Epidermal Growth Factor Receptor Mutants from Human Lung Cancers Exhibit Enhanced Catalytic Activity and Increased Sensitivity to Gefitinib

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Abstract

Somatic mutations within the epidermal growth factor receptor (EGFR) kinase domain are detected in 10% to 30% of human non–small cell lung cancers and are correlated with striking clinical responses in a subset of patients treated with EGFR kinase inhibitors, such as gefitinib and erlotinib. Cell-based studies suggest that these mutant EGFRs promote increased autophosphorylating activity on a subset of EGFR COOH-terminal tyrosines and the consequent engagement of a subset of downstream effectors. Because EGFR function is regulated at multiple levels *in vivo*, and it is therefore difficult to assess the direct consequences of these mutations on EGFR enzyme function, we measured EGFR catalytic activity in *in vitro* kinase assays using purified recombinant proteins corresponding to the cytoplasmic domain of wild-type and two frequently detected EGFR mutants (DelL747-P753insS and L858R). Both mutants exhibit substantially increased autophosphorylating activity relative to wild-type EGFR, and they exhibit distinct reaction kinetics. In addition, the mutant kinases are more sensitive to kinase inhibition by gefitinib, which seems to reflect their increased drug affinity. These findings suggest that the altered signaling properties and drug sensitivity of these EGFR mutants that have been observed *in vivo* largely result from differences in the catalytic properties of the kinase. In addition, we find that the T790M secondary "drug resistance mutation" of EGFR, which frequently arises in relapsed patients that initially responded to treatment, confers enhanced kinase activity to primary activating EGFR alleles and may, therefore, be oncogenic in some contexts. [Cancer Res 2007;67(5):2325–30]

Introduction

The epidermal growth factor receptor (EGFR) is frequently overexpressed in solid tumors (1). Recently, a novel class of EGFR mutations has been identified in ~10% to 30% of non–small cell lung cancer (NSCLC). Mutations arise in the kinase domain (2–4) and consist of small in-frame deletions, missense substitutions, and small in-frame insertions/duplications. Nearly half of all cases involve the substitution L858R. These mutations are correlated with striking clinical responses to EGFR kinase inhibitors [Iressa (gefitinib) and Tarceva (erlotinib)] in NSCLC patients (2–4). However, most responders eventually develop drug resistance, frequently associated with a secondary EGFR mutation, T790M, which disrupts drug binding while preserving catalytic function (5–7). Interestingly, the T790M allele is also detected at low frequency in untreated NSCLCs, and germ line T790M mutations have been detected in a family that exhibits inherited predisposition to lung adenocarcinoma (8). Thus, the T790M "resistance" mutation may also confer oncogenic activity to EGFR.

Cell culture studies suggest that mutant EGFRs exhibit enhanced autophosphorylating activity and increased activation of downstream signaling proteins and are sufficient to render NIH3T3 fibroblasts tumorigenic (9) and confer IL-3–independent growth to BAF3 cells (10, 11). However, EGFR is subject to multiple levels of regulation *in vivo*, including ligand binding, dimerization, ubiquitination, and trafficking (12, 13). Consequently, cell-based analysis might reflect multiple aspects of EGFR regulation. Therefore, we compared the enzymatic activities of wild-type and two frequently detected EGFR mutants using purified recombinant proteins. Both mutants exhibit substantially increased autophosphorylating activity and increased gefitinib sensitivity, suggesting that the altered signaling properties and drug sensitivity observed *in vivo* largely reflect differences in catalytic properties of the kinase.

Materials and Methods

Plasmid construction. The EGFR intracellular domain corresponding to wild-type and mutant human proteins was amplified using the following oligonucleotides: 5′-GCTCTAGAGGAGAAGCTCCCAAC-3′ and 5′-CGGGTACCTCATGCTCCAATAAA-3′. Fragments were excised and subcloned into the pVLFlag baculovirus vector. Resulting inserts were confirmed by DNA sequencing.

Cell culture. Serum-free adapted *Spodoptera frugiperda* (Sf9) insect cells were from Invitrogen (Frederick, MD). Cells were cultivated in complete sf-900 II serum-free medium optimized for insect cells (Invitrogen) without antibiotics at 27°C.

Baculovirus production. Sf9 cells were transfected with baculovirus vector containing EGFR inserts using the BD BaculoGold Transfection Kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Viral containing supernatants were collected 3 to 4 days postinfection and were used to infect 10-cm plates of Sf9 cells to increase viral titer. This was repeated three to four times. The presence of recombinant protein was confirmed by immunoblotting with anti-FLAG antibody.

Protein purification. Cultures of log-phase Sf9 cells were infected with viral supernatant. After 3 days, cells were treated with 22 μmol/L gefitinib for 5 h to dephosphorylate EGFR. Cells were centrifuged at 2000 × *g* for 5 min, and pellets were rinsed in PBS followed by centrifugation. Pellets were resuspended in 5 mL cold homogenization buffer (HB) containing 25 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, and 10% glycerol

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(including protease inhibitors). After 30 min, cells were dounced 30 times, and cleared supernatant was incubated for 2 h with 200 μL slurry of M2 agarose beads (Sigma, St. Louis, MO). M2 beads were pelleted and rinsed six times in HB containing 500 mmol/L NaCl. EGFR proteins were competed from beads using 1× FLAG peptide (Sigma). SDS-PAGE was used to assess the protein purity.

Larger scale protein production was done using a Wave Bioreactor System 20/50 (Wave Biotech, LLC, Somerset, NJ). In these cases, dephosphorylation was accomplished using *Yersinia* tyrosine phosphatase (YopH). After cell lysis, YopH was added to the cleared supernatant during overnight incubation with M2 agarose beads at 4°C. Complete dephosphorylation was observed using antiphosphotyrosine antibody (4G10; Upstate, Charlottesville, VA).

Kinase assays. About 20 ng of purified protein was used per reaction. Reactions were carried out in 10 mmol/L HEPES, 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, 10% glycerol, and 10 μmol/L ATP (except where noted otherwise). Reactions were done at 34°C and terminated using either Laemmli sample buffer or 10 mmol/L EDTA after 20 min, unless otherwise indicated. Radiolabeled proteins were electrophoresed on 10% SDS-PAGE, and gels were exposed on autoradiographic film.

EGFR autophosphorylation by rapid chemical quench. Rapid chemical quench studies were done as previously described (14). After various times (0.02–60 s), the reaction was quenched with 67 μL of 50 mmol/L EDTA. Zero time points were determined by mixing enzyme solution with 2× substrate solution without ATP at the longest reaction times. Quenched reaction products were resolved by 10% SDS-PAGE and analyzed by blotting with phosphospecific antibodies. Phosphorylation intensity was quantified using Labworks software.

Equilibrium fluorescence titrations to determine gefitinib affinity to proteins. Equilibrium fluorescence measurements were made using an SLM 4800C spectrofluorimeter (SLM Instruments, Rochester, NY). The emission spectra (300–450 nm) of EGFR were obtained upon excitation at 285 nm. All measurements were made at 25°C with 2-nm slits for excitation and 4-nm slits for emission. Aliquots of 0.5 μL of gefitinib (10 μmol/L) were added to 0.6 mL of enzyme solution containing 200 nmol/L EGFR, 20 mmol/L Tris-Cl (pH, 7.4), 150 mmol/L NaCl, 10 mmol/L $MnCl_2$, and 10% glycerol until no further decrease in fluorescence was observed. Observed fluorescence at 340 nm was then plotted versus total gefitinib concentration. Data were then fitted to the following expression relating observed fluorescence to gefitinib concentration [IR]:

$$F = F_{\max} - (F_{\min} \times [\mathrm{IR}])/(K_d + [\mathrm{IR}]) \qquad (A)$$

F is the observed fluorescence, $F_{\max}$ is fluorescence in the absence of gefitinib, $F_{\min}$ is the observed fluorescence in the presence of the saturating concentration of gefitinib, and K_d is the dissociation constant.

Protein immunodetection. For experiments in Sf9 cells, cells were infected with equivalent amounts of baculovirus. After 3 days, they were treated with the indicated amounts of gefitinib for 3 h and then lysed in sample buffer. Equal amounts of lysates were analyzed by 10% SDS-PAGE, followed by transfer to polyvinylidene difluoride membranes. After 1 h of filter blocking in 5% bovine serum albumin, membranes were incubated overnight with antibody. A chemiluminesence reagent (Perkin-Elmer, Boston, MA) was used to reveal the presence of tagged proteins. About 25% of the kinase reaction mixture was diluted in 140 μL of TBS and spotted on a nitrocellulose membrane using a Bio-Dot SF Microfiltration apparatus from Bio-Rad (Hercules, CA). Membranes were blocked in Odyssey blocking solution (LI-COR International, Lincoln, NE) for 1 h, followed by overnight incubation in primary antibody. Membranes were scanned and quantified using the Odyssey IR imager.

Antibodies. The following antibodies were used: anti-FLAG (Sigma); p-Tyr (Transduction Labs, San Jose, CA); Tyr^{845} and Tyr^{1173} (Cell Signaling Technologies, Danvers, MA); Tyr^{992}, Tyr^{1086}, and Tyr^{1148} (Invitrogen); and Tyr^{1045} and Tyr^{1068} (Abcam, Cambridge, MA). Both mouse and rabbit secondary antibodies used were from either Cell Signaling Technologies (Western blotting) or Rockland Inc. (Gilbertsville, PA; dot blotting).

Results and Discussion

Purified EGFR mutant kinases exhibit increased kinase activity and increased sensitivity to gefitinib. To examine the catalytic properties of mutant EGFR kinases, we generated recombinant baculoviruses encoding the cytoplasmic domain of wild-type and two common EGFR lung cancer mutants (L858R and DelL747-P753S, hereafter referred to as Del747-753; Fig. 1*A*). Upon insect cell infection, each form of EGFR is expressed at comparable levels; however, the two mutants exhibit substantially increased total tyrosine phosphorylation (Fig. 1*B*). Phosphospecific antibodies revealed that the mutant EGFRs exhibit increased phosphorylation on multiple sites, and phosphorylation is suppressed by gefitinib (Fig. 1*B*).

Next, we purified the EGFR intracellular domains for more detailed *in vitro* enzyme assays (Fig. 2*A*), and the Del747-753 and L858R EGFR kinases exhibited increased levels of tyrosine phosphorylation compared with wild-type EGFR (Fig. 2*B* and *C* and Supplementary Table S1). Similar to previous cell-based assays, purified mutant EGFRs exhibit about 10-fold increased sensitivity to gefitnib/erlotinib (2, 4); the EC_{50} for the purified wild-type kinase (22.8 nmol/L) is significantly higher than that observed for the Del747-753 (5.9 nmol/L) and L858R (7.5 nmol/L) mutants (Fig. 2*D* and *E*). These findings indicate that the increased

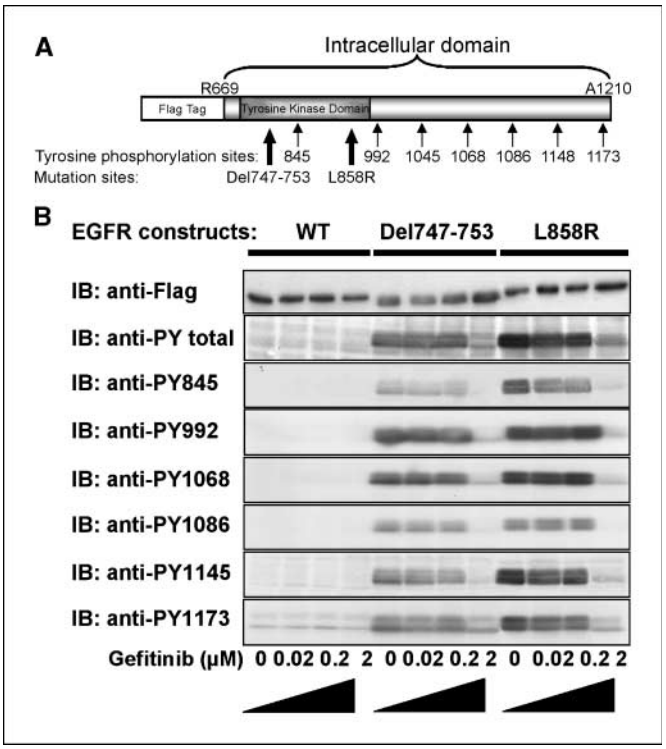

Figure 1. Increased autophosphorylating activity of EGFR mutants Del747-753 and L858R in insect cells. *A,* schematic representation of the recombinant protein constructs corresponding to the intracellular domain of EGFR. *Large arrows,* the sites corresponding to the relevant mutations, namely, DelL747-P753insS (Del747-753) and L858R. *Small arrows,* tyrosines that can be modulated by phosphorylation. *B,* Sf9 cells were infected with baculovirus expressing either wild-type or mutant EGFR intracellular domains. The phosphorylation of EGFR from cell lysates collected 3 d postinfection was determined by immunoblotting with antibodies directed against either total phosphotyrosine or specific antibodies that recognize the indicated phosphorylated tyrosine residues of the EGFR COOH-terminal domain. Where indicated, Sf9 cells were treated with gefitinib for 3 h at the indicated concentrations preceding cell lysis.

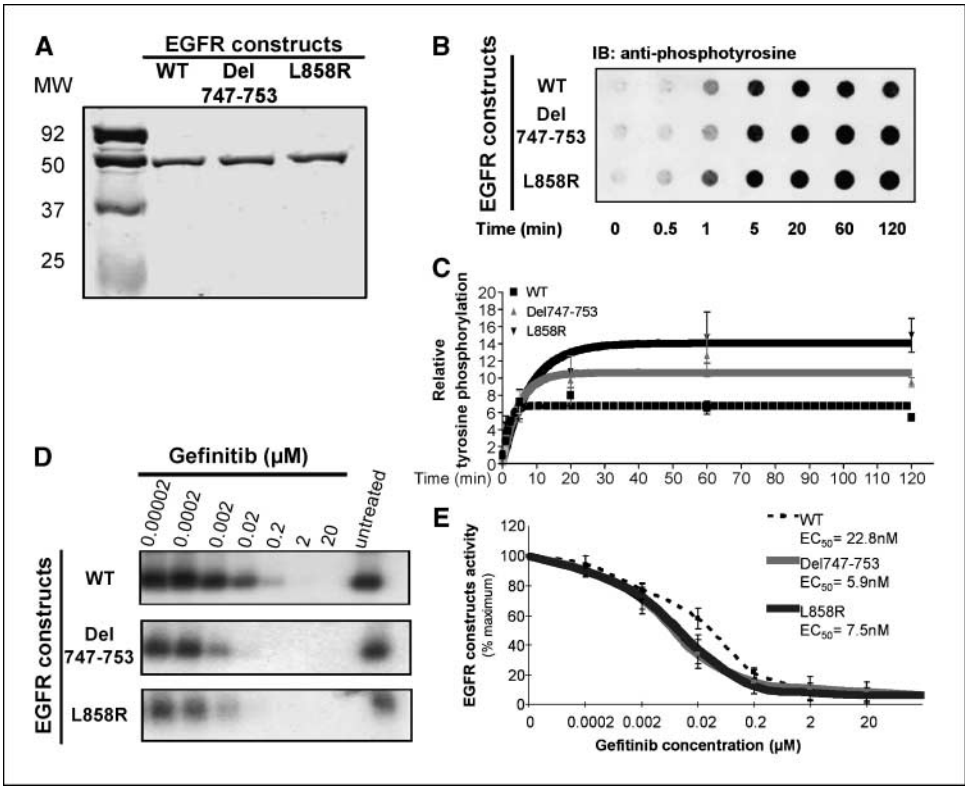

Figure 2. Increased kinase activity and gefitinib sensitivity of purified recombinant EGFR mutants. *A,* purified intracellular domains of EGFR proteins, namely, wild-type, Del747-753, and L858R subjected to 10% SDS PAGE, and the gel was then stained with Coomassie blue. The proteins seem to be purified to homogeneity. Molecular weight markers are indicated. *B,* representative immuno–dot blot of reaction products following an *in vitro* kinase assay using the purified EGFR proteins, following detection with anti-phosphotyrosine antibody. The assays were done at the indicated time points. *C,* graphical depiction of three independent experiments corresponding to (*B*), in which results were averaged. *D,* representative *in vitro* kinase assays done with purified EGFR proteins in the presence of ^{32}P γ-ATP for 20 min. Reaction products were analyzed by 10% SDS-PAGE followed by autoradiography. The indicated concentrations of gefitinib were included in the reaction. *E,* graphical representation of the findings from three independent experiments corresponding to (*D*). *Points,* means; *bars,* SE. The EC_{50} value is indicated for each EGFR protein.

autophosphorylating activity and drug sensitivity observed *in vivo* reflects a direct consequence of mutation on catalytic properties of the EGFR kinase domain.

Increased enzyme activity and altered substrate preference of mutant EGFR proteins. Cell-based studies revealed that mutant EGFRs selectively engage a subset of downstream signaling pathways (15). Therefore, we did *in vitro* enzyme assays with purified EGFR kinases, followed by immunoblotting of reaction products with phosphospecific antibodies. Most residues examined are more highly phosphorylated in the mutant proteins (Fig. 3). For all tyrosines examined, except Y1173, Del747-753 exhibits higher phosphorylation than wild-type EGFR (Supplementary Table S1). The L858R mutant protein exhibits increased phosphorylation on Y845, Y1045, Y1086, and Y1173. Importantly, in all cases, the E_{max} values were lower for wild-type enzyme. These findings suggest that the mutants exhibit differential reactivity with individual tyrosine-containing peptides within the EGFR cytoplasmic domain. Notably, these two classes of EGFR mutants each exhibit increased enzyme activity and increased drug sensitivity, despite the fact that they would be predicted to have substantially different structural consequences on the EGFR catalytic pocket.

Increased catalytic rates of mutant EGFR kinases. Our *in vitro* enzyme assays revealed that the two mutant EGFRs exhibit increased autophosphorylating activity on several COOH-terminal sites. However, the elevated activity in these assays was considerably reduced relative to differences detected in Sf9 cells, raising the possibility that in the *in vitro* assays, a relatively rapid saturation of all sites occurs on all three forms of EGFR in the time frame examined. Indeed, previous kinetic studies on recombinant fibroblast growth factor receptor (FGFR) and ErbB2 showed that autophosphorylation occurs in the millisecond to second time frame (14).[3] Therefore, we used rapid chemical quench methodology to compare reaction kinetics of wild-type and mutant kinases. Between 0.02 and 60 s, we observed substantial differences in the autophosphorylation profile between wild-type and mutant kinases (Fig. 3*B*). For wild-type EGFR, Y992 is the only site detectably phosphorylated in a 20-ms to 1-s time frame. Phosphorylation of the other sites is not detected under these conditions until the 10- to 60-s time domain, during which Y1068 seems to be phosphorylated. In contrast, the L858R mutant is autophosphorylated on Y845, Y1045, and Y1068 residues at the 10-s time point. Interestingly, all four tyrosine residues examined are phosphorylated at 0.02 s on the Del747-753 mutant. These findings are consistent with a substantially increased catalytic rate for the two mutant EGFR kinases relative to the wild-type protein and additionally indicate that the deletion and missense mutants exhibit distinct properties with regard to their autophosphorylation reaction rates.

These findings suggest a specific ordering of autophosphorylation for activated EGFR, which may be perturbed by mutation. Transient kinetic studies of the ErbB2 and FGFR kinases have similarly pointed to rapidly ordered autophosphorylation as a likely regulatory mechanism (14, 16). Moreover, the temporal dynamics of receptor phosphorylation have also been suggested to be important for EGFR signaling (17). If such ordering is influenced by the conformation of the catalytic pocket, it is conceivable that activating EGFR mutations could impact the ordered phosphorylation of COOH-terminal tyrosines and consequently affect signaling function.

[3] K.S. Anderson, unpublished results.

There may not always be direct parallels between *in vitro* model systems and ligand-stimulated cells. Indeed, the *in vitro* studies described here revealed some differences from previous cell-based studies regarding the "preferred" autophosphorylation sites of mutant EGFRs (15). Notably, the potential contribution of receptor dimerization to kinase activity and substrate selectivity of mutant EGFRs cannot be readily addressed *in vitro*. Therefore, efforts are under way to examine rapid kinetics of wild-type and mutant EGFRs immediately following ligand stimulation in cell culture.

Mutant EGFR kinases exhibit increased gefitinib affinity. One mechanism by which increased kinase activity could arise is that mutant enzymes, due to structural change within the catalytic pocket, exhibit increased ATP affinity. We did *in vitro* kinase assays using a range of ATP concentrations. Both the Del747-753 and the L858R mutants remain quite active even at the lowest ATP concentration tested (Supplementary Fig. S1*A*). However, at higher ATP concentrations, approaching physiologic levels, it is not possible to discriminate between the activity of wild-type and mutant kinases, suggesting that differences in ATP affinity may not be significant *in vivo*.

Next, we directly examined the affinity of wild-type and mutant EGFR kinases for gefitinib by conducting equilibrium fluorescence titration experiments. We previously showed in studies with the HER-2 kinase that there is a conserved tryptophan near the active site that can be used to monitor nucleotide/inhibitor binding affinity (16). For this analysis, an *in vitro* assay of purified EGFRs was used in which the change in intrinsic tryptophan fluorescence can be monitored at a range of gefitinib concentrations to determine the direct binding affinity (K_d; Supplementary Fig. S1*B*). The K_d for the wild-type EGFR was ~5 times that of

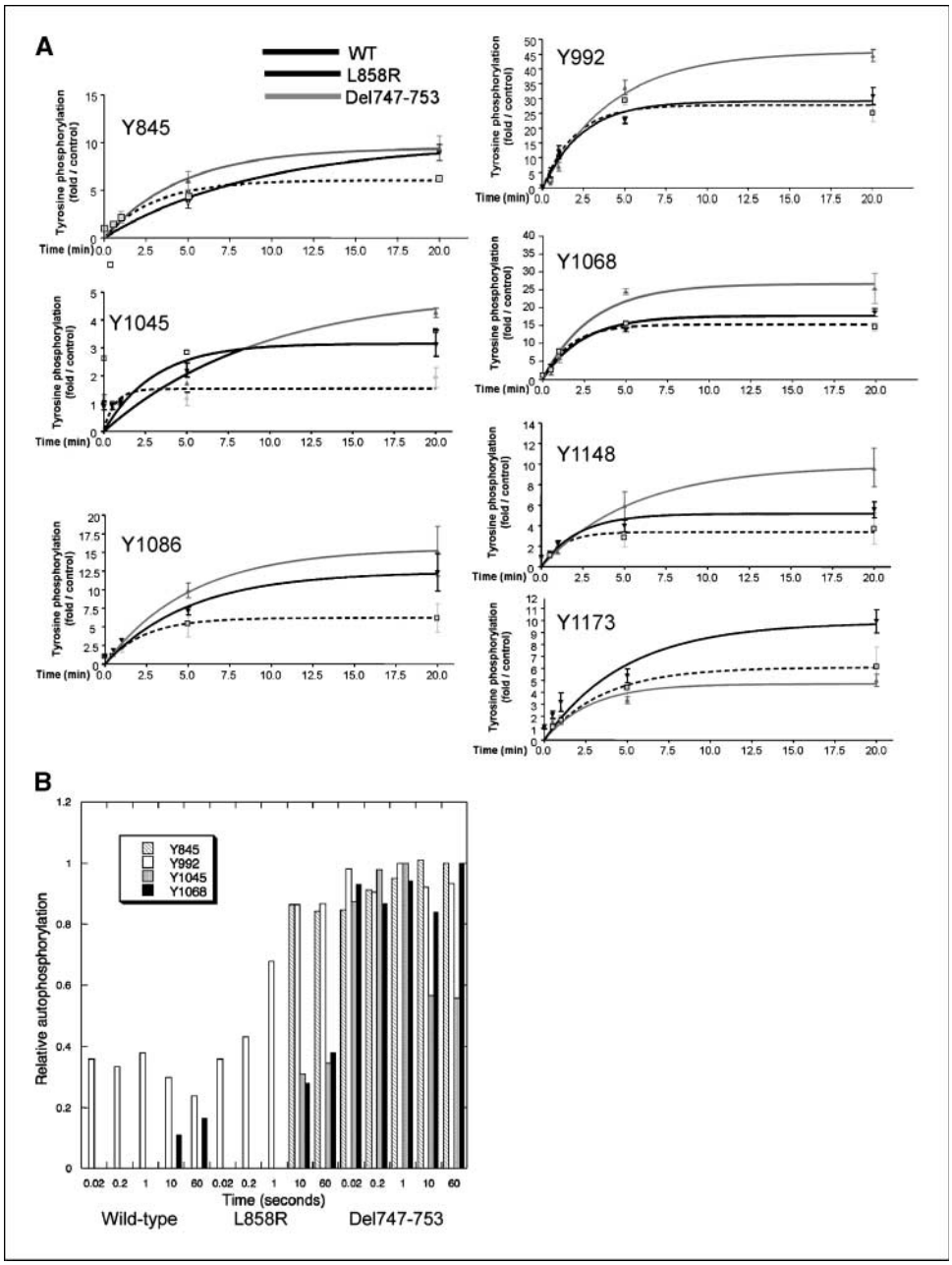

Figure 3. Autophosphorylation kinetics of EGFR mutants. *A,* graphical depiction of immuno–dot blot assays of the reaction products following kinase assays using the purified wild-type and mutant EGFR proteins. Antibodies directed against pY845, Y992, Y1045, Y1068, Y1086, Y1145, and Y1173 were used to detect reaction products following kinase assays at the indicated time points. $T = 0$ is before the addition of ATP, where none of the proteins are phosphorylated. In all cases, the results represent the average of three independent experiments; *bars,* SE. *B,* rapid chemical quench assays of wild-type and mutant EGFR kinases. *In vitro* kinase assays of the purified wild-type and mutant EGFRs using a Rapid Chemical Quench apparatus. After various reaction times (0.02–60 s), the reactions were quenched with EDTA, and quenched products were resolved by 10% SDS-PAGE and analyzed by immunoblotting with phosphospecific antibodies directed against EGFR Y845, Y992, Y1045, and Y1068. The intensities of phosphorylated EGFR were quantified using software Labworks.

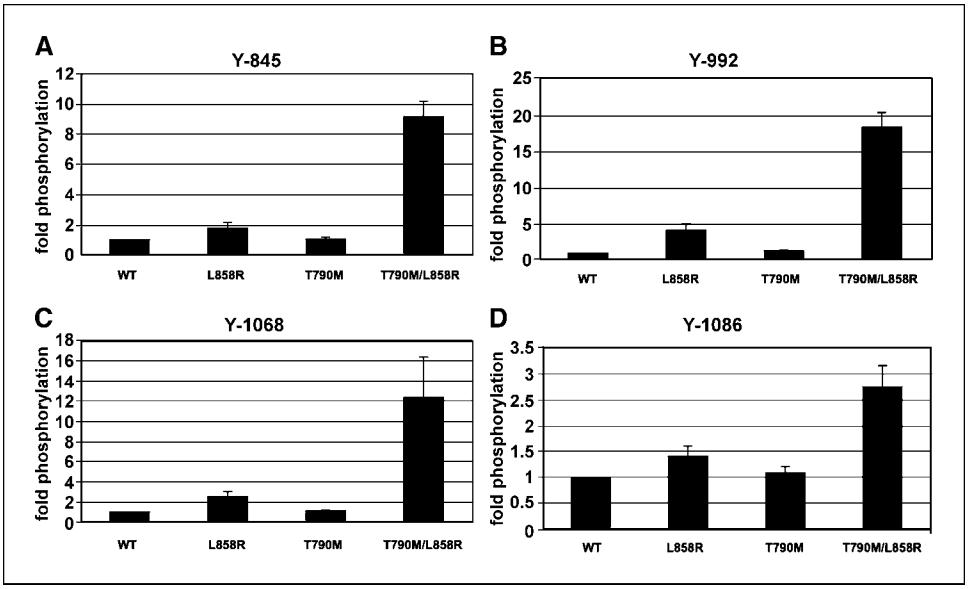

Figure 4. Increased autophosphorylation of the T790M/L858R double mutant. Sf9 cells were infected with baculovirus expressing either wild-type (WT), L858R, T790M, or L858R/T790M mutant EGFR intracellular domains. The phosphorylation of EGFR from cell lysates collected 3 d postinfection was determined by immunoblotting with specific antibodies directed against Y845 (*A*), Y992 (*B*), Y1068 (*C*), and Y1086 (*D*). Graphs represent the average of three independent experiments normalized to protein expression levels and quantified using the Odyssey IR imager. *Columns,* means; *bars,* SE.

the L858R mutant and ~13 times that of the Del747-753 mutant (Supplementary Fig. S1*C*). Thus, these mutants exhibit significantly increased gefitnib affinity *in vitro*, possibly contributing to their increased drug sensitivity in tumors.

Our observation that mutant EGFR kinases are more effectively inhibited by gefitinib is consistent with our equilibrium fluorescence titrations to establish that the mutant kinases exhibit increased affinity for gefitinib, which competes for ATP binding in the catalytic pocket. A recent report concluded that these mutant EGFRs do not differ detectably from wild-type EGFR in gefitnib affinity (18). However, in that study, assays were done with the isolated kinase domain expressed on a bacteriophage particle and lacked the COOH-terminal autophosphorylation sites. Another recent report suggests that, similar to our findings, the Del746-750 mutant exhibits increased gefitnib affinity (19). A recent study of purified EGFR cytoplasmic domains corresponding to the same mutants described here also concluded that the mutant EGFRs exhibit increased catalytic activity and erlotinib sensitivity (20). In their assay, the mutants exhibited an increased K_m for ATP for peptide substrate phosphorylation, potentially accounting for increased drug sensitivity.

The T790M mutation causes increased phosphorylation on several tyrosine residues. The T790M secondary mutation in EGFR has been detected in many patients with acquired resistance to gefitinib treatment (5, 7). Because this mutation has also been detected in some tumors in combination (in *cis*) with additional activating EGFR kinase mutations (8), we tested the possibility that such "double mutants" exhibit increased catalytic activity. We expressed recombinant EGFRs containing either T790M alone or coupled with the L858R mutation in insect cells and assayed phosphorylation. EGFR T790M exhibits tyrosine phosphorylation levels comparable to wild-type EGFR, whereas the T790M/L858R double mutant exhibits a substantial increase in phosphorylation on several sites (Fig. 4). Thus, the T790M resistance mutation, when combined with activating EGFR kinase domain mutations, confers significantly enhanced catalytic phosphorylating activity, suggesting that these mutations "cooperate" to produce a more potent kinase, and potentially explaining the additional role of T790M in predisposing to tumorigenesis. The fact that T790M has been linked to the acquisition of resistance to EGFR inhibitors (5, 7) and to inherited lung cancer predisposition (8) suggests a dual role in cancer, both as an oncogene and in conferring drug resistance.

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References

1. Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. Oncogene 2000;19:6550–65.
2. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
3. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306–11.
4. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
5. Balak MN, Gong Y, Riely GJ, et al. Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. Clin Cancer Res 2006;12:6494–501.
6. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:e73.
7. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non–small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
8. Bell DW, Gore I, Okimoto RA, et al. Inherited susceptibility to lung cancer may be associated with the T790M drug resistance mutation in EGFR. Nat Genet 2005;37:1315–6.

9. Greulich H, Chen TH, Feng W, et al. Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants. PLoS Med 2005;2:e313.

10. Kobayashi S, Ji H, Yuza Y, et al. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor. Cancer Res 2005; 65:7096–101.

11. Jiang J, Greulich H, Janne PA, Sellers WR, Meyerson M, Griffin JD. Epidermal growth factor-independent transformation of Ba/F3 cells with cancer-derived epidermal growth factor receptor mutants induces gefitinib-sensitive cell cycle progression. Cancer Res 2005;65:8968–74.

12. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res 2003;284:31–53.

13. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000;103:211–25.

14. Furdui CM, Lew ED, Schlessinger J, Anderson KS. Autophosphorylation of FGFR1 kinase is mediated by a sequential and precisely ordered reaction. Mol Cell 2006; 21:711–7.

15. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.

16. Jan AY, Johnson EF, Diamonti AJ, Carraway IK, Anderson KS. Insights into the HER-2 receptor tyrosine kinase mechanism and substrate specificity using a transient kinetic analysis. Biochemistry 2000;39:9786–803.

17. Johnson SA, Hunter T. Phosphoproteomics finds its timing. Nat Biotechnol 2004;22:1093–4.

18. Fabian MA, Biggs WH III, Treiber DK, et al. A small molecule-kinase interaction map for clinical kinase inhibitors. Nat Biotechnol 2005;23:329–36.

19. Sakai K, Yokote H, Murakami-Murofushi K, Tamura T, Saijo N, Nishio K. In-frame deletion in the EGF receptor alters kinase inhibition by gefitinib. Biochem J 2006;397:537–43.

20. Carey KD, Garton AJ, Romero MS, et al. Kinetic analysis of epidermal growth factor receptor somatic mutant proteins shows increased sensitivity to the epidermal growth factor receptor tyrosine kinase inhibitor, erlotinib. Cancer Res 2006;66:8163–71.

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