The application of EFC technology to study forward trafficking of GPCRs

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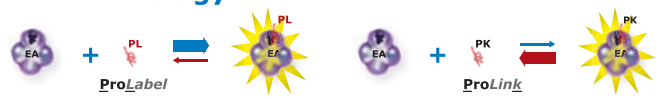
Abstract

DiscoveRx® has pioneered a novel enzyme complementation system to monitor cellular events such as protein translocation, protein interactions, and degradation in an HTS friendly, cell-based format. This unique technology provide a basis for the generation of novel cellular assays for intractable targets, and simplify the detection of cellular signaling events using a simple one-step chemiluminescent protocol. We have recently applied this technology to monitoring receptor trafficking along biosynthetic routes, in response to pharmacochaperone activity, as well as internalization in response to agonist activation. In this work, these tools were applied to defining pharmacochaperone activity on a mutant B2AR as a proof-of-principle for the detection of molecules that would facilitate folding and trafficking of receptors to the cell surface.

EFC Technology

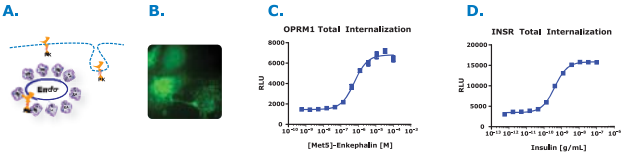


PathHunter® assays utilize the well established Enzyme Fragment Complementation (EFC) technology pioneered by DiscoveRx. This robust technology is based on the complementation of two inactive enzyme fragments, EA which encodes the majority of the enzyme, and a small peptide termed ProLabel. The ProLabel tag has a high affinity for EA whereas the ProLink peptide has been engineered to have a weakened complementation ability. This allows for mass-action driven complementation. In these translocation systems, the EA is localized to a specific subcellular compartment and the protein if interest is tagged with the ProLink tag. An increase in concentration of the ProLink tag in the vicinity of EA results in enhanced complementation.

Protocol and Detection

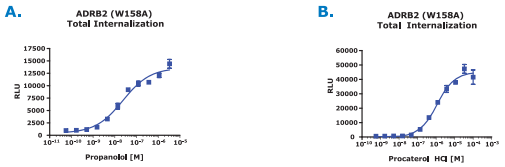
The cells are seeded in 384-well plates and incubated overnight in assay media. The following day the cells are treated with the indicated agonists. After compound incubation, the PathHunter® Detection reagents are added as a single mix-and-read reagent which includes a lysis buffer and substrate mixture optimized for enzyme complementation. After a short incubation, the PathHunter® chemiluminescent signal can be detected using any standard luminometric plate reader.

Endocytosis



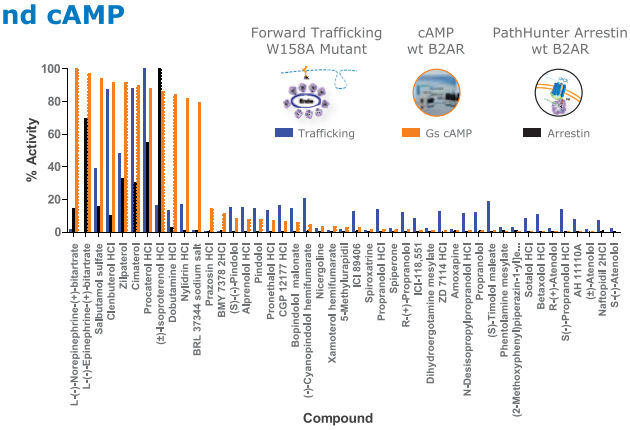
The PathHunter Total Internalization Assay is a cell-based assay designed to detect transmembrane receptors internalized in endosomes. (A) Receptors tagged with ProLink (PK) on the intracellular tail are expressed in a parental cell line expressing EA localized to endosomes. The internalization of PK tagged receptors co-localizes PK and EA on the endosomes and generates active enzyme. (B) Endosome localization of PK tagged receptors is revealed by an antibody to PK. (C) PK tagged OPRM1 and (D) the Insulin receptor are expressed in the PathHunter Total Internalization parental cell line. Exposure to agonist induces receptor internalization and an increase in EFC activity.

Pharmacochaperones



(A) ADRB2(W158A)-PK, a ProLink tagged variant of the β2-adrenergic receptor known to get trapped in the ER, is expressed in the Total Internalization parental cell line. The cell line is exposed to propranolol, a known ADRB2 antagonist. Propranolol stabilizes the misfolded ADRB2(W158A), which then traffics to the membrane. Constitutive recycling of the cell membrane leads to internalization of the receptor. (B) The agonist procaterol also chaperones the mutant receptor to the membrane.

Comparison of Pharmacochaperone, Arrestin, and cAMP



The Adrenergic screen was repeated on the wt B2AR in two signaling formats 1) cAMP accumulation using the HitHunter cAMP detection kit 2) the PathHunter Arrestin assay. The cAMP compound incubation time was 30 min. and for the arrestin assay the compound incubation time was 90 minutes. The results from each of these screens were normalized to the largest value from each screen (set equal to 100%) and the DMSO negative controls (0%). The values were sorted such that the compounds positive in cAMP (agonists) are displayed on the left hand side of the graph. The trafficking assay shows a wider range of hits since this assay detects agonsits as well as antagonists.

Hit Profiles

				Trafficking	Arrestin	cAMP	CHO control
Well	Compound	Compound class	[uM]	S/B	S/B	S/B	S/B
C3	Naftopidil 2HCl	Alpha 1 adrenergic antagonist.	10	6.8	1.0	1.0	1.0
E6	(S)-Timolol maleate	Beta 1 adrenergic antagonist.	10	15.6	0.9	1.2	1.3
E9	Procaterol HCl	**Beta 2 adrenergic agonist.**	10	78.2	21.3	15.2	1.2
G6	(-)-Cyanopindolol hemifumarate	**Beta adrenergic antagonist.**	10	17.1	1.0	1.8	1.2
G10	Propranolol HCl	**Beta adrenergic antagonist.**	10	12.0	0.9	1.3	1.0
Well	Compound	Compound class	[uM]	S/B	S/B	S/B	S/B
E11	BRL 37344 sodium salt	Beta 3 adrenergic agonist.	10	2.2	1.1	13.8	1.2
F7	Dobutamine HCl	**Beta 1 and Beta 2 adrenergic agonist.**	10	11.3	1.9	14.6	1.1
H9	Nylidrin HCl	**Beta adrenergic agonist.**	10	14.2	1.1	14.2	1.1

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F7	Dobutamine HCl	**Beta 1 and Beta 2 adrenergic agonist.**	10	11.3	1.9	14.6	1.1
H9	Nylidrin HCl	**Beta adrenergic agonist.**	10	14.2	1.1	14.2	1.1

Examples of compounds showing different phenotypes in the comparison screen. The top half of the table shows an example of the behavior of a typical agonist (green) which is positive in each assay tested. The far right column is the CHO negative control that was tested for cAMP accumulation in response to each of the ligands. The red values indicate the response of known antagonists. These compounds fail to cause signal in the arrestin and cAMP assays as expected, but bind the receptor and are capable of scoring in the trafficking assay. Three compounds were obtained from the screen that exhibited a full agonist cAMP response but were negative or only weakly positive response in the arrestin assay, these are shown in the table above. Further analysis of these compounds in the trafficking assay indicate that at least two of them, the dobutamine and the nylidrin are able to bind the B2AR and traffic it to the cell surface providing a positive measure of binding. These results are in good agreement with the existing literature on nylidrin and dobutamine where dobutamine exhibits weak partial agonist activity (however due to receptor reserve effects appears as a full agonist in cAMP), and only weak arrestin recruitment (S. Rajagopal et al. Quantifying Ligand Bias at Seven-Transmembrane Receptors. Mol Pharm 2011).

Summary

In this work we have adapted our internalization system to monitoring the trafficking of mutant GPCRs to the cell surface and subsequently to the endosomal compartment. Using a well-characterized mutant of B2AR we show that using a simple PathHunter chemiluminescent detection method we can monitor the ability of compounds to traffic the receptor to the cell surface. By comparing the results obtained with the trafficking assay and the more traditional arrestin and cAMP approaches we observed that the trafficking assay was able to identify both agonists and antagonists out of a small focused library with high signal to noise ratios. These attributes indicate that the PathHunter trafficking system could be applied to the identification of novel trafficking modulators of known and mutant GPCRs. Moreover this may represent an interesting tool for receptor deorphanization that has the potential to detect binding compounds without the need for a radioligand.

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