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Monitoring Interactions between Receptor Tyrosine Kinases and Their Downstream Effector Proteins in Living Cells Using Bioluminescence Resonance Energy Transfer[S]

Philip K. Tan, Jean Wang, Pey-Lih H. Littler, Kenneth K. Wong, Timothy A. Sweetnam, William Keefe, Norman R. Nash, Esther C. Reding, Fabrice Piu, Mark R. Brann, and Hans H. Schiffer

ACADIA Pharmaceuticals, Inc., San Diego, California

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ABSTRACT

A limited number of whole-cell assays allow monitoring of receptor tyrosine kinase (RTK) activity in a signaling pathway-specific manner. We present the general use of the bioluminescence resonance energy transfer (BRET) technology to quantitatively study the pharmacology and signaling properties of the receptor tyrosine kinase (RTK) superfamily. RTK BRET-2 assays monitor, in living cells, the specific interaction between RTKs and their effector proteins, which control the activation of specific downstream signaling pathways. A total of 22 BRET assays have been established for nine RTKs derived from four subfamilies [erythroblastic leukemia viral (v-erb-b) oncogene homolog (ErbB), platelet-derived growth factor (PDGF), neurotrophic tyrosine kinase receptor (TRK), vascular endothelial growth factor (VEGF)] monitoring the interactions with five effectors (Grb2, p85, Stat5a, Shc46, PLCγ1). These interactions are dependent on the RTK kinase activity and autophosphorylation of specific tyrosine residues in the carboxyl terminus. RTK BRET assays are highly sensitive for quantifying ligand-independent (constitutive), agonist-induced, or antagonist-inhibited RTK activity levels. We studied the signaling properties of the PDGF receptor, α polypeptide (PDGFRA) isoforms (V561D; D842V and Δ842–845) carrying activating mutations identified in gastrointestinal stromal tumors (GIST). All three PDGFRA isoforms are fully constitutively activated, insensitive to the growth factor PDGF-BB, but show differential sensitivity of their constitutive activity to be inhibited by the inhibitor imatinib (Gleevec). Epidermal growth factor receptor (EGFR) BRET structure-function studies identify the tyrosine residues 1068, 1114, and 1148 as the main residues mediating the interaction of EGFR with the adapter protein Grb2. The BRET technology provides an assay platform to study signaling pathway-specific RTK structure-function and will facilitate drug discovery efforts for the identification of novel RTK modulators.

Receptor tyrosine kinases (RTKs) represent a broad class of cell surface receptors that transduce signals across the cell membrane and regulate cell proliferation, survival, differentiation and migration (Schlessinger, 2000). Activation or overexpression of most of the known RTKs has been linked to some form of cancer (Sawyer et al., 2003; Krause and Van Etten, 2005). Although the RTKs are prime targets for treatment of cancer, only a small number of therapeutics has been identified despite massive drug discovery efforts. Many novel cancer drugs show only a limited response rate and cannot be applied to treat a wide spectrum of cancer types (Sawyers, 2004; Pao and Miller, 2005). One possible reason for these outcomes has been that the majority of methods used to identify kinase inhibitors are biochemical tyrosine kinase

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ABBREVIATIONS: RTK, receptor tyrosine kinase; BRET, bioluminescence resonance energy transfer; PI3K, phosphatidyl inositol 3-kinase; PLCγ1, phospholipase Cγ1; PKC, protein kinase C; STAT, signal transducer and activator of transcription; Luc, luciferase; GFP, green fluorescence protein; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; EGFR, epidermal growth factor receptor; PDGFRA, platelet-derived growth factor receptor, α polypeptide; PBS, phosphate-buffered saline; BDNF, brain-derived neurotrophic factor; HEK, human embryonic kidney; GPCR, G protein-coupled receptor; FPRL1, formyl peptide receptor-like 1; VEGF, vascular endothelial growth factor; GIST, gastrointestinal stromal tumors; erlotinib, 4-(3-ethynylphenylamino)-6,7-bis(2-methoxyethoxy)quinazoline hydrochloride; imatinib, 4-(4-methylpiperazin-1-ylmethyl)-*N*-[4-methyl-3-[4-(3-pyridyl) pyrimidin-2-ylamino]phenyl]benzamide methanesulfonate; K252a, (+)-10(*R*)-hydroxy-9(*S*)-methyl-1-oxo-9,12(*R*)-epoxy-2,3,9,10,11,12-hexahydro-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid methyl ester; AG1478, *N*-(3-chlorophenyl)-N-(6,7-dimethoxyquinazolin-4-yl) amine; PD153035, 4-(3-bromophenylamino)-6,7-dimethoxyquinazoline; PD158780, 4-(3-bromophenylamino)-6-(methylamino)pyrido[3,4-*d*] pyrimidine; PD168393, *N*-[4-(3-bromophenylamino)quinazolin-6-yl]-2-propenamide; PD174265, *N*-[4-(3-bromophenylamino)-6-quinazolinyl]propionamide.

assays (Olive, 2004; Minor, 2005). A shift toward the use of more whole-cell-based RTK assays is expected to lead to a better prediction of the clinical outcome of new drug candidates. Furthermore, cancer drugs are increasingly designed to target specific cell-signaling pathways, because cancer types show signaling pathway-specific deregulation signatures (Bild et al., 2006). The development of whole-cell-based RTK assays, which allow discriminating pathway specific signals, is important to facilitate this process.

We used the bioluminescence resonance energy transfer (BRET) technology (Xu et al., 1999; Angers et al., 2000; Pfleger and Eidne, 2006) and developed new whole-cell receptor tyrosine kinase assays, which enabled us to monitor in living cells the ligand-induced recruitment of downstream effector proteins to various members of the RTK superfamily. Many of the RTK-effector protein interactions depend on the autophosphorylation of specific tyrosine residues in the intracellular carboxyl terminus of an RTK. They control the assembly of larger protein complexes that are involved in building, shaping, and directing specific RTK signaling pathways (illustrated in Fig. 1a) (Schlessinger, 2000). We included in our study RTK effector proteins from different signaling pathways: the adapter proteins Grb2 and Shc46 in the Ras/mitogen-activated protein kinase pathway; p85, the regulatory subunit of phosphatidyl inositol 3-kinase (PI3K) in the PI3K/Akt pathway; phospholipase Cγ1 (PLCγ1) in the PLCγ1/PKC pathway; and the Stat5a protein in the STAT pathways. The central role of protein-protein interactions for RTK activation and signaling makes the BRET technology a method of choice to study RTK function in living cells in a signaling pathway-specific modus. RTK BRET-2 assays are highly sensitive and precisely dissect and quantify the pharmacological responses and signaling properties of RTKs. Earlier BRET studies analyzed the interactions of the insulin receptor with the insulin receptor substrate-1 (Blanquart et al., 2006), protein tyrosine phosphatase-1B (Blanquart et al., 2005), or the adapter protein Grb14 (Nouaille et al., 2006a,b). We demonstrate here that the BRET technology is universally applicable to the entire RTK superfamily and discuss the advantages of this technology compared with other methods that measure RTK activity.

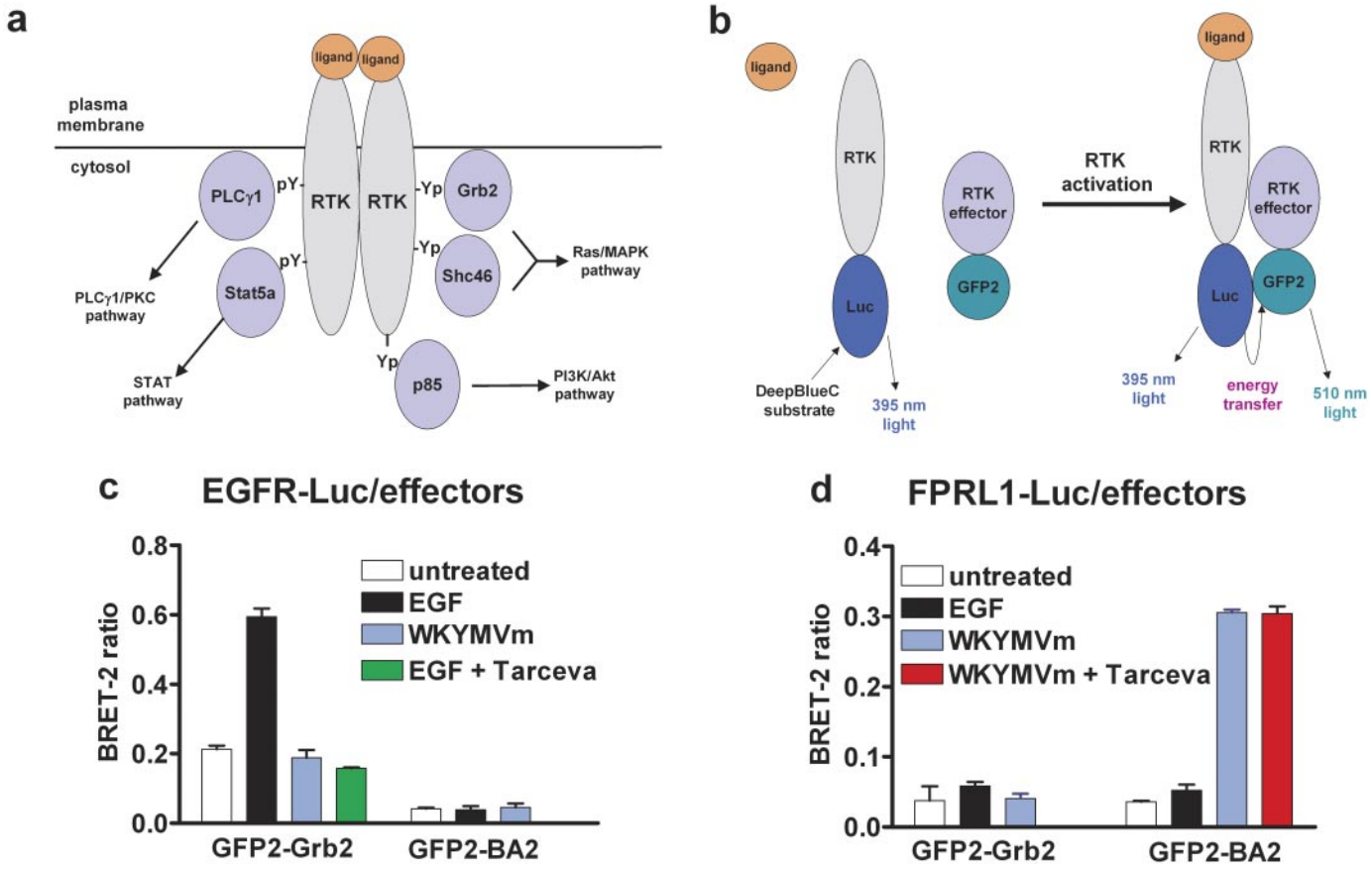

Fig. 1. The RTK whole-cell BRET-2 assay. a, illustration of RTK activation and signaling. Ligand-induced activation of RTK led to autophosphorylation of intracellular tyrosine residues, recruitment of effector proteins (e.g., Shc46, Stat5a, p85, Grb2, PLCγ1), and activation of downstream signaling pathways. b, measuring RTK activation in living cells using the BRET-2 technology. *R. reniformis* luciferase-tagged RTK-Luc (bioluminescence donor) and GFP2-tagged effector proteins (fluorescence acceptor) are transiently coexpressed in HEK293T cells. Activation of luciferase with membrane permeable substrate coelenterazine 400A (DeepBlueC), but without RTK activation, result primarily in blue light emission (395 nm). In contrast, RTK activation brings the RTK and the effector protein in close proximity so that activation of luciferase leads to energy transfer to GFP2, causing excitation of GFP2 and additional emission of green light (510 nm) by GFP2. The increase of the BRET-2 signal is measured as an increase in the ratio of green and blue light and correlates with increased RTK effector interactions. c and d, effector and ligand specificity of RTK BRET-2 assay. HEK293T cells were transiently cotransfected with luciferase-tagged RTK EGFR (EGFR-Luc, c) or with the luciferase-tagged GPCR FPRL1 (FPRL1-Luc, d) along with either GFP2-tagged RTK effector Grb2 (GFP2-Grb2) or GFP2-tagged GPCR effector β-arrestin-2 (GFP2-BA2). Transfected cells were incubated for 10 min with 16.7 nM EGF, an EGFR agonist (black bars), 0.33 μM WKYMVm, an FPRL1 agonist (blue bars), 16.7 nM EGF in the presence of 3.3 μM erlotinib, an EGFR kinase inhibitor (green bars), or 0.33 μM WKYMVm in the presence of 3.3 μM erlotinib. Open bars indicated no-ligand controls. BRET-2 measurements were performed and analyzed as described under *Materials and Methods*.

Materials and Methods

Cell Culture and Transfection. HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and 1× penicillin-streptomycin-L-glutamine solution (HyClone Laboratories Inc., Logan, UT) at 37°C and 5% CO_2. Cells grown in 10-cm^2 dishes were transfected at 80 to 90% confluence using the calcium phosphate DNA precipitation method (Jordan et al., 1996). The following DNA amounts were used for the RTK-BRET-2 assays: 1 μg of RTK-Luc DNA and 20 μg of GFP2-effector DNA, except where noted. The GPCR FPRL1 BRET-2 assay was performed by cotransfecting 1 μg of FPRL1-Luc DNA and 20 μg of GFP2-BA2 DNA. The ratio of 1:20 was predetermined in saturation experiments to be optimal for obtaining the best ligand induced increase in the BRET-2 signals. These DNAs in water were mixed with 80 μl of 2.5 M $CaCl_2$ and 1.7 ml of 2× HBS (50 mM HEPES, pH 7.05, 280 mM NaCl, and 1.5 mM Na_2HPO_4) and incubated at room temperature for 3 to 5 min. The media was removed from the cells, and 10 ml of fresh media was added to the transfection mixture, which was then transferred onto the cells. After 2 to 4 h of incubation at 37°C, the medium was replaced with 10 ml of fresh media. On the next day, the cells were serum starved by replacing the media with 10 ml of Dulbecco's modified Eagle's medium supplemented with 0.1% fetal bovine serum and 1× penicillin-streptomycin-L-glutamine for 16 to 20 h before harvesting.

Plasmids. BRET-2 vectors expressing *Renilla reniformis* luciferase (pRluc-N) and green fluorescent protein 2 (pGFP2-N and pGFP2-C) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Human cDNAs encoding RTKs or RTK effector proteins were obtained by standard reverse transcription-polymerase chain reaction on poly-A-RNA isolated from various human tissues or human cell lines. Genes were amplified without a stop codon when appropriate and subcloned in frame into the BRET-2 vectors. For expressing the amino- or carboxyl-terminally GFP2-tagged tandem SH2 domains as fusion protein SH2(PLCγ1)-GFP2 or GFP2-SH2(PLCγ1), the bases encoding amino acids 507 to 790 of human PLCγ1 were polymerase chain reaction-amplified from GFP2-PLCγ1 cDNA and inserted into pGFP2-N and pGFP2-C. Epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor, α polypeptide (PDGFRA) mutants were produced by standard site-directed mutagenesis methods from EGFR-Luc and PDGFRA-Luc, respectively. Accuracy of the sequences of all constructs used in this study has been confirmed.

RTK BRET-2 Measurement. Transfected cells were rinsed once with Dulbecco's PBS, detached with Dulbecco's PBS containing 5 mM EDTA, and resuspended at 4 to 8 × 10^6 cells/ml in BRET buffer (PBS containing 0.1% D-glucose and 1 mM sodium pyruvate). All ligand serial dilutions were prepared in BRET buffer containing 0.1% BSA. For agonist assays, 50 μl of 3-fold concentrated ligand dilutions were dispensed into wells of white, flat-bottomed, 96-well plates (Costar; Corning Life Sciences, Acton, MA). For antagonist assays, 25 μl of 6-fold concentrated agonist and 25 μl of 6-fold concentrated antagonist were added together per well. Ligands were incubated for 10 to 20 min with 50 μl of cell suspension to stimulate the interaction of RTK-Luc (bioluminescence donor) with GFP2-tagged downstream effector (fluorescence acceptor). The BRET-2 signal was detected directly after injecting 50 μl of 15 μM coelenterazine 400A (DeepBlueC; PerkinElmer Life and Analytical Sciences) diluted in PBS per well using the POLARstar OPTIMA plate reader (BMG Labtech GmbH, Offenburg, Germany) or the Mithras LB 940 plate reader (Berthold Technologies, Bad Wildbad, Germany). After 1 s of plate-shaking, luminescence emissions for *R. reniformis* luciferase and GFP2 were recorded through BRET-optimized filters (luciferase peakm 410 nm; GFP2 peak, 515 nm) for 1 to 2 s in well mode. The time from adding coelenterazine 400A to the plate well until the reading start was sufficient to fully activate luciferase (data not shown). The BRET-2 signal was calculated as the ratio between the luciferase and the GFP2 emission corrected by the background emission of cells transfected with RTK-Luc alone. Nonlinear regression analysis was performed with the software Prism (GraphPad Software Inc., San Diego, CA) to obtain dose response curves and IC_{50}/EC_{50} values. Throughout the text, EC_{50} and IC_{50} values are expressed as pEC_{50} or pIC_{50} [molar] values, which are calculated as $-\log_{10}$ of the EC_{50} or IC_{50} [molar]. Experiments were repeated two to three times with each data point, performed in triplicate, and expressed as mean ± S.E.M. The ligands EGF, heregulin-β1, platelet-derived growth factor BB (PDGF-BB), and brain-derived neurotrophic factor (BDNF) were purchased from Peprotech (Rocky Hill, NJ), VEGF-C was purchased from R&D Systems (Minneapolis, MN), and WKYMVm was purchased from Tocris Cookson Inc. (Ellisville, MO). Erlotinib (Tarceva) and imatinib (Gleevec) were synthesized by ACADIA Pharmaceuticals, Inc. (San Diego, CA).

Immunoblotting. Transfected cells in BRET buffer were incubated without or with EGF for 10 min and then lysed by adding a 10-fold volume of protein sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.005% bromphenol blue, 5% β-mercaptoethanol, and 1 mM sodium orthovanadate). Lysates were electrophoresed through 10% polyacrylamide gels and transferred to nitrocellulose for Western blotting. Luciferase- or GFP2-tagged fusion proteins were detected using monoclonal luciferase antibody 4410 (Chemicon, Temecula, CA) or polyclonal GFP antibody (Cell Signaling Technology, Danvers, MA). Proteins carrying phosphotyrosine residues were detected using the monoclonal antibody 4G10 (Upstate, Charlottesville, VA). Horseradish peroxidase-conjugated secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) were used for developing Western blots.

Results

The RTK BRET Assay. The BRET technology was applied to monitor ligand-induced changes in RTK-effector interactions in eukaryotic cells (e.g., HEK293T) by transiently coexpressing two fusion proteins: the RTK of interest carboxyl-terminally tagged with *R. reniformis* luciferase (RTK-Luc; bioluminescence donor) and a full-length RTK effector protein amino- or carboxyl-terminally tagged with green fluorescence protein 2 (GFP2-effector; fluorescence acceptor) (illustrated in Fig. 1b). Activation of receptors by incubation of the transfected cells with appropriate RTK ligands, leads to recruitment of GFP2-effectors to RTK-Luc fusion proteins. These interactions between RTKs and effector fusion proteins are indirectly quantified by measuring the luciferase (emission peak at 395 nm) and GFP2 (emission peak at 510 nm) light emissions after activation of *R. reniformis* luciferase with the membrane permeable luciferase substrate coelenterazine 400A (DeepBlueC). The GFP2 emission is due exclusively to energy transfer between activated luciferase and GFP2 and strictly depends on the proximity (<100 Å) and orientation of both proteins. The size of the BRET-2 signal is expressed as the ratio between GFP2 and luciferase emissions (see *Materials and Methods*), which correlates with the extent of recruitment of the effector protein to the RTK and therefore reflects RTK activation. It is noteworthy that the RTK BRET-2 signal is strongly affected by the expression level of each fusion protein and requires initial control experiments to determine the optimal transfection conditions (Supplemental Fig. 1, online).

First, we applied the BRET technology to study the interaction between the most extensively studied RTK, the EGFR, and the effector protein Grb2 (Fig. 1c). EGFR-Luc and amino-terminal GFP2-tagged Grb2 (GFP2-Grb2) were transiently coexpressed in HEK293T cells. After a 10-min incubation of

the cells with 16.7 nM EGF, we detected a 3-fold increase in the BRET-2 ratio from 0.21 ± 0.02 to 0.59 ± 0.04, indicating EGFR activation and recruitment of GFP2-Grb2 (Fig. 1c). Cotreatment of EGF with 3.3 μM erlotinib, an EGFR inhibitor, completely reversed the EGF-induced increase in the BRET-2 ratio (Fig. 1c). Furthermore, these cells yielded a BRET-2 ratio (0.16 ± 0.005) slightly lower than that obtained for untreated cells (0.21 ± 0.02), suggesting a low level of constitutive EGFR activity in untreated cells (discussed below). Similar results were also obtained for the EGFR tyrosine kinase inhibitors AG1478, PD153035, PD158780, PD168393, and PD174265 (data not shown).

The RTK BRET-2 assay responses are based on specific ligand-induced RTK effector interactions. To demonstrate ligand specificity, we tested the peptide WKYMVm, an agonist for the G protein-coupled receptor (GPCR) FPRL1 (formyl peptide receptor-like 1) in the EGFR/Grb2 BRET-2 assay (Fig. 1c). WKYMVm did not produce a response in the EGFR/Grb2 BRET-2 assay but did stimulate a 7-fold response in the FPRL1/BA2 BRET-2 assay (no ligand, 0.04 ± 0.002; WKYMVm, 0.30 ± 0.07), which is monitoring the interaction between the luciferase-tagged FPRL1 and GFP2-tagged β-arrestin-2 (BA2) protein (Fig. 1d). As expected, neither EGF nor erlotinib affected the WKYMVm-induced BRET-2 ratio in the FPRL1/BA2 BRET-2 assay (Fig. 1d). To demonstrate that the observed ligand-induced BRET-2 responses were based on specific protein interactions, we coexpressed EGFR-Luc with GFP2-BA2, or FPRL1-Luc with GFP2-Grb2, and stimulated the cells with EGF or WKYMVm, respectively. Neither ligand induced a response in these BRET assays, because the coexpressed receptors and effectors do not specifically interact in vivo (Fig. 1, c and d).

We next tested other EGFR effectors in EGFR BRET-2 assays and quantitatively studied the pharmacological properties of EGFR. The GFP2-tagged effector proteins Grb2, Shc46, p85, PLCγ1, and Stat5a were individually coexpressed with EGFR-Luc in HEK293T cells, and their BRET-2 responses were detected after incubation of these cells with variable EGF concentrations. We observed a dose-dependent increase in the BRET-2 signal in all EGFR BRET-2 assays that was efficiently inhibited with the EGFR inhibitor erlotinib in a dose-dependent manner (Fig. 2, a–e, and Table 1). Our results show that the different EGFR BRET-2 assays are highly sensitive in detecting responses to the native EGFR agonist EGF, with EC_{50} values ranging from 30 to 80 pM (summarized in Table 1). It is noteworthy that EGFR-Luc showed significant levels of constitutive activity in the interaction with the downstream effectors Grb2, Shc46, and p85, as indicated by the significantly higher baselines before er-

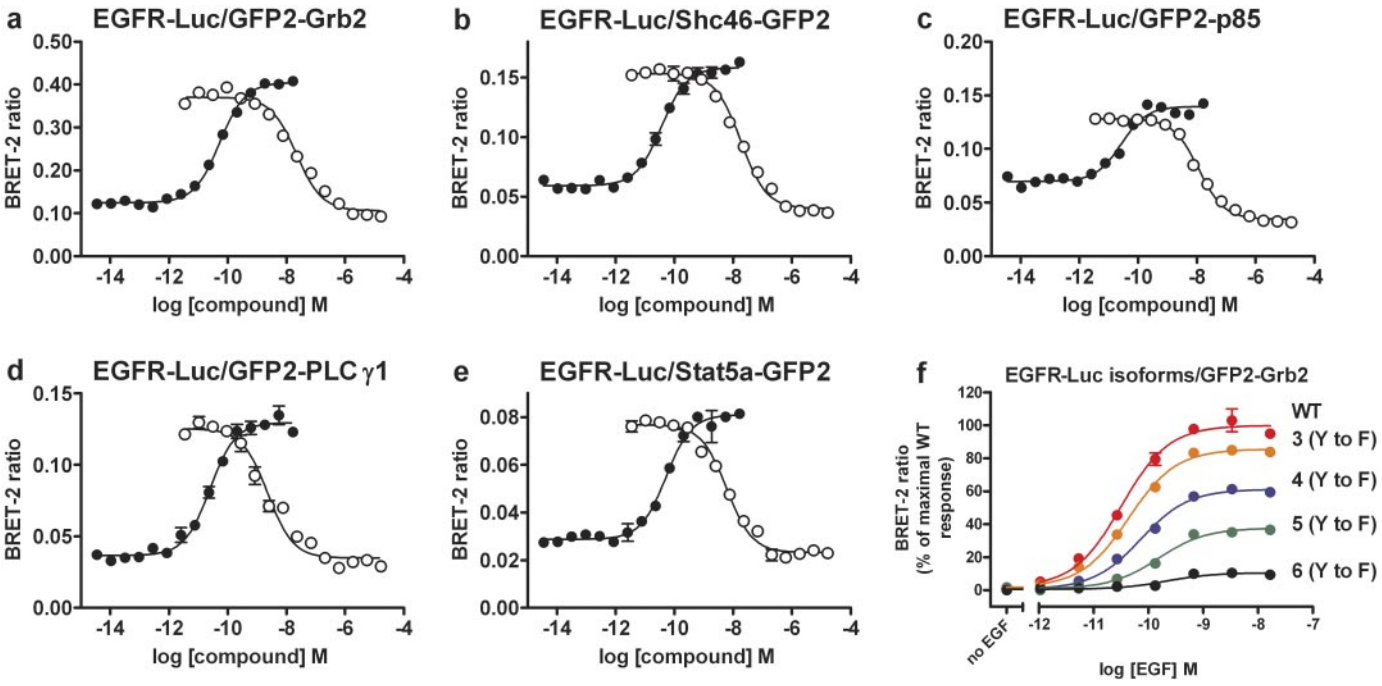

Fig. 2. Signaling pathway-specific EGFR BRET-2 responses are dependent on tyrosine phosphorylation. a–e, dose response curves for agonist EGF-stimulated responses in EGFR BRET-2 assays testing downstream effector Grb2, Shc46 (MAP kinase pathway), p85 (PI3K/Akt pathway), PLCγ1 (PLCγ1/PKC pathway) and Stat5a (STAT pathways). The HEK293T cells used in these EGFR-BRET-2 assays were cotransfected with EGFR-Luc and either GFP2-Grb2 (a), Shc46-GFP2 (b), GFP2-p85 (c), GFP2-PLCγ1 (d), or STAT5A- GFP2 (e). In agonist assays, EGFR BRET-2 dose-responses are stimulated by incubating the cells for 10 min with different amounts of EGF (●). The EGFR tyrosine kinase inhibitor erlotinib efficiently inhibited the observed EGF-stimulated BRET-2 responses (○; a–e). In these antagonist assays, cells were incubated for 10 min in the presence of 16.7 nM EGF and increasing concentrations of erlotinib. We observed constitutive wild-type EGFR activity mainly in the EGFR Grb2/shc/p85 BRET-2 assays (previously confirmed by Schiffer et al., 2007), indicated by a significant difference between the BRET-2 signal of unstimulated cells and the BRET-2 signals of the same cells after treatment with a high dose of EGF in the presence of the EGFR inhibitor erlotinib (a–c). EGFR BRET-2 responses are dependent on phosphorylation of specific tyrosine residues in the intracellular EGFR carboxyl terminus. Site-directed mutagenesis (Tyr to Phe) was performed on EGFR tyrosines 1068, 1086, 1101, 1114, 1148, and 1173, which are directly or indirectly involved in interaction with effector Grb2. Wild-type EGFR-Luc or EGFR-Luc isoforms were coexpressed with GFP2-Grb2 in HEK293T cells and tested in the EGFR/Grb2 BRET-2 assay (f). f, dose-response curves for the EGFR agonist EGF (10-min incubations) are shown normalized to the maximum of the wild-type EGFR response. EGFR isoform 3 (Tyr to Phe) contains mutations Y1086F, Y1101F, and Y1173F; 4 (Tyr to Phe) contains mutations Y1086F, Y1101F, Y1114F, and Y1173F; 5 (Tyr to Phe) contains mutations Y1068F, Y1086F, Y1101F, Y1114F, and Y1173F; and 6 (Tyr to Phe) contains mutations Y1068F, Y1086F, Y1101F, Y1114F, Y1148F, and Y1173F.

lotinib inhibition (Fig. 2, a–c). We previously confirmed that these differences are due to variable levels of constitutive wild-type EGFR activity in the different EGFR signaling transduction pathways (Schiffer et al., 2007). Our results presented in Fig. 2, d and e, suggest that there might be also a low level of constitutive EGFR activity in the Stat5a and PLCγ1/PKC pathways, but this has not been further explored or confirmed. Each of the presented EGFR BRET-2 assays monitors only one specific receptor-protein interaction that is involved in activating/modulating one specific downstream signaling pathway. Thus, these EGFR BRET-2 assays represent signaling pathway-specific, whole-cell based assays.

RTK BRET-2 Assays Are Dependent on Autophosphorylation of Specific Tyrosine Residues. Phosphorylated tyrosine residues localized in the intracellular carboxyl terminus of EGFR (Heldin, 1995) and specific phosphotyrosine binding or SH2 domains in the effector proteins (Schlessinger and Lemmon, 2003) mediate all the EGFR effector interactions we studied in Fig. 2. EGFR tyrosines 1068, 1086, 1101, 1114, 1148, and 1173 are involved in direct or indirect binding of the effector Grb2 (Schulze et al., 2005). We mutated these tyrosine residues to phenylalanine to verify that the EGFR/Grb2 BRET-2 signal is dependent on their phosphorylation. Introducing all six Tyr-to-Phe alterations into EGFR-Luc abolished the EGF-induced BRET-2/Grb2 response by 90 ± 0.9% compared with wild-type EGFR-Luc (Fig. 2f). We observed 66 ± 0.9% and 42 ± 1.0% impairment of the BRET-2/Grb2 responses for EGFR-Luc isoforms carrying five (Y1068F, Y1086F, Y1101F, Y1114F, Y1173F) or four (Y1086F, Y1101F, Y1114F, Y1173F) of the six Tyr-to-Phe changes, respectively (Fig. 2f). Three Tyr-to-Phe changes (Y1086F, Y1101F, Y1173F) caused only a 16 ± 1.1% inhibition of the BRET-2/Grb2 response (Fig. 2f). In contrast to the results from the BRET-2/Grb2 assays, abolishing phosphorylation at the six tyrosine residues only partially affected EGF-induced responses in the EGFR BRET-2/p85/STAT5a or /PLCγ1 assays (data not shown). Consistent with our results, a kinase-deficient EGFR-Luc isoform carrying the kinase domain mutation K721M completely abolished all BRET-2 responses with the effector proteins tested (data not shown). Finally, we performed EGFR BRET-2 assays using the two tandem repeat SH2 domains of PLCγ1 as the effector and found that the phospho-tyrosine binding domains of the effector PLCγ1 were sufficient for generating an EGFR BRET-2 response (Supplemental Fig. 2). Our experiments show that the EGFR BRET-2 assays are mediated by interactions between specific autophosphorylated tyrosine residues on activated EGFR and specific phosphotyrosine binding domains in RTK effectors. Furthermore, we demonstrated the sensitivity, reproducibility, and robustness of the RTK BRET-2 assay, which makes it an ideal tool to detect small functional differences in structure-function studies. It has been reported that the kinase domain of activated RTKs also *trans*-phosphorylates tyrosines on recruited effectors such as Grb2 and PLCγ1 (Schlessinger, 2000). The results from our EGFR-BRET-2 assays, therefore, suggest that tyrosine phosphorylation in the GFP2-tagged effectors should increase upon ligand treatment. Western blotting of lysates from EGFR BRET-2 assay cells confirmed this prediction (Supplemental Fig. 3).

Enabling the RTK Superfamily in BRET-2 Assays. Fifty-eight human RTKs have been described in the human genome (Robinson et al., 2000). We tested whether the RTK BRET-2 assay was applicable to study the pharmacological and signaling properties of RTKs other than EGFR. Indeed, we were able to measure RTK activation of other members of the RTK superfamily in BRET-2 assays (Fig. 3, a–d). Heregulin-β1 stimulated GFP2-Grb2 recruitment to ErbB4-Luc, another member of the EGFR family of growth factor receptors (Fig. 3a). PDGF-BB stimulated a dose-dependent increase of the BRET-2 ratio in a platelet-derived growth factor receptor, β polypeptide BRET-2/Grb2 assay (Fig. 3b). BDNF stimulated the neurotrophic tyrosine kinase receptor (Trk) family member TrkB in a TrkB/Shc46 BRET-2 assay (Fig. 3c). VEGF-C activated VEGF receptor 3 in a BRET-2/Grb2 assay (Fig. 3d). We enabled additional RTKs from these subfamilies

TABLE 1
RTK pharmacology in BRET-2 assays

RTK	Effector	Agonist	pEC50 [M]	Antagonist	pIC50 [M]
EGFR	Grb2	EGF	10.52 ± 0.10	Erlotinib	7.89 ± 0.04
	p85	EGF	10.46 ± 0.19	Erlotinib	7.96 ± 0.04
	Shc46	EGF	10.30 ± 0.23	Erlotinib	7.75 ± 0.06
	PLCγ1	EGF	10.42 ± 0.31	Erlotinib	8.56 ± 0.11
	Stat5a	EGF	10.09 ± 0.26	Erlotinib	8.12 ± 0.09
ErbB4	Grb2	HRG-β1	9.67 ± 0.09	N.P.	N.P.
	p85	HRG-β1	9.86 ± 0.19	N.P.	N.P.
PDGFRA	Grb2	PDGF-BB	9.20 ± 0.27	Imatinib	6.80 ± 0.39
	p85	PDGF-BB	9.58 ± 0.17	Imatinib	6.62 ± 0.13
	PLCγ1	PDGF-BB	9.89 ± 0.52	Imatinib	6.18 ± 0.12
PDGFRB	Grb2	PDGF-BB	8.93 ± 0.27	Imatinib	6.49 ± 0.35
	PLCγ1	PDGF-BB	9.08 ± 0.30	Imatinib	5.93 ± 0.12
Kit	Grb2	SCF	9.86 ± 0.34	Imatinib	6.18 ± 0.16
	p85	SCF	9.71 ± 0.63	N.P.	N.P.
TrkA	Shc46	NGF	9.28 ± 0.24	K252a	7.36 ± 0.13
	PLCγ1	NGF	9.73 ± 0.18	K252a	7.35 ± 0.09
	p85	NGF	9.34 ± 0.33	K252a	7.19 ± 0.13
TrkB	Shc46	BDNF	9.14 ± 0.16	K252a	7.22 ± 0.07
	PLCγ1	BDNF	9.13 ± 0.26	K252a	7.45 ± 0.15
	p85	BDNF	8.53 ± 0.11	K252a	7.53 ± 0.10
TrkC	Shc46	NT-3	9.42 ± 0.29	K252a	7.74 ± 0.09
VEGFR3	Grb2	VEGF-C	7.41 ± 0.01	N.P.	N.P.

N.P., not performed; pEC_{50}/IC_{50}, $-\log EC_{50}/IC_{50}$ [M]; HRG-β1, heregulin β1; SCF, stem cell factor; NGF, nerve growth factor; NT-3, neurotrophin-3.
Results are presented as the mean ± S.E.M. from three or four separate experiments.

in RTK BRET-2 assays (Table 1). Most of the RTKs analyzed in this study did interact with multiple effector proteins, which correlated well with published results about their signal transduction (Table 1). The sensitivity of all RTK BRET-2 assays for activation by their endogenous in vivo ligands was in the nanomolar range, which is in agreement with results from other methods and reflects the high in vivo potency of these growth factors. We have also shown that tyrosine kinase inhibitors with specificity for the tested RTKs efficiently inhibit their agonist-induced BRET-2 responses (summarized in Table 1). All together, we demonstrate that the RTK BRET-2 technology can measure activity of members from four subfamilies of RTKs. Therefore, it is possible that the BRET-2 technology could be used to monitor the activity of most, if not all, known RTKs.

Detection and Quantification of Constitutive PDGF Receptor Activities in RTK-BRET-2 Assays. Next, we applied the BRET-2 assay in a structure-function study to demonstrate its sensitivity and precision in characterizing RTK activity. Several somatic activating mutations have been identified in the PDGFRA gene in a small percentage of gastrointestinal stromal tumors (GIST) are believed to participate in their pathogenesis (Heinrich et al., 2003a,b). We studied two mutations causing a nonsynonymous amino acid alterations (V561D and D842V) and a four amino acid deletion mutation Δ842–845 with PDGFRA BRET-2 assays (Fig. 3e). In the absence of the agonist PDGF-BB, all mutant PDGFRA-Luc isoforms exhibited higher BRET-2 ratios compared with wild-type PDGFRA-Luc (Fig. 3e, no ligand), which is indicative of constitutive activity in the mutant isoforms. Addition of PDGF-BB produced a dose-dependent increase of the BRET-2 ratio for the wild-type receptor, which reaches a maximum at the level of the untreated mutated PDGFRA-Luc isoforms. The BRET-2 ratios of all PDGFRA-Luc isoforms remained unchanged in the presence of PDGF-BB (Fig. 3e). These findings suggest that the mutant PDGFRA-Luc isoforms are fully constitutively activated and ligand-insensitive receptors. Although, all three mutant isoforms show similar levels of constitutive activity, they showed different sensitivities to the PDGFRA inhibitor imatinib (Gleevec). Imatinib reduced the PDGF-BB-induced BRET-2 ratio of wild-type PDGFRA-Luc with a pIC_{50} = 6.62 ± 0.12 M. The constitutive activity of the mutant isoforms V561D and Δ842–845, in the absence of PDGF-BB, was inhibited by imatinib with similar potencies (V561D pIC_{50} = 6.98 ± 0.13 M and Δ842–845 pIC_{50} = 7.15 ± 0.10 M), whereas the mutant D842V isoform was approximately 30-fold less sensitive to imatinib (D842V pIC_{50} = 5.13 ± 0.10 M)

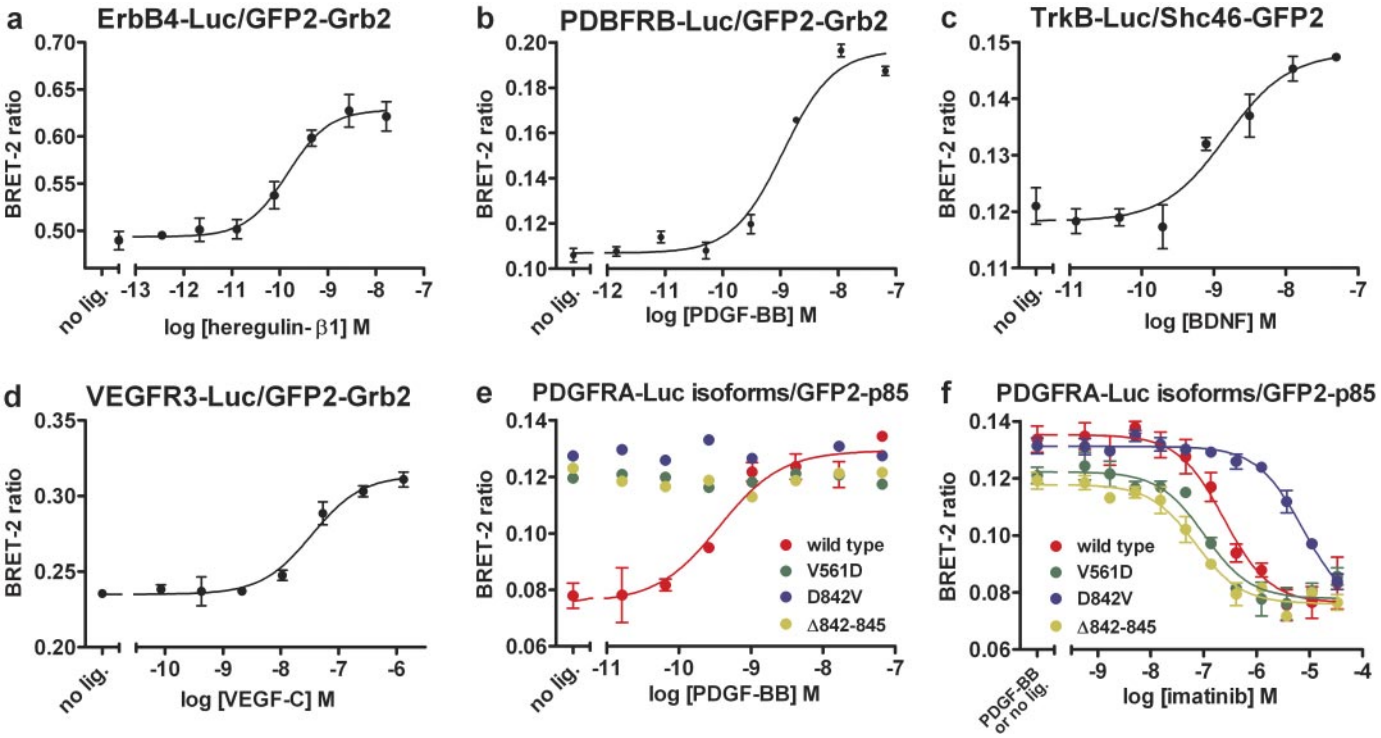

Fig. 3. Application of the BRET-2 technology to study pharmacology, signaling, and structure-function relationships in the RTK superfamily. Several RTKs from different RTK subfamilies have been tested in the BRET-2 assay. HEK293T cells are cotransfected with an RTK-Luc and a GFP2 effector. BRET-2 assay dose-response curves obtained for specific RTK agonists (10-min incubations) are shown: ErbB4-Luc + GFP2-Grb2 with ligand heregulin-β-1 (a), PDGFRB-Luc + GFP2-Grb2 with ligand PDGF-BB (b), TrkB-Luc + Shc46-GFP2 with ligand BDNF (c), and VEGF receptor 3-Luc + GFP2-Grb2 with ligand VEGF-C (d). PDGFRA BRET-2 assays reveal constitutive receptor activation and altered drug sensitivity in mutant PDGFRA identified in GIST. Wild-type PDGFRA-Luc or the mutated PDGFRA-Luc isoforms (PDGFRA V561D; PDGFRA D842V; PDGFRA Δ842–845) were cotransfected in HEK293T cells, and BRET-2 assays were performed using the GFP2-tagged downstream effector p85. In agonist assays, wild-type PDGFRA/p85 BRET-2 dose-responses are obtained by incubating the cells for 20 min with different amounts of PDGF-BB. Mutant PDGFRA isoforms are fully constitutively activated, as indicated by the dramatically increased BRET-2 signal baseline for the no-ligand control points and the lack of agonist PDGF-BB–dependent increase of the signal. e, the tyrosine kinase inhibitor imatinib efficiently inhibited the observed ligand-induced or ligand-independent (constitutive) BRET-2 responses of wild-type and mutant PDGFRA isoforms. The three mutant PDGFRA isoforms showed large differences in sensitivity to be inhibited by imatinib. f, in the wild-type PDGFRA/p85 BRET-2 antagonist assays, cells were incubated for 20 min in the presence of 16.7 nM PDGF-BB and increasing concentrations of imatinib. Increasing concentrations of imatinib in the absence of PDGF-BB inhibited constitutive activity of mutant PDGFRA isoforms.

(Fig. 3f). These results demonstrate that the RTK BRET-2 assays can be used to quantitatively characterize the signaling properties and pharmacology of constitutively active wild-type and mutant RTK isoforms, which is important for identifying the signaling pathways, principally driving a cancer pathogenesis and the design of treatment strategies.

Discussion

Our results present BRET as a powerful technology to study pharmacology and signaling properties of the RTK superfamily in living cells. We developed 22 RTK BRET assays for nine RTKs from four different subfamilies, suggesting that RTK BRET assays can be developed for most, if not all, of the 58 human RTKs. BRET-2 assays are conveniently performed in 96- and 384-well plate formats and produce GFP2 and luciferase emissions that can be easily measured using BRET-enabled multifunctional plate readers. The BRET-2 signals are calculated as a ratio between both reporter emissions, which eliminates assay variation as a result of different numbers of cells per well and facilitates automation and integration of the BRET assays into high-throughput screening formats (data not shown). RTK BRET assays are highly sensitive in detecting constitutive or ligand-induced receptor activity (Figs. 2 and 3) and deliver precise, quantitative pharmacological data for the study of agonist or antagonists. It is noteworthy that in contrast to many other methods, BRET-2 assays evaluate the pharmacological properties of ligands closer to a steady-state equilibrium for ligand receptor interactions. However, changing the experimental design of the RTK BRET-2 assays also allows monitoring of the kinetic properties of these interactions in real time (data not shown). RTK BRET assays preserve typical RTK activation and signaling properties, despite the fact that the receptors or effector proteins are tagged with luciferase or GFP2, respectively. For example, we demonstrated in the EGFR/Grb2 BRET-2 assay that Grb2 recruitment to activated EGFR was dependent on EGFR kinase activity and autophosphorylation of specific EGFR tyrosine residues and that activation led to downstream phosphorylation of EGFR effector proteins (Supplemental Fig. 3). It is noteworthy that in contrast to most RTK assays in use today, each RTK BRET-2 assay measures effector-specific receptor activity. This unique feature allows establishment and comparison of multiple signaling pathway-specific assays for one receptor, each studying a different effector RTK interaction. For example, we established and compared BRET-2 assays for five different EGFR effector interactions, which play an important role in transducing EGFR signals into four different RTK signaling pathways (Fig. 2 and Table 1). We recently applied these EGFR BRET-2 assays to quantitatively study the pharmacological and signaling properties of somatic mutations in EGFR identified in lung cancer and found strong constitutive activation of mutant EGFR receptors preferentially signaling through the PI3K/Akt pathway (Schiffer et al., 2007). The high sensitivity of the RTK BRET assay precisely dissects and characterizes RTK structure-function relationships. We confirmed and quantified the contribution of 6 EGFR tyrosine residues in the recruitment of the adapter protein Grb2 (Fig. 3). Furthermore, we demonstrated that different mutant PDGFRA isoforms identified in GIST are fully constitutively active but show different sensitivities to the inhibitor imatinib. These results were previously not detected in experiments using phosphotyrosine antibodies. RTK BRET-2 assays may be useful in the future to dissect the pharmacological properties of somatic mutations in oncogenic RTKs and help to define better treatment strategies in cancer. In conclusion, RTK BRET assays represent a novel assay platform that will facilitate the characterization of RTK pharmacology and signaling and strengthen ongoing research efforts to identify and develop novel drugs targeting RTKs.

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Address correspondence to: Hans H. Schiffer, ACADIA Pharmaceuticals, 3911 Sorrento Valley Blvd., San Diego, CA 92121. E-mail: hschiffer@acadia-pharm.com