The Juxtamembrane Region of the EGF Receptor Functions as an Activation Domain

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SUMMARY
In several growth factor receptors, the intracellular juxtamembrane (JM) region participates in autoinhibitory interactions that must be disrupted for tyrosine kinase activation. Using alanine scanning mutagenesis and crystallographic approaches, we define a domain within the JM region of the epidermal growth factor receptor (EGFR) that instead plays an activating—rather than autoinhibitory—role. Mutations in the C-terminal 19 residues of the EGFR JM region abolish EGFR activation. In a crystal structure of an asymmetric dimer of the tyrosine kinase domain, the JM region of an acceptor monomer makes extensive contacts with the C lobe of a donor monomer, thus stabilizing the dimer. We describe how an uncharacterized lung cancer mutation in this JM activation domain (V665M) constitutively activates EGFR by augmenting its capacity to act as an acceptor in the asymmetric dimer. This JM mutant promotes cellular transformation by EGFR in vitro and is tumorigenic in a xenograft assay.

INTRODUCTION
The EGF receptor (EGFR) has served as an important model for elucidating the activation mechanisms of receptor tyrosine kinases (RTKs) and is also the target for several cancer therapeutics in current clinical use (Ciardiello and Tortora, 2008; Sergina and Moasser, 2007). Like other RTKs (Linggi and Carpenter, 2006), EGFR comprises an extracellular ligand-binding region, a single transmembrane helix, and a cytoplasmic region that contains the tyrosine kinase domain (TKD). Activation of the TKD is essential for cellular responses to growth factors, and mutations that enhance EGFR tyrosine kinase activity are frequently oncogenic in human tumors (Lee et al., 2006; Sergina and Moasser, 2007; Sharma et al., 2007). For example, deletions or substitutions in the extracellular region activate EGFR in glioblastoma, and small deletions or point mutations within the TKD activate EGFR in non-small cell lung cancer (NSCLC).

Structural analyses of extracellular regions have shown how ligand binding facilitates EGFR dimerization (Lemmon, 2009), a critical step for ligand-dependent RTK activation (Schlessinger, 2000). Crystallographic and mutational studies suggest that this leads to allosteric activation of the TKD through the formation of an asymmetric dimer (Zhang et al., 2006) in which the C lobe of one TKD abuts the N lobe of its dimerization partner and promotes conformational changes that stabilize an activated state (Zhang et al., 2006).

The TKD accounts for only ~50% of the EGFR intracellular region. The remainder comprises the 225 amino acid carboxy terminal (CT) region (residues 961–1186) and the 38 amino acid cytoplasmic juxtamembrane (JM) region (residues 1064–1102). The CT region harbors most of the autophosphorylation sites and contains a putative autoinhibitory domain (Walton et al., 1990) that has not been characterized. The JM region of EGFR contains two threonines (T654 and T669) that are known to be phosphorylated with inhibitory consequences (Heisermann et al., 1990; Welsh et al., 1991). In addition, the JM region includes receptor-trafficking signals (Bao et al., 2000; He et al., 2002; Hsu and Hung, 2007; Morrison et al., 1995), a putative calcium-binding site (Martin-Nieto and Villalobo, 1998), and a polybasic region (Alfa et al., 2003; McLaughlin et al., 2005). In previous studies using large deletion mutations, we found that a portion of the JM region is required for kinase activation (Thiel and Carpenter, 2007). Our observations suggest that the JM region of EGFR plays an activating role, contrasting starkly with the autoinhibitory function described for JM regions of other RTKs (Hubbard, 2004). Here, we define the minimal JM activation domain (JMAD) using alanine scanning mutagenesis and evaluate reported lung cancer mutations in this region for their influence on EGFR activity. We also describe a crystal structure of the EGFR TKD that contains the entire JM region. This structure reveals key interactions responsible for function of the JMAD in TKD activation and suggests structural mechanisms for EGFR activation by JM mutations in NSCLC.

RESULTS AND DISCUSSION
Alanine Scanning Mutagenesis of the EGF Receptor Juxtamembrane Region
We previously showed that deletions in the JM region abolish both EGF-induced activation of intact EGFR and...
Figure 1. Effect of Alanine Scanning Mutagenesis of the JM Region on Tyrosine Phosphorylation

(A) Schematic of full-length EGFR, with the transmembrane (TM), tyrosine kinase domain (TKD), and carboxy-terminal (CT) regions marked. The intracellular domain (ICD) construct includes the JM, TKD, and CT regions. An alignment of JM region sequences across the human ErbB receptor family is shown, with the putative JM activation domain (JMAD) indicated.

(B) Relative Py/EGFR-CD

(C) 642 Molecular Cell 34, 641–651, June 26, 2009 ©2009 Elsevier Inc.
autophosphorylation of an intracellular domain (ICD) construct (Figure 1A) overexpressed in Cos-7 cells (Thiel and Carpenter, 2007). Deleting amino acids 645–662 or 645–678 from the JM region reduced ICD autophosphorylation in cells by ~95%. These JM residues are absent from published crystal structures of the EGFR TKD, which utilized constructs beginning at residue 672 (Stamos et al., 2002; Wood et al., 2004; Zhang et al., 2006). To determine which amino acids within the JM region contribute to TKD activation, we used alanine scanning mutagenesis in the context of an epitope-tagged ICD construct (Figure 1A). The influence of mutations on ICD autophosphorylation should reflect only alterations of TKD activation. Indirect effects arising from mutation of JM-trafficking motifs will not complicate interpretation of these studies as they might with intact EGFR (He et al., 2002; Kil et al., 1999; Lin et al., 2001; Kil and Carlin, 2000). We showed previously that mutating K721 (thus abrogating ATP binding) abolishes in vivo tyrosine autophosphorylation of EGFR ICD constructs (Thiel and Carpenter, 2007), illustrating that tyrosine phosphorylation of the ICD reflects intrinsic kinase activity levels.

Of the 36 alanine substitutions made in the JM region, 19 led to a significant reduction (≥50%) of ICD tyrosine phosphorylation. Fifteen of these “sensitive” positions are located in the C-terminal section of the JM (Figure 1B and Figure S1 available online), between residues 664 and 682—in a region encoded by exon 18 (which also encodes a portion of the TKD N lobe). Within this sequence, T669 (a known MAP kinase phosphorylation site; see below) is the only site at which alanine substitution does not impair kinase activity. Interestingly, four of the loss-of-function mutations are either similar (L664A and V665A) or identical (P675A and L680A) to changes present in the JM region of ErbB-3 (Figure 1A), a member of the EGFR family thought to have an inactive TKD (Guy et al., 1994). As shown in Figure 1A, the boundary between exon 17 and exon 18 is conserved in all ErbB receptors, and the four receptors share more identity in the part of the JM region encoded by exon 18 than by exon 17.

**Analysis of JM Mutations in Full-Length EGFR**

We also analyzed three JM mutations in full-length EGFR—V650A, V665A, and L680A (Figure 1C)—alongside catalytically inactive K721R EGFR (to establish a baseline for loss of function). Each EGFR variant was transiently expressed in NIH 3T3 cells, and tyrosine phosphorylation at Y1173, a known autophosphorylation site, was measured by western blotting in the absence and presence of EGFR. The V650A mutant resembles wild-type EGFR in its EGF-dependent autophosphorylation (Figure 1C), consistent with the failure of this mutation to reduce ICD autophosphorylation (Figure 1B). By contrast, activation of the V665A and L680A EGFR mutants is significantly compromised (as seen in the ICD context), and autophosphorylation of K721R EGFR is undetectable. To control for the possibility that reduced activation of V665A and L680A EGFR arises from impaired trafficking of the receptor to the cell surface, we bound Alexa-Fluor-conjugated EGF to 293 cells expressing each mutated receptor (or wild-type EGFR) and assessed cell-surface fluorescence using flow cytometry. The full-length receptor mutants were expressed on the cell surface at levels within 10% of the wild-type receptor (Figure S2A).

**The JM Region Contributes Directly to EGFR Dimerization**

In an effort to understand how the JM region exerts its positive influence on EGFR activation, we crystallized a form of the EGFR TKD that extends from residue 645 to 998 (EGFR645–998(K721M)) and includes the entire JM region. Previous crystallographic studies of EGFR TKD utilized protein that lacks residues 645–671 (Stamos et al., 2002; Wood et al., 2004; Yun et al., 2007; Zhang et al., 2006). The best crystals were obtained using a form of EGFR645–998(K721M) was solved to 2.8 Å resolution using molecular replacement as described in the Experimental Procedures (Table 1).

As shown in Figure 2A, EGFR645–998(K721M) forms the same asymmetric dimer in crystals as that seen repeatedly (Figure 2B) for active conformations of the EGFR TKD (Stamos et al., 2002; Yun et al., 2007; Zhang et al., 2006) and the ErbB4 TKD in both active (Qiu et al., 2008) and inactive (inhibitor-bound) states (Wood et al., 2008). In this asymmetric dimer, the C lobe of a “donor” TKD (yellow) abuts the N lobe of an “acceptor” TKD (green), contacting a surface of the acceptor made up from elements of the xC helix, the β4/β5 strand, and an N-terminal extension of the N lobe (marked in Figure 2B) that includes part of the JM region. Remodeling of these structural elements upon dimerization leads to allosteric activation of the acceptor (Zhang et al., 2006). Well-defined electron density was seen for EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain. The extended JM region of the acceptor TKD appears to “cradle” the C lobe of the donor in the asymmetric dimer of EGFR645–998(K721M) beginning at R653, so this structure lacks only the sequence 645RRHIVRK652 that immediately follows the transmembrane domain.

Although nearly all of the donor/acceptor interactions seen in previous active structures (Zhang et al., 2006) are precisely maintained in the EGFR645–998(K721M) dimer (Figure 2A), it is important to note that the K721M TKD itself adopts an inactive-like conformation (Wood et al., 2004; Zhang et al., 2006). This is not unexpected because K721 is a catalytically crucial residue in EGFR that must form an ion pair with a glutamate (E738) in the xC helix (and contact ATP) in the normal active kinase (Huse and Kuriyan, 2002). Loss of the K721/E738 ion pair in EGFR645–998(K721M) is likely to disrupt the
Table 1. Data Collection and Refinement Statistics

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a Rsym = Σ|h_c − <h_c>/Σh_c, wherein <h_c> = average intensity over symmetry equivalent measurements.

b Highest-resolution shell data are shown in parentheses.

c R factor = Σ|Fo − Fc|/ΣFo, wherein summation is over data used in the refinement; Rfree includes 10% of the data excluded from the refinement.

d Ramachandran plots were calculated using RAMPAGE (Lovell et al., 2003).

Percentage denotes residues in allowed regions.

communication of interactions required for normal allosteric activation upon formation of the asymmetric TKD dimer. Indeed, there are poorly defined regions of electron density within the P loop (residues 696–699), within the loop that connects strand β3 and the αC helix (residues 724–729), and within the activation loop (residues 833–851), suggesting significant disorder in these regions as a result of reduced restraints on the αC helix in the K721M mutant. Thus, the K721M-mutated acceptor TKD in Figure 2A maintains an inactive-like conformation despite retaining nearly all interactions with the donor that normally promote allosteric activation. This is evident from the slightly different relationships between the N and C lobes of each monomer in the structures shown in Figures 2A and 2B. The fact that EGFR645–998 (K721M) forms the asymmetric dimer shown in Figure 2 despite adopting an inactive conformation suggests no (or little) inhibitory effect of alanine substitution (T654–T669) on the donor C lobe and may also alter local structure by breaking an E666/R662 ion pair. Similarly, alanine substitution at P667, P670, or G672 could alter local structure (and/or disrupt van der Waals interactions) and thus impair JM interactions with the donor C lobe. The data in Figure 1B show that each of these changes impairs EGFR ICD autophosphorylation, as the structure would predict. Of equal importance, where Figure 1B indicates no (or little) inhibitory effect of alanine substitution (T654–E663 and T669), Figure 2C shows that the side chains in the JM region are not engaged in contacts with the donor C lobe. These “insensitive” side chains are colored light gray in Figure 2C.

The x helix at the N terminus of the acceptor JM region (residues 653–663) makes no direct contact with the donor C lobe. Its axis is almost perpendicular to the C lobe surface in Figures 2A and 2C, and it makes contacts with symmetry-related molecules in the crystal. This helix is part of a reported binding site for calmodulin that encompasses residues 645–660 (Martin-Nieto and Villalobo, 1998). The independence of this N-terminal helix in the structure presented here and the disorder of the polybasic region that precedes it (residues 645–653) aguments against the reported interactions of the EGFR JM region with calmodulin (Martin-Nieto and Villalobo, 1998) and with negatively charged membrane surfaces (McLaughlin et al., 2005) could occur coincident with the cradling of the donor C lobe seen in Figure 2A. Thus, proposed modes of JM-mediated EGFR regulation that involve these interactions (McLaughlin et al., 2005) may occur in parallel with the JM contributions to donor/acceptor interactions seen here.

Effects of Thrreonine Phosphorylation within the JM Region

The JM region contains two threonines that are known to be phosphorylated in presumed regulatory feedback loops. T654 is phosphorylated by protein kinase C (Hunter et al., 1984), leading to impaired EGF-induced EGFR phosphorylation. We showed previously (Thiel and Carpenter, 2007) that a phosphomimetic mutation at this position (T654D) reduces ICD autophosphorylation, whereas a T654A mutation elevates ICD phosphorylation (Figure 1B). T669 phosphorylation by MAP kinase also negatively regulates EGFR kinase activity (Li et al., 2008; Northwood et al., 1991; Takishima et al., 1991). As shown in Figure 3A,
a phosphomimetic T669D mutation again prevents ICD tyrosine phosphorylation, whereas a T669A mutation leads to elevated tyrosine phosphorylation compared to the wild-type ICD (Figures 3A and 1B). Thus, phosphorylation of T669 is likely to exert the same influence on EGFR ICD activity as T654 phosphorylation, supporting a mechanism for EGFR regulation by posttranslational modification of T669 within the putative JM activation domain.

Because the side chains of both T654 and T669 are solvent exposed in Figure 2C and make no contacts with the donor C lobe, the inhibitory effect of their phosphorylation does not reflect a simple (direct) disruption of JM-mediated interactions between receptors. In the case of T669, phosphorylation could have an indirect effect by altering JM structure or stability. For T654 phosphorylation, the structure presented here does not suggest a clear possibility. Interestingly, alignment of EGFR (ErbB-1) and ErbB-3 JM sequences (Figure 1A) shows that the EGFR T669, which is conserved in both ErbB-2 and -4, is an aspartate in ErbB-3, consistent with reports that ErbB-3 has little or no kinase activity (Guy et al., 1994).

**JM Interactions Are Required for L834R Tyrosine Phosphorylation**

Clinical studies of patients with NSCLC have identified a number of mutations in the EGFR TKD that are associated with increased sensitivity to EGFR-targeted tyrosine kinase inhibitors (Sharma et al., 2007). Many of these mutations cause constitutive (ligand-independent) activation of the EGF receptor when studied in reconstituted cellular systems (Choi et al., 2007; Jiang et al., 2005) and appear to do so by disrupting autoinhibitory interactions within the TKD itself (Zhang et al., 2006). The L834R mutation, located in the kinase activation loop, is one of the most clinically frequent and well-characterized examples. To determine whether EGFR activation by such mutations requires the JM-mediated interactions shown in Figure 2C, we combined...
the L834R mutation with a loss-of-function JM region mutation (L680A). A doubly mutated ICD construct containing L834R plus the JM L680A mutation was expressed in Cos-7 cells. Figure 3B shows that kinase activation due to the L834R mutation is blocked by the L680A mutation, indicating that the oncogenic activated (L834R) kinase remains dependent on JM function and, presumably, on formation of the asymmetric TKD dimer.

Activating EGFR JM Mutations in Non-Small Cell Lung Cancer

In addition to the TKD mutations, several publications have described relatively rare EGFR exon 18 JM point mutations in NSCLC patients (Chou et al., 2005; Pallis et al., 2007; Tsao et al., 2005). However, the impact of these mutations on receptor activity was not assessed, and in some cases, normal patient tissue was not examined for presence of the mutation. Thus, their relationship to NSCLC is currently not clear. To determine how these patient-derived JM mutations affect receptor kinase activity, equivalent changes were made in ICD constructs and expressed in Cos-7 cells (Figure 4A). The L834R mutation was analyzed in parallel as a positive control. As shown in Figure 4A, most of the patient-derived JM mutations (L664P, P667S, L668P, P670L/S, and N676D) reduced ICD autophosphorylation, suggesting that they may not be relevant in NSCLC. However, two mutations (V665M and L679F) significantly increased ICD tyrosine phosphorylation to levels similar to those seen with the L834R mutation.

We also introduced the V665M, L668P, and P670L mutations into full-length EGFR and measured tyrosine phosphorylation of transiently expressed receptor in the absence and presence of EGF (Figures 4B and 4C). Consistent with the ICD studies, the V665M mutation promotes ligand-independent EGFR autophosphorylation so that, even without ligand, V665M EGFR is as heavily phosphorylated as the fully activated wild-type receptor. This effect cannot be explained by differences in cell-surface expression of the mutant compared to wild-type receptor (Figure S2A). Thus, the V665M mutation resembles other activating NSCLC mutations in its effects on full-length EGFR (Choi et al., 2007; Jiang et al., 2005), suggesting that it could represent an oncogenic JM mutation with relevance in NSCLC. By contrast, the L668P mutation reduced EGF-induced receptor autophosphorylation, consistent with its effect in the ICD context. The P670L mutation had a less-marked negative effect on full-length EGFR than on ICD autophosphorylation, suggesting a possible effect of this proline substitution on ICD stability.

V665M-Mutated EGFR Promotes Cellular Transformation and Tumorigenesis

To evaluate the proliferative and oncogenic potential of the V665M mutation, we tested NIH 3T3 cells stably expressing wild-type, V665M, L834R, or D813A (catalytically inactive) EGFR for colony formation in soft agar in the absence and presence of EGF. As shown in Figure 5A, in the presence of EGF, the V665M EGFR mutant supports anchorage-independent growth more effectively than the wild-type receptor. Moreover, cells expressing V665M EGFR formed more colonies following EGF addition than cells expressing the well-described NSCLC mutant L834R.

To determine whether the observed increase in anchorage-independent growth by the V665M mutation reflects oncogenic
transformation, we injected NIH 3T3 cells stably expressing equivalent levels of wild-type or V665M EGFR subcutaneously into nude mice. The results, shown in Figure 5B, demonstrate that, 3 months after injection, cells expressing V665M EGFR formed more (and larger) tumors than cells expressing wild-type EGFR, regardless of the number of cells injected. These results indicate that the JM activation domain is a significant factor in the regulation of EGFR receptor kinase activity at the biological, as well as biochemical, level. The appearance of the V665M mutation in NSCLC patients also indicates that mutations in the JM activation domain are clinically important.

Mechanism of EGFR Activation by the V665M Mutation

In the mechanism for EGFR TKD activation proposed by Zhang et al. (2006), the C lobe of the donor contacts the N lobe of the acceptor in the asymmetric dimer (Figure 2B) and promotes conformational changes in the acceptor that lead to its allosteric activation. A crucial question is whether the V665M mutation promotes constitutive EGFR TKD activation by augmenting the function of the donor or acceptor. We took advantage of mutations (Zhang et al., 2006) that force receptor molecules to act solely as either donor monomers (I682Q) or acceptor monomers (V924R) to ask whether the V665M-mutated EGFR ICD functions more effectively than wild-type as donor, acceptor, or both. In the experiment shown in Figure 6, the donor ICD is myc tagged, and the acceptor ICD is Flag tagged. Alone, neither becomes autophosphorylated (Figure 6, lanes 3 and 4), but substantial activity is seen when the two are coexpressed (Figure 6, lane 5). Lanes 8–10 (Figure 6) demonstrate that maximal ICD autophosphorylation (greater than wild-type levels) is seen when the V665M mutation is present in the acceptor monomer (V924R/V665M), regardless of whether it is coexpressed with the I682Q donor or the doubly mutated (I682Q/V665M) donor. If the V924R/V665M acceptor is expressed without a donor monomer (Figure 6, lane 7), there is no activation, indicating that the large increase in activation observed in lanes 9 and 10 requires donor/acceptor interaction. In contrast, coexpression of an I682Q/V665M donor with a V924R acceptor (Figure 6, lane 8) does not increase tyrosine phosphorylation compared to the control (Figure 6, lane 5). These data establish that the V665M JM mutation enhances the capacity of the ICD to act as an acceptor monomer but does not greatly influence its ability to function as a donor. This is consistent with our previous finding that an intact JM region is required for EGFR ICD to function as an acceptor in the asymmetric dimer (Thiel and Carpenter, 2007). The V665M mutation seen in NSCLC appears to promote JM function in this context.

Structural Explanation for EGFR Activation by V665M and L679F Mutations

Details of the interface between the JM region of the acceptor and C lobe of the donor (Figure 2C) suggest a possible structural basis for the EGFR activation by the V665M and L679F mutations found in NSCLC patients. The side chain of V665 projects from the cells indicated above were evaluated 3 months after s.c. injection. Open circles represent single mice, with a total of four mice per cell type analyzed.
The V665M mutation must be present in the acceptor molecule to activate EGFR

ICD constructs containing mutations that limit them to functioning only as a donor (I682Q) or acceptor (V924R) in the asymmetric TKD dimer were used to assess the mechanism by which the V665M mutation activates EGFR. The noted combinations of ICD constructs were coexpressed in 293 cells as indicated, and lysates were subjected to SDS-PAGE and immunoblotting for phospho-EGFR (with anti-EGFR-pY1173) and for protein levels with anti-Myc and anti-Flag as appropriate. Autophosphorylation levels are greater than wild-type (lane 2) only when the V665M mutation is present in the acceptor TKD (lanes 9 and 10).

into a cavity on the surface of the C lobe that is lined by aliphatic portions of Q788 and Y789 from helix E and Q825 from the β7/β8 loop (between the catalytic loop and activation loop of the TKD). The V665 side chain does not fill this cavity (Figure 7A). However, substituting V665 with a methionine, as modeled in the right panel of Figure 7A, would fill the cavity completely and is likely to stabilize the association of the acceptor’s JM region with the donor C lobe at this location. We also considered the possibility that projection of a methionine side chain into the cavity shown in Figure 7A might alter the positions of its lining residues (Q788, Y789, and Q825). In particular, because Q825 lies between two key regulatory elements in the kinase, such alterations could allosterically regulate kinase activity. However, in overlays of all known active and inactive EGFR TKD structures (including the structure described here), the positions of these three residues do not change. A direct allosteric effect of the V665M mutation therefore seems unlikely.

A second activating mutation in the JM region among those found in NSCLC patients is seen at L679 in a region that has been visualized in all active EGFR TKD structures (Stamos et al., 2002; Yun et al., 2007; Zhang et al., 2006) and is unaltered in the structure presented here. Mutation of L679 to phenylalanine could improve packing of the JM region, with a POPOP sequence between helices g6 and dH in the C lobe of the donor (Figure 7B). Again, this region is identically placed in the active and inactive configurations of the kinase.

Conclusions

Crystallographic studies together with mutagenesis and biochemical data have shown that, for several RTKs (c-Kit, EphB2, and Flt3), the intracellular JM region plays an autoinhibitory role, sterically hindering substrate access to the nucleotide-binding pocket (Chan et al., 2003; Griffith et al., 2004; Wybenga-Groot et al., 2001). In these RTKs, phosphorylation of key tyrosines within the JM region results in a conformational shift that reverses the autoinhibition, leading to kinase activation (Binns et al., 2000; Mol et al., 2003).

We show here that the JM region of the EGF receptor instead plays an activating (rather than autoinhibitory) role. Alanine substitutions at most positions in the C-terminal half of the JM region (encoded by exon 18) lead to a loss of kinase activity. We term this part of the JM region the JM activation domain (JMAD). By solving the crystal structure of an EGFR TKD with its JM region intact, we showed that the entire 19 amino acid JMAD (residues 664–682) of an acceptor in the asymmetric TKD dimer makes intimate contact with the C lobe of the donor. The most straightforward explanation for how the JMAD contributes to EGFR activation is that it enhances formation of the asymmetric dimer, thus promoting allosteric activation of the acceptor TKD. Indeed, although wild-type EGFR645–998 is monomeric at concentrations of up to 50–100 μM (data not shown; Zhang et al., 2006), adding the JM region causes EGFR645–998 to aggregate in AUC studies (data not shown). This might reflect JMAD-stabilized formation of head-to-tail TKD polymers similar to those seen in crystals. Phosphorylation of T669 within the JMAD is likely to disrupt its local structure, thus inhibiting EGFR activity by reducing the strength of acceptor/donor interactions.

Identification of an important regulatory region within the ICD but outside of the TKD itself is of particular significance because regions outside of the kinase domain are rarely examined for mutations in clinical screens (Sharma et al., 2007). Where broader screens have been performed, a limited number of apparently rare JM mutations in clinical tumor samples have been reported, but not characterized. We found that two of these previously reported clinical EGFR JM mutations (V665M and L679F) activate the receptor. In both cases, the degree of EGFR activation is similar to that seen for well-characterized oncogenic mutations (such as L834R) in the TKD itself (Sharma et al., 2007). Biochemical studies and structural arguments suggest that the V665M mutation stabilizes acceptor/donor interactions. The existence of such a clinically observed mutation within the JMAD that we also show to be activating and transforming highlights the biological importance of this domain in regulating the EGF receptor kinase activity and cellular responsiveness. This activation domain is encoded by the same exon as a significant portion of the N lobe of the TKD and could be considered as a part of the TKD proper.

Experimental procedures

Reagents, Antibodies, Cell Lines, and Plasmids
NIH 3T3, Cos-7, and 293 cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) at 37°C at 5% CO2. EGF was purchased from R&D Systems.
Figure 7. Mechanism of Activating JM Mutations

(A) Contacts between the acceptor JM domain and donor C lobe are shown with the V665 side chain represented as space-filling spheres. The V665 side chain fails to fill a cavity on the donor C lobe formed by the side chains of Q788, Y789, and Q825. However, as shown in the right panel, a methionine (red) at position 665—modeled for this figure—would completely fill the cavity, increasing van der Waals contacts between acceptor and donor.

(B) Similarly, the L679F mutation is likely to promote interactions between the JM region (green) and the donor C lobe, as indicated by modeling a phenylalanine at this position.

Expression and Purification of EGFR

A PCR-amplified cDNA fragment encoding amino acids 645–998 of EGFR was subcloned into pFastBac1, modifying the amino terminus to include a hexahistidine tag in the N-terminal sequence MHHHHHHHGR... (in which the final R is R645 of EGFR). The K721M mutation was incorporated using the QuikChange kit (Stratagene). Baculovirus generation and protein expression in Sf9 cells employed the Bac-to-Bac expression system (Invitrogen) as recommended by the manufacturer. Baculovirus-infected Sf9 cells were harvested 3 days after infection, were lysed by sonicating in 50 mM Tris (pH 8.0), 150 mM NaCl, and 10% glycerol, containing 5 mM β-mercaptoethanol and 1 μM protease inhibitor cocktail (Roche). Cell debris was removed by centrifugation at 40,000 x g for 30 min. The supernatant was incubated with Ni-NTA agarose (Qiagen) for 1 hr at 4°C, and bound protein was eluted using stepwise

In Vivo Tumorigenicity

To determine the capacity of NIH 3T3 cells to form tumors in vivo when stably expressing pBabe-puro vector, wild-type, or V665M EGFR, nude mice (four mice per cell type) received four dorsal s.c. injections, one in each quadrant, expressing pBabe-puro vector, wild-type, or V665M, L834R, or D813A, or vector alone (m.o.i. = 0.1) were used for infection. Cells expressing equal concentrations of receptor were sorted via FACS by staining with a non-ligand-interfering ectodomain antibody (anti-EGFR Ab-3, LabVision) and then seeded at 8 x 10^3 cells per well in a 12-well plate. Cells were cultured in 10% FBS with or without 50 ng/ml EGF. Medium was changed every 2 days, and colonies were counted at 3 weeks using an Oxford Optronix Gelcount.

Cell Culture and Transfections

Transient transfection for expression in mammalian cells was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Of the expression plasmid, 2 μg was used per 60 mm dish. Transient expression of full-length EGFR receptor in NIH 3T3 cells was carried out using a DNA: Fugene6 (Roche) ratio of 1.5 μg:4.5 μl, according to the manufacturer’s instructions. To assess ligand-dependent EGFR activation, transfected cells were serum starved overnight and treated with 50 ng/ml EGF.

Cell Lysis and Immunoblotting

Cells at 80%–90% confluence were either untreated or treated with EGF as indicated and then washed with phosphate-buffered saline. Cells were scraped and lysed in ice-cold lysis buffer containing 1% Triton, 10% glycerol, 50 mM HEPES (pH 7.2), and 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, and 25μg/ml leupeptin. Lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4°C. SDS sample buffer was added to lysates, and samples were boiled for 5 min. Samples were then subjected to SDS-PAGE on 8% polyacrylamide gels, transferred to PVDF, and immunoblotted.

Retrovirus-Mediated Gene Transduction and Colony Formation Assay

The pBabe-puro retroviral vector containing the coding sequences for wild-type EGFR, V665M, L834R, or D813A were used to produce virus-containing supernatants from transfected Phoenix cells. Viral supernatant was used to transduce NIH 3T3 cells in the presence of 5 μg/ml polybrene. Equal amounts of retrovirus-containing wild-type EGFR, V665M, L834R, or D813A were used to produce virus-containing supernatants from transfected Phoenix cells. Viral supernatant was used to transduce
imidazole washes. Eluted protein was bound to a cation exchange column (S2: Bio-Rad) in 20 mM Tris (pH 8.0), 200 mM NaCl, and 2 mM DTT and was eluted with a linear gradient to 1 M NaCl in the same buffer. Fractions containing EGFR TKD were diluted in 10 mM phosphate buffer (pH 8), loaded on a hydroxyapatite column (CHT2-T: Bio-Rad), and eluted with a linear gradient to 500 mM Na/K phosphate (pH 8.0). Fractions containing protein were concentrated and gel filtered on a Superdex 200 column (GE Healthcare) in 20 mM Tris (pH 8.0), 250 mM NaCl, and 2 mM DTT.

Crystallization and Structure Determination

EGFR<sup>645–998</sup>(K721M) was concentrated to 5 mg/ml and crystallized using the hanging-drop method. Crystals (~10 μm × 10 μm × 40 μm) grew in 1 day in 100 mM Tris (pH 8.5), 100 mM KCl, and 10% PEG3350 at 21°C. Crystals were cryoprotected in mother liquor containing 15% PEG and 20% glycerol and frozen in liquid nitrogen. Diffraction data were collected at the CHESS F1 beamline, where crystals diffracted to 2.8 Å (Table 1). Data were processed using HKL2000 (Otwinowski and Minor, 1997), and the structure was solved by molecular replacement (MR) using Phaser implemented in the CCP4 package (Storoni et al., 2004). The N and C lobes of the inactive EGFR TKD (PDB code 2G57) were found in simultaneous but independent searches. Density for the JM region of EGFR<sup>645–998</sup> (K721M) was immediately visible, and the structure of the protein was built and rebuilt using the program O (Emsley and Cowtan, 2004). Manual model building was alternated with successive rounds of refinement using the programs DM and Refmac in CCP4 (CCP4, 1994) and inspection of composite omit maps generated using CNS (Brunger et al., 1998). TLS refinement was incorporated in the final stages (Winn et al., 2001). Figures for publication were made using MacPymol (DeLano, 2002).

ACCESSION NUMBERS

The atomic coordinates for EGFR<sup>645–998</sup> (K721M) have been deposited in the RCSB Protein Data Bank under ID code 3GOP.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00388-8.

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Note Added in Proof
Contributions of the juxtamembrane region to EGFR dimerization and activa-
tion are detailed in a related study: Jura, N., Endres, N.F., Engel, K., Deindl, S.,
Mechanism for activation of the EGF receptor catalytic domain by the juxta-