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Monitoring interactions between receptor tyrosine kinases and their downstream effector proteins in living cells using Bioluminescence Resonance Energy Transfer (BRET)

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

ACADIA Pharmaceuticals, Inc.

San Diego, California 92121, USA

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Running title: Receptor tyrosine kinase BRET assays

Address correspondence to:

Hans H. Schiffer

ACADIA Pharmaceuticals

3911 Sorrento Valley Blvd.

San Diego, California, 92121, USA

Tel: 858 320-8625

Fax. 858 558-2872

E-mail: hschiffer@acadia-pharm.com

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Non-standard abbreviations:

BRET, bioluminescence-resonance-energy-transfer; GFP2, green fluorescence protein 2; PLCγ1, phospholipase gamma 1; RTK, receptor tyrosine kinase; Luc, luciferase

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Abstract

A limited number of whole-cell assays are available that allow monitoring receptor tyrosine kinase (RTK) activity in a signaling pathway-specific manner. Here we present the general use of the bioluminescence resonance energy transfer (BRET) technology to quantitatively study the pharmacology and signaling properties of the RTK super family. RTK BRET-2 assays monitor, in living cells, the specific interaction between RTK's and their effector proteins, which control the activation of specific downstream signaling pathways. A total of 22 BRET assays have been established for 9 RTK's derived from 4 subfamilies (ErbB, PDGF, TRK, VEGF), monitoring the interactions with 5 effectors (Grb2, p85, Stat5a, Shc46, PLCγ1). These interactions are dependent on the RTK kinase activity and autophosphorylation of specific tyrosine residues in their carboxy-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 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. 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.

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Introduction

Receptor tyrosine kinases (RTKs) represent a broad class of cell surface receptors that transduce signals across the cell membrane, which regulate cell proliferation, survival, differentiation and migration (Schlessinger, 2000). Activation or over expression of most of the known RTK's has been linked to some form of cancer (Sawyer et al., 2003; Krause and Van Etten, 2005). While the RTK's are prime targets for treatment of cancer, only a small number of therapeutics have been identified in spite of 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 associated with the fact that the majority of methods used to identify kinase inhibitors are biochemical tyrosine kinase assays (Olive, 2004; Minor, 2005). It is expected that a shift towards the use of more whole-cell-based RTK assays will lead to a better prediction of the clinical outcome of new drug candidates. Furthermore, cancer drugs are increasingly designed to target specific cell-signalling 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 utilized 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

super family. Many of the RTK-effector protein interactions depend on the autophosphorylation of specific tyrosine residues in the intracellular carboxy-terminus of a RTK. They control the assembly of larger protein complexes which 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/MAPK 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 RTK's. Earlier BRET studies analyzed the interactions of the insulin receptor with the insulin receptor substrate-1 (IRS-1) (Blanquart et al., 2006), protein tyrosine phosphatase-1B (PTP-1B) (Blanquart et al., 2005) or the adapter protein Grb14 (Nouaille et al., 2006a; Nouaille et al., 2006b). We demonstrate here that the BRET technology is universally applicable to the whole RTK super family and discuss the advantages of this technology compared to other methods that measure RTK activity.

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Materials and Methods

Cell culture and transfection.

HEK293T cells were maintained in DMEM supplemented with 10% FBS and 1 X penicillin-streptomycin-L-glutamine solution (HyClone Laboratories Inc., USA) at 37°C and 5% CO_2. Cells grown in 10 cm^2 dishes were transfected at 80-90% confluency 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 RTK-Luc DNA and 20 μg GFP2-effector DNA, except where noted. The GPCR FPRL1 BRET-2 assay was performed by co-transfecting 1 μg FPRL1-Luc DNA and 20 μg 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 DNA's in water were mixed with 80 μl 2.5 M $CaCl_2$ and 1.7 ml 2X HBS (50 mM HEPES pH 7.05, 280 mM NaCl, 1.5 mM Na_2HPO_4) and incubated at room temperature for 3 to 5 minutes. 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 2-4 hours incubation at 37°C, the medium was replaced with 10 ml fresh media. On the next day, the cells were serum starved by replacing the media with 10 ml DMEM supplemented with 0.1% FBS and 1x penicillin-streptomycin-L-glutamine for 16-20 hours prior to harvesting.

Plasmids.

BRET-2 vectors expressing *Renilla* luciferase (pRluc-N) and green fluorescence protein 2 (pGFP2-N and pGFP2-C) were purchased from Perkin Elmer, USA. Human cDNA's encoding RTK's or RTK effector proteins were obtained by standard RT-PCR on poly-A-

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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-terminally or carboxy-terminally GFP2-tagged tandem SH2 domains as fusion protein SH2(PLCγ1)-GFP2 or GFP2-SH2(PLCγ1), the bases encoding amino acids 507-790 of human PLCγ1 were PCR amplified from GFP2-PLCγ1 cDNA and inserted into pGFP2-N and pGFP2-C. EGFR and 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 D-PBS, detached with D-PBS containing 5 mM EDTA, and resuspended at 4-8 X 10^6 cells/ml in PBS containing 0.1% D-glucose and 1 mM sodium pyruvate (BRET buffer). All ligand serial dilutions were prepared in BRET buffer containing 0.1% BSA. For agonist assays, 50 µl of three-fold concentrated ligand dilutions were dispensed into wells of white flat bottom 96-well plates (Costar, USA). For antagonist assays, 25 µl of six-fold concentrated agonist and 25 µl of six-fold concentrated antagonist were added together per well. Ligands were incubated for 10-20 minutes 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, Biotium Inc.) diluted in PBS per well using the POLARstar OPTIMA plate reader (BMG Labtech, Germany) or the Mithras LB 940 plate reader (Berthold

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Technologies, Germany). After one-second plate shaking, luminescence emissions for *Renilla* luciferase and GFP2 were recorded through BRET optimized filters (luciferase: peak 410 nm and GFP2: 515 nm) for 1-2 seconds in well mode. The time from adding coelenterazine 400A to the plate well to the reading start was sufficient to fully activate luciferase (data not shown). The BRET-2 signal is calculated as the ratio between the luciferase and the GFP2 emission corrected by the background emission of cells transfected with RTK-Luc alone. Non-linear regression analysis was performed with the software PRISM (GraphPad software INC, CA, USA) to obtain dose response curves, IC_{50} and EC_{50} values. Throughout the text, EC_{50} and IC_{50} values are expressed as pEC_{50} [M] or pIC_{50} [M] values, which are calculated as $-\log_{10}$ of the EC_{50} [M] or IC_{50} [M]. Experiments were 2-3 times repeated with each data point performed in triplicates and expressed as mean +/- SEM. The ligands EGF, heregulin-β1, PDGF-BB, and BDNF were purchased from Peprotech (USA), VEGF-C was purchased from R&D Systems (USA), and WKYMVm was purchased from Tocris (USA). Tarceva and imatinib were synthesized by ACADIA Pharmaceuticals, Inc.

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% bromophenol 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, USA), or polyclonal GFP antibody (Cell Signaling

Technology, USA). Proteins carrying phosphotyrosine residues were detected using the monoclonal antibody 4G10 (Upstate, USA). HRP-conjugated secondary antibodies from Santa Cruz Biotechnology (USA), and SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) were used for developing western blots.

Compounds

Tarceva, 4-(3-Ethynylphenylamino)-6,7-bis(2-methoxyethoxy)quinazolinehydrochloride;

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-1H-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.

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Results

The RTK BRET assay

The BRET technology was applied to monitor ligand-induced changes in RTK-effector interactions in eucaryotic cells (e.g. HEK293T) by transiently co-expressing two fusion proteins: the RTK of interest carboxy-terminally tagged with *Renilla reniformis* luciferase (RTK-Luc; bioluminescence donor) and a full-length RTK effector protein amino- or carboxy-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 ligand(s) leads to recruitment of GFP2-effectors to RTK-Luc fusion proteins. These interactions between RTK's 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 *Renilla* luciferase with the membrane permeable luciferase substrate coelenterazine 400A (DeepBlueC, DBC). The GFP2 emission is exclusively due 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 methods), which correlates with the extent of recruitment of the effector protein to the RTK and therefore reflects RTK activation. Importantly, 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 Figure 1, online).

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First, we applied the BRET technology to study the interaction between the most extensively studied RTK, the epidermal growth factor receptor (EGFR), and the effector protein Grb2 (Fig. 1c). EGFR-Luc and amino-terminal GFP2-tagged Grb2 (GFP2-Grb2) were transiently co-expressed in HEK293T cells. After a 10-minute incubation of the cells with 16.7 nM of epidermal growth factor (EGF), we detected a three-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). Co-treatment of EGF with 3.3 μM of the EGFR inhibitor Tarceva 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) that is slightly lower than 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 stimulated a seven 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 beta-arrestin-2 (BA2) protein (Fig. 1d). As expected, neither EGF nor Tarceva affected the WKYMVm-induced BRET-2 ratio in the FPRL1/BA2 BRET-2 assay (Fig. 1d). To demonstrate that

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the observed ligand-induced BRET-2 responses were based on specific protein interactions, we co-expressed 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 co-expressed receptors and effectors do not specifically interact *in vivo* (Figs. 1c and 1d).

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 co-expressed 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, which was efficiently inhibited with the EGFR inhibitor Tarceva in a dose-dependent manner (Figs. 2a-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}'s ranging from 30 to 80 picomolar (summarized in Table 1). Interestingly, 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 prior to Tarceva inhibition (Figs. 2a-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 figures 2d and 2e 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

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only one specific receptor-protein interaction, which 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 carboxy-terminus of EGFR (Heldin, 1995) and specific phospho-tyrosine binding PTB or SH2 domains in the effector proteins (Schlessinger and Lemmon, 2003) mediate all the EGFR effector interactions we studied in figure 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 (Y) residues to phenylalanine (F) to verify that the EGFR/Grb2 BRET-2 signal is dependent on their phosphorylation. Introducing all six Y to F alterations into EGFR-Luc abolished the EGF-induced BRET-2/Grb2 response by 90 % ± 0.9 % compared to 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 Y to F changes, respectively (Fig. 2f). Three Y to F 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 are sufficient for generating an EGFR BRET-2 response (Supplemental Figure 2, online). Our experiments show that the EGFR BRET-2 assays are mediated by interactions between specific autophosphorylated tyrosine residues on activated EGFR and specific phospho-tyrosine binding domains in RTK effectors. Furthermore, we demonstrate that 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 RTK's also trans-phosphorylates tyrosines on recruited effectors like 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 Figure 3, online).

Enabling the RTK super family in BRET-2 assays

58 human RTK's have been described in the human genome (Robinson et al., 2000). We tested if the RTK BRET-2 assay is applicable to study the pharmacological and signaling properties of other RTK's than EGFR. Indeed, we were able to measure RTK activation of other members of the RTK super family in BRET-2 assays (Figs. 3a-d). Heregulin-β1 stimulated GFP2-Grb2 recruitment to ErbB4-Luc, another member of the EGFR family of growth factor receptors (Fig. 3a)**.** Platelet-derived growth factor BB (PDGF-BB)

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stimulated a dose-dependent increase of the BRET-2 ratio in a PDGFRB BRET-2/Grb2 assay (Fig. 3b). Brain derived growth factor (BDNF) stimulated the neurotrophic tyrosine kinase receptor (Trk) family member TrkB in a TrkB/Shc46 BRET-2 assay (Fig. 3c). VEGF-C activated vascular endothelial growth factor 3 receptor (VEGFR3) in a BRET-2/Grb2 assay (Fig. 3d). We enabled additional RTK's from these sub-families in RTK BRET-2 assays (Table I). Most of the RTK's analyzed in this study did interact with multiple effector proteins, which correlated well with published results about their signal transduction (Table I). The sensitivity of all RTK BRET-2 assays for activation by their endogenous *in vivo* ligands was in the nano-molar range, which is in agreement with results from other methods and also reflects the high *in vivo* potency of these growth factors. We have also shown that tyrosine kinase inhibitors with specificity for the tested RTK's 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 sub-families of RTK's. Therefore, it is possible that the BRET-2 technology can be used to monitor the activity of most if not all known RTK's.

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), (Heinrich et al., 2003b). We studied two mutations causing a

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non-synonymous 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 to 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} [M] = 6.62 ± 0.12. 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} [M] = 6.98 ± 0.13 and Δ842-845 pIC_{50} [M] = 7.15 ± 0.10), while the mutant D842V isoform was around 30 fold less sensitive to imatinib (D842V pIC_{50} [M] = 5.13 ± 0.10) (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 pathway(s) mainly driving a cancer pathogenesis and the design of treatment strategies.

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Discussion

Our results present BRET as a powerful technology to study pharmacology and signaling properties of the RTK super family in living cells. We developed 22 RTK BRET assays for 9 RTK's from 4 different sub-families, suggesting that RTK BRET assays can be developed for most, if not all, of the 58 human RTK's. BRET-2 assays are conveniently performed in 96 and 384 multi-well plate formats and produce GFP2 and luciferase emissions, which can be easily measured using BRET enabled multi-functional plate readers. The BRET-2 signals are calculated as a ratio between both reporter emissions, which eliminates assay variation due to different numbers of cells per well, and facilitates automation and integration of the BRET assays into high throughput-screening (HTS) formats (data not shown). RTK BRET assays are highly sensitive in detecting constitutive or ligand-induced receptor activity (Figs. 2 and 4) and deliver precise, quantitative pharmacological data for the study of agonist or antagonists. Importantly, 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 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 demonstrate in the EGFR/Grb2 BRET-2 assay that Grb2 recruitment to activated EGFR is dependent on EGFR kinase activity and autophosphorylation of specific EGFR tyrosine residues, and that activation leads to downstream phosphorylation of EGFR effector proteins (Supplemental Figure 3, online). Importantly,

in contrast to most RTK assays in use today, each RTK BRET-2 assay measures effector specific receptor activity. This unique feature allows establishing and comparing 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 RTK's and help to define better treatment strategies in cancer. In conclusion, RTK BRET assays represent a novel assay platform, which will facilitate the characterization of RTK pharmacology and signaling, while strengthening ongoing research efforts to identify and develop novel drugs targeting RTK's.

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Legends for figures

Figure 1. The RTK whole-cell BRET-2 assay (**a**) Illustration of RTK activation and signaling. Ligand induced activation of RTK lead 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. *Renilla* luciferase-tagged RTK-Luc (bioluminescence donor) and GFP2-tagged effector proteins (fluorescence acceptor) are transiently co-expressed in HEK293T cells. Activation of luciferase with membrane permeable substrate coelenterazine 400A (DeepBlueC), but without RTK activation, primarily result 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, d**) Effector and ligand specificity of RTK BRET-2 assay. HEK293T cells were transiently co-transfected with luciferase-tagged RTK EGFR (EGFR-Luc, **c**) or with 1 μg of the luciferase-tagged GPCR FPRL1 (FPRL1-Luc, **d**) along with either GFP2-tagged RTK effector Grb2 (GFP2-Grb2) or GFP2-tagged GPCR effector beta-arrestin-2 (GFP2-BA2). (**c, d**) Transfected cells were incubated for 10 minutes with 16.7 nM of the EGFR agonist EGF (black bars), 0.33 μM of the FPRL1 agonist WKYMVm (blue bars), 16.7 nM EGF in the presence of 3.3 μM of the EGFR kinase inhibitor

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Tarceva (green bars) or 0.33 μM of the FPRL1 agonist WKYMVm in the presence of 3.3 μM of the kinase inhibitor Tarceva No ligand controls (open bars). BRET-2 measurements were performed and analyzed as described in methods.

Figure 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 co-transfected 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 minutes with different amounts of EGF (filled circles). The EGFR tyrosine kinase inhibitor Tarceva efficiently inhibited the observed EGF-stimulated BRET-2 responses (open circles; a-e). In these antagonist assays, cells were incubated for 10 minutes in the presence of 16.7 nM EGF and increasing concentrations of Tarceva. We observed constitutive wild type EGFR activity mainly in the EGFR Grb2/shc/p85 BRET-2 assays (previously confirmed in (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 Tarceva (a-c). EGFR BRET-2 responses are dependent on phosphorylation of specific tyrosine residues in the intracellular EGFR carboxy-terminus. Site directed mutagenesis (Y-F) was performed on EGFR tyrosines 1068, 1086, 1101, 1114, 1148 and 1173, which are direct or indirectly

involved in interaction with effector Grb2. Wild type EGFR-Luc or EGFR-Luc isoforms were co-expressed with GFP2-Grb2 in HEK293T cells and tested in the EGFR/Grb2 BRET-2 assay (2f). (f) Dose-response curves for the EGFR agonist EGF (10 minute incubations) are shown normalized to the maximum of the wild type EGFR response. EGFR isoform 3 (Y to F) contains mutations Y1086F, Y1101F and Y1173F; 4 (Y to F) contains mutations Y1086F, Y1101F, Y1114F and Y1173F; 5 (Y to F) contains mutations Y1068F, Y1086F, Y1101F, Y1114F and Y1173F; 6 (Y to F) contains mutations Y1068F, Y1086F, Y1101F, Y1114F, Y1148F and Y1173F.

Figure 3. Application of the BRET-2 technology to study pharmacology, signaling and structure-function relationships in the RTK superfamily. Several RTK's from different RTK subfamilies have been tested in the BRET-2 assay. HEK293T cells are co-transfected with an RTK-Luc and a GFP2-effector. BRET-2 assay dose-response curves obtained for specific RTK agonists (10 minute 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), VEGFR3-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 co-transfected in HEK293T cells and BRET-2 assays 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 minutes with different amounts of PDGF-BB. Mutant PDGFRA isoforms are fully

constitutively activated 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 minutes 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.

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Table 1. RTK pharmacology in BRET-2 assays

RTK	effector	agonist	pEC50 [M]	antagonist	pIC50 [M]
EGFR	Grb2	EGF	10.52 ± 0.10	Tarceva	7.89 ± 0.04
	p85	EGF	10.46 ± 0.19	Tarceva	7.96 ± 0.04
	Shc46	EGF	10.30 ± 0.23	Tarceva	7.75 ± 0.06
	PLCγ1	EGF	10.42 ± 0.31	Tarceva	8.56 ± 0.11
	Stat5a	EGF	10.09 ± 0.26	Tarceva	8.12 ± 0.09
ErbB4	Grb2	HRG-β-1	9.67 ± 0.09	n.p	n.p
	p85	HRG-β-1	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

Results are presented as the mean ± SEM. from 3-4 separate experiments.
n.p. not performed; pEC_{50}/IC_{50} = -log EC_{50}/IC_{50} [M]

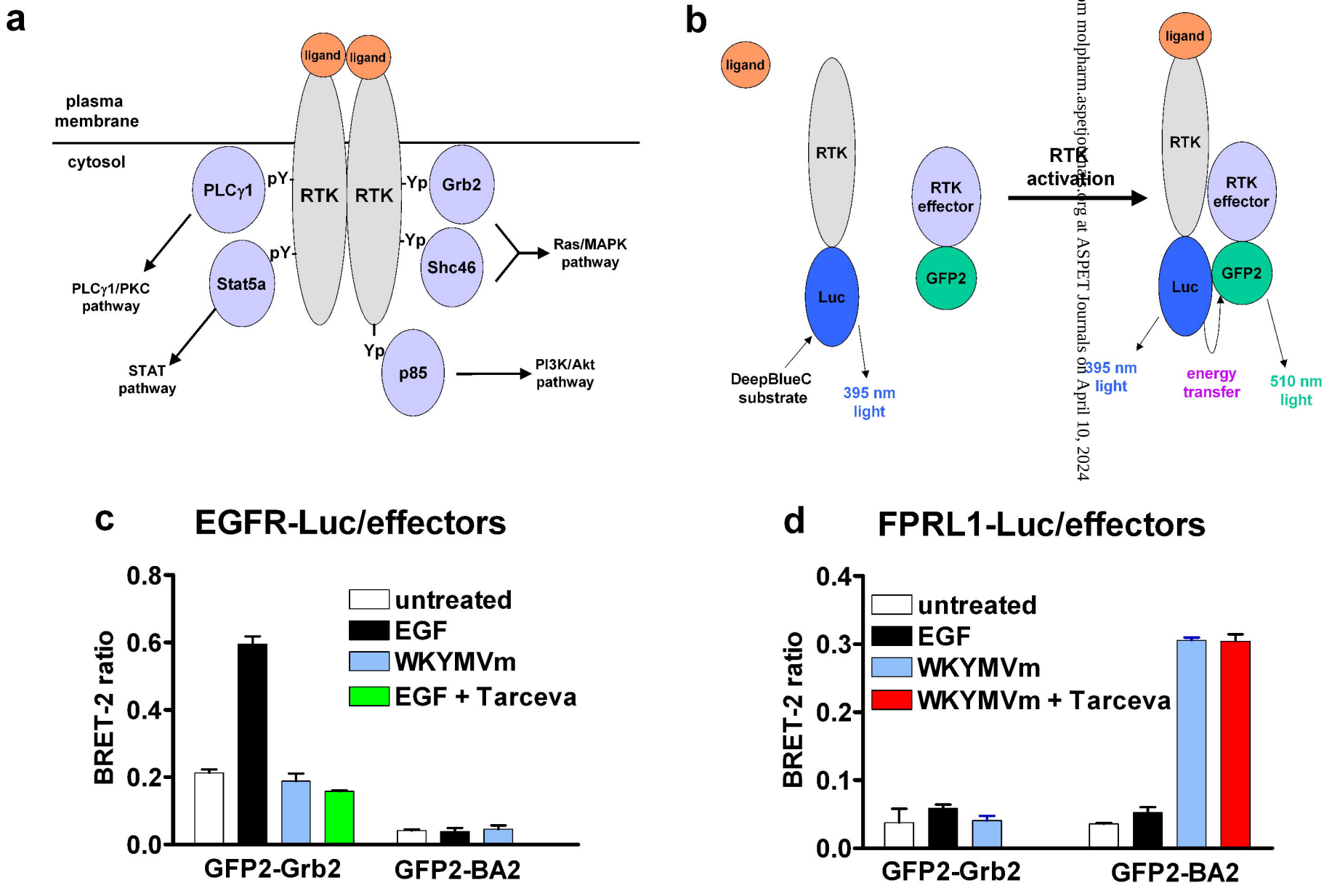

Figure 1

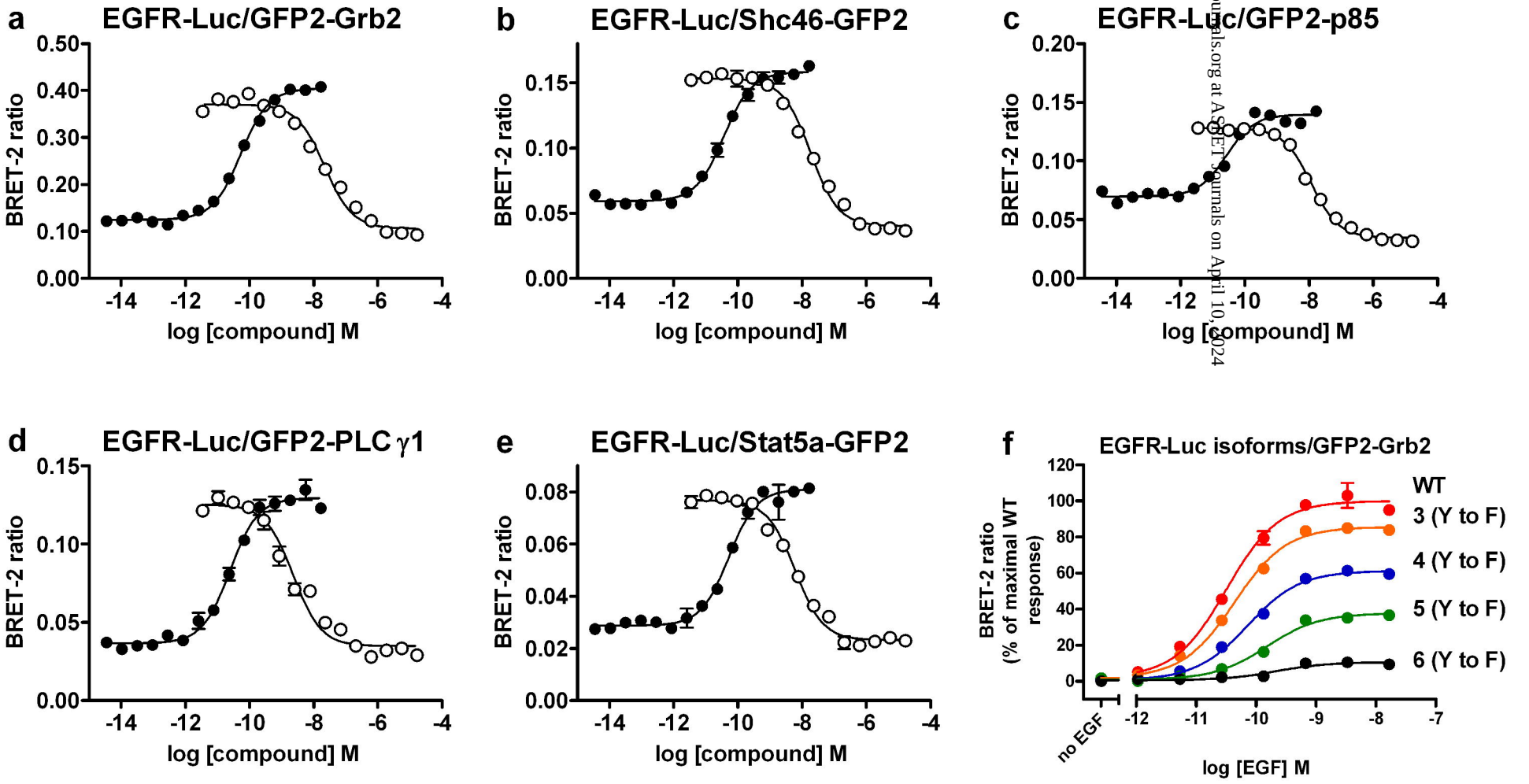

Figure 2

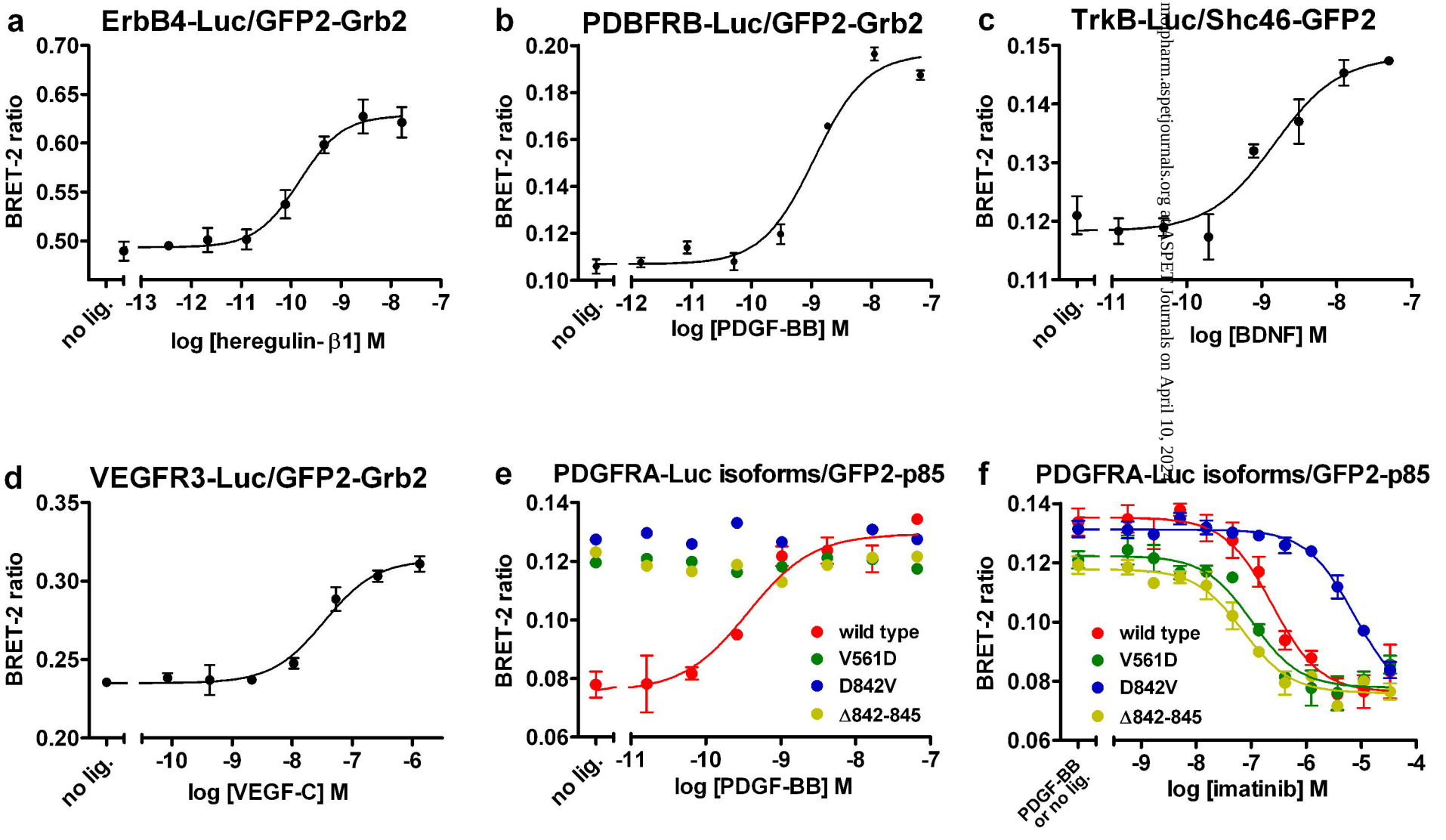

Figure 3