The genetic design of signaling cascades to record receptor activation

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We have developed an experimental strategy to monitor protein interactions in a cell with a high degree of selectivity and sensitivity. A transcription factor is tethered to a membrane-bound receptor with a linker that contains a cleavage site for a specific protease. Activation of the receptor recruits a signaling protein fused to the protease that then cleaves and releases the transcription factor to activate reporter genes in the nucleus. This strategy converts a transient interaction into a stable and amplifiable reporter gene signal to record the activation of a receptor without interference from endogenous signaling pathways. We have developed this assay for three classes of receptors: G protein-coupled receptors, receptor tyrosine kinases, and steroid hormone receptors. Finally, we use the assay to identify a ligand for the orphan receptor GPR1, suggesting a role for this receptor in the regulation of inflammation.

cellular assays | G protein-coupled receptor | protein interaction

All cells have evolved mechanisms to respond to rapid changes in the environment. Extracellular signals are detected by transmembrane receptors that translate binding into intracellular signaling events. Most signaling systems that respond to environmental cues exhibit adaptation mechanisms that afford the cell a facile response to rapid changes in their surroundings. Mechanisms to assure the rapid but transient response to environmental cues are of obvious advantage to the cell but seriously limit most assays for receptor function. We have genetically modified receptors such that transient responses to ligand result in the stable transcription of a reporter gene. The transformation of a transient intracellular response to a stable amplifiable readout provides a sensitive and quantitative assay for receptor function.

We have developed an assay for receptor activation and more generally for protein–protein interaction that involves the fusion of a membrane receptor with a transcriptional activator. The membrane-bound receptor and transcription factor sequences are separated by a cleavage site for a highly specific viral protease. A second gene encodes a fusion of the viral protease with a cellular protein that interacts only with activated receptor. Ligand binding to the receptor will stimulate this protein–protein interaction, recruiting the protease to its cleavage site. Site-specific cleavage will release the transcriptional regulator that can now enter the nucleus and activate reporter genes. Recently, a similar principle, based on the complementation of split tobacco etch virus (TEV) protease fragments, has been used to monitor protein interactions (1). Our experimental scheme derives conceptually from the mechanism of action of the Notch receptor in which ligand binding elicits proteolytic cleavage events in the receptor to release a Notch intracellular domain that translocates to the nucleus and modulates transcription of downstream target genes (2, 3) (Fig. 1*A*).

The assay we have developed relies solely on exogenous genes introduced into the cell to create a novel signaling pathway. As a consequence, reporter gene activity will be independent of the activation of any endogenous cell signaling events that frequently confound existing assays for receptor function. This approach transforms transient rapidly adapting signaling events into more stable and amplifiable cellular responses by virtue of the irreversible release of a membrane-anchored transcription factor. Because this assay requires the association of two proteins to stimulate a response, we call this the Tango assay. We have developed Tango assays to monitor the activity of three different classes of receptors: G protein-coupled receptors (GPCRs), receptor tyrosine kinases, and steroid hormone receptors.

Results

Tango Assay for GPCRs. In initial experiments, we designed a Tango assay to monitor the activation of GPCRs (Fig. 1*B*). We generated a fusion protein consisting of the human arginine vasopressin receptor 2 (AVPR2) joined at its cytoplasmic C terminus to the transcriptional activator, tTA (4). Interposed between the receptor and tTA sequences, we introduced a specific 7-aa cleavage site for a highly specific protease, the N1a protease from TEV (5). We then constructed a second fusion protein consisting of the TEV protease linked to human β-arrestin2. These receptor and arrestin fusion genes were transiently introduced into a cell line that contains a tTA-dependent reporter gene.

Ligand activation of GPCRs results in the phosphorylation of specific serine and threonine residues at the cytoplasmic C terminus of the receptor by a class of GPCR kinases (6). Ligand-occupied phosphorylated receptor then recruits arrestin, preventing further G protein activation (7). In the Tango assay, the ligand-dependent recruitment of arrestin-TEV protease to the receptor fusion and the subsequent proteolytic cleavage frees tTA to enter the nucleus and activate reporter genes. The association of arrestin with ligand-bound receptor is observed for most GPCRs, a feature that allowed us to extend the Tango assay to multiple receptors within this class.

We tested a series of variant TEV protease cleavage sites previously shown to be cleaved by the protease at reduced efficiency (8) and identified one mutant site that maximized the signal-to-background ratio [supporting information (SI) Fig. 5].

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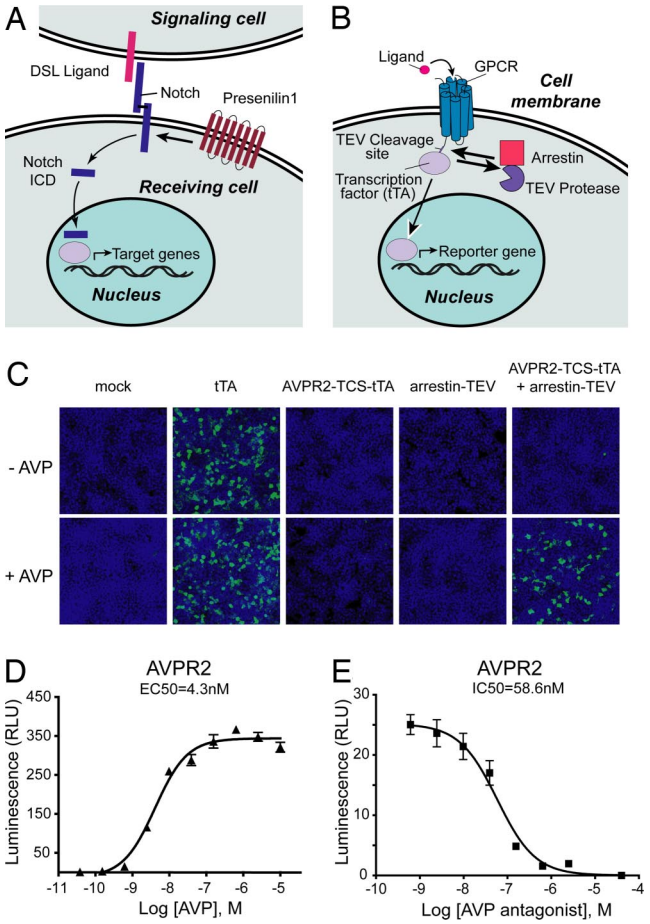

Fig. 1. Design of the Tango assay. (*A*) A schematic of the Notch signaling pathway. The binding of Delta/Serrate/Lag-2 (DSL) ligands to Notch leads to a proteolytic cleavage of Notch by a protein complex involving presenilin. This cleavage releases a Notch intracellular domain (ICD) that translocates to the nucleus and modulates expression of target genes. (*B*) Schematic of the Tango assay method to monitor GPCR–arrestin interactions. Ligand binding to the target receptor stimulates recruitment of the arrestin-TEV protease fusion, triggering the release of the tethered transcription factor tTA. Free tTA then enters the nucleus and stimulates reporter gene activity. (*C*) Tango assay for the arginine vasopressin receptor AVPR2. HTL cells (an HEK293T-derived cell line containing a stably integrated tTA-dependent luciferase reporter) were transiently transfected with expression plasmids encoding the arginine vasopressin receptor 2 (AVPR2)-TEV cleavage site-tTA (AVPR2-TCS-tTA) and β-arrestin2-TEV protease (Arrestin-TEV) fusion proteins, as indicated. Control cells were transfected either with a tTA expression plasmid (tTA) or with empty parental expression plasmid (mock). Luciferase expression was detected by immunostaining with anti-luciferase (green), and cell nuclei were visualized with the DNA dye TOTO-3 (blue). Western blot analysis confirmed that receptor and arrestin components were expressed at similar levels in each transfection (data not shown). (*D* and *E*) Dose-response curves generated with a Tango assay for the human AVPR2. HTLA cells (an HEK293-derived cell line containing stable integrations of a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene) were transiently transfected with an AVPR2-TCS-tTA fusion gene. (*D*) Response to varying doses of the AVPR2 agonist vasopressin. (*E*) Response to varying doses of an AVPR2 antagonist, added 15 min before treatment with an EC80 dose (17 nM) of vasopressin. All errors bars represent SD (n = four measurements).

Cells transfected with the AVPR2-TEV cleavage site (TCS)-tTA and arrestin-TEV fusions exhibited a 200-fold increase in reporter gene activity after overnight incubation with AVP, the receptor agonist (Fig. 1*D*). Reporter gene activity in response to AVP exhibits a dose–response profile that corresponds with that of the native arginine vasopressin receptor in a second messenger assay (9). Agonist-induced stimulation of reporter gene activity was blocked by treatment with a peptide analogue receptor antagonist (Fig. 1*E*).

When the receptor-tTA and arrestin-TEV fusions were expressed individually, reporter activity was negligible in the presence or absence of the ligand (Fig. 1*C*). Moreover, AVP-induced reporter gene stimulation was not detected when the receptor-TCS-tTA fusion protein was co-expressed with the native TEV protease or with TEV protease fused to the SH2 domain of phospholipase Cγ, a protein not known to associate with GPCRs (data not shown). These data indicate that reporter gene activity in this assay is a specific and quantitative measure of the ligand-induced interaction between GPCR and arrestin. We provide a more detailed analysis of the establishment and properties of this assay and have extended the Tango assay to additional members of the family of GPCRs that signal through all known classes of G proteins (see below).

A Tango Assay for Receptor Tyrosine Kinases. We next asked whether the conceptual design of the Tango assay could be modified to monitor the activation of receptor tyrosine kinases. Binding of ligand to members of this receptor family results in the phosphorylation of specific tyrosine residues in the receptor cytoplasmic domain (10, 11). These phosphotyrosine motifs then serve as binding sites for the recruitment of signaling proteins containing Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains (12). We therefore constructed a receptor tyrosine kinase fusion with the TEV protease cleavage site (TCS) and the transcriptional activator tTA linked to the receptor C terminus. A second fusion gene consisting of a PTB or SH2 domain linked to the TEV protease mediates the ligand-dependent cleavage and release of tTA. As a model, we used the insulin-like growth factor 1 receptor (IGF1R) that interacts with the PTB domain of the adaptor protein, Shc1 (13, 14). The IGF1R-TCS-tTA fusion was expressed along with the Shc1-TEV protease fusion in a cell line containing a tTA-responsive reporter gene. Exposure of this cell to IGF1 resulted in a 10-fold increase in reporter gene activity (Fig. 2*A*). In control experiments, transfection of the IGF1R-TCS-tTA fusion alone or in the presence of the native TEV protease gene failed to elicit significant induction of reporter gene activity by IGF1 (data not shown). The application of the Tango approach to another tyrosine kinase receptor, EGFR, revealed similar results (data not shown), demonstrating that this assay affords a specific and quantitative approach to monitor the activity of the large family of receptor tyrosine kinases. We have further extended the assay concept to the family of interferon receptors that also signal through tyrosine phosphorylation (data not shown).

Tango Assay for Steroid Hormone Receptors. We next asked whether the regulated interaction of two cytoplasmic receptor proteins can also be measured using the Tango approach. Two distinct estrogen-activated intracellular receptors are present in the mammalian genome, ERα and ERβ. Free estrogen receptors are sequestered in the cytoplasm in an inactive state in a multiprotein complex. The binding of estrogen results in the formation of homo- or heterodimers that enter the nucleus and interact with specific DNA response elements to modulate target gene transcription (15).

We adapted the Tango technology to monitor the ligand-induced interaction of cytoplasmic estrogen receptor monomers (Fig. 2*B*). We anchored one interaction partner, ERα, to the inner face of the cell membrane by fusing its N terminus to the transmembrane protein CD8 (16). The C terminus of the anchored ERα was fused to the transcription factor tTA with an intervening TEV protease cleavage site (TCS). This strategy immobilizes the transcription factor tTA at the plasma membrane preventing nuclear entry until released by proteolytic cleavage. We monitored ERα/ERα ho-

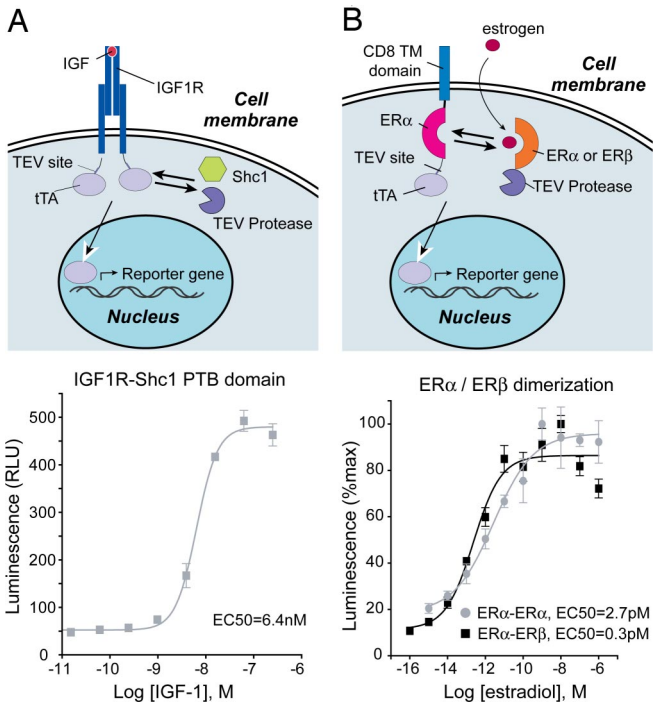

Fig. 2. Tango assays for tyrosine kinase and steroid hormone receptors. (*A*) Receptor tyrosine kinase signaling. HTL cells were transfected with an IGF-1 receptor (IGF1R)–TCS-tTA fusion construct and a Shc1 PTB domain-TEV protease fusion plasmid. Luciferase activity in this IGF1R–Shc1 interaction assay was stimulated by IGF-1. (*B*) Ligand-induced homo- and heterodimerization of estrogen receptors (ERs). HTL cells were transfected with a CD8-ERα-TCS-tTA fusion and either an ERα- or ERβ-TEV protease fusion to measure ERα homodimerization (light circles) or ERα/ERβ heterodimerization (dark squares) as illustrated. Responses were normalized to the maximal response for each receptor. All error bars represent SD (n = four measurements).

modimerization by coexpression of the membrane-anchored CD8-ERα-TCS-tTA fusion with a free cytoplasmic ERα fused to the TEV protease. In parallel, we monitored ERα/ERβ heterodimerization by expressing the anchored CD8-ERα-TCS-tTA fusion with a fusion of TEV protease to ERβ. Stimulation with estradiol resulted in a 10-fold induction in reporter gene activity in the homo- and heterodimerization assays (Fig. 2*B*) with EC_{50} values in accord with those of the native receptors (17). Transfection of the CD8-ERα-TCS-tTA alone, or with native TEV protease, resulted in background activity that was not stimulated by estradiol (data not shown). These results demonstrate that the Tango protein interaction methodology can be readily applied to monitoring ligand-induced signaling events that involve both transmembrane and intracellular receptors.

Extending Tango to the Family of GPCRs. Experiments to extend Tango to a larger repertoire of GPCRs revealed that the extent of agonist-induced reporter gene stimulation varied among different GPCRs. Individual members of the GPCR family differ in their affinity for arrestin (18), a characteristic that is largely determined by the phosphorylation at serine and threonine sites in the C-terminal tail of the receptor (9). To extend the use of the Tango assay to GPCRs that weakly recruit arrestin upon activation, we examined whether the addition of the C-terminal tail of a receptor that forms stable complexes with arrestin could enhance assay performance for those receptors that responded poorly in the Tango assay. We observed that the addition of a short fragment from the C-terminal tail of AVPR2 enhanced assay performance for multiple receptors, including the κ-opioid receptor and the D2 dopamine receptor (SI Fig. 6) but had no discernible effect on the ligand specificity of the recipient receptor (SI Fig. 7 and SI Table 1). This C-terminal modification of the receptor fusion gene extends the Tango assay to receptors that weakly recruit arrestin molecules upon activation and has allowed us to develop assays for 89 GPCR family members representing all known signaling classes (data not shown).

Agonist and Antagonist Specificity for the Adrenergic Receptor Family. We next examined the profile of the agonist and antagonist selectivity of a family of related GPCRs using the Tango assay. We established assays for a panel of five well characterized α- and β-adrenergic receptors and monitored receptor activation and inhibition by selective agonists and antagonists. In the Tango assay, α- and β-adrenergic receptors displayed the predicted specificity for subtype selective agonists and antagonists (Fig. 3 and SI Table 2). In addition, these Tango assays, with signal-to-background ratios that can exceed 1,000-fold, afford greater sensitivity over a larger dynamic range than comparable second messenger assays, in which 2- to 5-fold responses are typically observed. These results illustrate that monitoring arrestin recruitment in the Tango assay provides an accurate measure of receptor activation and demonstrate the general utility of this method to evaluate the ligand selectivity profiles of a panel of GPCRs.

To determine whether the Tango assay mirrored the quantitative effects of agonists and partial agonists observed in assays of the native GPCR, we examined the activation of the β2-AR by a series of β-adrenergic partial agonists, compounds that elicit submaximal receptor responses at saturating doses. We observed that each agonist elicits different levels of activity at saturation (Fig. 3*C*). The weakest partial agonist, nylidrin, elicited a maximal response ≈100 times lower than that produced by a full agonist, isoproterenol. Moreover, the relative responses of the β2-AR to these partial agonists in the Tango assay were in accord with data obtained in second messenger assays that report native signaling pathways (19). Thus, the stimulation of reporter gene activity in the Tango assay is not binary; rather, it distinguishes intermediate levels of receptor activation induced by partial agonists.

Target Selectivity of the Tango Assay. The Tango assay reconstructs a receptor-mediated signaling cascade with three exogenous genes. Thus, in contrast to assays of second messenger accumulation, the Tango assay should be immune to signaling elicited by activation of receptors endogenous to the cell. We compared Tango with an assay that monitors receptor-mediated elevations in intracellular calcium. The Tango assay was performed with the AVPR2-TCS-tTA fusion in HEK293 cells that express endogenous muscarinic acetylcholine and purinergic P2Y receptors (20, 21). In the Tango assay, luciferase reporter gene activity was induced only by vasopressin that activates the exogenous AVPR2-TCS-tTA receptor and not by agonists for the endogenous receptors (SI Fig. 8*A*). In contrast, assays for intracellular calcium reveal a response to vasopressin as well as to carbachol and ATP, agonists for the muscarinic and P2Y receptors, respectively (SI Fig. 8*B*).

In a second experiment, we introduced AVPR2-TCS-tTA into a cell along with an unmodified κ-opioid receptor and observed reporter gene activity only by agonists selective for AVPR2 (SI Fig. 8*C*). Similarly, cells transfected with κ-opioid receptor-TCS-tTA along with an unmodified AVPR2 responded solely to κ-opioid agonists. Thus, the Tango assay, unlike most second messenger assays, selectively measures the activation of a specific exogenous receptor-TCS-tTA fusion without interference from endogenous receptor-mediated signaling pathways.

The selectivity of the Tango assay should permit us to monitor agonists for a given receptor in unfractionated tissue extracts that often contain agonists for endogenous receptors. We ex-

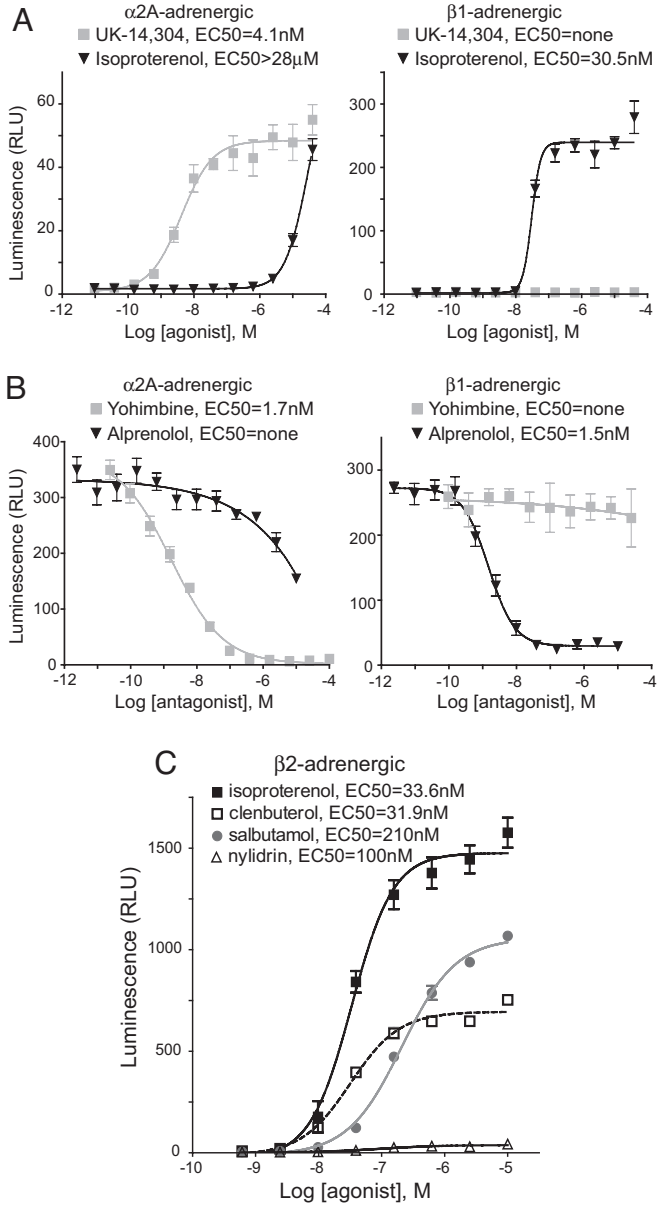

Fig. 3. Using the Tango assay to profile adrenergic receptor agonists and antagonists. (*A* and *B*) Agonist and antagonist selectivity profiling using Tango assays for human adrenergic receptors. HTLA cells were transiently transfected with α2A-, α2B-, α2C-, β1-, or β2-adrenergic receptor-TCS-tTA fusions, each of which contained the C-terminal tail sequence from AVPR2. (*A*) In these representative Tango agonist assays, α-adrenergic receptors were preferentially activated by the α-selective agonist UK14,304, and β-adrenergic receptors were preferentially activated by the β-selective agonist isoproterenol. (*B*) In representative antagonist assays, α- and β-ARs displayed the expected selectivity to the antagonists yohimbine and alprenolol, respectively. All error bars represent SD (n = four measurements). See SI Table 2 for complete results. (*C*) Stimulation of luciferase reporter gene activity in the β2-adrenergic receptor Tango assay by a full agonist, isoproterenol, and partial agonists salbutamol, clenbuterol, and nylidrin. Agonist-stimulated luciferase activity was measured in an HEK293T-derived cell line containing stably integrated luciferase reporter, β-arrestin2-TEV, and β2-AR-TCS-tTA (with AVPR2 C-terminal modification) constructs.

amined this hypothesis by monitoring activation of the human κ-opioid receptor by crude extracts of bovine hypothalamus. Hypothalamic extract produced dose-dependent increases in reporter gene activity, with a maximum induction >500 times background (SI Fig. 8*D*). Pretreatment with a selective κ-opioid antagonist, nor-binal torphimine, completely blocked the response to the hypothalamic extract in this assay, indicating that the response we observe derives solely from activation of the exogenous κ-opioid receptor. Thus, Tango provides a selective bioassay to monitor the activation of a receptor by endogenous agonists present in biological extracts.

Identifying Ligands for Orphan GPCRs. We exploited the sensitivity and specificity of the Tango assay to identify a ligand for the orphan GPCR, GPR1 (22). GPR1 is most closely related to CMKLR1 (23, 24), a member of a group of leukocyte chemoattractant receptors. The endogenous ligand recognized by GPR1 has not been identified, and thus the biological function of this receptor remains unknown. Given the sequence similarity between GPR1 and CMKLR1, we used the Tango assay to examine whether GPR1 responds to the leukocyte chemattractant chemerin, the previously reported endogenous ligand for CMKLR1 (23, 24). We found that GPR1 responded in the Tango assay to recombinant chemerin protein with an EC_{50} of 240 pM, compared with 3 nM for CMKLR1 (Fig. 4*A*). A C-terminal fragment representing amino acids 145–157 of the mature chemerin protein, previously shown to activate CMKLR1 (25), also activated GPR1 with an EC_{50} of 1 nM, compared with 24 nM for CMKLR1 (Fig. 4*B*).

We next examined the ability of chemerin to activate GPR1 and CMKLR1 using a calcium mobilization assay. In this assay, addition of chemerin to cells expressing GPR1 resulted in an elevation of intracellular calcium to a level 30% of that observed in cells expressing CMKLR1 (Fig. 4*C*). Thus, in the Tango assay, chemerin is a more potent agonist for GPR1 than for CMKLR1, whereas in the calcium mobilization assay, chemerin is more effective in stimulating CMKLR1. The relatively weak response of GPR1 to chemerin using a calcium mobilization assay may explain a previous study that did not detect chemerin-mediated activation of GPR1 (23).

To confirm the identification of GPR1 as a potential chemerin receptor, we performed radioligand-binding studies using an iodinated C-terminal peptide fragment of chemerin (25). In these experiments, the labeled peptide was added to cells transfected with GPR1 or CMKLR1 as well as to untransfected cells in the presence of either full length chemerin (Fig. 4*D*) or unlabeled peptide (Fig. 4*E*) as competitors. We observed specific binding of the chemerin peptide to cells transfected with GPR1 as well as to cells transfected with CMKLR1, whereas untransfected cells showed no specific binding (data not shown). Saturation-binding analysis performed on GPR1-transfected cells revealed a single binding site with a calculated K_d of 5.3 nM (Fig. 4*F*), compared with 4.9 nM for CMKLR1-transfected cells (Fig. 4*G*). Thus, both GPR1 and CMKLR1 appear to bind the chemerin C-terminal peptide with similar affinity, whereas the Tango assay, an assay of receptor function, suggests that the chemerin peptide may be a more potent activator of GPR1 than of CMKLR1. These experiments illustrate that the Tango assay may be particularly advantageous for the analysis of receptors with second messenger pathways that are either weak or unknown.

Discussion

The Tango assay introduces three exogenous genes into a cell to construct a receptor-mediated signaling cascade. The concept derives from the mechanism of action of the Notch receptor and requires the sequestration of a transcription factor to the cell membrane by physically linking it to a receptor. Activation of the receptor fusion results in the recruitment of a signaling protein fused to a protease that then cleaves and releases the transcription factor to activate reporter genes in the nucleus. This experimental strategy was modified to develop assays for three classes of receptors: GPCRs, receptor tyrosine kinases, and

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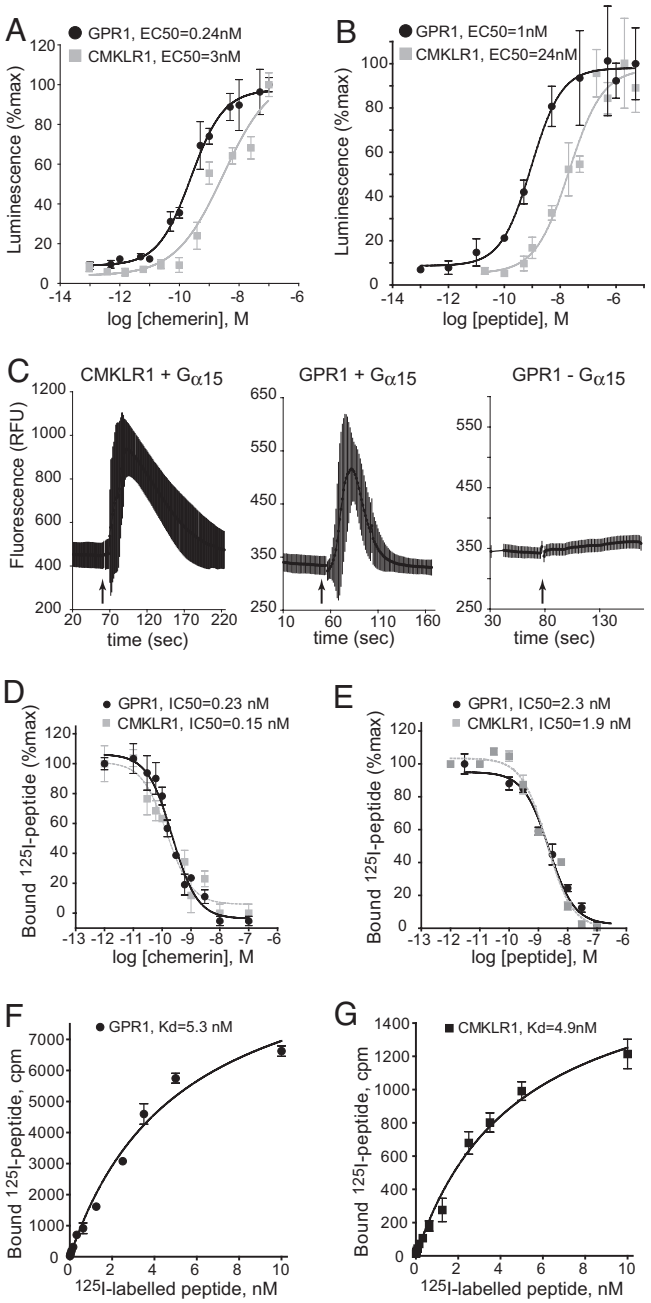

Fig. 4. Identification of an agonist for the orphan receptor GPR1. (*A* and *B*) Dose-response profiles of GPR1 and CMKLR1 Tango assays in response to recombinant human chemerin protein (*A*) and a peptide fragment of chemerin (chemerin 145–157) (*B*). Responses were normalized to the maximal response for each receptor. (*C*) Calcium mobilization assay showing activation of CMKLR1 and GPR1 by chemerin peptide (chemerin 149–157, at 1 μM) in the presence of the promiscuous G protein $G_{\alpha 15}$. Time of ligand addition is indicated by the arrows. The responses of seven representative cells were averaged. All error bars represent SD. (*D–G*) Binding of radiolabeled chemerin C-terminal peptide to GPR1-and CMKLR1-transfected cells. Shown is displacement of iodinated chemerin C-terminal peptide ($chemerin_{149-157}$) binding to GPR1- (black circles) and CMKLR1-expressing cells (gray squares) by full-length chemerin peptide (*D*) or unlabeled $chemerin_{149-157}$ (*E*). Shown is saturation binding of ^{125}I-$chemerin_{149-157}$ to GPR1-transfected cells (*F*) and CMKLR1-transfected cells (*G*).

steroid hormone receptors. By using a signaling cascade composed of exogenous components, the Tango assay is largely independent of endogenous second messengers or adaptation mechanisms. This experimental strategy affords features that distinguish Tango from other assays that measure receptor function. The genetic modifications of the receptor in the Tango assay transform a transient receptor-mediated cellular response into the stable transcription of a reporter gene. The amplification inherent in transcriptional activation provides a sensitive and quantitative assay of receptor function. Moreover, reporter gene activity is immune to signaling events that result from activation of endogenous receptors, a feature that can confound existing assays of receptor function.

The Tango assay for GPCRs monitors receptor activation by exploiting ligand-mediated arrestin binding. Because virtually all GPCRs associate with arrestin upon activation, we exploited the generality of arrestin recruitment to develop assays for a wide range of GPCRs that include receptors that activate all known G protein classes. GPCR signaling has been assayed previously by monitoring the subcellular redistribution of a fluorescently labeled arrestin in response to GPCR stimulation (26, 27). Tango provides a more quantitative assay that monitors arrestin recruitment solely to the modified receptor of interest and is therefore unaffected by the signaling of endogenous receptors. Tango therefore affords a general but selective assay for all GPCRs, independent of the nature of the G protein that they activate. Thus, it is particularly advantageous when G protein activation of second messengers is weak or if the identity of the downstream G protein is unknown.

The approach we have devised to monitor the hormone-evoked dimerization of extracellular estrogen receptors demonstrates that Tango can be used more broadly to detect protein–protein interactions. Recently, a protein interaction technique based on the complementation of split TEV protease fragments has been described (1). Like the Tango method described here, this split TEV approach involves a cleavage step catalyzed by TEV protease, thereby converting transient interactions into a long-lasting signaling readout. Our data indicate that splitting TEV protease is not required to limit proteolytic activity before interaction. Instead, we observe that regulated localization within the cell is sufficient to discriminate between free and associated TEV- and transcription factor-fused partners. Because Tango therefore obviates the need to engineer complementary TEV fragments with low inherent affinity, it affords enhanced sensitivity and greater generality.

The sensitivity of Tango, coupled with the ability to monitor activity of a receptor of interest without interference from endogenous receptor signaling, provides a suitable bioassay for identifying endogenous ligands for orphan receptors. We have exploited this feature to identify a candidate endogenous ligand for the orphan GPCR, GPR1. GPR1 is most closely related to CMKLR1, a recently described receptor for the inflammation-associated leukocyte chemoattractant chemerin. Using the Tango assay, we found that GPR1 is also activated by chemerin, and we confirmed these observations by an independent Ca^{2+} mobilization assay and by radioligand-binding studies. The existence of a second, previously unrecognized receptor for chemerin raises the possibility that at least some of the activities of chemerin in inflammation may be mediated through GPR1. These observations illustrate the utility of the Tango methodology as a bioassay to discover endogenous ligands for elusive orphan receptors.

Methods

Materials. Arg^8-Vasopressin, [Adamantaneacetyl^1, O-Et-D-Tyr^2, Val^4, Aminobutyryl^6, $Arg^{8,9}$]-Vasopressin, dopamine HCl, U-69593, U.K.-14,304, isoproterenol, alprenolol, carbachol, ATP, nor-binaltorphimine dihydrochloride, recombinant human IGF-1, and 17-β estradiol were obtained from Sigma. Yohimbine was from Tocris. The adrenergic compound library was obtained from Biomol. Anti-luciferase was from Promega and was used at a dilution of 1:500. TOTO-3 was from Molecular Probes (Invitrogen) and was diluted

1:1,000. Recombinant human Chemerin was from R&D Systems, and Chemerin peptides were from Phoenix Pharmaceuticals.

Plasmid Constructs. See *SI Text* for details of plasmid construction. Constructs were generated by PCR using high-fidelity DNA polymerases [Platinum Taq High Fidelity (Invitrogen) or Expand High Fidelity (Roche)]. All constructs were verified by sequencing both DNA strands.

Cell Culture and Transfections. Adherent HEK293T cells were cultured in DMEM (Specialty Media/Millipore), supplemented with 10% FBS (HyClone), 2 mM L-glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin. See *SI Text* for details of stable cell line generation and transfection methods. Twenty-four hours after transfection, cells were dissociated by using TrypLE Express (Invitrogen) and plated in assay plates or cryopreserved in freezing medium (Specialty Media/Millipore).

Reporter Gene Assays. Growing or cryopreserved cells were plated in white 96-well assay plates at 10,000–20,000 cells per well or in 384-well assay plates at 6,000 cells per well in DMEM, supplemented with 10% FBS, glutamine, penicillin, and streptomycin. For Tango assays with chemerin peptides, cells were plated instead in serum-free medium (Cambrex Biowhittaker Pro-293). For estrogen receptor Tango assays, cells were plated in phenol red-free DMEM without serum addition. Test agonists were added 0–6 h after plating. Test antagonists were added 15 min before addition of an EC_{80} dose of agonist. Cells were cultured for 8–24 h before measuring reporter gene activity. β-Galactosidase activity was determined by using the Luminescent Beta-Galactosidase Detection Kit II (BD Biosciences), and luciferase activity was determined by using the Bright-Glo luciferase assay system (Promega), using the manufacturers' protocols. Luminescence was measured by using an LMAX II-384 (Molecular Devices), MicroBeta Jet 1450 (Wallac/Perkin–Elmer), or Victor3 (Perkin–Elmer) luminometer.

Calcium Imaging Assays. Unmodified GPR1 and CMKLR1 expression constructs or an AVPR2-tTA fusion construct were transiently transfected into adherent HEK293T cells, together with a $G_{\alpha-15}$ expression construct at a ratio of 4:1 receptor:G protein, using Fugene 6 (Roche). After a 24-h incubation period, cells were washed and loaded with a calcium-sensitive fluorescent dye (FLIPR Calcium 3 assay kit, Molecular Devices), following the vendor's protocol. Fluorescence imaging was performed with a Zeiss inverted fluorescence microscope equipped with a Lambda DG-4 wavelength switcher (Sutter Instruments) and an Orca II digital camera (Hamamatsu). Data were analyzed using MetaFluor imaging software (Molecular Devices).

Tissue Extracts. For κOPR assays, bovine hypothalamus extract was prepared by using a boiling water/acid extraction method. See *SI Text* for details.

Radioligand-Binding Assays. A 9-aa peptide (YFPGQFAFS), corresponding to amino acid residues 149–157 of full-length chemerin, was radioiodinated on tyrosine (Phoenix Pharmaceuticals). See *SI Text* for details of binding studies.

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