

Pharmacology and Signaling Properties of Epidermal Growth Factor Receptor Isoforms Studied by Bioluminescence Resonance Energy Transfer

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Received June 7, 2006; accepted September 11, 2006

ABSTRACT

We have developed a new assay for measuring epidermal growth factor receptor (EGFR) activation using the bioluminescence resonance energy transfer (BRET) technology, which directly measures the recruitment of signaling proteins to activated EGFR. Our results demonstrate that EGFR BRET assays precisely measure the pharmacology and signaling properties of EGFR expressed in human embryonic kidney 293T cells. EGFR BRET assays are highly sensitive to known EGFR ligands [pEC_{50} of epidermal growth factor (EGF) = 10.1 ± 0.09], consistent with previous pharmacological methods for measuring EGFR activation. We applied EGFR BRET assays to study the characteristics of somatic EGFR mutations that were recently identified in lung cancer. In agreement with recent reports, we detected constitutively active mutant EGFR isoforms, which predominantly signal through the phosphatidylinositol-3-kinase/Akt pathway. The EGFR inhibitors Iressa or Tarceva are

severalfold more potent in inhibiting constitutive activity of mutant EGFR isoforms compared with wild-type EGFR. Notable, our results reveal that most of the mutant EGFR isoforms tested were significantly impaired in their response to EGF. The highest level of constitutive activity and nearly complete loss of epidermal growth factor responsiveness was detected in isoforms that carry the activating mutation L858R and the secondary resistance mutation T790M. In summary, our study reveals that somatic mutations in EGFR quantitatively differ in pharmacology and signaling properties, which suggest the possibility of differential clinical responsiveness to treatment with EGFR inhibitors. Furthermore, we demonstrate that the EGFR BRET assays are a useful tool to study the pharmacology of ligand-induced interaction between EGFR and signaling pathway-specifying adapter proteins.

Overexpression and activation of the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK), plays an important role in the etiology of non-small-cell lung cancer (NSCLC) (Pao and Miller, 2005). Therefore, EGFR is recognized as a key target for the development of NSCLC therapies (Hynes and Lane, 2005). Two drug development strategies focusing on EGFR inhibition are currently pursued: 1) the identification of reversible or irreversible small molecule drugs that inhibit the intracellular tyrosine kinase

activity of EGFR by competitively binding to the ATP-binding site of the kinase domain, and 2) the identification of humanized monoclonal antibodies (mAbs) that interact with extracellular EGFR domains interfering with ligand binding (e.g., epidermal growth factor; EGF) or EGFR dimerization (Hynes and Lane, 2005). The reversible small molecule EGFR inhibitors Iressa (gefitinib) (Herbst et al., 2004) and Tarceva (erlotinib) (Minna and Dowell, 2005) and the antibody drug Erbitux (cetuximab; IMC-C225) (Goldberg, 2005) have already been marketed in the United States for NSCLC. However, early clinical studies observed only a 10 to 15% response rate in a U.S. population of unselected NSCLC

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.106.027656.

ABBREVIATIONS: EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; NSCLC, non-small-cell lung cancer; mAb, monoclonal antibody; EGF, epidermal growth factor; BRET, bioluminescence resonance energy transfer; Grb, growth factor receptor-bound protein; ERK, extracellular signal-regulated kinase; PLC, phospholipase C; Stat/STAT, signal transducer and activator of transcription; Luc, luciferase; GFP, green fluorescent protein; MAP, mitogen-activated protein; HEK, human embryonic kidney; PBS, phosphate-buffered saline; DPBS, Dulbecco's phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; AG-1478, 4-(3'-chloroanilino)-6,7-dimethoxy-quinazoline; WT, wild-type; Ab, antibody; CL-387,785, *N*-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butanamide; HKL-272, (*E*)-*N*-{4-[3-chloro-4-(2-pyridinylmethoxy)anilino]-3-cyano-7-ethoxy-6-quinolinyl}-4-(dimethylamino)-2-butanamide; PD168393, 4-[(3-bromophenyl)amino]-6-acrylamido quinazoline.

patients treated with both drugs (Fukuoka et al., 2003; Perez-Soler et al., 2004). A partial explanation for this low response rate was recently obtained by the discovery of somatic EGFR mutations in only a small subset of NSCLC patients. They clustered in exons 18 to 21 of the EGFR gene, which encode the intracellular EGFR tyrosine kinase domain (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a). The presence of these mutations is significantly associated with a clinical response to treatment with Iressa or Tarceva, giving hope that they could be used as biomarkers to predict drug responsiveness (Pao and Miller, 2005). The somatic mutations cluster in the activating loop of the EGFR kinase domain and have been more frequently found in females, Asians, nonsmokers, and adenocarcinomas (Pao and Miller, 2005). These mutations include point mutations that change single amino acids and small in-frame deletions (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a). A particularly high incidence has been observed in nonsmokers that have adenocarcinomas with bronchoalveolar features (Pao and Miller, 2005). It is noteworthy that cancer cell lines, which endogenously express high levels of the mutated EGFRs, are significantly more sensitive to growth inhibition when treated with Iressa or Tarceva (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a; Sordella et al., 2004). Several in vitro studies show that the somatic mutations identified in EGFR are activating mutations, causing aberrant activation of downstream EGFR signaling pathways and an increase in cell proliferation and survival (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a; Sordella et al., 2004). It is thought that inhibition of these activities with Iressa or Tarceva might contribute to a more beneficial clinical treatment outcome. However, some NSCLC patients who respond to treatment with Iressa or Tarceva lack these somatic mutations (Pao and Miller, 2005), whereas some patients that contain somatic EGFR mutations do not respond to treatment with Iressa (Cappuzzo et al., 2005), indicating that additional mechanism(s) exist that promote sensitivity to these drugs. Furthermore, some NSCLC patients who harbor somatic EGFR mutations in the EGFR gene and initially respond to Iressa or Tarceva eventually develop drug resistance, which coincides with the occurrence of a secondary resistance mutation T790M in the EGFR kinase domain (Kwak et al., 2005; Pao et al., 2005). In vitro cancer cell lines, which express EGFR harboring both the T790M mutation and a somatic activating mutation, show a 100-fold loss of sensitivity to growth inhibition by Iressa compared with the activating mutation alone (Kwak et al., 2005). More recent data indicate that irreversible inhibitors (e.g., HKI-357, HKI-272, and CL-387,785) can effectively inhibit EGFR kinase activity despite the presence of the T790M mutation (Kobayashi et al., 2005; Kwak et al., 2005). The development of new technologies that overcome limitations of current receptor tyrosine kinase screening assays is expected to lead to the discovery of novel EGFR inhibitors with broader response rate in humans and higher receptor selectivity. We used the bioluminescence resonance energy transfer (BRET) technology and developed a new cell-based assay that enabled us to monitor in living cells and in real time, the ligand-induced recruitment of signaling proteins (e.g., Grb2) to the EGFR. These protein interactions are key events in the assembly of larger signaling complexes, which leads to the activation of specific EGFR signaling pathways. The EGFR BRET assays have enabled a detailed

and comprehensive assessment of the pharmacology and signaling properties of somatic mutations in the EGFR.

Materials and Methods

Cloning and Plasmids. Human cDNAs encoding EGFR, Grb2, p85, phospholipase C (PLC) γ 1, and Stat5A were obtained by standard reverse transcription-polymerase chain reaction on poly-A RNA isolated from various human tissues or tumor cell lines. Identities of all cDNAs were confirmed by completely sequencing the open reading frames. EGFR isoforms containing somatic mutations or changes of tyrosine codons to phenylalanine codons were generated by standard mutagenesis methods. EGFR and isoforms were in-frame subcloned into the vector pRLuc-N (PerkinElmer Life and Analytical Sciences, Boston, MA) to generate a chimeric cDNA expressing the EGFR-*Renilla*-luciferase fusion protein (EGFR-Luc). The cDNAs encoding the EGFR signaling molecules (Grb2, Stat5A, PLC γ 1, and p85) were subcloned into the vector pGFP2-N or pGFP2-C (PerkinElmer Life and Analytical Sciences) to generate chimeric cDNAs expressing the corresponding fusion proteins: green fluorescent protein (GFP)2-Grb2, GFP2-p85, GFP2-PLC γ 1, and STAT5A-GFP2.

Drugs, Compounds, and Antibodies. The *Renilla reniformis* luciferase substrate coelenterazine 400A (DeepBlueC; DBC) was obtained from Biotium (Hayward, CA). ACADIA Pharmaceuticals synthesized Tarceva and Iressa. CL-387,785 was purchased from Calbiochem (San Diego, CA). Antibodies were purchased from Chemicon International (San Diego, CA) (*R. reniformis* luciferase antibody MAB4410) or Cell Signaling Technology Inc. (Beverly, MA) (p44/42 MAP kinase antibody 9102 and phospho-p44/42 MAP kinase antibody 9101). Recombinant human EGF protein (100-15) was purchased from Peptrotech (Rocky Hill, NJ).

EGFR BRET Assay. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (with 4500 mg/l D-glucose and glutamine, without sodium pyruvate) (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) supplemented with penicillin-streptomycin-glutamine solution (Invitrogen). Ten-centimeter plate cultures were transiently cotransfected with plasmid DNAs expressing a bioluminescence donor (1 μ g of plasmid DNA expressing EGFR-luciferase) and one of the following fluorescence acceptors—40 μ g of plasmid DNA encoding GFP2-Grb2, GFP2-p85, GFP2-PLC γ 1, or Stat5A-GFP2—depending on the signaling pathway studied. The ratio of 1:40 was predetermined in saturation experiments to be optimal for obtaining the best ligand-induced increase in BRET signal. Two days after transfection, cells were harvested and resuspended in phosphate buffered-saline (PBS), pH 7.5, with glucose and sodium pyruvate to a concentration of 2×10^6 – 4×10^6 cells/ml, depending on transfection efficiency. Transfection was performed with Polyfect (QIAGEN, Valencia, CA) as described by manufacturer. One day after transfection, cells were serum-starved for 24 h in Dulbecco's modified Eagle's medium, 0.1% fetal bovine serum supplemented with penicillin-streptomycin-glutamine solution. Drug dilutions were prepared in Costar 3912, nontreated, white polystyrene 96-well plates (Corning Life Sciences, Acton, MA). For agonist assays, 50 μ l of any drug concentration tested was incubated with 50 μ l of the cell suspension for 5 min to establish the ligand-induced recruitment of GFP2-tagged EGFR signaling proteins to the intracellular carboxyl terminus of EGFR-luciferase. For antagonist assays, 25 μ l of any antagonist concentration tested was incubated for 10 min with 50 μ l of the cell suspension followed by additional 20-min incubation time after addition of 25 μ l of the used agonist. For both types of assay, 50 μ l of *R. reniformis* luciferase substrate DBC (5 μ M final concentration) was added to activate the luciferase. Luciferase and GFP2 emissions were measured after DBC addition and a 1-s shaking step for 1 s each. Note that GFP2 is excited through BRET between activated luciferase and GFP2 but strictly dependent on proximity (<100 Å). The time after addition of coelenterazine 400A is sufficient to reach equilibrium

with luciferase activity (data not shown). Injection of DBC and recording of luminescence kinetics are automatically performed by the multiplate reader Mithras 940LB (Berthold Technologies, Bad Wildbad, Germany). The plate reader is equipped with filters to detect GFP2 emission (505–525 nm) and *R. reniformis* luciferase emission (375–445 nm). The BRET signal is calculated as the ratio between the *R. reniformis* luciferase emission and the GFP2 emission corrected by the background emissions of nontransfected cells. The first 5 min of the time course of EGF induced BRET-2 signal increase in the EGFR/Grb2 BRET-2 assay (Fig. 1B) was generated by a timely coordinated injection of EGF and DBC, performed by the multiplate reader Mithras 940LB (Berthold Technologies). Dose-response curves and nonlinear regression analysis are performed with the software Prism (GraphPad Software Inc., San Diego, CA) to obtain IC_{50} and EC_{50} values.

Cell Extract Formation and Western Blot. Transfected BRET assay cells were harvested and resuspended in DPBS ($2 \times 10^6 \times 10^6$ cells/ml dependent on transfection efficiency). Whole cell extracts were prepared after incubation with 25 nM EGF prepared in DPBS or with DPBS alone in radioimmunoprecipitation assay buffer (including proteinase inhibitor cocktail). Samples were solubilized in loading buffer and directly separated on 12% SDS-PAGE. Western blot analysis was performed as recommended for the antibodies purchased from Cell Signaling Technology Inc.

Results

Signaling Pathway-Specific EGFR BRET-2 Assays.

The rapid ligand-stimulated autophosphorylation of specific tyrosine residues in the intracellular carboxyl terminus of RTKs is an obligatory event in RTK signaling (Schlessinger, 2000; Schlessinger, 2002). The phosphorylated tyrosine residues serve as docking sites for a diverse set of proteins, which are involved in building, shaping, and directing the specific RTK downstream signaling pathways (Schlessinger, 2000, 2002). RTK pharmacology and signaling have traditionally been studied quantitatively using mainly immunological methods that detect RTK phosphorylation (Olive, 2004) or downstream effects on proliferation. We designed a functional RTK assay that uses the BRET-2 technology (Gales et al., 2005). We applied this technology to quantitatively monitor, in living cells, the recruitment of various EGFR signaling proteins, which interact with activated EGFR to link the receptor to the four main RTK signaling

pathways. The various signaling proteins we studied include the adapter proteins Grb2 and Shc (MAP kinase proliferation pathway); Stat5A (STAT pathway), PLC γ 1 (PLC γ 1-protein kinase C/calcium pathway); and p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K-Akt survival pathway). The human EGFR protein was in frame carboxyl-terminal tagged with *R. reniformis* Luc, whereas GFP2 was fused in-frame to the amino termini of Grb2, PLC γ 1, and p85 and the carboxyl terminus of Stat5A (see *Materials and Methods*). To perform an EGFR BRET assay, EGFR-Luc and a GFP2-tagged signaling protein were transiently coexpressed in HEK293T. Cells were incubated for 20 min with variable concentrations of EGF to activate EGFR and stimulate recruitment of the GFP2-tagged signaling molecule to EGFR-Luc. BRET-2 signals were measured by detecting luciferase and GFP2 emissions, after addition of the cell-permeable *R. reniformis* luciferase substrate DeepBlueC, and by calculating the ratio between the detected GFP2 and Luc emissions (see *Materials and Methods*). The BRET between activated luciferase (luminescence donor) and GFP2 (fluorescence acceptor) causes excitation of GFP2, but this signal is strictly dependent on the proximity of both proteins, so that the BRET-2 signal in this assay directly correlates with the activation of EGFR and recruitment of GFP2-tagged EGFR effector proteins. A recent report showed that the BRET technology can also be applied to monitor in living cells the recruitment of insulin receptor substrate-1 or protein tyrosine phosphatase-1B to the insulin receptor (Laursen and Oxvig, 2005).

Figure 1 shows agonist and antagonist dose-response curves in cells cotransfected with wild-type EGFR-Luc and GFP2-Grb2. Application of variable concentrations of the potent agonist EGF caused a dose-dependent BRET-2 signal increase in the wild-type EGFR BRET-2 assay ($pEC_{50} = 10.1$; Fig. 1A), demonstrating the high sensitivity of the assay. Already 10 s after applying a high dose of EGF (17 nM), the EGFR BRET-2 signal reached around 90% of the maximal response (see time course in Fig. 1B). The EGF-induced BRET-2 signal peaked by 5 min and persisted for more than 20 min (Fig. 1B; data not shown). The observed increase in the Grb2/BRET-2 signals was dependent on phosphorylation

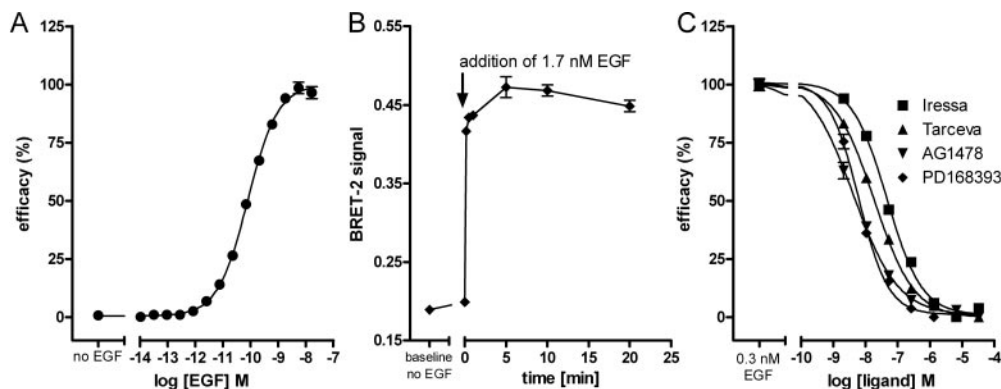


Fig. 1. A, dose-response curve for agonist EGF in EGFR/Grb2 BRET-2 assay. Wild-type EGFR is coexpressed with GFP2-Grb2 in HEK293T cells and then analyzed in the EGFR/Grb2 BRET-2 assay to monitor MAP kinase signaling (see *Materials and Methods*). B, time course of EGF-induced BRET-2 signal increase in the EGFR/Grb2 BRET-2 assay. Wild-type EGFR is coexpressed with GFP2-Grb2 in HEK293T cells and then analyzed in the EGFR/Grb2 BRET-2 assay to monitor the increase of the EGFR BRET-2 signal over a time period of 20 min after addition of 17 nM EGF (see *Materials and Methods*). C, Antagonism of 0.33 nM EGF-induced EGFR responses in EGFR BRET/Grb2 assays. Wild-type EGFR is coexpressed with GFP2-Grb2 in HEK293T cells and then analyzed in the EGFR/Grb2 BRET-2 assay to monitor MAP kinase signaling (see *Materials and Methods*). Dose responses for EGFR inhibitors Iressa (■), Tarceva (▲), AG1478 (▼), and PD168393 (◆) are compared.

of multiple EGFR tyrosine residues in the intracellular carboxyl terminus. Changing the tyrosine (Y) residues 1068, 1086, 1101, 1148, and 1173 to phenylalanine (F) in EGFR-Luc resulted in $70 \pm 0.6\%$ reduction of the EGF-induced BRET-2 signal in the Grb2/BRET-2 assay [EGFR wild type, 0.23 ± 0.006 (–EGF) and 0.60 ± 0.006 (+EGF) versus EGFR quintuple mutant EGFR isoform, 0.19 ± 0.002 (–EGF), 0.32 ± 0.002 (+EGF); data not shown]. A mutant EGFR isoform carrying the Y1086F, Y1101F, Y1114F, and Y1173 mutations showed only a 45% reduction of the EGF-induced Grb2/BRET-2 signal (data not shown). At least six phosphorylated tyrosine residues have been identified in the carboxyl terminus of EGFR that mediate multiple direct or indirect interactions of the adapter protein Grb2 with EGFR (Schulze et al., 2005). Our results are consistent with previous results that demonstrate that the recruitment of EGFR adapter proteins is dependent on tyrosine phosphorylation (Schlessinger, 2000, 2002). The EGF stimulated recruitment of the GFP2-tagged signaling proteins to EGFR-Luc could be effectively inhibited through the coapplication of various EGFR kinase domain inhibitors [e.g., EGFR Grb2/BRET-2 assay in Fig. 1C: AG1478, $pIC_{50} = 8.38$ (5 nM); PD168393, $pIC_{50} = 8.21$ (6.3 nM); Iressa, $pIC_{50} = 7.33$ (46 nM); and Tarceva, $pIC_{50} = 7.73$ (19 nM)] or neutralizing EGFR antibodies (data not shown). In contrast, incubation of the cells with varying concentrations of the kinase domain inhibitor Iressa in the absence of exogenous EGF slightly reduced the BRET-2 signal, suggesting the presence of constitutive activity in wild-type EGFR (Fig. 2A, Iressa, ○). The BRET-2 signal was reduced to around 0.19 for wild type at the highest concentrations of Iressa, which is likely to be the baseline BRET-2 signal for the EGFR/Grb2 BRET-2 assay. In contrast to EGF-stimulated EGFR activity, the constitutive activity of the wild-type EGFR could not be neutralized with anti-human EGF antibodies (data not shown).

EGFR has previously been carboxyl-terminally tagged with GFP isoforms, which did not change half-life, ligand-induced tyrosine phosphorylation, or internalization properties (Hayes et al., 2004). Interaction of EGFR and Grb2 in living cells has also been visualized by fluorescence resonance energy transfer microscopy (Sorkin et al., 2000). In addition, EGFR proteins have been shown to recruit yellow fluorescent protein-tagged Grb2 or GFP-tagged PLC γ 1 from the cytoplasm to the plasma membrane (Sorkin et al., 2000;

Hayes et al., 2004). Our wild-type EGFR-luciferase chimera did show pharmacological and signaling properties similar to reported data for untagged EGFR receptors (Fry et al., 1998; Hynes and Lane, 2005), suggesting that the luciferase tag did not severely alter the function of EGFR.

The activation of EGFR signaling pathways is controlled by the recruitment of specific signaling molecules to phosphorylated tyrosine residues in EGFR domains of the intracellular carboxyl terminus. The EGFR BRET-2 experiments described in Fig. 1 were focusing on the recruitment of Grb2, an adapter protein that transduce EGFR activity into the MAP kinase pathway. We also studied wild-type EGFR pharmacology in BRET-2 assays using the signaling molecules p85, PLC γ 1, or Stat5A (Table 1). All wild-type EGFR BRET-2 assays showed a high sensitivity to the agonist EGF (mean $pEC_{50} = 10.1 \pm 0.11$; Table 1). These results are in close agreement with previous methods for measuring wild-type EGFR activity, thereby validating the EGFR BRET-2 assay. Thus, we used these EGFR BRET-2 assays to study the pharmacology and signaling properties of somatic EGFR mutations in lung cancer and in particular compared the pharmacological activities of the EGFR inhibitors Iressa and Tarceva.

Somatic Mutations in EGFR Cause Constitutive Activity and Affect Responsiveness to EGF. Somatic EGFR mutations have been identified in NSCLC, which activate EGFR signaling (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a). We studied four somatic EGFR mutations in the EGFR BRET-2 assays: L858R, the most common point mutation observed in NSCLC patients (exon 21) is localized in the activation loop of the EGFR TK domain; G719C (exon 18); localized in the nucleotide phosphate binding loop (P-loop), and the deletion mutations $\Delta 752-759$ and $\Delta 747-749$ A750P (exon 19), both localized close to the ATP binding region. EGFR-Luc isoforms carrying these mutations were cotransfected with GFP2-Grb2 to evaluate the effects of these somatic mutations on MAP kinase pathway signaling. The presence of the described mutations in EGFR-Luc did not significantly affect the expression levels compared with wild-type EGFR-Luc (data not shown; Fig. 5, bottom). In the absence of exogenously added EGF, we observed significant constitutive activity for all four mutations tested (compare no ligand baselines in Fig. 2, A–E), which is reflected by the higher Grb2/BRET-2 assay signal of the mutants compared

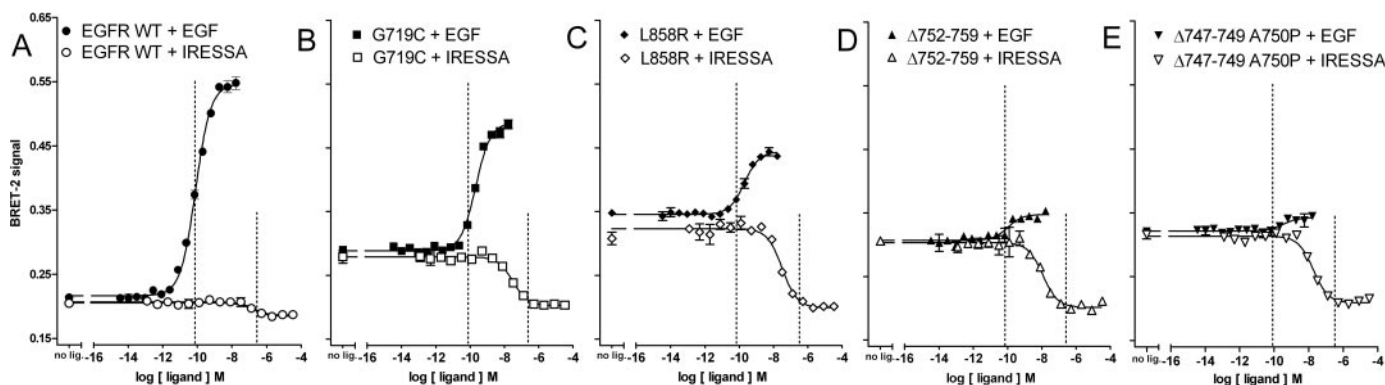


Fig. 2. Constitutive and EGF-induced activities of mutant EGFR isoforms and inhibition of their constitutive activities by small molecule EGFR inhibitor Iressa. Wild-type EGFR-Luc or mutant EGFR-Luc isoforms are coexpressed with GFP2-Grb2 in HEK293T cells and then analyzed in the EGFR/Grb2 BRET-2 assay to monitor MAP kinase signaling (see *Materials and Methods*). A, EGFR WT. B, EGFR G719C. C, EGFR L858R. D, EGFR $\Delta 752-759$. E, EGFR $\Delta 747-749$ A750P. Dashed lines in A to E indicate EC_{50} for EGF responses and IC_{50} for Iressa responses at the wild-type EGFR. Open symbols, EGF; closed symbols, Iressa; no lig., no ligand.

with the wild-type EGFR. Although wild-type EGFR exhibited a BRET-2 signal of 0.21 in the absence of EGF, the L858R mutant receptor showed the highest level of constitutive activity with a BRET-2 signal of 0.33. All constitutive activities were efficiently inhibited both by Iressa (Fig. 2, A–E) and Tarceva (data not shown; Table 1). The P-loop G719C mutation (exon 18) had the lowest constitutive activity of the four mutations tested, whereas the three other mutants localize in two mutation hotspots in exons 19 and 21 with the highest constitutive activity. Our results indicate that the somatic mutations tested cause an increase in the constitutive interaction between mutant EGFR-Luc isoforms and GFP2-Grb2. Iressa and Tarceva effectively inhibit this increase in the BRET-2 signal, probably by competing with ATP binding at the intracellular catalytic tyrosine kinase domain. Similar results were obtained in the EGFR/Shc BRET-2 assay, which monitored the recruitment of the adapter protein Shc42 to EGFR-Luc (data not shown). Our results suggest that the studied somatic mutations in EGFR cause a constitutive increase in MAP kinase pathway signaling (Fig. 5; see below).

It is noteworthy that treating the various mutant EGFR isoforms with EGF revealed dramatic differences in their respective EGF responsiveness. EGF is a very potent agonist for wild-type EGFR ($pEC_{50} = 10.1$; Table 1; Figs. 1A and 2A, ●) in the EGFR/Grb2 BRET-2 assay, demonstrating the sensitivity of the EGFR BRET-2 assay. Whereas all mutant EGFR isoforms are only slightly less potent in responding to EGF, they show more dramatic differences in efficacy compared with wild-type EGFR (Table 1; Fig. 2, A–E, closed symbols). For the wild-type receptor, the BRET-2 signal increased from 0.21 in the absence of EGF to 0.55 in the presence of the maximal effective dose of EGF (Fig. 2A, ●). However, for the EGFR point mutants, the signal with EGF increased to only 0.50 for G719C and 0.45 for L858R (Fig. 2, B and C, closed symbols, respectively). The EGF signal was further impaired in the deletion mutants, which showed only a slight ligand induced increase in the BRET-2 signal to 0.35 (Fig. 2, D and E, closed symbols), indicating a strong impairment in transducing EGF signals into the MAP kinase pathway signaling. Therefore, none of the tested constitutively active EGFR mutants reached wild-type EGFR activity level after EGF stimulation. This reduced response to EGF has

previously not been recognized because other methods did not clearly separate constitutive activity from EGF-stimulated responses (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a). We could show that somatic mutation in EGFR dramatically increases the level of constitutive receptor activity and decreases the responsiveness to EGF. These results demonstrate that EGFR BRET assays are a useful pharmacological tool that allows the separate study of ligand-dependent and ligand-independent EGFR function.

Iressa and Tarceva Effectively Inhibit Constitutive Activity of EGFR Isoforms. Although the constitutive activity of wild-type EGFR in the EGFR/Grb2 BRET-2 assay is inhibited by Iressa at a pIC_{50} of 6.59 (Table 1; Fig. 2A, ○), the constitutive activities of the somatic mutants as measured by the same assay are 5- to 10-fold more sensitive to inhibition by Iressa (Fig. 2, B–D, open symbols), with pIC_{50} values ranging from 7.41 to 7.59 (Table 1). Tarceva produced similar results (Table 1). These results are consistent with previous findings in cell proliferation assays of cancer cell lines endogenously expressing EGFR mutants (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004b; Sordella et al., 2004) and provide one possible explanation for the high clinical responsiveness of NSCLC patients who harbor somatic EGFR mutations and are treated with these EGFR inhibitors. No significant differences were observed between Iressa and Tarceva acting at the various EGFR mutants, except that Tarceva seems slightly more potent in inhibiting the wild-type and mutant EGFR isoforms ($\Delta IC_{50} = 0.41 \pm 0.14$ for EGFR/Grb2 BRET-2 assays). It is surprising that all of the constitutively active mutations increase inhibitor potency. In many other receptor systems, constitutively active mutations decrease the potency of inhibitors. This may reflect fundamental differences in the receptor interaction with signal transduction inhibitors (e.g., Iressa and Tarceva) versus traditional receptor antagonists and inverse agonists (Spalding et al., 1995).

Constitutive Activity of EGFR Isoforms Is Predominantly Transduced through the PI3K/Akt Survival Pathway. Based on the results from the EGFR/Grb2 BRET-2 assay, we decided to study the effect of the L858R and the $\Delta 752-759$ mutations on other EGFR signaling pathways. The results of all experiments are summarized in Table 1 and presented in Fig. 3. For each receptor isoform and

TABLE 1

Pharmacology of Iressa and Tarceva on constitutive active EGFR isoforms

The EGF EC_{50} and Iressa and Tarceva IC_{50} values for wild-type and mutant EGFR isoforms were compared and tested for statistically significant differences by a two-tailed unpaired *t* test. The mean and S.E.M. for all values are shown; log values are given in molar concentration.

	EC_{50} Log [EGF]	<i>n</i>	IC_{50} Log [Iressa]	<i>n</i>	IC_{50} Log [Tarceva]	<i>n</i>	EGFR Signaling Pathway
EGFR WT	10.14 ± 0.01	32	6.59 ± 0.08	15	6.83 ± 0.08	15	MAP kinase (Grb2)
EGFR L858R	$9.63 \pm 0.03^*$	8	$7.59 \pm 0.05^*$	4	$8.04 \pm 0.04^*$	4	
EGFR $\Delta 752-759$	$9.71 \pm 0.06^*$	8	$7.88 \pm 0.06^*$	4	$8.23 \pm 0.05^*$	4	
EGFR $\Delta 747-749$ A750P	$9.69 \pm 0.08^*$	7	$7.64 \pm 0.03^*$	3	$8.00 \pm 0.06^*$	4	
EGFR G719C	$9.78 \pm 0.04^*$	8	$7.41 \pm 0.10^{\dagger}$	4	$8.05 \pm 0.06^*$	4	
EGFR WT	9.97 ± 0.09	6	N.D.	3	N.D.	3	STAT (STAT5A)
EGFR L858R	9.91 ± 0.05	3	8.46 ± 0.10	3	8.88 ± 0.03	3	
EGFR $\Delta 752-759$	9.79 ± 0.17	3	8.45 ± 0.01	3	8.74 ± 0.19	3	
EGFR WT	10.22 ± 0.02	6	8.00 ± 0.15	6	8.03 ± 0.03	7	PI3K/Akt (p85)
EGFR L858R	$9.36 \pm 0.21^{\ddagger}$	3	8.33 ± 0.11	3	$8.79 \pm 0.06^*$	3	
EGFR $\Delta 752-759$	$9.25 \pm 0.18^*$	3	$8.56 \pm 0.09^{\S}$	3	$8.60 \pm 0.09^*$	3	
EGFR WT	10.15 ± 0.03	9	N.D.	4	N.D.	4	PLC γ 1-calcium (PLC γ 1)
EGFR L858R	$9.89 \pm 0.02^*$	5	8.04 ± 0.15	4	8.50 ± 0.11	4	
EGFR $\Delta 752-759$	9.93 ± 0.07	5	8.32 ± 0.10	5	8.42 ± 0.14	5	

N.D., not determined.

Statistically significant differences are labeled as follows: $^*P < 0.0001$; $^{\dagger}P = 0.0002$; $^{\ddagger}P = 0.0006$, and $^{\S}P = 0.041$.

signaling pathway studied, the BRET-2 signals are transformed in percentage and normalized to the wild-type EGFR response. The 100% BRET-2 signal of wild-type EGFR represents the sum of EGF-stimulated wild-type EGFR activity plus the constitutive activity of wild-type EGFR [determined by Iressa inhibition and indicated as a closed bar for wild-type (WT) EGFR in Fig. 3, A–E]. With the exception of the Stat5A effector, EGF as agonist showed a slight loss of potency for activating the two mutants EGFR isoforms compared with the wild-type EGFR (Table 1). Consistent with the EGFR/Grb2 BRET-2 assay (Figs. 2 and 3A), both mutant EGFR isoforms (L858R and $\Delta 752$ -759) show constitutive activity (in the absence of EGF) for all pathways (Fig. 3, A–D, open bars), but with quantitative differences. It is noteworthy that all EGFR mutants tested predominantly signal through the PI3K/Akt survival pathway (Fig. 3C). For the L858R mutant, the constitutive activity (Fig. 3C, L858R, open bar) is approximately 70% of the total wild-type response (Fig. 3C, WT, closed bar). This observation correlates well with previous studies that detected increased Akt phosphorylation in the cancer cell line H-1975 that endogenously express EGFR L858R (Sordella et al., 2004). Meanwhile, the corresponding constitutive signaling activity of the L858R mutant through the MAP kinase, STAT, and PLC γ 1-calcium signaling pathways ranged only between 28 and 40% of the total wild-type responses (L858R in Fig. 3, A, B, and D, open bars). The deletion mutant EGFR $\Delta 752$ -759 showed a similar profile for constitutive activity and coupling to the different signaling pathways, with 54% activity in the PI3K/Akt pathway ($\Delta 752$ -759 in Fig. 3C, open bar) and lower levels of activity 30 to 35% in the other pathways ($\Delta 752$ -759 in Fig. 3, A, B, and D, open bar). It will be interesting to determine in the future with the EGFR/p85 BRET-2 assay whether all other described EGFR mutants show high constitutive activation of the PI3K/Akt pathway and whether this activity

directly correlates with increased drug sensitivity. A recent study used an EGF independent Ba/F3 cell transformation assay to show constitutive activity of the EGFR L858R and G719S isoforms and a differential sensitivity of both isoforms to small molecule inhibitors (Jiang et al., 2005). We also found differences in the EGF responsiveness between the L858R and $\Delta 752$ -759 mutants. When the L858R isoform was treated with EGF in BRET assays monitoring STAT, PI3K/Akt, or PLC γ 1-calcium signaling, we detected in all assays a slightly reduced EGF responsiveness compared with wild-type EGFR (Fig. 3, B–D, compare differences between open and closed bars). This result was similar to the slightly reduced EGF responsiveness of EGFR L858R detected in the Grb2/BRE-2 assay (Figs. 2C and 3A). It is noteworthy that the deletion mutant EGFR $\Delta 752$ -759 showed a large quantitative difference in the EGF-stimulated response in a comparison of the Stat5A/BRET-2 and Grb2/BRET-2 assays (compare difference between open and closed bars for $\Delta 752$ -759 in Fig. 3, A and B). EGF-stimulated Stat5A-GFP2 recruitment to EGFR-Luc $\Delta 752$ -759, but it did not significantly stimulate the recruitment of GFP2-Grb2. It is possible that EGFR receptors, which carry this mutation, are impaired in transducing the EGF signal downstream into the MAP kinase pathway, but that they are still able to activate the STAT pathway. These results suggest that a specific somatic EGFR kinase domain mutation can differentially affect EGFR signaling pathways.

In contrast to the differential constitutive activity and EGF responsiveness of the mutant EGFR isoforms, the EGFR inhibitors Iressa or Tarceva do not show a preference to inhibit the constitutive activity of a specific mutant EGFR isoform (Table 1). However, Iressa and Tarceva are in general more potent in inhibiting the constitutive activity of mutant EGFR isoforms than of the wild-type EGFR. This observation is consistent with the increase in drug sensitivity

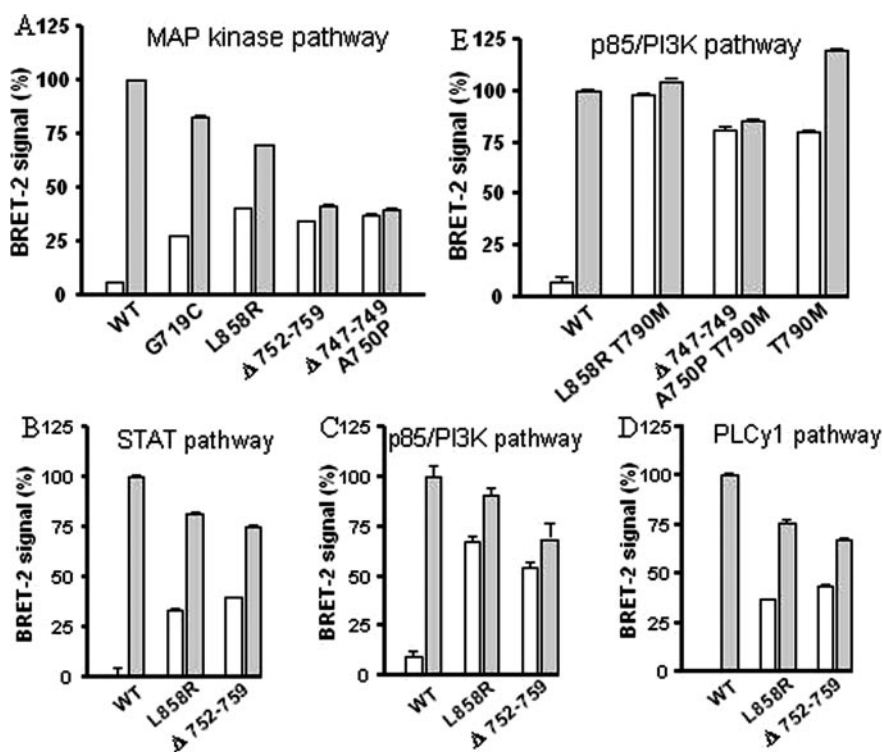


Fig. 3. Differential affects of somatic EGFR mutations on constitutive EGFR signaling and EGF-induced signaling. The indicated wild-type or mutant EGFR isoforms are coexpressed with GFP2-Grb2 (A), Stat5A-GFP2 (B), GFP2-p85 (C and E), or GFP2-PLC γ 1 (E) in HEK293T cells and analyzed as described under *Materials and Methods*. The EGF-induced signal (0.33 nM EGF) plus the constitutive activity for the wild-type EGFR (closed bars) is normalized to 100%. The BRET-2 signal of the mutant EGFR isoforms is expressed as a percentage of wild-type EGFR responses and derived from ratios between the *R. reniformis* luciferase emission and the GFP2 emission corrected by the background emissions of nontransfected cells. Open bars, constitutive EGFR activity; closed bars, constitutive EGFR activity plus EGF-induced activity. The following EGF-stimulated wild-type EGFR BRET-2 signals for the studied EGFR signaling pathways were observed: MAP kinase pathway: 0.21 ± 0.06 (–EGF), 0.55 ± 0.02 (+EGF); p85/PI3K pathway: 0.11 ± 0.03 (–EGF), 0.21 ± 0.03 (+EGF); STAT5 pathway: 0.08 ± 0.008 (–EGF), 0.17 ± 0.09 (+EGF); and PLC γ 1 pathway: 0.09 ± 0.009 (–EGF), 0.14 ± 0.03 (+EGF).

to inhibit proliferation of cancer cell lines that harbor these mutations compared with cancer cell lines that express only wild-type EGFR (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004a; Sordella et al., 2004). We could not detect constitutive activity in the wild-type EGFR with the EGFR/Stat5a or EGFR/PLC γ 1 BRET-2 assays, which prevented us from quantitating the increase of drug sensitivity. Because our results are derived from heterologous EGFR expression in HEK293T cells, it suggests that the presence of somatic mutations in EGFR is principally sufficient to increase drug sensitivity of cancer cells that express mutant EGFR isoforms.

Impact of T790M Mutation on Inhibition of Constitutive EGFR Activity by Tarceva and Iressa. Many lung cancer patients will relapse and acquire drug resistance to both Iressa and Tarceva during their treatment regiment. Acquisition of drug resistance is a complex process involving multiple poorly characterized pathways, with one pathway involving the occurrence of resistance mutations (Dean et al., 2005). Several studies have reported the identification of an acquired secondary resistance mutation (T790M) in the EGFR kinase domain of patients who were treated and initially responded to Iressa and Tarceva (Kobayashi et al., 2005; Kwak et al., 2005; Pao et al., 2005). The T790M mutation was only found in the presence of an activating EGFR mutation in tumor samples, although only in a small fraction of the cells within the entire tumor. The mutation has also been found in patients that did not undergo treatment with

Iressa and Tarceva. It is noteworthy that a recent report identified T790M as an inherited germline EGFR mutation potentially associated with susceptibility to late onset NSCLC (Bell et al., 2005).

We analyzed a mutant EGFR variant bearing the T790M mutation alone and mutant EGFRs that carry T790M in combination with the L858R or Δ 747-749 A750P mutations in the EGFR/p85 BRET-2 assay. Our results show that the T790M mutation alone generates a highly constitutively active EGFR receptor (Fig. 3E, open bar). In addition, we analyzed the double mutant EGFRs L858R T790M and Δ 747-749 A750P T790M, both reported to occur in patients that developed drug resistance in NSCLC (Kobayashi et al., 2005; Kwak et al., 2005; Pao et al., 2005). Both double mutants showed the highest levels of constitutive activity of all mutants tested in this report. The constitutive activity of the EGFR L858R T790M receptor (Figs. 3E, open bar, and 4A, open circle) reached the same maximal activity level as the wild-type EGFR treated with EGF, whereas the constitutive activity level of EGFR Δ 747-749 A750P T790M reached 80% of the maximal EGF response for wild-type EGFR (Fig. 3E, open bar) similar to the constitutive activity of the T790M mutation alone. Meanwhile, the EGF responsiveness of both double mutants was nearly completely impaired (Figs. 3E, compare differences between open and filled bars, and 4A). Our results strongly indicate that the development of drug resistant cells carrying the T790M mutation is accompanied by a dramatic increase in constitutive activation of the PI3K/

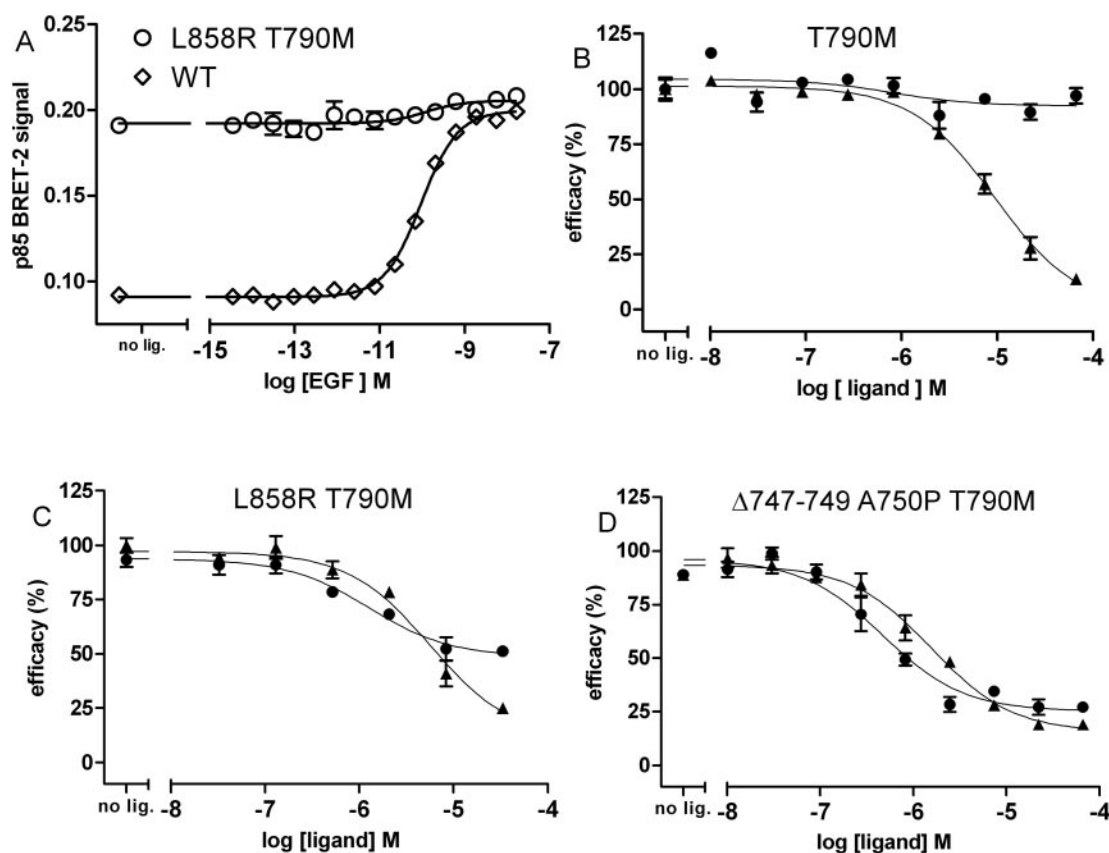


Fig. 4. Iressa and Tarceva are partial inhibitors of high constitutively active EGFR receptors that bear the resistant mutation T790M in combination with the L858R or Δ 747-749 A750P mutations. Mutant EGFR isoforms are coexpressed with GFP2-p85 in HEK293T cells and analyzed in the EGFR/p85 BRET-2 assay, monitoring PI3K/Akt pathway signaling. Dose responses for the activation of EGFR WT and EGFR L858R T790M with EGF are shown in A. Dose responses for the inhibition of constitutive receptor activity with the small molecule inhibitor Iressa (\blacktriangle) and Tarceva (\bullet) are compared in B and C. A, EGFR WT and EGFR L858R T790M. B, EGFR T790M. C, EGFR L858R T790M. D, EGFR Δ 747-749 A750P.

Akt pathway (Figs. 3E and 4A). We found similar robust constitutive activation of the MAP kinase pathway by the double mutant receptors (data not shown).

We next analyzed the effect of Iressa and Tarceva on EGFR isoforms carrying the T790M mutation. Tarceva was not effective in inhibiting the constitutive activity of the EGFR T790M isoform (Fig. 4B, ●). However, despite similarities with Tarceva in structure and mode of action, Iressa inhibited the constitutive activity of the T790M isoform (85% inhibition with 33 μ M high dose), but with a lower potency compared with the other somatic mutants ($pIC_{50} = 5.3 \pm 0.033$) (Fig. 4B, closed triangles). In contrast to the prediction from the T790M mutation alone, we observed significant but partial inhibition (50–75%) of the double mutants by Iressa or Tarceva (Fig. 4, C and D). These results are in good agreement with experiments in which the proliferation of the cancer cell line NCI-H1975, which express the double mutant EGFR L858R T790M isoform, is significantly suppressed by high concentration of Iressa. Immunoblot data suggest that EGFR tyrosine 1068 and Akt phosphorylation is suppressed in this cell line at high micromolar concentrations of Iressa (Kobayashi et al., 2005). It is noteworthy that Tarceva was slightly more potent in inhibiting EGFR L858R T790M activity than Iressa in our BRET/p85 BRET-2 assay (Tarceva $pIC_{50} = 5.89 \pm 0.06$ versus Iressa $pIC_{50} = 5.25 \pm 0.07$). A similar potency difference was observed for the deletion mutant EGFR $\Delta 747-749$ A750P T790M (Tarceva $pIC_{50} = 6.33$ versus Iressa $pIC_{50} = 5.80$). This analysis of the EGFR T790M mutation in the EGFR BRET-2 assay correlates well with the known resistance of T790M-bearing tumors to Iressa and Tarceva and show that it is important to develop new strategies for inhibiting the drug resistant cell clones because of their more aggressive properties. Recent studies demonstrate that irreversible EGFR inhibitors are more effective in inhibiting the constitutive activity of these EGFR double mutants (Carter et al., 2005; Kobayashi et al., 2005; Kwak et al., 2005).

We tested the irreversible inhibitor CL-387,785 in the EGFR/p85 BRET-2 assay with EGFR L858R T790M and EGFR $\Delta 747-749$ A750P T790M and observed complete inhibition of constitutive activity with an pIC_{50} of 6.86 ± 0.14 and 6.81 ± 0.12 , respectively (data not shown). Wild-type EGFR showed a higher sensitivity for inhibition with a pIC_{50} of 9.4 ± 1.1 . Similar results were obtained in the EGFR/Grb2 assay (data not shown). Very early treatment of primary tumors with irreversible inhibitors might reduce the risk to develop T790M-related drug resistance and relapses. In addition, the lack of EGF responsiveness and the decreased sensitivity for the EGFR inhibitor Iressa and Tarceva suggest that concurrent EGF treatment could rescue patients from toxicities of EGFR inhibitors that would not be tolerated otherwise.

Mutant EGFR Isoforms Constitutively Activate Downstream ERK1/2 in the EGFR/Grb2 BRET-2 Assay. We explored how the signaling through the MAP kinase pathway downstream of the adapter protein Grb2 is affected by somatic EGFR mutations in our EGFR/Grb2 BRET-2 assays. ERK1/2 are the prototypic MAP kinases and are activated downstream of Grb2. We studied ERK1/2 protein expression and phosphorylation in BRET-2/Grb2 assay cells by Western blotting (Fig. 5). Cells expressing GFP2-Grb2 in combination with the wild-type EGFR-Luc, the mutated

EGFR-Luc isoform L858R, and the mutated EGFR-Luc isoform L858R T790M were either left untreated or treated with EGF and then analyzed. We found similar levels of EGF induced (T202/Y204) phosphorylation of ERK1/2 (molecular mass 42 and 44 kDa) in all BRET-2 samples analyzed (Fig. 5, lanes 2, 4, 6, and 8), including the no receptor control (Fig. 5, lane 2). HEK293T cells endogenously express a low level of EGFR, which is sufficient for ERK1/2 activation in nontransfected cells (Fig. 5, lane 2). Introduction of the wild-type EGFR-Luc or of the two mutant EGFR isoforms did not enhance the EGF-induced phosphorylation of ERK1/2, which are detected as 42/44-kDa protein bands by Western blotting (Fig. 5, compare lanes 2, 4, 6, and 8). However, our blots additionally detected an increase of phosphorylated ERK1/2 proteins in the form of a reduced mobility band (Fig. 5, lanes 3–8), which was absent in cells lacking transfected EGFR-Luc isoforms (Fig. 5, lane 2). Western blotting using an antibody against *R. reniformis* luciferase shows that the reduced-mobility phospho-ERK1/2 bands migrate at the same

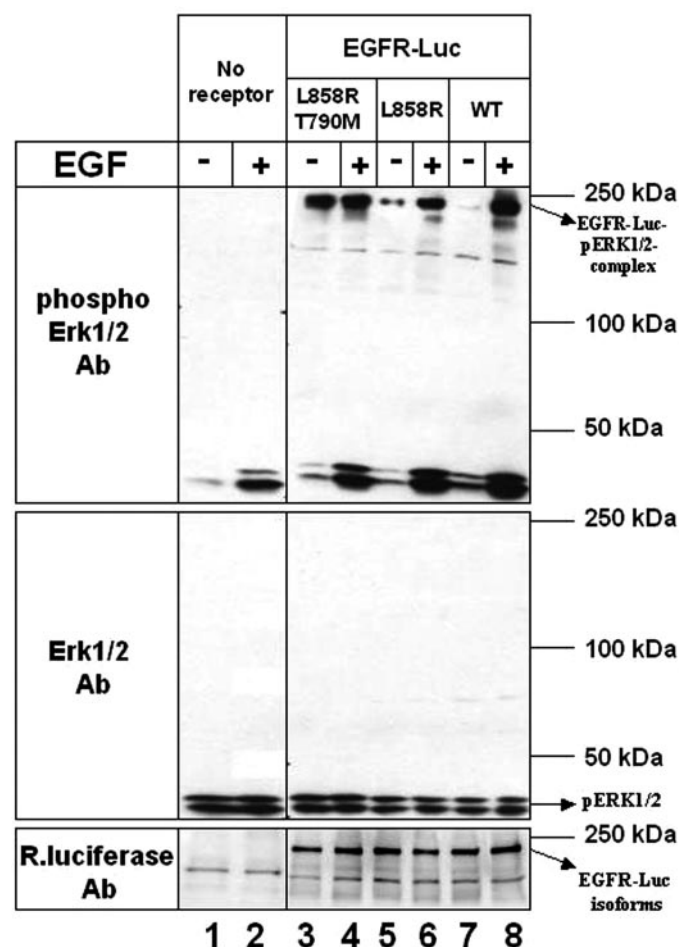


Fig. 5. Constitutive activity of mutant EGFR isoforms leads to downstream ERK1/2 activation. Mutant EGFR isoforms are coexpressed with GFP2-Grb2 in HEK293T cells. Cells were either untreated (–) or treated (+) with 25 nM EGF for 5 min, lysed, and analyzed by 12% SDS-polyacrylamide gel electrophoresis and Western blotting using antibodies recognizing human phosphorylated ERK1/2, ERK1/2 protein, or *R. reniformis* luciferase. Lanes 1 and 2, no receptor control (GFP2-Grb2 alone); lanes 3 and 4, EGFR-Luc L858R T790M; lanes 5 and 6, EGFR-Luc L858R; lanes 7 and 8, wild-type EGFR-Luc. Phospho-ERK1/2 antibody (Ab) (top), control protein ERK1/2 Ab (middle), and control *R. reniformis* luciferase Ab (lower).

blot location as the EGFR-Luc isoforms (Fig. 5, bottom). These observations suggest the formation of a complex between phosphorylated ERK1/2 and activated EGFR and are consistent with the results of Habib et al. (2003), who demonstrated that the overexpression of EGFR in HEK293 cells or cancer cell lines (e.g., A431) leads to formation of a stable complex between EGFR and phosphorylated ERK1/2 (Habib et al., 2003). We could not detect this complex using the ERK1/2 protein antibody in Western blotting, possibly because of masking of the ERK1/2 antibody epitope in the complex (Fig. 5, middle). It is noteworthy that the observed EGFR-Luc-phospho-ERK1/2 complex formation was enhanced by treatment with EGF, particularly in the wild-type EGFR-Luc-transfected cells (Fig. 5, compare lanes 7 and 8). Furthermore, the EGFR L858R and L858R T790M mutants, which show constitutive activity in the BRET-2/Grb2 assays (Figs. 2–4), showed increased amounts of this reduced mobility phospho-ERK1/2 complex compared with wild-type EGFR in the absence of EGF (Fig. 5, top, compare lanes 3 and 5 with lane 7). For the double mutant L858R T790M, the amount of this complex could not be further enhanced with EGF (Fig. 5, lanes 3 and 4), consistent with the maximal constitutive activation observed with this mutant in the BRET-2/Grb2 assay (Fig. 3E). Our results are consistent with a recent report demonstrating increased MAP kinase signaling by showing constitutive phosphorylation of the adapter protein Shc in cells expressing EGFR L858R (Greulich et al., 2005). In contrast Sordella et al. (2004) showed that the somatic EGFR mutation L858R does not affect ERK1/2 phosphorylation despite an increase in autophosphorylation of tyrosine residue 1068, which is involved in Grb2 binding (Sordella et al., 2004). Overall, the observed activation of downstream MAP kinase signaling events in the form of an activated EGFR-ERK1/2 complex correlates well with the pharmacological data collected with our BRET-2 assays. Overexpression of EGFR is commonly observed in various cancer types and is involved in cell transformation and cancer progression (Pao and Miller, 2005). Understanding the pharmacology and signaling properties of overexpressed mutated EGFR isoforms might help to obtain a better prognosis about the disease outcome and improve the decision about treatment options.

Discussion

We developed a quantitative BRET-2 assay that allowed us to effectively study the pharmacology and signaling properties of somatic mutations in EGFR. Although our EGFR BRET assays are based on the heterologous overexpression of luciferase-tagged EGFR isoforms as a luminescence donor and GFP2-tagged adapter proteins as fluorescence acceptors (e.g., GFP2-Grb2) in HEK293T cells, they proved to be valuable in assessing the pharmacological properties of EGFR agonists and antagonists and in determining the level of constitutive activity. It is possible that tagging EGFR and the signaling proteins alters their protein conformations and influences their interactions. However, it seemed not to prevent efficient EGF-induced recruitment of the adapter proteins to EGFR in our BRET assays (Figs. 1 and 2; Table 1). It is important to note that all adapter proteins used in our BRET-2 assays were full-length protein moieties. It is noteworthy that despite the use of tagged proteins in the EGFR Grb2/BRET-2 assay, we were able to detect downstream

modulation of ERK1/2 protein kinases in the MAP kinase pathway (Fig. 5). The level of constitutive activity from different mutant EGFR isoforms was mirrored in the levels of ERK1/2 phosphorylation (Fig. 5). In this specific case, overexpression of luciferase-tagged EGFR also caused a ligand-induced interaction of phosphorylated ERK1/2 kinases with EGFR-Luc (Fig. 5), which has been previously observed in cancer cell lines (Habib et al., 2003). Therefore, our EGFR BRET-2/Grb2 assays let us study both EGFR pharmacology and EGFR-ERK1/2 complex formation side by side in one system and will facilitate research to understand the role of this complex in cancer. The results from our BRET assays are consistent with published data from other studies, suggesting that monitoring the recruitment of EGFR signaling proteins by BRET is a useful tool to analyze EGFR signaling.

Besides the demonstration of EGFR BRET assays as a pharmacological tool, our results reveal important new insights into the pharmacology and signaling properties of somatic EGFR mutations in lung cancer. As previously reported, our results also show that somatic mutations in EGFR are sufficient to increase constitutive activity and enhance the sensitivity to the EGFR inhibitors Iressa or Tarceva. However, in contrast to recent studies, which detected constitutive activity of mutated EGFR isoforms in growth or foci formation assays (Greulich et al., 2005), we were able to analyze constitutive EGFR activity in selected signaling pathways. In particular, our BRET assays detected a preferentially constitutive activation of the PI3K/Akt-survival pathway for all mutant EGFR isoforms tested. These results are consistent with the previously reported strong activation of the PI3K pathway in cancer cell lines, which express these mutated EGFR isoforms (Pao and Miller, 2005). However, these earlier studies could not distinguish between ligand dependent and independent activation. It is noteworthy that we show that the level of constitutive EGFR activity and the response of EGFR isoforms to EGF are differentially affected by these mutations. The impairment of EGF responsiveness of mutated EGFR isoforms has previously not been recognized. In particular, the deletion mutations (e.g., $\Delta 752-759$) seem to weaken the EGF responsiveness of the MAP kinase signaling pathway more severely than somatic mutations that only change single amino acids (e.g., L858R). The severe loss of EGF responsiveness in the deletion mutants might interfere with the function of the autocrine EGF-EGFR loop that is known to contribute to cancer cell growth and maturation (Tateishi et al., 1990). It is noteworthy that the $\Delta 752-759$ mutant showed only a partial impairment in EGF responsiveness in the STAT pathway, in contrast to results obtained for the MAP kinase pathway. This disparity may be caused by a structural change in the tyrosine kinase domain, which differentially affects the EGF-stimulated autophosphorylation at specific tyrosine residues and hence differentially affects the recruitment of Grb2 and Stat5A to the EGF-activated receptors. Thus, somatic EGFR mutations in lung cancer cannot be viewed as identical with respect to their EGF responsiveness. This finding could have important clinical implications for disease prognosis and drug response of individual NSCLC patients.

It is currently unclear why the EGFR inhibitors Iressa and Tarceva, which are believed to share the same mechanism of action, show differences in the clinical efficacy affecting overall survival in NSCLC (Tyagi, 2005). We extensively com-

pared the pharmacology for both drugs acting on the wild-type and mutant EGFR isoforms in all four major EGFR signaling pathways using the EGFR BRET assays. No comprehensive data set has previously been reported that compared the pharmacology of Iressa and Tarceva acting on several mutated EGFR isoforms and the main EGFR signaling pathways. Both drugs showed very similar pharmacological properties, except that Tarceva is slightly more potent than Iressa in inhibiting EGFR signaling pathways (Table 1). The effective steady-state plasma concentrations reported for Iressa (0.4–1.4 μM) and Tarceva (3 μM) reached in the clinic (Hidalgo et al., 2001; Baselga et al., 2002) are significantly higher than required to inhibit EGF-stimulated EGFR signaling in vitro BRET-2 assays. The results from our BRET-2 assays imply that both drugs would saturate mutant EGFR isoforms and inhibit their constitutive activity during treatment if these concentrations were reached in tumors. It is noteworthy that skin rash and gastrointestinal side effects occur more commonly with Tarceva than with Iressa. The development of skin rash is dose-dependent and seems to be correlated to the clinical response and survival, thus making rash a potential surrogate marker of activity (Perez-Soler et al., 2005). Therefore, it might be possible that clinical doses of Iressa do not always saturate EGFR in the skin and maybe also in the tumor tissue to explain the difference in clinical efficacy of both drugs.

Acquisition of resistance to the treatment with Iressa or Tarceva has been observed in the clinical treatment of NSCLC. In some patients, the occurrence of resistance has been correlated with the presence of the secondary resistance mutation T790M in EGFR. The T790M mutation has recently also been found in the germline of lung cancer families (Bell et al., 2005). Structural models of the EGFR kinase domain bearing the T790M mutation predict a steric hindrance for Tarceva or Iressa binding to the ATP-binding site (Kobayashi et al., 2005). We analyzed the pharmacological properties of the T790M mutation alone and the T790M mutation in combination with primary activating mutations with our EGFR BRET-2 assays. Our data reveal high levels of constitutive activity and EGF responsiveness for the mutant EGFR isoform T790M (T790M in Fig. 3B, open bar). Carrying this mutation as the only EGFR mutation in the germline might be sufficient to promote lung cancer, which would explain the cosegregation of lung cancer and the presence of this mutation in an extended family (Bell et al., 2005). The occurrence of the T790M mutation in the background of activated mutant EGFR isoforms (e.g., L858R or $\Delta 747\text{--}749$ A750P) has been correlated with the development of drug resistance to Iressa or Tarceva and the recurrence of tumor tissue in NSCLC. Both double mutant EGFR isoforms: L858R T790M and $\Delta 747\text{--}749$ A750P T790M are highly constitutively active and do not respond to EGF (Fig. 3E). In particular, the constitutive activity of EGFR L858R T790M reached the level of a fully EGF-stimulated wild-type EGFR. We speculate that cells expressing EGFR L858R T790M might be very aggressive cancer cells, which are drug-resistant and less dependent on EGF, and these cells therefore might give rise to a less favorable disease prognosis during relapse. The development of new EGFR inhibitors that efficiently target the T790M-containing receptor isoforms might facilitate the development of new

effective drugs that will reduce the risk of drug resistance and relapse. It might be important to administrate these drugs already during treatment of the primary tumor, before a potential relapse occurs. It is important to note, that the IC_{50} of inhibiting the double mutant EGFRs in our in vitro cell based BRET-2 assay is similar to the effective concentrations of Tarceva and Iressa reported in the human plasma of treated NSCLC patients (Hidalgo et al., 2001; Baselga et al., 2002). Therefore, small differences in the plasma concentrations of Iressa or Tarceva could cause significant changes in their efficacy to inhibit the EGFR PI3K/Akt pathway in the double mutants. The lack of complete inhibition might explain the acquisition of drug-resistant cells. Large differences in human plasma concentrations for Iressa or Tarceva have been reported between different NSCLC patients (Hidalgo et al., 2001; Baselga et al., 2002). However, occurrence of the T790M mutation in activated cancer cell lines reflects only one of several mechanisms of acquiring drug resistance (Dean et al., 2005). It is completely unknown when and how the T790M mutation arises.

The results from our study demonstrate that EGFR BRET assays are a powerful tool to study pharmacology and signaling properties of EGFR. Due to the similar mechanism of activation and signal-transduction between EGFR and other RTK's, the BRET technology should be adaptable to study pharmacology and signaling properties of the whole RTK family.

Acknowledgments

We thank Andria Lee Del Tredici for critical reading of the manuscript.

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