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A pH-Sensitive Fluor, CypHer™ 5, Used to Monitor Agonist-Induced G Protein-Coupled Receptor Internalization in Live Cells

BioTechniques 33:1152-1157 (November 2002)

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ABSTRACT

G protein-coupled receptors (GPCRs) are the largest family of proteins involved in transmembrane signal transduction and are actively studied because of their suitability as therapeutic small-molecule drug targets. Agonist activation of GPCRs almost invariably results in the receptor being desensitized. One of the key events in receptor desensitization is the sequestration of the receptor from the cell surface into acidic intracellular endosomes. Therefore, a convenient, generic, and noninvasive monitor of this process is desirable.

A novel, pH-sensitive, red-excited fluorescent dye, CypHer™ 5, was synthesized. This dye is non-fluorescent at neutral pH and is fluorescent at acidic pH. Anti-epitope antibodies labeled with this dye were internalized in an agonist concentration- and time-dependent manner, following binding on live cells to a range of GPCRs that had been modified to incorporate the epitope tags in their extracellular N-terminal domain. This resulted in a large signal increase over background.

When protonated, the red fluorescence of CypHer 5 provides a generic reagent suitable for monitoring the internalization of GPCRs into acidic vesicles. This approach should be amenable to the study of many other classes of cell surface receptors that also internalize following stimulation.

INTRODUCTION

G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors in the human genome. Because a significant percentage of current medicines designed to alleviate diseases as widespread as cancer, osteoporosis, hypertension, and inflammation act at these receptors, they have been very actively studied and are considered targets for further, novel drug design (14,17).

GPCRs consist of seven transmembrane-spanning helices linked by intracellular and extracellular loops (3,12, 13). In all cases, the N-terminus is extracellular and the C-terminus is intracellular. Agonist stimulation of these receptors activates a range of second messenger pathways via the family of heterotrimeric G-proteins. Response to a GPCR agonist wanes over time. This process is described as desensitization. Such desensitization of GPCRs usually occurs within seconds to minutes after exposure to agonist. A common mechanism for desensitization is phosphorylation of the receptor, on serine and/or threonine residues, by members of a family of G-protein receptor kinases. This allows high-affinity binding of arrestin. Arrestins uncouple GPCRs from their G-proteins, resulting in attenuation of second messenger regulation (3,5,13).

Agonist activation also promotes a rapid sequestration of the GPCR away from the plasma membrane into the endosomal pathway, from which it is then sorted either into recycling endosomes or into lysosomes for proteolysis. Prolonged agonist activation of a GPCR results in its down-regulation. Down-regulation is the reduction of the total population of the GPCR in the cell (12,15).

Fluorescent techniques to monitor the cellular distribution of GPCRs and their interactions with other proteins are becoming increasingly popular (6).

Furthermore, several GPCRs have had the GFP from *Aequorea victoria* appended to their C-terminal tail. Following expression of such constructs in mammalian cells, agonist-induced receptor internalization has been studied using standard microscopy techniques (2,7). Incorporation of epitope tags into the extracellular N-terminal sequence of GPCRs has also become popular to allow the detection of the GPCR via immunoprecipitation or immunoblotting (4,10,11,16). This has also enabled the application of immunocytochemical techniques to study GPCR distribution (8,9). However, because of the need to subsequently identify the primary epitope tag antibody, this has been primarily restricted to fixed cells.

An assay has been developed that relies on the intrinsic ability of GPCRs and potentially other transmembrane receptors to internalize into acidic vesicles upon agonist stimulation. This relies on a novel, red-excited, pH-sensitive cyanine dye, designated CypHer™ 5 (Amersham Biosciences, Cardiff, UK). Therefore, it is ideally suited to report movement of a receptor from the cell surface into internal acidic endosomes. This study examines the use of CypHer 5-labeled anti-epitope tag antibodies to measure the agonist activation of a range of GPCRs.

MATERIALS AND METHODS

We used the following materials: FuGENE™ 6 (Roche Applied Science); Kreb's Ringer Buffer (KRB; 120 mM NaCl, 25 mM HEPES, pH 7.4, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, and 5 M NaOH); Thyrotropin Releasing Hormone (TRH) (Sigma); MAb Trap Kit (Amersham Biosciences); 1 M sodium hydrogen carbonate buffer, pH 8.3; PBS, pH 7.4; VSV-G clone P5D4 mouse ascites fluid (Sigma); CypHer 5 NHS ester (Amersham Biosciences); and IgG-free BSA (Sigma).

Construction of VSV-TRHR-1 and VSV-TRHR-1-GFP Gene Fusion Constructs

The construction of VSV-TRHR-1 (thyrotropin releasing hormone receptor 1) has been described previously (10). To obtain a VSV-TRHR-1-GFP gene fusion protein, the coding sequence of a modified triGFP (Amersham Biosciences) was amplified by PCR. A *Hin*dIII restriction site and a two-amino-acid spacer (Gly-Ala) were introduced in front of the initiator methionine of GFP using the amino terminal primer 5′-GAGAAGCTTGGAGCTATGGGATC-CAAAGGAGAAGAACTTTTCACT-3′. An *Xba*I restriction site was introduced after the stop codon in GFP using the carboxyl terminal primer 5′-TGCTCTA-GATTAACCGGTTTTGTATAGTTCA-TCC-3′. The VSV-TRHR-1 construct was digested to excise the carboxyl terminal *Hin*dIII/*Xba*I fragment. This was replaced with the *Hin*dIII-GFP-*Xba*I fragment, resulting in the construct VSV-TRHR-1-GFP. Both VSV-TRHR-1 and VSV-TRHR-1-GFP were digested with the restriction enzymes *Nhe*I and *Xho*I, and their 3′ recessed ends were filled in using DNA polymerase I, large (Klenow) fragment under standard conditions. Both fragments were inserted into the *Eco*RV restriction site of the cytomegalovirus-driven expression vector pCORON 2100 (Amersham Biosciences). The sequence integrity of both clones was confirmed before expression and ligand binding studies.

Cell Culture and Generation of Stable Cell Lines

Chinese hamster ovary (CHO) cells were grown in F12 Ham's medium supplemented with 2 mM glutamine, penicillin/streptomycin, and 10% FCS. Cells were seeded onto 6-well dishes and transfected when they were approximately 50% confluent, according to the manufacturer's instructions. Cells were transfected with 1.5 µg plasmid DNA and increasing amounts (2–6 µL) of FuGENE 6 in serum-free media. The DNA/FuGENE 6 complex was placed on the cells and left for 5–6 h. It was then removed and replaced with serum-containing media. Forty-eight hours after transfection, the cells were incubated with growth media containing 1 mg/mL G418. A control set of CHO cells was incubated with the same media to ensure that the nonresistant cells died. After cell death, the wells were trypsinized and seeded onto a 300-mm petri dish. Colonies were picked from these dishes and grown to a 162-cm^2 tissue culture flask. Cells were harvested in PBS, homogenized using a Dounce homogenizer in 10 mM Tris, pH 7.4, 5 mM EDTA buffer, centrifuged at 10 000× *g* at 4°C for 25 min, and then assayed for protein content using the Pierce protein assay.

Antibody Labeling

An anti-IgG was purified from the anti-VSV-G monoclonal antibody mouse ascites fluid (Sigma) and anti-c-myc antibody (clone 9E10) using a HiTrap Protein G affinity column (Amersham Biosciences) according to the manufacturer's instructions. The concentration of antibody was determined by its UV absorbance at 280 nm. Anti-FLAG M2 antibody (Sigma) was supplied as the IgG fraction; however, before use, it was dialyzed using PBS, pH 7.4, before dye conjugation. Both the antibody and a 60 molar excess of CypHer 5 NHS were incubated in 100 mM sodium carbonate buffer, pH 8.3, in PBS for 45 min at room temperature in the dark. The CypHer 5-conjugated antibody was separated from free CypHer 5 by dialysis in PBS overnight at room temperature. The molar concentration of antibody and dye in the final sample was then calculated by measuring the absorbance of the labeled antibody at 280 and 480 nm. The mean number of dye molecules coupled to the antibody was then determined. The CypHer 5-labeled antibody was diluted to 0.5 mg/mL with PBS plus 0.1% BSA and stored frozen at -20°C.

Antibody Binding Experiments

Cells were seeded the day before use into 96-well plates (Packard Viewplates) and were used at 70%–80% confluency unless otherwise stated. Cells were washed twice with KRB at room temperature and then incubated with CypHer 5-labeled anti-VSV-G antibody at the appropriate concentration for 10 min at room temperature. After this time, TRH was added to the well, and the cells were imaged after 30 min incubation at 37°C using either the IN Cell Analyzer (Amersham Biosciences) or the Zeiss LSM410 confocal microscope.

RESULTS AND DISCUSSION

A novel, red-excited fluorescent, pH-sensitive cyanine dye was designed and synthesized. It was designated CypHer 5. CypHer 5 has an estimated pKa of 6.1 (1). It is 95% non-fluorescent at pH greater than 7.4 and is maximally fluorescent at pH less than 5.5. Other design criteria include adequate aqueous solubility and the inclusion of a reactive N-hydroxy succinimidyl ester to facilitate its conjugation to biomolecules (Figure 1). Agonist activation of GPCRs expressed in mammalian cells almost invariably results in their translocation from the plasma membrane into acidic vesicles of the endosomal pathway. A range of GPCRs was modified to include well-characterized epitope-tag sequences within the extracellular N-terminal segment. These were expressed in either CHO or Hek293 cells. Commercially available monoclonal antibodies to the various epitope tags were labeled with CypHer 5 and added to cells expressing each GPCR. Cells were then exposed to either agonist ligands for the GPCR or to vehicle and then were subsequently visualized using a high-throughput confocal imaging system able to measure molecular translocations or fluorescence changes in living cells at high resolution (IN Cell Analyzer).

For each of a FLAG™-tagged IP prostanoid receptor, a *c-myc*-tagged δ-opioid receptor and a VSV-tagged TRHR-1, CypHer 5-labeled antibodies were internalized in an agonist-specific manner, as determined by the appearance of bright red, intracellular, perinuclear fluorescent patches (Figure 2). These GPCRs were selected as examples of the subfamilies that function predominantly via the elevation of intracellular cAMP (IP prostanoid), inhibition of cAMP production (δ-opioid), and elevation of intracellular Ca^{2+} levels (TRHR-1) and the epitope tags as three of the most popular in current use. Counterstaining of cell nuclei with Hoechst 33342 indicated both that there were as many cells in the fields visualized in the absence of receptor agonists and that the internalized GPCRs were not translocated to the nucleus (Figure 2). A degree of fluorescent signal was observed in the absence of agonist. This internalization of CypHer 5 in the absence of agonist is likely to arise from a combination of the receptor's constitutive activity and constitutive endocytosis of nonspecifically cell surface bound CypHer 5-labeled antibody. The relative contribution of these two elements remains to be explored in detail. Receptor function and internalization was examined in more detail for the VSV-tagged TRHR-1 stably expressed in CHO cells.

VSV-TRHR-1 clone 11 expressed approximately 2.3 pmol receptor/mg cell homogenate with an apparent K_d of 49.2 nM for TRH (results not shown).

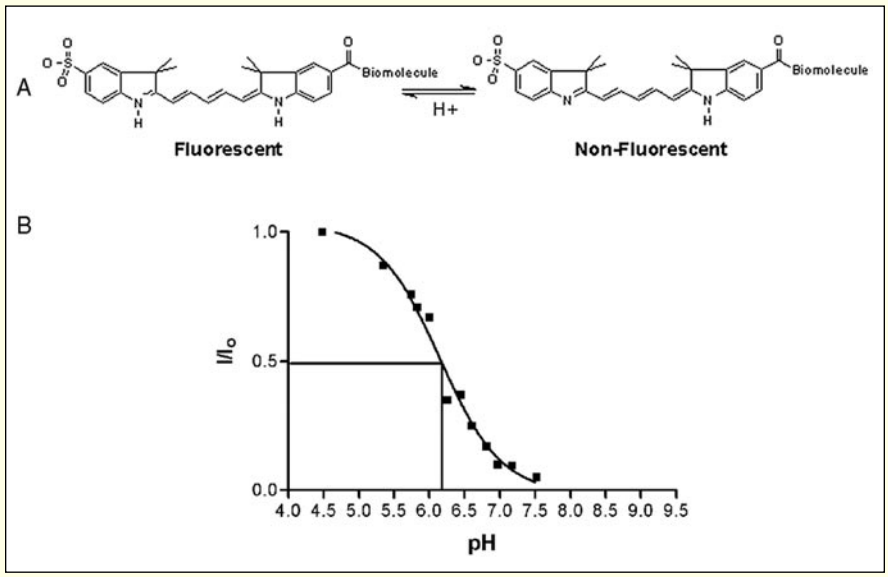

Figure 1. CypHer 5 construct and properties. (A) The structure of CypHer 5 in its protonated and non-protonated form. (B) Experimental data from which the pKa of CypHer 5 was calculated.

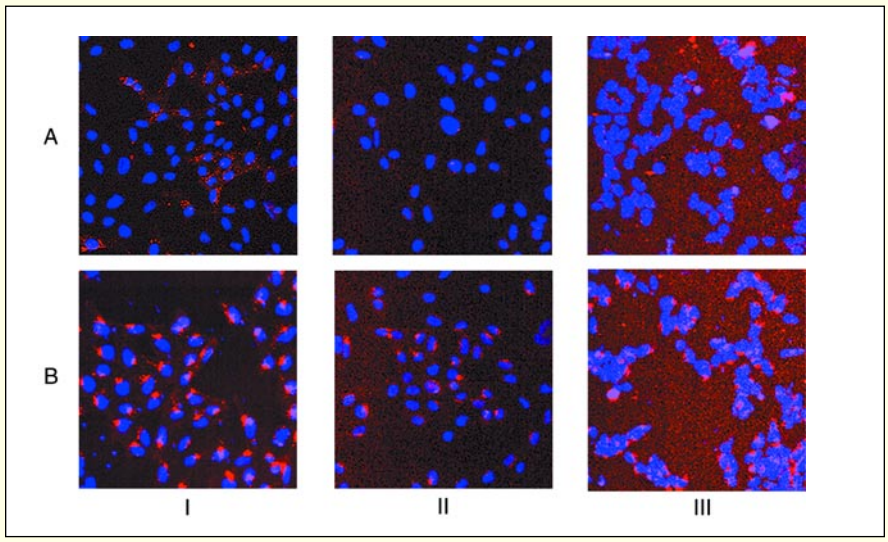

Figure 2. Agonist-mediated receptor internalization measured using CypHer 5-labeled antibodies. Cells expressing an epitope-tagged receptor were pre-incubated with 10 µg/mL CypHer 5-labeled antibody and 1 µM Hoechst 33342 nuclear stain for 10 min at room temperature, and then agonist was added for the appropriate length of time at 37°C. Cells were imaged with and without agonist. The top panel of images is without agonist (A), and the bottom panel is with agonist (B). (I) CHO cells expressing the VSV-TRHR-1 were pre-incubated with 10 µg/mL CypHer 5-labeled anti-VSV-G antibody and stimulated with 10 µM TRH for 30 min. (II) CHO cells expressing the c-myc δ-opioid receptor were pre-incubated with 10 µg/mL clone 9E10 antibody, labeled with CypHer 5, and stimulated with 1 µM D-Ala, D-Leu Enkephalin (DADLE) for 20 min. (III) Hek293 cells expressing an IP prostanoid receptor were pre-incubated with 10 µg/mL CypHer 5-labeled M2 anti-FLAG antibody and then stimulated with 1 µM iloprost for 45 min.

This is similar to the estimates for the binding affinity of this construct previously reported in Hek293 cells (2).

After pre-incubation with CypHer 5-labeled anti-VSV-G antibody, VSV-TRHR-1 CHO clone 11 cells were treated with various concentrations of TRH (0–10 μM) and then imaged on the IN Cell Analyzer. The images were analyzed using a proprietary algorithm that directly measures the presence and intensity of large perinuclear granules. A sigmoidal concentration-response curve with an EC_{50} value of 1.44 nM was observed (Figure 3A). To determine the time course of the receptor's internalization, VSV-TRHR-1 CHO clone 11 cells were treated with 10 μM TRH. The cells were then imaged using a Zeiss LSM410 confocal microscope at various time intervals. Very little red-excited fluorescence was observed at the time of 0 min. An increase in cell-associated fluorescence was observed as the receptor-associated dye entered acidic, perinuclear vesicles (Figure 3B). The signal reached a steady state within 20 min. No signal increase was observed in the absence of TRH or in cells not expressing the VSV-TRHR-1 (results not shown). Therefore, TRH induced concentration- and time-dependent internalization of CypHer 5-labeled anti-VSV-G antibody that was dependent on the presence of both agonist and receptor.

CONCLUSIONS

CypHer 5-labeled antibodies will track epitope-tagged GPCRs from the plasma membrane into the endocytic pathway. This approach can be widely used, as it is effective for three distinct but widely used epitopes, as well as for GPCRs that couple to each of the three major signal transduction pathways. A large dynamic range in signal was observed, which is due to the low background level fluorescence before receptor internalization. Therefore, CypHer 5 is a useful reagent to observe agonist occupancy of an epitope-tagged GPCR. To date, the GPCR family has been the most productive set of targets for small-molecule drug discovery. The natural ligands that activate a large number of GPCRs identified by genomic approaches remain to be elucidated. CypHer 5 technology is thus appropriate to examine ligand activation of such "orphan" GPCRs. CypHer 5 assay technology will likely provide a generic assay for GPCR internalization using a variety of epitope tags and should also be amenable to the study of native receptors to which high-affinity antibodies are available that bind to an external epitope. Non-GPCR cell surface receptors that internalize in response to external stimuli could also be studied.

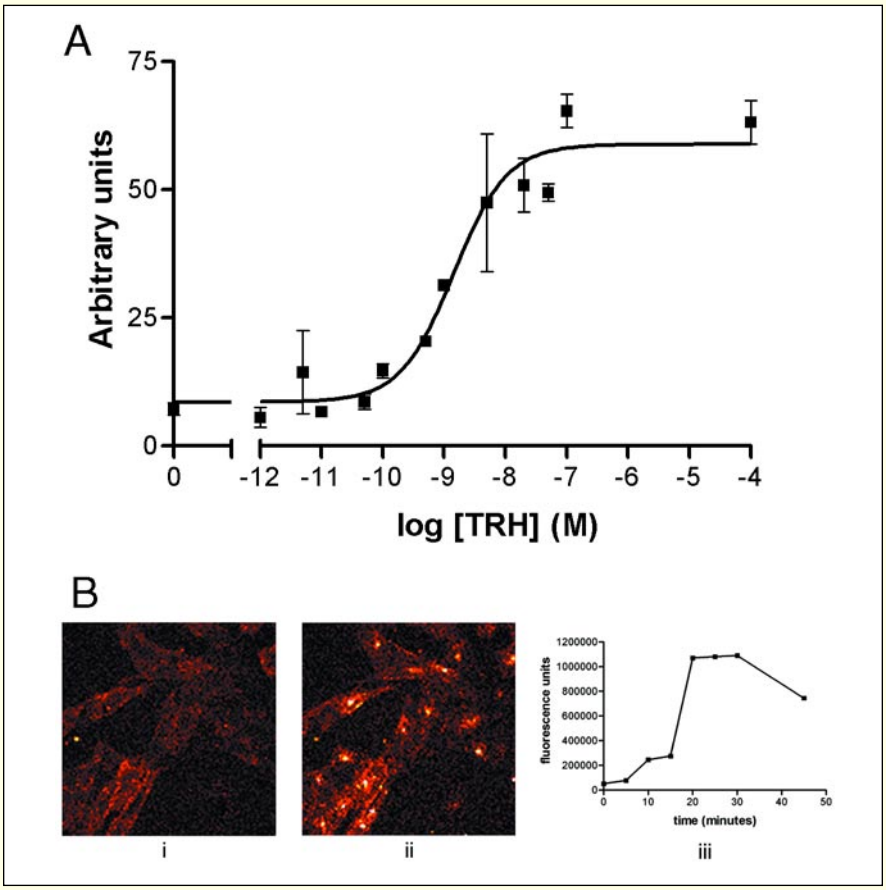

Figure 3. Characterization of agonist-mediated TRHR-1 internalization using CypHer 5-labeled anti-VSV-G antibodies. (A) The measurement of CypHer 5-labeled anti-VSV-G antibody internalization in response to increasing concentrations of TRH (0–10 µM) in CHO VSV-TRHR-1 11 cells. CHO VSV-TRHR-1 clone 11 cells were pre-incubated with 10 µg/mL CypHer anti-VSV-G antibody for 10 min at room temperature and then treated with increasing concentrations of TRH (0–10 µM) for 10 min at 37°C. The cells were then imaged on the IN Cell Analyzer. The data were quantified using a proprietary algorithm. The EC_{50} value for this experiment was approximately 1.44 nM. A further five experiments produced similar results (z-factor > 0.3). (B) A time course of 10 µM TRH treatment of CHO VSV-TRHR-1 11 cells, pre-incubated for 5 min at 37°C with CypHer5-anti-VSV-G antibody. Cells were seeded onto glass-bottomed petri dishes and kept on the heated stage (37°C) of a Zeiss LSM 410 microscope. CypHer 5 anti-VSV-G antibody (10 µg/mL) was added in 1 mL KRB for 5 min. After the pre-incubation with antibody, 10 µM TRH was added to the cells, which were then imaged at 633 nm excitation (665 nm emission) over time: (i) 0 min and (ii) 45 min (images not shown for intermediate time points). (iii) Quantification of the time course using Metamorph™ image analysis software.

The red fluorescence of CypHer 5 also offers the capacity to multiplex detection of other polypeptides tagged to fluorescent proteins that excite at shorter wavelengths. This would allow multiple readouts from a single cell, giving more information on the receptor's intrinsic functionality.

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