic internalization/lysosomal targeting motifs to the receptor trafficking machinery, thereby triggering receptor down-regulation processes. Receptors lacking internalization sequences can lead to cell transformation. Thus, the normally strict controls on cell proliferation may loosen when regulatory constraints intrinsic to the EGFR structure are relaxed by these mutations.

Mutations of the EGFR gene occur in many types of human tumors where growth deregulation is pervasive. In gial tumors of the central nervous system, abnormalities of the EGFR are restricted to grade III (anaplastic astrocytoma) and especially grade IV (glioblastoma multiforme) disease. Several clinical and histopathological studies have shown that such abnormalities are related to a shorter interval to relapse and decreased survival rates, suggesting that they play an important role in glioma malignancy progression. Nearly 50% of grade IV tumors have significantly amplified EGFR genes, and of these, the majority also show rearrange-
ments of the gene so that these gliomas express both wt EGFR and the mutant forms (16, 20, 21). The most common of the rearrangements are genomic alterations that eliminate a DNA fragment containing exons 2–7 of the gene and generate a mutant receptor (variously called ∆EGFR, de2–7 EGFR, or EGFRvIII) with an in-frame deletion of 267 amino acids from the extracellular domain (21–24). This specific genetic alteration is also found frequently in lung and breast cancers (15, 25).

In previous studies we found that expression of ∆EGFR in glioblastoma cells markedly enhanced tumor cell growth in nude mice (26), but the molecular mechanisms of the enhanced tumorigenicity have not been elucidated. Two defects have been described for the ∆EGFR, lack of EGF binding and constitutive autophosphorylation that was unaffected by ligand but whose extent was low compared with that of ligand-activated wt EGFR in the same cell. The importance of autophosphorylation as a marker of constitutive kinase activation of ∆EGFR to tumor growth was thus unclear.

Here we report that ∆EGFR are expressed on the cell surface but are defective in down-regulation due to low rates of receptor endocytosis. Both the intrinsic tyrosine kinase activity and each of the major sites of tyrosine autophosphorylation located in the regulatory carboxyl terminus are essential for the enhanced tumorigenesis characteristic of ∆EGFR. Unlike wt EGFR, a single point mutation in any of the major autophosphorylation sites is sufficient to abrogate the in vivo growth advantage conferred by ∆EGFR. Thus deletion of exons 2–7 abolished ligand binding but created a constitutively active EGFR that was strictly dependent for biological activity on intact autophosphorylation. Defective attenuation due to failure to down-regulate activated receptors appears to be the major mechanism that enhances tumorigenicity of these weakly phosphorylated EGFR mutants. Thus deletion of exons 2–7 results in a partially active EGFR that couples to mitogenic signal transduction but not to vesicular trafficking pathways.

EXPERIMENTAL PROCEDURES

Materials—Anti-EGFR antibodies included mouse monoclonals 528 and 225 (27) and 13A9 (28) directed against the membrane domain (provided by J. Kyte, University of California at San Diego, La Jolla, Ca), and mouse monoclonal C13 directed against residues 642–663 in the submembrane region of EGFR. Polyclonal sera, Ksm, directed against residues 225 (27) and 13A9 (28) directed against the ectodomain, rabbit antiserum 12.8% (v/v) glycerol, 1.3% 2-mercaptoethanol, 2 mM Na3VO4, 5 µM G418 per ml (26). 5 × 106 cells in 100 µl of staining buffer (phosphate-buffer saline (PBS), 1% calf serum, 0.1% sodium azide) were exposed (30–60 min) to anti-EGFR monoclonal antibody 528 which recognized both wild-type and mutant EGFR, or cells were exposed to monoclonal antibody D806 specific for mutant receptors, and then exposed to fluorescent isothiocyanate-conjugated goat anti-mouse immunoglobulin antibody (PharMingen, Inc.) for 30–60 min. Stained cells were analyzed with a FACSort (Becton Dickinson). For cell sorting, sodium azide was omitted from the staining buffer, and the brightest 5% of the stained cells were selected with a FACStar (Becton Dickinson). The sorted cells were cultured and reanalyzed. The number of DK and DY mutant receptors on the cell surface was estimated by comparing the fluorescence intensity of these cells with that of U87MG.

Immunoprecipitation and Western Blotting—Cells (4 × 106 cells per well in a 12-well plate or 2.5 × 106 cells per 100-mm dish) were cultured overnight in medium containing 10% dialyzed fetal bovine serum. The cells were exposed to 100 ng/ml EGF (Collaborative Biomedical Products) for 5 min at 37 °C. For Western blotting, cells were washed with PBS containing a PBS buffer containing 0.06% Tri-CI, pH 6.8. 1.3% SDS, 12.8% (v/v) glycerol, 1.3% 2-mercaptoethanol, 2 mM NaN3, 5 µg/ml leupeptin, 5 µg/ml antipain, 5 µg/ml pepstatin, and 0.25% bромophenol blue, and then boiled. The lysates were electrophoresed through SDS, 7.5% polyacrylamide gels and the proteins transferred to nitrocellulose membranes (Bio-Rad). For immunoprecipitation/Western blotting, cells were lysed in a PBS buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.04% sodium fluoride, and 5 µg/ml each of the protease inhibitors. Cellular debris was removed by centrifugation at 12,000 × g for 5 min at 4 °C. EGFR were immunoisolated using a ∆EGFR-specific monoclonal antibody, D1133, or anti-EGFR monoclonal antibody, 528, electrophoresed, and transferred to nitrocellulose membranes. Blots were probed with horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody PY20, and the proteins were visualized using the enhanced chemiluminescence detection system (Amersham Corp.). The blots were stripped and re-probed with the C13 anti-EGFR monoclonal antibody in the enhanced chemiluminescence system. EGFR concentration and phosphorylation were determined by scanning the autoradiograms with a densitometer. Multiple exposures were obtained to ensure linearity of response, and relative phosphorylation was calculated under these conditions. The level of EGFR phosphorylation was normalized to the protein concentration. The enhanced chemiluminescence employed was minimized to provide the possibility of residual signal when stripping blots probed with [32P]protein-A.

For additional quantitation of phosphorytrosine content, confluent monolayers of U87MG or U87MG wt EGFR cells were untreated or treated with EGF or 225 IgG (27), rinsed with PBS, and extracted with 500 µl of boiling SDS sample buffer (5% SDS, 50 mM Tri-CI, pH 6.8, 5% 2-mercaptoethanol, 1 mM Na3VO4). Equal amounts of protein were loaded on 5–15% gradient gels, transferred to nitrocellulose in the presence of 0.1 mM Na3VO4, and blocked with 2.5% BSA, 0.1 mM Na3VO4, and 0.005% Tween 20. Tyrosine phosphate was detected using 5 µg/ml affinity purified phosphotyrosine polyclonal

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2 H.-J. S. Huang, E. Stockert, and L. J. Old, unpublished data. CTC-3′ (antisense, nucleotides 3421–3437); (e) 5′-GGACGACGTAGACCA-3′ (sense, nucleotides 3421–3437); (f) 5′-GGCTGTAGTTGAAA-3′ (antisense, nucleotides 3503–3527); (g) 5′-AGAATCTCGTCTTACCACTAGCC-3′ (sense, nucleotides 3503–3527); and (h) GTGCTGCCGAGGGCTCATCT-3′ (antisense, nucleotides 3574–3590).
antibodies in conjunction with 50 ng/ml 125I-\(\beta\)2-M Protein A for 30 min. Dried blots were exposed to storage phosphor plates followed by imaging using a Bio-Rad G250 Molecular Imager. Profiles from the appropriate lanes were used to determine the difference between the phosphotyrosine pattern of either control or U87MG cells expressing \(\Delta\)EGFR using an analyzer software. Films were then developed in 0.1 M PBS and exposed simultaneously to the left or right flanks, respectively, of 4- to 5-wk-old female nude mice of BALB/c background. The growing tumors were measured twice a week with a caliper, and tumor volumes were calculated using width \(a\) and length \(b\) measurements \((a^2b/2\), where \(a < b\) \(\) (37). For intracerebral stereotactic implantation, \(1 \times 10^8 - 5 \times 10^9\) cells in 5 \(\mu\)l of PBS were inoculated into the corpus striatum in the right hemisphere of the nude mouse brain (38). Brains were removed 12 days post inoculation, embedded in OCT compound (Miles), frozen in liquid nitrogen, and stored at \(-80\)°C. Thin cryostat sections (5-6 \(\mu\)m) were stained with 528 antibody and counter-stained with hematoxylin. Tumors were microscopically measured, and the volumes of tumors were calculated as described above.

RESULTS

Defective Down-regulation of \(\Delta\)EGFR—The autophosphorylation of wt EGFR induced by ligand binding results in a conformational change proposed to expose cryptic endocytic and lysosomal targeting codes required for induced internalization and down-regulation (11, 39). Kinase-inactive EGFR are defective in ligand-induced down-regulation (40, 41), whereas deletion of the trafficking sequence codes in the carboxyl terminus of kinase-active EGFR results in ligand-dependent transformation and tumorigenesis by these attenuation-defective EGFR (12, 42). The extent of autophosphorylation of mutant \(\Delta\)EGFR was much less than that of ligand-activated wt EGFR (26), raising the question of whether these mutant receptors can undergo the endocytosis and down-regulation characteristic of activated EGFR. Receptor distribution was first analyzed by immunofluorescence microscopy using monoclonal antibodies directed to the fusion junction in the ectodomain of \(\Delta\)EGFR, to wt EGFR, and to phosphotyrosine. As shown in Fig. 1, both wild-type and \(\Delta\)EGFR were predominantly located at the cell surface of U87MG cells (Fig. 1a, a and c). Staining of permeabilized cells localized both wt EGFR and \(\Delta\)EGFR diffusely on the cell surface with enhancements at cell margins in agreement with previous reports (43, 44). After addition of EGF, wt EGFR were lost from the cell surface and appeared in perinuclear vesicles corresponding to endosomes and lysosomes (Fig. 1c). In contrast, the distribution of \(\Delta\)EGFR was not changed upon addition of EGF, and receptors remained at the cell surface (Fig. 1g). Cells expressing wt EGFR, phosphotyrosine staining was most marked in focal adhesions (Fig. 1b) and, after addition of EGF, increased phosphotyrosine co-localized with internalized EGFR in perinuclear vesicles (Fig. 1d). In cells expressing \(\Delta\)EGFR, phosphotyrosine was detected primarily with a punctate distribution at the cell surface which corresponded to the distribution of \(\Delta\)EGFR (Fig. 1f); after addition of EGF, phosphotyrosine staining at the cell surface remained unchanged (Fig. 1a).

To determine whether the phosphorylation detected in these assays could be primarily attributed to \(\Delta\)EGFR (Fig. 1, e–h), extracts from U87MG and U87MG.\(\Delta\)EGFR cells were analyzed by Western blotting with anti-phosphotyrosine antibodies. Phosphorimage quantitation indicated that a single peak corresponding to \(\Delta\)EGFR accounted for the majority of the measurable increase in phosphotyrosine (Fig. 2A). Identical results were obtained when these cells were chronically treated with EGF or with the antagonist monoclonal anti-EGFR antibody 225 (data not shown), confirming lack of effect of both ligand agonist and an antagonist monoclonal antibody on tyrosine kinase activity of \(\Delta\)EGFR. To confirm that the single peak corresponding to \(140–155\) kDa was indeed \(\Delta\)EGFR, the mutant receptors were repeatedly immunodepleted from U87MG.\(\Delta\)EGFR cell lysates (Fig. 2B, lanes 3–4) using a mono-
clonal antibody specific to ΔEGFR. In the ΔEGFR-depleted lysate the overwhelming majority of phosphotyrosine immunoreactivity in the 140–155 kDa region on Western blots had disappeared (Fig. 2B, lane 5), indicating that phosphotyrosine immunoreactivity detected at this region (Fig. 2B, lane 2) is ΔEGFR.

Because the rate-limiting step in down-regulation is internalization from the cell surface (9, 45), the rates of endocytosis of wt EGFR and ΔEGFR were directly measured using the 13A9 monoclonal antibody that recognizes both. Previous studies have indicated that the rate of endocytosis of monoclonal antibodies bound to the ectodomain of wt EGFR accurately reflects basal and ligand-stimulated internalization kinetics (9). The rate of endocytosis of ΔEGFR corresponded to the basal rate of endocytosis of non-activated and kinase-inactive EGFR (Fig. 3) (9) and was consistent with random entrapment in coated pits (9). In sharp contrast, ligand-activated wt EGFR exhibited a significantly higher rate of endocytosis. ΔEGFR were thus defective in down-regulation at least in part due to a low rate of constitutive internalization equivalent to that of non-activated wt EGFR.

Essential Sites of Autophosphorylation in ΔEGFR—The major phosphotyrosine containing protein in U87MG ΔEGFR cells was the ΔEGFR itself (Figs. 1 and 2) suggesting that autophosphorylation was critical to biological signaling by this mutant receptor. To determine the contribution of the intrinsic tyrosine kinase activity and autophosphorylation of ΔEGFR to tumorigenicity, several derivative ΔEGFR mutants were constructed (Fig. 4A). The kinase activity of ΔEGFR was inactivated by introducing a point mutation in the ATP-binding site which changed lysine 721 to methionine (8) (ΔK, Fig. 4A). Other ΔEGFR cDNAs were constructed with point mutations that altered the tyrosine phosphorylation sites identified in wt EGFR (Tyr-992, Tyr-1068, Tyr-1086, Tyr-1148, and Tyr-1173) (46–48), either singly (DY1, Y1173F) or in combinations (DY2–5). The constructs were introduced into U87MG cells individually by retroviral-mediated gene transfer and selected, and populations expressing levels of mutant receptors similar to that of U87MG ΔEGFR were sorted and expanded for further analysis (Fig. 4A). As a control, U87MG cells infected with viruses carrying the wt EGFR gene and expressing a similar level of wt EGFR were also selected by the same procedure. The presence of equivalent amounts of mutant (140–155 kDa) and wt receptors (170 kDa) in the cells was confirmed by probing
Western blots using anti-EGFR antibodies (Fig. 4B, lower panel).

While ΔEGFR was constitutively autophosphorylated (Fig. 4B, upper panel, lanes 3 and 4), DK EGFR was devoid of any significant tyrosine phosphorylation (Fig. 4B, upper panel, lane 5). Tyrosine phosphorylation of DK EGFR could not be restored by activation of the endogenous wt EGFR in the same cells; only a minor amount of DK EGFR phosphorylation was observed (Fig. 4B, upper panel, lane 6), indicating a lack of substantial cross-phosphorylation between ligand-activated wild-type and mutant receptors. This conclusion was supported by the lack of phosphorylation of wt EGFR which co-existed with the constitutively active ΔEGFR in the same cells (Fig. 4B, upper panel, lane 3). Autophosphorylation of mutant receptors was principally at tyrosine residues 1068, 1148, and 1173, because receptors mutated at these sites (DY3) showed almost complete loss of phosphorylation (Fig. 4B, upper panel, lanes 11 and 12). Mutants which retained one (DY2 and DY4) or two (DY1) of these residues were still phosphorylated (Fig. 4B, upper panel, lanes 7–10, 13, and 14). Receptors with mutations of all five tyrosine residues (DY5) were somewhat phosphorylated, albeit at significantly reduced levels (Fig. 4B, upper panel, lanes 15 and 16).

In order to quantify autophosphorylation of the various mutant EGFRs, receptors were immunosolated before probing with anti-phosphotyrosine antibody. Fig. 5A shows that the extent of phosphorylation per molecule of ΔEGFR was approximately 12% that of wt EGFR activated by EGF treatment. Analysis of the DY series of Tyr to Phe mutations indicated that Tyr-1173 appeared to be the major autophosphorylation site since a single mutation at this residue decreased phosphorylation to 35% that of ΔEGFR. Furthermore, mutant receptor DY4, which retained only the Tyr-1173 site, was phosphorylated to 42% that of ΔEGFR (Fig. 5B). Phosphorylation of mutant receptors with alterations in more than one autophosphorylation site was even lower, with decreases to 25 and 1% for DY2 and DY3, respectively (Fig. 5B). The phosphorylation level of DY5 was 3% that observed for ΔEGFR, suggesting that mutation of all five tyrosines resulted in accessibility of the intrinsic kinase to some minor phosphotyrosine site. In contrast, mutation of the kinase domain completely abolished autophosphorylation of ΔEGFR (Fig. 5B).

Autophosphorylation of EGFR tyrosine residues by ligand binding not only activates binding sites for signaling molecules but may also regulate the catalytic activity of the receptor by altering the conformation of the kinase domain and/or by changing the access of cellular substrates to the kinase active site (49, 50). This raised the question of whether mutations of the tyrosine residues in ΔEGFR affected the kinase activity. To evaluate the contribution of the kinase domain and the phosphorylation sites of ΔEGFR in the enhanced tumorigenic effect, the kinase activities of ΔEGFR containing mutations in tyrosine residues were determined. Cells overexpressing wild-type or mutant receptors were solubilized, and the receptors were immunosolated using anti-wt EGFR or anti-ΔEGFR specific antibodies. Receptor kinase activity was assayed in vitro using the synthetic substrate, 1173 peptide (51). While point muta-
Fig. 5. Quantitation of tyrosine phosphorylation of mutant EGFR. A, U87MG.wtEGFR cells and U87MG.ΔEGFR cells were treated without (−) or with (+) 100 ng/ml EGF for 5 min at room temperature prior to cell lysis. Mutant and wild-type EGFR were immunoprecipitated by anti-EGFR antibody, 528. Receptors loaded in each lane were isolated from 1 × 10^6 cells (wtEGFR lanes) or 3 × 10^5 cells (ΔEGFR lanes). Receptor tyrosine phosphorylation was detected by Western blot analysis as described in Fig. 4A, and autoradiograms were quantitated by densitometry. Tyrosine phosphorylation per wild-type receptor (wtEGFR lanes) or per mutant receptor (ΔEGFR lanes) is shown below each and calculated as a percentage of wild-type EGFR (100%). B, characterization of ΔEGFR autophosphorylation by mutational analysis. Receptors loaded in each lane were immunoprecipitated by the mutant receptor specific monoclonal antibody D113 (DY2 lane) or with parental U87MG cells (DY lane). Receptor tyrosine phosphorylation was detected by Western blot analysis as described in Fig. 4B and quantified by densitometry. Tyrosine phosphorylation per mutant receptor is shown below each lane and calculated as a percentage of ΔEGFR (100%).

Fig. 6. Effect of mutations of ΔEGFR on tumorigenic enhancement in subcutaneous implantations. A, tumorigenic activity of kinase-inactive mutant receptor (DK) expressed in U87MG cells. B, tumorigenic activity of tyrosine phosphorylation site mutants of ΔEGFR. 2 × 10^6 (+), n = 4; or 1 × 10^5 (●), n = 4) U87MG.ΔEGFR cells or 1 × 10^6 U87MG.DK cells (○, n = 4), U87MG.DY1 cells (□, n = 4), U87MG.DY3 cells (△, n = 4), U87MG.DY4 cells (▲, n = 4), U87MG.DY5 cells (●, n = 4), or U87MG.wtEGFR cells (□, n = 4) were inoculated into the right flank of nude mice while 1 × 10^6 U87MG cells (●, n = 4) or U87MG.DK cells (□, n = 4) were injected into the left flank of the same animals. Tumor size was quantitated at the indicated times. Similar results were obtained in two to three additional experiments.

U87MG.DK implantations expressed endogenous EGFR of 170 kDa as well as smaller species of 140–155 kDa corresponding to the DK receptor. These DK receptors were expressed at a level comparable with that of U87MG.DK cells grown in vitro
Deletion of amino acid residues 6–274 located in the ectodomain of EGFR creates a highly tumorigenic receptor tyrosine kinase as measured by growth of subcutaneously and intracerebrally implanted glioma expressor cells (26). Because this mutant receptor occurs in a variety of human tumors (13–15), the basis for its enhanced proliferative effects are of considerable interest. The ΔEGFR has several features in common with v-erbB, the principal oncogene of avian erythroblastosis virus (52), including failure to bind ligand and constitutive activation of intrinsic protein tyrosine kinase activity as assessed by autophosphorylation (53, 54). v-erbB resulted from retroviral insertion that reproducibly deleted most of the ectodomain of the avian EGFR (55), whereas ΔEGFR results from a common gene deletion that occurs naturally during malignant progression (18, 21, 24, 56). However, the transforming activity and tissue specificity of v-erbB is dependent on additional truncation and/or mutation of the carboxyl terminus (57, 58), raising the question of whether a small deletion of the ligand-binding domain in ΔEGFR alone is capable of eliciting any biological effect. The present studies confirm the hypothesis that the constitutively tyrosine kinase activity resulting from ectodomain deletion is essential to tumorigenic effects in human tumor cells, where this mutant receptor naturally occurs. Since the extent of autophosphorylation of ΔEGFR was small compared with ligand-activated wild-type EGFR, the basis for strong mitogenic signaling was uncertain. The present studies indicate that enhanced tumorigenesis of ΔEGFR is strictly dependent on intact autophosphorylation sites located in the regulatory carboxyl terminus. Our observation that ΔEGFR are defective in endocytosis and down-regulation suggests that the effects of the small extent of constitutive activation are greatly amplified due to failure of normal attenuation mechanisms.

Individual autophosphorylation sites in several tyrosine kinase receptors, an event particularly well studied in PDGF receptors, exhibit high specificity for binding SH2 and PTB domains (59); mutation of individual sites abrogates signaling via particular proteins (60). Autophosphorylation sites of EGFR appear to exhibit less specificity (2), although heterodimerization with and transphosphorylation of molecules such as erbB2 and erbB3 may provide coupling to specific downstream events (61). In ΔEGFR, Tyr-1173, the favored autophosphorylation site in ligand-activated wt EGFR (46), appears to be the major site of autophosphorylation. Mutation of Tyr-1173 reduced ΔEGFR function to enhance tumor formation in the animal model.
Tyr-1173 abolished detectable autophosphorylation. Mutation of any of these major autophosphorylation sites alone (Y1173F [DY1] Figs. 6 and 7, Y1068F [DY6], and Y1148F [DY8], data not shown) or in combination (DY3, DY4, DY5) abolished the enhanced tumorigenesis characteristic of ΔEGFR. Since these measurements reflect the average extent of phosphorylation per receptor, these values may reflect heterogeneity in the phosphorylation of any one receptor. The requirement that all major sites of tyrosine autophosphorylation be intact for enhanced tumorigenesis could reflect a combinatorial role for interaction of multiple SH2 and phosphotyrosine binding domain proteins with constitutively activated ΔEGFR. Alternatively, given the finding that ΔEGFR are autophosphorylated to only ~10% of the level of ligand-activated wild-type receptors, all autophosphorylation sites may be required to reach a critical threshold. These characteristics distinguish ΔEGFR from wt EGFR where it is necessary to mutate multiple tyrosine autophosphorylation sites to block ligand-dependent transformation (3).

There is evidence that ΔEGFR couple to the mitogen-activated protein kinase signaling pathway utilized by wt EGFR (62) but more weakly (63). Montgomery et al. (63) observed activation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase and mitogen-activated protein kinases in NIH 3T3 cells expressing ΔEGFR. The extent of activation was modest presumably due to enhanced tyrosine phosphatase activity. Further studies confirmed minimal activation of mitogen-activated protein kinases but could not detect increases in GDP-bound Ras (64). However, Prigent et al. (65) detected a 2-fold increase in GDP-bound Ras in U87MG cells expressing ΔEGFR and observed that anti-Ras antibodies inhibited DNA synthesis in these cells. Both Moscatello et al. (64) and Prigent et al. (65) observed constitutive association of Grb2 with ΔEGFR. Interestingly, while binding to Grb2 has been proposed to function in endocytosis of wt EGFR (66), ΔEGFR are defective in endocytosis despite binding Grb2. While ΔEGFR may couple to additional signaling pathways, it appears to use the same mitogenic signal transduction pathways as wt EGFR consistent with the low level constitutive activation of the mutant receptor. Moreover, it has been reported (64) that ΔEGFR is constitutively dimerized. We noted a small (<5%) extent of dimerization of ΔEGFR that was unaffected by addition of ligand. The extent of dimerization observed was roughly proportional to the extent of activation of ΔEGFR (~10%) and efficacy of cross-linking. ΔEGFR thus exhibits neither strong homo- nor hetero-dimerization.

Autophosphorylation of EGFR provides sites both for assembly of components of the signal transduction cascade and for exposure of cryptic sequences in the carboxyl terminus necessary for ligand-induced endocytosis and down-regulation. In the absence of the carboxyl terminus, ligand-activated EGFR exhibit enhanced tumorigenesis due to failure to increase both the absence of the carboxyl terminus, ligand-activated EGFR inhibit neither strong homo- nor hetero-dimerization. ΔEGFR displays tyrosine kinase activity, it may interact with the cellular trafficking machinery in the same way as wt EGFR.

The constitutive kinase activation, autophosphorylation levels set near the threshold for enhanced mitogenesis, and the defect in endocytosis may all reflect partial activation of ΔEGFR. Conformational changes sufficient to allow autophosphorylation to ~10% is sufficient for receptor mitogenic signaling but insufficient for exposure of endocytic sequence codes necessary for down-regulation and attenuation of signal transduction. The recent observation that the kinase activities of wt EGFR and ΔEGFR can be differentially blocked by various tyrosine kinase inhibitors is consistent with the idea that the conformational changes induced by ectodomain deletion in ΔEGFR differs from ligand-activated wt EGFR (72). We hypothesize that deletion of exons 2–7 removes inhibitory constraints sufficient to allow low level constitutive activation of the intrinsic protein tyrosine kinase activity of EGFR. Although specific autophosphorylation sites may engage specific phosphatases in wt EGFR (66), the intrinsic protein tyrosine kinase activity of EGFR at the cell surface, the apparent site of assembly of many signaling complexes, would prolong and enhance its low level of activity. ΔEGFR is thus seen as active for mitogenic signaling but escapes detection by the endocytic trafficking system so that signaling is unattenuated.

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