Activity and Cellular Localization of an Oncogenic Glioblastoma Multiforme-associated EGF Receptor Mutant Possessing a Duplicated Kinase Domain

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Abstract

A mutation of the epidermal growth factor receptor (EGFR) that results in a tandem kinase domain duplication (TKD-EGFR) has been described in glioblastoma multiforme biopsies and cell lines. Although the TKD-EGFR confers tumorigenicity, little is known about the molecular underpinnings of receptor dysregulation. Therefore, we transfected B82L mouse fibroblast cells devoid of endogenous EGFR to determine the molecular mechanisms of receptor activation when expressed in cells as well as the contribution of each duplicated kinase domain to receptor phosphorylation. The TKD-EGFR displayed chronically elevated basal autophosphorylation at five known phosphotyrosine sites. The chronically phosphorylated TKD-EGFR was also resistant to competitive inhibition of ligand-binding compared to wild-type-EGFR (WT-EGFR) and exhibited undetectable levels of basal dimerization, suggesting the TKD-EGFR escapes known mechanisms of receptor down-regulation. Immunofluorescence analyses revealed a substantial portion of the TKD-EGFR resides in the cytosol in an activated state, although surface-localized subsets of the receptor retain ligand-responsiveness. Kinase activity-deficient knockouts of the N-terminal or the C-terminal kinase domains generated TKD-EGFRs that recapitulate the autophosphorylation/localization patterns of a constitutively activated receptor versus a WT-like EGFR, respectively. Investigation of the molecular activity of the TKD-EGFR yields evidence for a unique mechanism of constitutive activity and dual kinase domain activation.

Keywords

EGF receptor (EGFR); glioblastoma multiforme; constitutive activity; duplicate kinase domains

INTRODUCTION

Growth factors and their receptors are important in the development and progression of various cancers. For example, the up-regulation of EGF, bFGF, PDGF, and VEGF, or their receptors, have been implicated in promoting the cell migration (Brockmann et al. 2003),
proliferation (Pollack et al. 1991), and angiogenesis (Jensen 1998) associated with the most malignant form of brain cancer, namely glioblastoma multiforme (GBM). GBM is a common form of adult primary brain tumor (Bondy et al. 2008), and the World Health Organization classifies GBM as a grade IV astrocytoma characterized by a preponderance to diffusely and aggressively invade both hemispheres of the brain parenchyma and to exhibit refractoriness to treatment (Collins 2004). Patient prognosis is dismal with median survival times ranging from 12–15 months after diagnosis (Wen and Kesari 2008).

The development of GBM is heterogeneous, but examination of the genetic profiles associated with it reveals two general pathways. Approximately 20% of GBMs arise secondarily, with the tumor progressing through lower grades before worsening to a grade IV glioma. In these instances, loss of the p53 tumor suppressor is a frequent event, although other genetic defects can occur, including a loss of the phosphatase PTEN and a loss of heterozygosity (LOH) in chromosome 16. In contrast, 80% of GBMs arise de novo without evidence of earlier grade progression and often involve alterations in the EGFR (Ohgaki and Kleihues 2005). In primary GBMs, the EGFR is over-expressed and/or amplified in nearly 50% of cases (Ekstrand et al. 1991), and approximately half of these cases additionally possess receptor mutations (Frederick et al. 2000).

The EGFR is the archetypal member of the ErbB family of receptor tyrosine kinases and regulates many cellular processes, including proliferation, growth, and migration (Jorissen et al. 2003). Upon binding extracellular ligands, the receptor dimerizes with another EGFR or other ErbB family member and undergoes phosphorylation on its regulatory C-terminal tail, which activates the receptor and provides docking sites for the tyrosine phosphorylation of downstream signaling effectors (Edwin et al. 2006).

EGFR over-expression, amplification, and mutation have been described in multiple cancers, including those of the brain and lung (Sihto et al. 2005). The most common EGFR mutation in GBMs is EGFRvIII, wherein a portion of the extracellular ligand-binding domain is deleted and which exhibits ligand-independent signaling (Huang et al. 1997). The EGFRvIII escapes known regulatory mechanisms, including homo-dimerization (Chu et al. 1997) and down-regulation by internalization (Grandal et al. 2007). Constitutive activity induced by this mutation and others appears to be a common mechanism of aberrant signaling in cancers possessing EGFR mutations (Riese et al. 2007).

One EGFR mutation detected in GBM patient-derived samples and cell lines (Fenstermaker et al. 1998; Ciesielski and Fenstermaker 2000; Fenstermaker et al. 2007) involves an in-frame, high-fidelity duplication of residues 664-1030, comprising a tandem kinase domain duplication (TKD-EGFR). The TKD-EGFR has been detected in two GBM biopsy panels (Fenstermaker et al. 1998; Frederick et al. 2000), but little is known about the incidence of this mutation in GBM, and its existence in other cancers is unclear. Soft agar assays using NR6 mouse fibroblasts devoid of endogenous EGFR but transfected with the TKD-EGFR demonstrated anchorage-independent growth both in the presence and absence of ligand (Ciesielski and Fenstermaker 2000). Furthermore, nude mice injected with TKD-EGFR-transfected cells displayed significant tumor growth after 40 days compared to wild type (WT) and non-expressing controls (Ciesielski and Fenstermaker 2000). The TKD-EGFR
revealed little difference in ligand-induced internalization rates, but the authors noted a relative paucity of high-affinity receptors compared to normal and an apparent elevated basal kinase activity (Ciesielski and Fenstermaker 2000). Beyond exploring the relative ligand affinities and internalization rates, little is known about the molecular mechanics of the TKD-EGFR.

Using B82L mouse fibroblast cells containing negligible endogenous EGFR, we examined the expression and autophosphorylation of WT- and TKD-EGFRs. Furthermore, we generated kinase domain knockout mutants of the TKD-EGFR to elucidate the contribution of each kinase domain to receptor activity. We observed constitutive kinase/autophosphorylation activity and altered basal localization of the TKD-EGFR, for which the C-terminal duplicated kinase domain was primarily responsible. This observation has important implications for understanding EGFR activation, presents a unique activation mechanism in proteins with duplicated functional domains, and lends insight into a tumorigenic mutation involved in GBM development.

**MATERIALS AND METHODS**

**Generation of plasmid vectors**

The pLXIN plasmids containing empty vector (EV) or the TKD-EGFR sequences were generously provided by M.J. Ciesielski and R.A. Fenstermaker (Roswell Park Cancer Institute, Buffalo, NY). Generation of WT-EGFR was accomplished by removing the duplicated region at a duplicated Bgl-II restriction site and re-ligating using T4 DNA ligase. Two non-conservative point mutations from this WT-like sequence were mutated to generate their original protein sequence (E907D and T1171A) using the QuickChange site-directed mutagenesis kit (Stratagene). DNA plasmids were amplified in competent TOP10F′ cultures and amplified using Midiprep kits (Qiagen). Sequences were confirmed using the BigDye sequencing protocol (UW Biotechnology Center).

**Generation of kinase domain dead vectors**

A plasmid containing the WT-EGFR was mutated to generate a methionine at the active site lysine (K721M) using the QuickChange kit. The forward primer was CCCCCTGGCTATCATGGAATTAAGAAGC and the reverse primer was GCTTCTCTTAATTCCATGATAGCGACGGG. Plasmid containing TKD-EGFR was digested at a duplicated Bgl-II site and inserted into a FLAG-CMV vector. Both the FLAG-insert and the WT-like plasmid had point mutations introduced by site-directed mutagenesis as described above. The mutated insert was reintroduced into WT-like plasmid to generate the C-terminal kinase domain mutation (K1088M-TKD) whereas mutated WT-like plasmid was re-ligated with either an unmodified or mutated insert to generate the N-terminal kinase domain mutation (K721M-TKD) or the kinase domain knockout (K721M/K1088M or KIIM-TKD), respectively. Plasmids were amplified and sequenced as described above. Insertion was further confirmed by analysis of restriction fragment size from an Apa-I restriction site flanking the kinase domain and insert orientation was confirmed by analysis of restriction fragment size from a duplicated Nsi-I restriction site.
Stable EGFR expression in B82L mouse fibroblasts

B82L mouse fibroblast cells were plated and grown to 50–80% confluency on the day of transfection. Lipofectamine or Lipofectamine 2000 (Invitrogen) was used according to protocol and cell selection began two days post-transfection using 500 μg/ml G418 sulfate until a population of stably transfected cells was generated (typically 2–3 weeks). G418 sulfate selection was maintained to prevent loss of receptor expression.

Immunoblotting for EGFR phosphorylation

B82L cell transfections were serum-starved for ≥4 hours and treated with 10 nM EGF or 20 mM HEPES unless otherwise indicated. Cells were washed twice with HBSS and harvested with Laemmli buffer. Cell lysates were sonicated for 10% duty cycle 1.75 on a Branson 250 sonifier and centrifuged at 4°C and 14,000 rpm for 10 minutes to remove cell debris and nuclear material. Total protein levels were quantified using the MicroBCA total protein analysis assay (Thermo Scientific). Samples were boiled for 5 minutes and 50–60 μg of protein were loaded onto 6% SDS-PAGE gels prior to transferring to PVDF membranes. Immunoblotting with pY992, pY1068, pY1086, pY1148, and pY1173 phosphospecific EGFR antibodies (Biosource) or pan-reactive antibodies against the extracellular domain (Clone H11, Labvision) or the kinase domain (SC03, Santa Cruz) was performed in 5% milk in TBS-T. Loading controls were obtained by immunoblotting for STAT3 or STAT5 (Santa Cruz). Blots were incubated with HRP-tagged goat anti-rabbit or anti-mouse IgG (Santa Cruz) and developed with Super-Signal-West-Dura chemoluminescence agent (Thermo Scientific).

Inhibition of EGFR by monoclonal antibody 528

Monoclonal antibody 528 (mAb 528) was affinity-purified from hybridoma cell line HB 8509 (ATCC, Rockville, MD). Cells were serum-starved for ≥4 hours then incubated with 1 μg/ml mAb 528 for four additional hours prior to EGF stimulation. Cells were subsequently processed and immunoblotted as outlined above.

Crosslinking for EGFR dimerization

Cells were serum-starved for ≥4 hours and subsequently treated with 10 nM EGF or 20 mM HEPES. Cells were then treated with 10 μM 1-ethyl-3-(3-dimethylpropyl) carbodiimide (EDAC, Sigma) for 15 minutes prior to processing and immunoblotting as outlined above (100 μg per sample). To prevent internalization in some experiments, cells were pre-treated with 1 mM ammonium chloride (Sigma) or 3 mM amiloride (Sigma) for one hour prior to EGF treatment.

Immunohistochemical staining to determine EGFR localization

Cells were plated onto coverslips in 6-well plates at a density of 5×10⁴ cells per well and grown overnight. Cells were serum-starved for 4 hours prior to treatment with 10 nM EGF or 20 mM HEPES for 15 minutes. Cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized in 0.1% Triton-X 100. Endogenous Biotin Blocking kit (Invitrogen) was used prior to blocking in PBS supplemented with 4% normal donkey serum (NDS, Jackson Immunological), and 1% BSA (Sigma). Cells were incubated with anti-
EGFR antibody clone H11 (Labvision: 1 mg/ml, 1:500) and/or pY1086 anti-phosphospecific EGFR antibody (Upstate: 200 μg/ml, 1:100) diluted in 1% NDS/1% BSA for one hour followed by Alexa 488-conjugated donkey anti-mouse secondary antibody (Invitrogen: 2 mg/ml, 1:10,000) and/or BiotinXX-conjugated goat anti-rabbit antibody (Invitrogen: 2 mg/ml, 1:10,000). Cells were then incubated with Alexa 568-conjugated streptavidin (Invitrogen: 1 mg/ml, 1:10,000) for one hour. 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) was included either in the secondary or tertiary incubation steps to stain nuclear material. Coverslips were mounted with ProLong Gold (Invitrogen) and examined for fluorescence using a Nikon A1 confocal microscope. Images were obtained and processed using Nikon Elements software.

**Statistical Analysis**

All statistical analyses were computed using R version 1.16. Both the Mann-Whitney nonparametric analysis and Student’s T-test were employed, and criteria for significance were based on p-values lower than 0.05/n, where n was the number of comparisons tested.

**RESULTS**

**The TKD-EGFR is constitutively tyrosine phosphorylated**

The TKD-EGFR is an insertion mutation wherein the kinase domain and the CAIN domain, spanning residues 664 to 1030 and representing exons 18–26 of the gene, are inserted directly adjacent to residue 1030, thereby providing a tandem, in-frame duplication of 367 residues (Fig. 1). Important domains and residues duplicated in this region include the kinase domain, the tyrosine phosphorylation site at Y992, as well as the CAIN domain containing internalization motifs and the hinge region separating the kinase domain from the regulatory C-terminal tail.

To study EGFR activation, B82L mouse fibroblast cells with negligible endogenous ErbB family member expression were stably transfected with the pLXIN vector containing either no insert (EV), full length wild type (WT), or the TKD-EGFRs (Fig. 1). A distinct difference in migration was observed on immunoblotting (Fig. 1 inset) between WT-(170 kDa) and TKD-EGFR (190 kDa), consistent with the increased size of the TK-EGFR, whereas little to no signal was detected in EV controls. Furthermore, although antibodies directed against an EGFR extracellular domain epitope revealed similar relative levels of expression, antibodies directed against the kinase domain depicted a large increase in signal for the TKD-EGFR, consistent with the multiplication of the antibody epitope.

To determine TKD-EGFR activity, the transfected B82L cells were serum-starved then treated with 10 nM EGF or vehicle. Receptor phosphorylation was assessed by individually immunoblotting for major sites of tyrosine autophosphorylation, including Y992, Y1068, Y1086, Y1148, and Y1173 (Fig. 2a). For both WT and TKD-EGFR-expressing cells, all phosphotyrosine sites revealed a similar pattern of EGF-induced phosphorylation. However, in contrast to WT-EGFR, the TKD-EGFR displayed elevated levels of basal autophosphorylation at the major tyrosine sites tested (Table 1). With all but the phosphorylation site at Y992, basal activation of TKD ranged from 21–50% of EGF-induced phosphorylation.
phosphorylation, compared to ≤5% basal activation of WT-EGFR. A Wilcoxon rank sum test applied to the band densities from pY1173 also showed a statistically significant elevation in basal signal in TKD-EGFR compared to WT-EGFR (p=0.007) and EV (p=0.0006). These observations were consistent through three to four independent rounds of transfection, and also persisted for at least 24 hours (Fig. 2b).

**TKD-EGFR constitutive activity is resistant to a ligand-competitive blocking antibody**

Autocrine/paracrine release of ligand is a common mechanism of constitutive activation of the EGFR (Ekstrand et al. 1991; Tang et al. 1997). To test this mechanism of constitutive activity, we pre-treated serum-starved EV-, WT-, and TKD-EGFR-expressing B82L cells for four hours with monoclonal antibody 528 (mAb 528), which is a competitive antagonist of the ligand-binding domain (Gill et al. 1984; Sato et al. 1983), prior to 10 nM EGF stimulation. Because there was no observable preference with regards to site-specific tyrosine phosphorylation, we chose to limit immunoblotting to phospho-Y1173 and phospho-Y1068.

Treatment of WT-EGFR-expressing cells with mAb 528 reduced EGF-stimulated activity at each phosphorylation site by 58–70% (Fig. 3). Although the antibody inhibited receptor phosphorylation in the EGF-treated TKD-EGFR-expressing cells to a lesser degree (43–59%), the elevated basal phosphorylation levels of the TKD-EGFR were relatively unaffected by the presence of the ligand-blocking antibody, remaining at 19–24% of activated TKD EGFR for phospho-Y1068 and 30–36% for phospho-Y1173.

**Constitutive activity of the TKD-EGFR appears independent of dimerization**

Upon EGFR activation, dimerization rapidly occurs to propagate signaling and initiate internalization. To delineate the relationship between constitutive receptor activation and dimerization, we cross-linked the EGFR by treating EGF-stimulated and unstimulated B82L transfected cells with 15 mM EDAC and blotting for both total and Y1173-phosphorylated EGFR (Fig. 4). The TKD-EGFR, similar to WT-EGFR, was capable of forming phosphorylated/activated dimers upon EGF stimulation. Although there continued to be constitutively phosphorylated/active monomers in the unstimulated TKD-EGFR-expressing cells, no basal level of TKD-EGFR dimerization was observed. Furthermore, because EGFR phosphorylation usually leads to internalization, and because EDAC is only cell-surface accessible, we also pre-treated cells for one hour with 1 mM ammonium chloride or 3 mM amiloride, which impair internalization or promote cell surface recycling, but found no increase in detectable basal dimerization levels (data not shown).

**The TKD-EGFR resides primarily in an activated state within the cell**

Constitutive activity can occur if the EGFR escapes internalization and subsequent down-regulation (Grandal et al. 2007). However, previous reports have shown little difference in the kinetics of TKD-EGFR internalization after EGF stimulation (Ciesielski and Fenstermaker 2000), suggesting little inherent receptor resistance to ligand-induced internalization. To evaluate TKD-EGFR localization, we used immunofluorescence to observe total and phosphorylated receptor with and without EGF treatment (Fig. 5). Because of the relationship between phospho-Y1086 and receptor internalization (Jiang et al. 2003),

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the localization of activated receptor was tracked using antibodies directed against phospho-Y1086. We detected an absence of signal in EV-transfected cells (Figs 5a, 5d), whereas WT-EGFR resided on the cell surface (Fig 5b) and became phosphorylated/activated and largely internalized after EGF treatment (Fig 5e). In contrast, a substantial portion of the TKD-EGFR already resided within the intracellular compartment and appeared phosphorylated at Y1086 without EGF addition (Fig 5c), with limited redistribution of activated receptor occurring after EGF treatment (Fig 5f). Furthermore, unpermeabilized cells consistently demonstrated surface expression of WT-and TKD-EGFR in the absence of added EGF (data not shown).

**Kinase domain knockouts of the TKD-EGFR reveal distinctive autophosphorylation dynamics for each kinase domain**

The kinase domain of the TKD-EGFR is duplicated with very high fidelity, but the relative functionality of each domain is unknown. Using site-directed mutagenesis, kinase domain knockouts of both WT- and TKD-EGFR were generated by altering the active site lysine at residue 721 (as well as residue-1088 in the TKD-EGFR) to methionine. This mutation compromises ATP binding by the EGFR and attenuates substrate phosphorylation (Chen et al. 1987; Honegger et al. 1987). For the TKD-EGFR, both active site lysines at residue-721 (N-terminal kinase domain, K721) and the duplication at residue-1088 (C-terminal kinase domain, K1088) were mutated alone (K721M-TKD and K1088M-TKD) as well as together (K721M/K1088M- or KIIM-TKD) to determine the contribution of each kinase domain to receptor phosphorylation and activity.

Immunoblotting for EGFR phospho-Y1068 and phospho-1173 with and without EGF stimulation revealed that the kinase domain knockouts of WT- (K721M-WT) and TKD-EGFR (KIIM-TKD, i.e., both kinase domains) abrogated EGF-stimulated autophosphorylation activity by 92–96% and 90–94%, respectively (Fig. 6). Basal and stimulated WT- and TKD-EGFR phosphorylation levels were similar to those previously observed in Fig. 2.

Mutations of the individual active site lysines demonstrated disparate phosphorylation dynamics of each kinase domain in the TK-EGFR. When the N-terminal kinase domain was mutated (K721M TKD), the TKD-EGFR maintained constitutive phosphorylation of Y1068 and Y1173 but, unlike unmodified TKD-EGFR, this phosphotyrosine level was only moderately inducible upon EGF-stimulation (3–9% basal and 5–12% activated compared to WT-EGFR). In contrast, mutation of the C-terminal kinase domain (K1088M TKD) resulted in a receptor phenotype analogous to WT-EGFR wherein basal phosphorylation was nearly non-existent (≤1% of activated WT-EGFR), requiring EGF-stimulation for robust autophosphorylation (26–38% of activated WT-EGFR).

To verify the autophosphorylation activity of each kinase domain after EGF stimulation, TKD-, K721M TKD-, and K1088M TKD-EGFR-expressing cell lines were treated with EGF over the course of an hour and evaluated for phosphorylation at Y1173 (Fig. 7). Each of the TKD variations evidenced EGF-induced activity, although the K1088M EGFR (with the N-terminal kinase intact) activated nearly three times more robustly than the K721M TKD-EGFR (with just the duplicated kinase domain intact), whereas the K7 2 1 M T K D-
EGFR consistently displayed ligand-independent basal autophosphorylation. Furthermore, although the phosphorylation of K1088M TKD-EGFR is reduced by more than 50% of peak levels over the one-hour period, the TKD-and the K721M TKD-EGFRs maintain 60% and 96%, respectively, of their peak phosphorylation levels (observed after ten minutes of EGF stimulation).

The kinase domain mutations recapitulate WT- and TKD-like internalization and activation patterns

Given the observed similarities of K1088M TKD-EGFR and K721M TKD-EGFR to WT- and TKD-like autophosphorylation patterns, respectively, immunofluorescence was used to determine whether the single- and double-kinase domain mutations adhered to the same localization patterns established for the unmodified WT- and TKD-EGFR-expressing cells (Fig. 8). Both of the complete kinase domain knockouts, K721M WT-EGFR (Figs 8a, 8e) and KIIM TKD-EGFR (Figs 8d, 8h) retained surface-localized unactivated/unphosphorylated receptor under all tested conditions. The WT-like K1088M TKD EGFR variant expressed non-activated receptor at the surface (Fig 8c) that became phosphorylated and internalized upon EGF stimulation (Fig 8g) whereas the TKD-like K721M TKD-EGFR exhibited predominately phosphorylated intracellular populations (Fig. 8b) with little change in internalization and activation patterns upon EGF stimulation (Fig 8f). Furthermore, all of the mutants were capable of EGF-induced dimerization but displayed no detectable basal dimerization (data not shown).

Lastly, we pre-treated TKD-, K721M TKD-, and K1088M TKD-EGFR-expressing cells with or without the ligand-blocking mAb 528 and immunoblotted for phospho-Y1173. Both the TKD- and the K721M TKD-EGFR-expressing cells retained their constitutive phosphorylation in the presence of the mAb 528 (20–40% and 8–10%, respectively, of stimulated TKD levels), and EGF-induced levels were only moderately susceptible to mAb 528 addition (43% and 23% inhibition from uninhibited levels, respectively), whereas the phosphorylation of the K1088M TKD-EGFR was abrogated by 75% from uninhibited levels and nearly completely absent basally (1–2% of stimulated TKD-EGFR levels), similar to the behavior of WT-EGFR (Fig. 9).

DISCUSSION

In many cancers, the EGFR is over-expressed, amplified, and/or mutated (Sihto et al. 2005). Recently, an EGFR mutant wherein the kinase domain is duplicated (the TKD-EGFR mutation) was identified in GBM tumor biopsies and a cell line (A172). Earlier reports found this mutant to be highly tumorigenic in vivo and in vitro, but little is known about its molecular mechanism of tumorigenesis. Here we have delineated the expression, localization and activation patterns, as well as the contributions of the individual kinase domains, of this TKD-EGFR mutant.

Transfection of B82L mouse fibroblast cells, which express negligible levels of endogenous ErbB family members, revealed that the TKD-EGFR is constitutively phosphorylated at five known sites of tyrosine phosphorylation. Although previous reports have suggested constitutive phosphorylation of the TKD-EGFR under certain conditions, this study
expounds upon earlier findings by taking advantage of the development of increasingly sensitive phosphospecific antibodies to demonstrate constitutive activity at individual tyrosine phosphorylation sites under serum-starvation conditions. We found that there is no preferential site of receptor phosphorylation/activation and that it is not simply a feature of increased phosphorylation by other kinases, such as Src, given that several of the elevated TKD-EGFR phosphorylation sites are not Src targets.

Immunofluorescence revealed that the TKD resides primarily in the intracellular space and that these intracellular pools are constitutively phosphorylated. Consistent with this concept, the basal activation of the TKD receptor is resistant to inhibition by the ligand-competitive antagonist mAb 528, and EGF-stimulated TKD-EGFR activity is not inhibited below the elevated basal levels. Furthermore, basal levels of dimerization were not detected, even when cells were pre-treated with agents that attenuate internalization. Despite these high intracellular pools, B82L cells expressing the TKD-EGFR retained some degree of cell surface expression and EGF-responsiveness as observed by EGF-induced C-terminal phosphorylation and receptor internalization after EGF-stimulation.

These data demonstrate that the TKD-EGFR is capable of EGF-induced stimulation and internalization as well as ligand- and dimerization-independent constitutive activity. This observation suggests that there is a subset of the TKD-EGFR that remains localized to the intracellular compartment and another subset that is accessible at the cell surface, given that neither EGF nor the ligand-competitive antagonist mAb 528 are known to cross the cell membrane to activate or inhibit the receptor, respectively.

Mutating each of the two active site lysines (K721 and/or K1088) revealed distinctive roles for each of the duplicated kinase domains. Abrogating kinase activity in the C-terminal kinase domain (K1088M), which preserves N-terminal kinase activity (K721), resulted in a WT receptor-like profile wherein the receptor retains EGF-responsiveness but has little to no detectable basal activity. In contrast, mutating the N-terminal kinase domain (K721M), thereby retaining the C-terminal kinase activity (K1088), resulted in a receptor with elevated basal activity but only marginal responsiveness to EGF stimulation.

This difference in kinase activity was also observed following inhibition with mAb 528, which delineated a WT-like inhibition profile of the K1088M TKD-EGFR in contrast to the K721M TKD-EGFR that, similar to unmodified TKD-EGFR, exhibited mAb-attenuated phosphorylation only to the elevated basal level. Furthermore, immunofluorescence demonstrated a similar localization and phosphorylation pattern of the K721M and K1088M mutants, analogous to the TKD-EGFR and WT-EGFR, respectively. Both TKD-EGFR kinase domains also demonstrated EGF-inducible phosphorylation of the receptor over a period of an hour, although the K721M TKD-EGFR was far less responsive to EGF stimulation. Finally, abrogation of both kinase domains (KIIM TKD), similar to WT-EGFR kinase domain knockouts (K721M WT), eliminated nearly all of the constitutive activity and much of the ligand-induced activity, which was also observed on immunofluorescence in the context of decreased intracellular pools and phosphorylation. It should be noted that immunoblotting sometimes revealed a lower band in the K1088M TKD-EGFR-expressing cells after autophosphorylation, which was attributed to a degradation product or construct-
specific mechanism as WT-like sequences were not observed in the original plasmids, both by DNA sequencing as well as by a lack of characteristic restriction digestion patterns using ApaI and NsiI (data not shown).

The observed difference in kinase domain phosphorylation dynamics has important implications regarding the spatial arrangement of the kinase domains relative to regulatory regions of the EGFR, such as the juxtamembrane domain and the C-terminal tail. Alterations in the juxtamembrane domain have previously been associated with increased autophosphorylation and are directly coupled to conformational changes induced by the extracellular ligand-binding domain (Poppleton et al. 1999), thereby translating EGF stimulation and subsequent dimerization into intracellular changes that promote C-terminal tail autophosphorylation and downstream activation. The C-terminal tail is instead implicated in inhibition of EGFR activity until phosphorylated, and this inhibition is in part thought to occur by steric or chemical interactions with the kinase domain (Bertics and Gill 1985; Landau et al. 2004). Duplication of sterically relevant residues, such as those in the region of 955–957/8, may result in the altered phenotype observed in our results.

Thus, despite the high sequence fidelity in the duplicated kinase domains, which might predict similar kinase activities of each domain, the overall functionality of the kinase domains appears dependent upon the surrounding regulatory elements. In particular, the N-terminal kinase domain appears to be under the regulatory controls of the ligand-binding domain and the subsequent steric shifts in the juxtamembrane domain, as well as the inhibition by the C-terminal tail. The C-terminal kinase domain, in contrast, seems to escape many of the known regulatory elements and therefore remains constitutively activated. However, because there is little difference between unstimulated and EGF-stimulated receptor phosphorylation, and because this kinase domain mutation localizes primarily into the intracellular compartment, this strongly suggests that much of this receptor population remains activated and internally localized, thereby making it relatively inaccessible to both EGF-stimulation as well as 528 mAb exposure. The duplication of the CAIN domain and relevant internalization motifs, such as the di-leucine motif at 1010–1011, warrant further exploration regarding their effect on the intracellular localization pattern of the TKD-EGFR.

The TKD-EGFR appears activated and localized to the intracellular compartment in the absence of detectable dimerization, which is distinct from WT-EGFR. Perhaps the TKD-EGFR forms an in cis asymmetric dimer with its duplicated kinase domains that could promote the open conformation of the C-terminal kinase domain similar to those observed in EGFR homodimerization (Zhang, Gureasko et al. 2006). This scenario would suggest that when WT-EGFR dimerizes, the relative position of the kinase domain in relation to its dimerization partner could also be a factor in determining whether the kinase domain is in an open or a closed state. Alternatively, the C-terminal kinase domain of the TKD-EGFR may not be fully accessible to proximal phosphatase action thereby allowing its basal kinase activity to proceed relatively unopposed.

The work on this receptor also has important implications for understanding the activity of proteins possessing duplicated kinase domains that, to our knowledge, represent a mechanism of action that has not previously been observed. Unlike the JAK family of
kinases, which possess a duplicated “pseudokinase” domain responsible for kinase activity regulation, the duplicated kinase domain of the TKD-EGFR does not appear to serve a similar suppressive function (Saharinen and Silvennoinen 2002). The TKD-EGFR is also distinct from the dual kinase domains in PDGFR, VEGFR, and FGFR receptor tyrosine kinases (Blume-Jensen and Hunter 2001), which possess a linker region that bisects the two halves of an otherwise singular kinase domain. Finally, the TKD is also unique from the AGC family of proteins, including RSK (Anjum and Blenis 2008) and MSK (Smith et al. 2004), which require activation of one kinase domain to either directly phosphorylate the second kinase domain or recruit other kinases for activation, although the TKD-EGFR shares some similarity with the RSK4 isoform with respect to constitutive activity and intracellular localization (Dümmler et al. 2005). However, in this family, both kinases are required for full activity of the protein, in contrast to the distinctive roles of each TKD-EGFR kinase domains. Thus, the TKD-EGFR is interesting with respect to the observation that evolutionary events frequently involve large-scale insertions and duplications and presents an intriguing glimpse into the nature and consequences of functional domain duplications.

In conclusion, the TKD-EGFR appears constitutively active and has a high degree of intracellular localization. Furthermore, we have delineated differences in the kinase activity of each of the duplicated kinase domains that sheds light on the receptor’s ability to bypass its conventional regulatory elements, which may reflect its role in brain tumor development and may have implications on the susceptibility of certain GBM patients to EGFR-targeted treatments.

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ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| EGFR         | epidermal growth factor receptor |
| EV           | empty vector |
| WT           | wild type |
| TKD-EGFR     | tandem kinase domain duplication mutant |

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Figure 1.
Diagram of the regions of the EGFR highlighting important domain boundary residues (normal script numbers), domain sizes (parenthetical numbers), and tyrosine phosphorylation sites (italicized numbers). The insertion point and relevant amino acid information of the TKD-EGFR-specific region (designated TKD and spanning from 1031–1397) is indicated at the site of insertion at residue 1030. The insets are representative immunoblots using antibodies against the extracellular and the kinase domain of EGFR and show the approximately 20 kDa shift of the TKD-EGFR (TKD) from 170 kDa to 190 kDa. 10-minute EGF stimulation (+) had little effect on overall receptor expression. Equal loading was verified by immunoblotting for STAT5 (α-STAT5).
2a.

B82L Cell Line
10 nM EGF (10′)

|         | EV  | WT  | TKD |
|---------|-----|-----|-----|
| α-pY1173 EGFR  |     |     |     |
| α-pY1086 EGFR  |     |     |     |
| α-pY1068 EGFR  |     |     |     |
| α-pY1148 EGFR  |     |     |     |
| α-pY992 EGFR   |     |     |     |
| α-STAT3        |     |     |     |
2b. Figure 2.
Immunoblotting of basal and EGF-induced receptor activities at five phosphotyrosine sites. (a) B82L mouse fibroblast cells transfected with empty vector (EV), wild type-EGFR (designated WT), or TKD-EGFR (designated TKD) were serum-starved and treated with 10 nM EGF (+) or 20 mM HEPES volume control (−) for 10 minutes. Samples (50–60 μg of total protein) were run on 6% SDS-PAGE gels and immunoblotted with phosphospecific anti-EGFR antibodies (Biosource) for phospho-Y992 (α-pY992), phospho-Y1068 (α-pY1068), phospho-Y1086 (α-pY1086), phospho-Y1148 (α-pY1148), and phospho-Y1173 (α-pY1173) (n=2–7). (b) Constitutive and EGF-induced activity of the five phosphotyrosine activation sites were monitored on immunoblots over a 24-hour period after serum-starvation (n=1). Equal loading was verified by immunoblotting for STAT3 (α-STAT3).
Figure 3.
Immunoblots of receptor activity after inhibition by the ligand-competitive antagonist mAb 528. B82L cells transfected with either empty vector (EV) or wild-type-EGFR (designated WT) or TKD-EGFR (designated TKD) were serum-starved and incubated with (+) or without (−) 1 μg/ml 528 mAb for 4 hours prior to stimulation with 10 nM EGF (+) or 20 mM HEPES volume control (−). Samples (50–60 μg of total protein) were electrophoresed using 6% SDS-PAGE gels and immunoblotted with phosphospecific anti-EGFR antibody for phospho-Y1173 (α-pY1173) or phospho-Y1068 (α-pY1068) (Biosource) and STAT5 (α-STAT5) (Santa Cruz) as a loading control (n=2–3).
Figure 4.
Immunoblots of cross-linked EGFR at unstimulated and EGF-stimulated conditions. B82L cells transfected with either empty vector (EV) or wild-type-EGFR (designated WT) or TKD-EGFR (designated TKD) were serum-starved and treated with (+) and without (−) 10 nM EGF for 10 minutes. Cells were subsequently cross-linked using 100 mM EDAC for 15 minutes. Samples (100 μg of total protein) were loaded on 5% SDS-PAGE gels, electrophoresed and immunoblotted with phosphospecific anti-EGFR antibody phospho-Y1173 (α-pY1173) or pan-reactive anti-EGFR (H11) (α-EGFR) antibody (n=3–4).
Figure 5.
Immunofluorescent analysis of EGFR localization and activation patterns. Transfected B82L cells containing empty vector (B82L EV) (a, d), wild-type EGFR (B82L WT) (b, e), and TKD-EGFR (B82L TKD) (c, f) were serum-starved and treated for 15 minutes without (a–c) or with (d–f) 10 nM EGF to ensure internalization. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked, and stained for pan-reactive EGFR (Clone H11, Labvision, green), EGFR phosphorylated at Y1086 (pY1086, Upstate, red), and nuclear material (DAPI, Molecular Probes, blue). Colocalization of total receptor (green) and phosphophorylated receptor (red) appears yellow in these merged images. Images were captured at 60X magnification (n=3).
Figure 6.
Immunoblotting of EGFR activation using kinase domain knockouts of WT-and TK-EGFR. (a) B82L cells were stably transfected with empty vector (EV), wild-type-EGFR (WT), TKD-EGFR (TKD), and kinase domain knockout EGFR variants (K721M WT, K721M TKD, K1088M TKD, KIIM TKD). Cells stably expressing these constructs were then assessed for receptor expression via immunoblotting with pan-reactive anti-EGFR (H11). (b) The stably transfected B82L cells were serum-starved prior to 10-minute treatment with 10 nM EGF (+) or vehicle (20 mM HEPES) (−). Samples (50–60 μg of total protein) were loaded on 5 or 6% SDS-PAGE gels and immunoblotted with phosphospecific anti-EGFR antibodies for phospho-Y1173 (α-pY1173) and phospho-Y1068 (α-pY1068), as well as pan-reactive anti-EGFR antibody against the kinase domain (n ≤15). Immunoblotting for STAT5 (α-STAT5) was used as a loading control for all blots.
Figure 7.
Time course of TKD-EGFR kinase domain mutants over one hour of ligand treatment. B82L cells stably transfected with TKD-EGFR (TKD), K721M TKD-EGFR (K721M TKD), or K1088M TKD-EGFR (K1088M TKD), were serum-starved and then treated with 10 nM EGF for 5, 10, 30, and 60 minutes (a 20 mM HEPES volume control was performed for the 60 minute time point). Cell lysates (50–60 μg of total protein) were loaded on 6% SDS-PAGE gels, electrophoresed and immunoblotted with a phosphospecific anti-EGFR antibody directed towards phospho-Y1173 (α-pY1173). Data from immunoblots (a) were normalized to EGFR expression and a STAT5 loading control (assessed via immunoblotting with anti-STAT5 (α-STAT5), and all signals were normalized against stimulation of TKD-EGFR at 10 minutes and plotted on a scatter plot (b). Error bars represent standard errors of the mean (n=4).
Figure 8.
Immunofluorescent analysis of localization and activation of EGFRs containing kinase domain knockouts. B82L cells transfected with K721M WT- (a, e), K721M TKD-(b, f), K1088M TKD- (c, g), or KIIM TKD- (d, h) EGFRs were treated for 15 minutes either with vehicle (20 mM HEPES) (a–d) or 10 nM EGF (e–h) and immunostained for total EGFR expression (green) or phospho-Y1086 activated EGFR (red), as well as for nuclear material using DAPI (blue). Colocalization of total receptor (green) and phosphorylated receptor (red) appears yellow in these merged images. Images were captured at 60X magnification (n=3).
Figure 9.
Immunoblots of tyrosine phosphorylation of EGFR at Y1173 in the presence or absence of ligand-competitive inhibition by mAb 528. B82L cells transfected with TKD-EGFR, K721M TKD-EGFR, or K1088M TKD-EGFR were serum-starved and then pre-treated with (+) and without (−) 1 μg/ml mAb 528 for four hours prior to a 10-minute stimulation with 10 nM EGF (+) or vehicle (20 mM HEPES) (−). Samples (50–60 μg of total protein) were loaded on 6% SDS-PAGE gels and immunoblotting was performed for phosphorylated EGFR at Y1173 (α-pY1173) and total EGFR (α-EGFR) (n=4). Immunoblotting for STAT5 (α-STAT5) was used as a loading control.

| Cell Line                  | B82L TKD | B82L K721M TKD | B82L K1088M TKD |
|---------------------------|----------|----------------|-----------------|
| 10 nM EGF (10m)           | − + − +  | − + − +        | − + − +         |
| 1 μg/ml 528 (4h)          | − − + +  | − − + +        | − − + +         |

α-pY1173 EGFR

α-EGFR

α-STAT5
Table 1

Phosphorylation Level of the Major Autophosphorylation Sites of the WT-EGFR and the TKD-EGFR in the Absence of Added Ligand. The basal phosphorylation state of each of five major EGFR autophosphorylations sites were assessed by immunoblotting as detailed under Materials and Methods and expressed as a percent of the EGF-stimulated phosphorylation state achieved at each site.

| Autophosphorylation Sites | WT-EGFR | TKD-EGFR | p-value     |
|--------------------------|---------|----------|-------------|
| pY1173                   | 5.5%    | 38%      | <0.0005 (n=9) |
| pY1148                   | <0.1%   | 35%      | NS* (n=2)   |
| pY1086                   | <0.1%   | 23%      | <0.05 (n=2) |
| pY1068                   | 3.6%    | 52%      | <0.0005 (n=5) |
| pY992                    | 16%     | 5.3%     | NS* (n=4)   |

*NS: not significant