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A proximity based general method for identification of ligand and receptor interactions in living cells

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ABSTRACT

Autocrine based selections from intracellular combinatorial antibody and peptide libraries have proven to be a powerful method for selection of agonists and identification of new therapeutic targets. However, success requires a case-by-case construction of a robust selection system which is a process that can be time consuming and expensive. Here we report a general system that takes advantage of the chemical rate acceleration caused by approximation of a membrane tethered ligand and its receptor. The system uses an artificial signal transduction and is, thus, agnostic to the endogenous signal transduction of the receptor–ligand system. This method allows analysis of receptor–ligand interactions and selection of molecules from large libraries that interact with receptors when they are in their natural milieu.

1. Introduction

Intracellular combinatorial libraries offer great promise for generation of novel receptor agonists that perturb cellular physiology. However, there is a need for a general-purpose reporter system that is quantitative over a large dynamic range. We reasoned that one could take advantage of proximity effects, operating through effective molarity, to achieve this goal. The concept of effective molarity relates to the kinetic advantage of an intramolecular reaction relative to its intermolecular counterpart [1,2].

To understand the central idea, it is useful to contrast how reporter systems are constructed nowadays versus what could be achieved if one had a general way to harness effective molarity. Today, selection of agonist ligands in solution generally depends on interaction with receptors to change the receptor state so that they activate intracellular signal transductions linked to expression of the reporter gene. Thus, each reporter system requires a separate construct whose nature depends on specific information about the cell and molecular biology of the system in question. In contrast, proximity-based reporter systems could rely solely on the extent to which two molecules interact and would be independent of the specific requirements of the natural pathways. Instead, the system could be linked to a universal reporting system.

Two recent advances in our laboratory have put us in position to test these ideas. The first is the advent of large intracellular combinatorial libraries in which as many as 1.0×10^8 different antibodies or peptides encoded by lentivirus are expressed in cells that also express the receptor of interest [3–8]. Since each cell has both the receptor and a different potential agonist, each cell becomes a reaction compartment unto itself. The second advance is that potential agonist and receptor are co-localized in cellular compartments [4,5]. This allows the interacting molecules to sequester and reach a higher effective molarity than would be achieved had they interacted in bulk solution. Arguably, the most important case is when the receptor and potential agonist are co-localized in the plasma membrane [5].

The construction of a general reporter system would take advantage of this molecular interaction in the plasma membrane and report on its occurrence. To achieve this, we employed an enzyme-substrate system that is similar to one used in the Arrestin-GPCR reporter system [9]. However, we constructed it in a way that would be generalizable to any molecular interaction in the plasma membrane [5–9]. In our system the ligand and receptor are anchored and co-localized in the plasma membrane, and because of this we could engineer them so that each carries only one component of the enzyme-substrate reporter system. When the interaction between a receptor and its ligand cause them to approximate, a proteolytic reaction releases transcription factor that enters the nucleus to activate expression of the reporter gene. Two advantages of this system are that molecular interactions of large numbers of potential ligands with receptor are determined in the physiologically relevant milieu of cell and one can use this method to search for ligands to receptors where the mechanism of signal transduction is unknown.

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2. Materials and methods

2.1. Cell lines

HEK293 (ATCC cat No. CRL-1573) or HEK293T (ATCC cat No. CRL-3216) cells were maintained in DMEM containing 10% (vol/vol) FBS, penicillin and streptomycin and transfected using Lipofectamine 2000 (Life technologies). Mammalian cell antibiotics geneticin and hygromycin were from Invivogen. Luciferase Assay Reagent was obtained from Promega (E1500).

2.2. Construction of lentiviral vectors of ligand-transmembrane-tobacco etch virus (TEV) protease

The ligand was fused to the N-terminus of the Platelet-derived growth factor receptor (PDGFR) transmembrane domain (TM; amino acids 514–561) while the TEV protease was fused to the C-terminus of the transmembrane domain. Different numbers of GGGGS linker sequences were added between the ligand and transmembrane domain. The ligand-transmembrane-TEV was introduced into the lentiviral vector under control of the Ubiquitin C (UBC) promoter.

2.3. Construction of vectors of receptor-TEV cleavage site-GAL4-VP16

The yeast transcription activator protein GAL4 DNA-binding domain was fused to the Herpes simplex virus VP16 C-terminal activation domain to generate an artificial transcription factor that is orthogonal to mammalian cells. The coding region of Thrombopoietin receptor(TpoR), Glucagon-like peptide 1 receptor (GLP1R) or C-X-C chemokine receptor type 4 (CXCR-4) was followed by the different TEV protease cleavage sites and the GAL4-VP16 transcription factor. The assembled gene was cloned into pcDNA5 with Cytomegalovirus (CMV) promoter. The vector has a geneticin selection cassette, for the generation of stable cell lines.

2.4. Upstream activator sequence reporter gene vector (UAS)

The UAS-reporter gene vector contains 9 repeats of GAL4 UAS and the adenovirus late promoter. This sequence drives the transcription of the luciferase luc2P or tdTomato reporter gene in response to binding of GAL4-VP16 transcription factor. The vector has a hygromycin selection cassette, for the generation of stable cell lines.

2.5. Generation of stable reporter cell line

Stable cell lines were generated by transfection HEK293 cells with UAS-reporter gene vector first using Lipofectamine. The transfected cells were selected in 200 μg/mL hygromycin. After two weeks, the selected cells were harvested and transfected with the specific receptor-TEV cleavage site-GAL4-VP16 transcription factor vector. The cells were selected in medium containing 800 μg/mL geneticin and 100 μg/mL hygromycin for two weeks.

2.6. Package of lentivirus

Virus was produced in HEK293T cells by cotransfection of lentiviral vectors with the pCMVD8.9 and pVSVg viral packaging vectors at ratio of 1:1:1. Supernatants containing virus were collected 48 h post transfection and filtered through a 0.22 μm membrane filter unit (Millipore). The titer of lentivirus prep was determined using Lenti-X p24 ELISA (Clontech).

2.7. Proximity based reaction assay

The stable cell line harboring both the UAS-reporter gene and receptor-cleavage site-transcription factor was plated in a 96 well plate at 20,000 cells per well. Cells were infected by lentivirus carrying the ligand-PDGFR TM-TEV protease to achieve Multiplicity of infection (MOI) equal to 1. Cells were cultured for 24–72 h before measuring the reporter gene activity. Luciferase activity was determined by using a luciferase assay system (Promega) while expression of tdTomato was observed by fluorescent microscopy.

3. Results

3.1. System construction

We constructed an enzyme-substrate system where the proteolytic reaction is inefficient so that at basal concentrations it proceeds slowly. We used the TEV protease and a highly specific cleavage site. The protease was added to the C-terminus of the PDGFR TM domain of the membrane tethered ligand constructs (Fig. 1). The cleavage site was located between the cytoplasmic portion of the receptor and an artificial transcription factor. After cleavage, the transcription factor was released and activated the expression of a reporter gene (Fig. 1). Significantly, because the potential ligand and its receptor are both anchored in the plasma membrane, any interaction between them can cause approximation and activation of the intracellular reactants to which they are linked to and facilitate the generation of a signal.

In this system the effective molarity operates on two levels. First, due to the receptors and ligands being co-localized in the plasma membrane there is a powerful effect of sequestration that favors interaction. Secondly, when these sequestered molecules interact they bring the enzyme and substrate components together, thereby greatly increasing the effective molarity of the signaling components of the system. It is important to note that this system only requires molecular interaction in the membrane and is largely independent of any specialized effects such as receptor conformational changes resulting from the interaction.

3.2. Concept validation

To validate the method, we studied both single and multiple pass membrane proteins. For the single pass receptors, we studied the natural thrombopoietin (TPO) as well as scFv (single-chain variable fragment) to the TpoR [7]. For the multiple pass membrane receptors, we studied the recognition of natural peptide Exendin-4 by GLP1R.

The hormone TPO or the TPOR binding antibody scFv, 3D9 or 14F12 [7], was displayed on the cell surface by fusing them to the N-terminus of the PDGFR TM domain. An irrelevant antibody was used as negative control. We reported that membrane tethered 3D9-Fc protein can activate the TpoR sie-bla reporter cell line [5], and, thus, it was used as a positive control. The antibodies or authentic TPO displayed at the plasma membrane increased the transcription factor mediated signal relative to the irrelevant antibody control (Fig. 2). An orthogonal experiment showed that display of TpoR antibodies or TPO did not increase the signal in cells bearing an unrelated receptor such as GLP1R. We also tested the effect of the linker length between the ligand and TM domain. TPO tethered to the plasma membrane by 3–10 tandem repeats of G-G-G-G-S generated similar signals, whereas in the case of scFv, longer distance resulted in higher activity (Fig. 2). Thus, the longer flexible linker may be of more general utility.

We next studied whether the method could be used for multiple pass membrane proteins. In a previous study, we found that

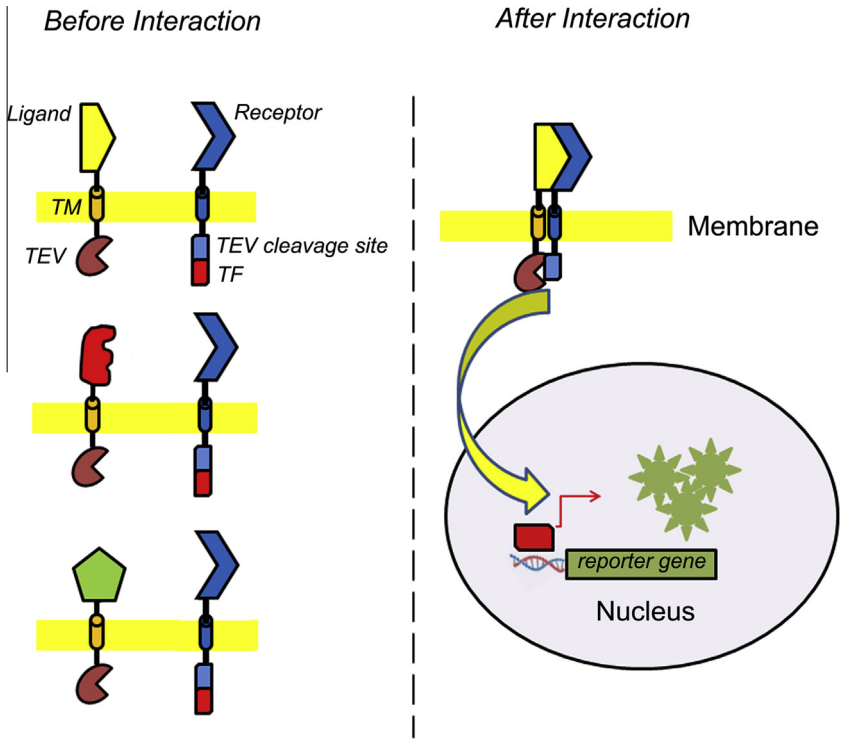

Fig. 1. Schematic of the proximity based method. Membrane tethered peptide or antibody ligand is coupled to the TEV protease on their intracellular sides. Secondly, membrane receptor has a TEV cleavage site and an artificial transcription factor added to its intracellular side. Interaction of co-located receptor and ligand approximates the TEV and TEV recognition site, which releases the transcription factor. The released transcription factor enters the cell nucleus and activates expression of the reporter gene.

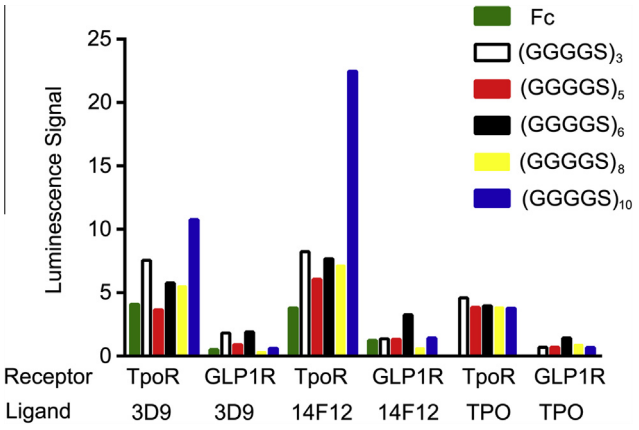

Fig. 2. Proximity based method to probe the interaction between TpoR and its ligands. Stable cell lines had the luciferase reporter gene under control of UAS and TpoR-TEV cleavage site-transcription factor. Cells were transduced with lentivirus to display TPO or the TpoR binding antibodies 3D9 or 14F12 on cell plasma membrane. Different linkers including human IgG1 Fc or 3,5,6,8 or 10 tandem repeats of GGGGS were placed between the ligand and PDGFR TM domain. Luciferase activities were measured 2 days post infection. The cell line harboring GLP1R-TEV cleavage site-transcription factor construct was used as negative control. The luminescence signals were normalized by dividing it by signals from irrelevant antibodies with the same linker type and length.

membrane tethered Exendin-4 can activate the GLP1R. Thus, we used GLP1R and Exendin-4 as a receptor–ligand pair for this study. GLP1R belongs to the Class B GPCR receptors. The binding model for a Class B receptor ligand envisions two sets of interactions: the C-terminal part of the peptide ligand interacts with the N-terminal ectodomain of the receptor and the N-terminus of the ligand interacts with transmembrane helices and connecting loops of the receptor.

We fused the full length GLP1R to the transcription factor. The membrane tethered Exendin-4 was fused to the TEV protease at its C-terminal intracellular domain. The interaction of the coexpressed receptor and Exendin-4 resulted in significantly increased signal starting from day 1 and continuing through day 3 post infections (Fig. 3A). We tested whether the C-terminal tail length of the GLP1R had an effect on the signal to noise (S/N) ratio. Previous studies demonstrated that the C-terminus could be truncated as far as the C-terminal Leu422 residue, without affecting GLP-1 potency or expression of the receptor [10]. We fused amino acids 1–426 of the GLP1R to the releasable transcription factor. The construct with the truncated receptor generated higher S/N ratio (Fig. 3A). As further controls, we studied non-specific peptide ligands of similar lengths. Non-specific peptides such as Vc1.1, which is a blocker of the nicotinicacetylcholine receptor (nAChR)/metabotropic GABA receptor (GABA) resulted in increased background (Fig. 3B). To reduce the noise while maintaining the strength of the signal, four TEV substrate sequences cleaved by TEV with different efficiencies were tested. TEV protease recognizes a linear epitope of the general form E-X-X-Y-X-Q-(G/S), with cleavage occurring between Q and G or Q and S [11]. The most efficient substrate was ENLYFQS (TEV1), which we used in all the experiments above.

We hypothesized that the substrate sequences that were less efficiently cleaved would generate lower background, while maintaining the strength of the signal. A systematic study demonstrated that many different amino acids could be accommodated in different positions with varying cleavage efficiencies (Kcat/km). We compared the S/N ratio of four different sequences ENLYFQS (TEV1), ENFYFQS (TEV 2), ENLYYQS (TEV 3) and ENLFFQS (TEV 4). Their respective kcats/km were 4.51 ± 0.65; 0.024 ± 0.001; 0.056 ± 0.005; 0.35 ± 0.041 mM^{-1} s^{-1}[11,12].

We monitored the effect of employing these sequences on reaction kinetics in the proximity based signaling system. The poor TEV2 substrate resulted in the highest S/N ratio even after 3 days post infection, while the relatively efficient TEV1 and TEV4 sub-

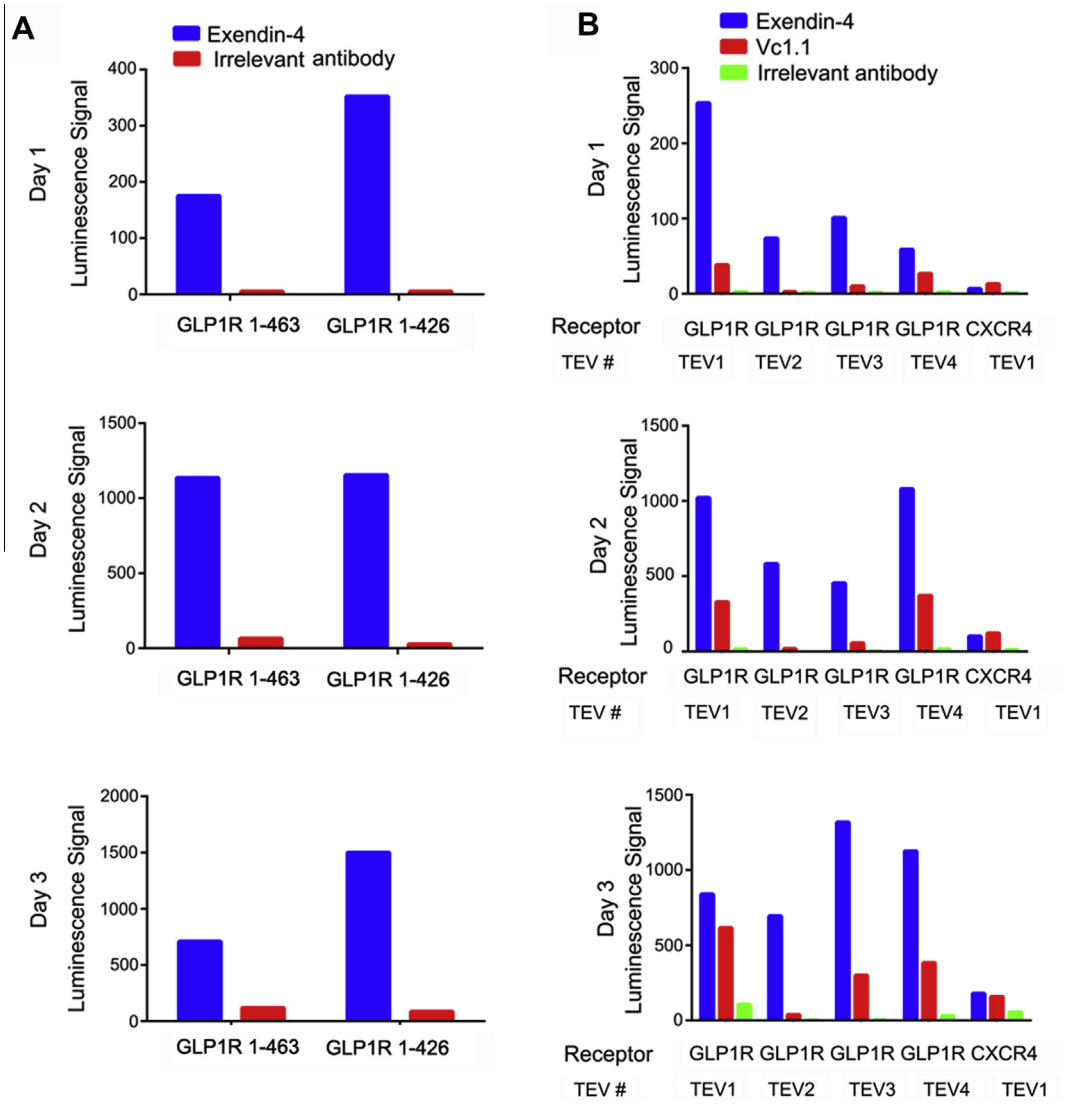

Fig. 3. Proximity based reaction for GPCR receptor GLP1R. (A) Full length GLP1R (1–463) and truncated GLP1R (1–426) were coupled to a TEV cleavage site and the transcription factor at their C-terminus. Stable cell lines harboring a luciferase reporter gene under control of UAS and different GLP1R constructs were established. Cells were transduced with lentivirus to display Exendin-4 on cell plasma membrane. Luciferase activities were measured 1–3 days post infection. The signals of infected cells were divided by the signals of corresponding uninfected cells to give the signal-to-noise ratio (S/N). (B) Enhancing S/N by varying TEV cleavage site. Four TEV cleavage sites that are cleaved with varying efficiencies were used for the GLP1R (1–426) constructions. Stable cell lines containing the GLP1R constructions were transduced with lentivirus encoding Exendin-4 as positive construct, or Vc1.1 or irrelevant antibodies as negative controls. Luciferase activities were measured 1–3 days post infection. The signals of infected cells were divided by the signals of corresponding uninfected cells.

strates resulted in high levels of background starting from day 2 (Fig. 3B). Thus, TEV2 is presently the optimal cleavage site for proximity enhanced reaction.

For the proximity based reaction to be used generally for the selection of receptor ligands, reporter genes such as fluorescent protein can be ideal when coupled with fluorescence activated cell sorting. In addition, fluorescence proteins are useful for dynamic cell assays in living cells, enabling the assessment of a signaling activity over time. We used tdTomato fluorescent protein as a reporter gene and observed expression of fluorescent protein. Consistent with the results with the luciferase reporter gene, membrane tethered Exendin-4 induced a high percentage of cells expressing fluorescent protein. Notably, weakened TEV2 and TEV3 cleavage sites exhibited much lower background (Fig. 4).

4. Discussions

Proximity effects, manifested as effective molarities, regulate much of the biology of the cell. Such proximity effects can be achieved by compartmentalization, adherence to scaffolding molecules, or sequestration in enzyme active sites. In a practical sense, one can think of effective molarity in terms of the concentration of a reactant that would be required for an intermolecular reaction to achieve the same rate as the intramolecular version of the same reaction. Effective molarities can be as large as hundreds to 10^{10} molar [1,2]. Our goal for the relevant experiment was to take advantage of this physical parameter to generate a robust and general reporter system that only functions when the ligand and receptor interact.

A critical feature when designing constructs where proteins are displayed in the plasma membrane is the linker sequence that connects the antibody or peptide to the transmembrane domain. For example, most of the membrane-tethered toxins generated thus far contain a stretch of 20 alternating glycine and asparagine residues. This linker provides the rotational flexibility and distance necessary for the peptide toxin to bind to its cognate ion channel [13]. However, we find that there is no general rule concerning linker length and it should be optimized for each case.

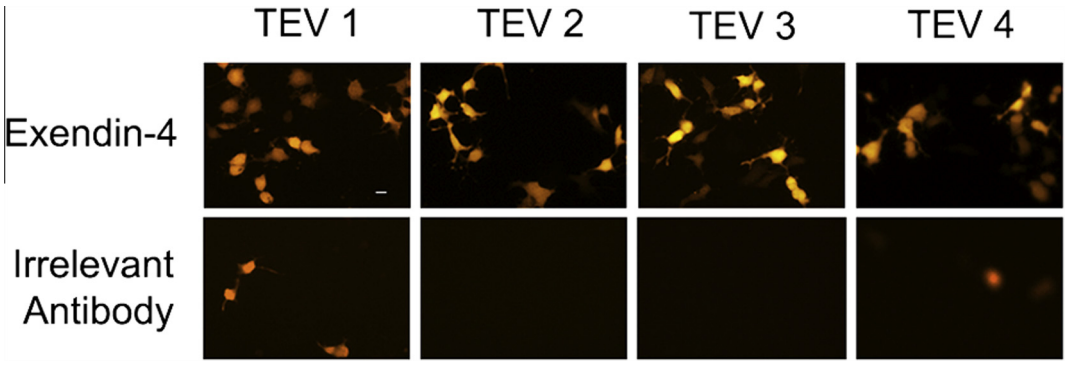

Fig. 4. Fluorescent proteins as reporter genes. Stable cell lines containing the tdTomato fluorescent protein reporter gene under control of UAS and GLP1R constructions were studied. The cells were transduced with lentivirus containing Exendin-4 as a positive construct or irrelevant antibodies as negative controls. Expression of fluorescent protein was monitored 2 days post infection.

We generated a system that is agnostic to the choice of the reporter gene or the assay format. This is important because each system has its own advantages and disadvantages. For example, the luciferase reporter system is highly sensitive and ensures the wide dynamic range of the assay, making it ideal for high-throughput screening studies. However, luciferase assays are end point assays. On the other hand, GFP or other fluorescent proteins are ideal for imaging or fluorescence activated cell sorting, making them suitable for selection [3–9].

Finally, our method should not be limited to the particular enzyme-substrate system used here. Perhaps conditional sensitive catalysis can be employed to monitor associated agonist dependent cellular changes such as pH or cation concentrations. This would add another level of specificity to the selections. Protein fragment complementation based assays may also be used to induce active enzyme formation, which works rapidly at the cell surface and thus avoids uncertainties related to interplay with genes in the nucleus.

In conclusion, this work provides a new and universal approach to study receptor–ligand interactions in living cells. It operates through an increased effective molarity when the proximity of receptor and membrane tethered ligand is enforced. This method allows analysis of receptor–ligand interactions when they are in their natural milieu and in situations where the downstream signaling is unknown.

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