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A G protein-coupled receptor (GPCR) in red: live cell imaging of the kappa opioid receptor-tdTomato fusion protein (KOPR-tdT) in neuronal cells

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Abstract

Introduction—In contrast to green fluorescent protein and variants (GFPs), red fluorescent proteins (RFPs) have rarely been employed for generation of GPCR fusion proteins, likely because of formation of aggregates and cell toxicity of some RFPs. Among all the RFPs available, tdTomato (tdT), one of the non-aggregating RFP, has the highest brightness score (about 3 times that of eGFP) and unsurpassed photostability.

Methods—We fused tdT to the KOPR C-terminus. The KOPR-tdT cDNA construct was transfected into Neuro2A mouse neuroblastoma cell line (Neuro2A cells) and rat cortical primary neurons for characterization of pharmacological properties and imaging studies on KOPR trafficking.

Results—KOPR-tdT retained KOPR properties (cell surface expression, ligand binding, agonist-induced signaling and internalization) when expressed in Neuro2A cells and rat primary cortical neurons. Live cell imaging of KOPR-tdT enables visualization of time course of agonist-induced internalization of KOPR in real time for 60 min, without photobleaching and apparent cell toxicity. U50,488H-induced KOPR internalization occurred as early as 4 min and plateaued at about 30 min. A unique pattern of internalized KOPR in processes of primary neurons was induced by U50,488H.

Discussion—tdT is an alternative to, or even a better tool than, GFPs for fusing to GPCR for trafficking studies, because tdT has higher brightness and thus better resolution and less photobleaching problems due to reduced laser power used. It also has advantages associated with its longer-wavelength emission including spectral separation from autofluorescence and GFPs, reduced cell toxicity the laser may impose, and greater tissue penetration. These advantages of tdT over GFPs may be critical for live cell imaging studies of GPCRs in vitro and for studying GPCRs in vivo because of their low abundance.

Keywords

GPCR; tdTomato; red fluorescent protein; live cell imaging; neurons; GFP

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Introduction

Green fluorescent protein and variants (GFPs) were widely used to generate GPCR fusion proteins for receptor studies and as drug discovery tools. In contrast, few red fluorescence proteins (RFPs) have been used for this purpose, likely attributed to formation of aggregation and cell toxicity of some RFPs. Recently developed RFPs have been of increasing interest for marking specific cell population in whole animals (e.g., Campbell et al., 2002; Shaner et al., 2004; Vintersten et al., 2004; Shuen et al., 2008; Nowotschin et al., 2009; Winters et al., 2012). This is due to their long-wavelength emissions and thus spectral separation from tissue autofluorescence and the GFPs, reduced cell toxicity, and greater tissue penetration. Among all the RFPs available, tdTomato (tdT), one of the non-aggregating RFP, has the highest brightness score (~3 and 6 times that of eGFP and mCherry, respectively) and highest photostability (Shaner et al., 2004; Shaner et al., 2005). tdT has no obvious cell toxicity and has been used increasingly to selectively label cells in transgenic mouse models (e.g., Muzumdar et al., 2007; Shuen et al., 2008; Morris et al., 2010; Winters et al., 2012). Recently, tdT has also been used successfully in a few fusion protein applications for visualizing non-GPCR proteins (e.g., Bjorkoy et al., 2005; Singh et al., 2009; Palanca & Sagasti, 2013). However, to the best of our knowledge, no studies on GPCR-tdT fusion proteins have been published.

The kappa opioid receptor (KOPR) belongs to the rhodopsin subfamily of GPCRs. Stimulation of KOPR activates Gi/Go proteins resulting in changes in downstream signals, such as inhibition of adenylyl cyclase, activation of p42/p44 mitogen-activated protein kinase (MAPK), increase in K⁺ conductance, and decrease in Ca²⁺ conductance (for a review, see Liu-Chen, 2004).

In this study, tdT was fused to the C-terminus of KOPR and KOPR-tdT fusion protein was transfected and characterized in Neuro2A mouse neuroblastoma cell line (Neuro2A cells) and rat cortical primary neurons. Live cell imaging of agonist-promoted trafficking of KOPR-tdT was performed.

Materials and Methods

More details on materials and the methods denoted with * are provided in Supplemental Experimental Procedures online.

Materials* were obtained from indicated sources as follows

FLAG-tagged mouse KOPR (FLAG-mKOPR) cDNA in pcDNA3 (Chu et al., 1997), Dr. Mark von Zastrow of University California San Francisco; ptdTomato-N1 and pEGFP-N3, Clontech (Mountain View, CA); Neuro2A cells stably-transfected with FLAG-tagged human KOPR were generated as described previously (Li et al., 2012).

Generation of the cDNA for KOPR-tdT and KOPR-eGFP fusion proteins

Briefly, by overlapping PCR, the cDNA of tdT or eGFP was fused to the C-terminus of mKOPR, with a linker sequence (GGC AGC ATT GCG ACC) encoding 5 amino acids (Gly Ser Ile Ala Thr) in between. This linker was used in the delta opioid receptor (DOPR)-eGFP fusion protein previously (Scherrer et al., 2006). The fusion cDNA was cut by KpnI and NotI and cloned into ptdTomato-N1 vector cut by the same two enzymes. Primer designs are as follows. mKOPR-tdT and mKOPR-eGFP shared the same primers below because the N-terminal and C-terminal sequences of tdT were replaced by those of eGFP (Shaner et al., 2004). 5' primer-KpnI-mKOPR: CGGGGTACCGCCACC ATG GAG TCC CCC ATT CAG; 3' primer-NotI-STOP-tdTomato (or eGFP): AAGGAAAAAAGCGGCCGC CTA CTT GTA CAG CTC GTC CAT G; Linker primer (+): GTA GGC AGC ATT GCG ACC

ATG GTG AGC AAG GGC GAG; Linker primer (–): CAT GGT CGC AAT GCT GCC
TAC TGG CTT ATT CAT CCC TCC.

Transient Expression of mKOPR-tdT, mKOPR-eGFP or FLAG-mKOPR in Neuro2A cells*

Cells were cultured as described (Li et al., 2012) and transfected with each cDNA (7–10 µg DNA) using Lipofectamine 2000 (30 µl) following the instructions of Invitrogen. About 48 hours after transfection, cells were detached and harvested in 1× PBS with 1mM EDTA for binding experiments and p44/42 phosphorylation assays. Saturation binding assays with [³H]diprenorphine (see below for detailed method) showed that K_d and B_{max} values of the three mKOPR constructs transiently transfected into Neuro2A cells were similar (0.4–0.5 nM and ~10 pmole/mg protein, respectively).

Stable Expression of mKOPR-tdT, mKOPR-eGFP or FLAG-mKOPR in Neuro2A Cells*

Clonal Neuro2A cells stably expressing each construct, with B_{max} values of about 1–3 pmol/mg protein, were established, as described previously (Li et al., 2012). For imaging studies, these stable clones were used, instead of transiently transfected cells, because the expression levels of KOPR in transiently transfected cells were too high.

Cultures and transfection of primary cortical neurons from E18 embryonic rats*

The experiments were performed as described previously with some modifications (Li et al., 2012).

Membrane Preparation*

Membranes were prepared from Neuro2A cells according to Huang et al. (Huang et al., 2012) with some modifications.

Receptor Binding*

Saturation binding of [³H]diprenorphine to membranes from Neuro2A cells transiently-transfected with mKOPR-tdT, mKOPR-eGFP or FLAG-mKOPR was performed with eleven concentrations of [³H]diprenorphine (ranging from 25 pM to 2 nM), and K_d and B_{max} values were determined (Huang et al., 2001). Competition inhibition by U50,488H or dynorphin A (1–17) of [³H]diprenorphine binding was performed with ~0.4–0.5 nM [³H]diprenorphine in the absence or presence of different concentrations of agonists shown in Fig. 1 and K_i values were determined (Huang et al., 2001).

Quantitation of % of total KOPR on cell surface by radioligand binding on intact cells

Binding was performed on intact cells in 1× PBS with 1mg/ml BSA at room temperature for 60 min according our previous report (Li et al., 1999). Total receptor levels were determined by binding with ~1 nM [³H]diprenorphine in the presence or absence of 10 µM naloxone. Cell surface receptors were assessed by binding with ~1 nM [³H]diprenorphine in the presence or absence of 1µM dynorphin A(1–17). Diprenorphine and naloxone, two hydrophobic ligands, bind to both cell surface and intracellular KOPR, whereas dynorphin A(1–17), a hydrophilic ligand, binds to surface KOPR only. Statistical analysis (one-way ANOVA) was performed using Prism software (GraphPad, La Jolla, CA).

p42/p44 MAP Kinase Phosphorylation

Neuro2A cells were transiently transfected and two days later were detached by 1×PBS with 1mM EDTA and washed with 1×PBS twice. Cells were re-suspended and incubated in the Opti-MEM medium for 60 min at 37°C, and then treated with or without U50,488H at 37°C for 5 min. Cells were lysed with 2×Laemmli sample buffer and subjected to 8% SDS-

PAGE. Phosphorylated p44/42 MAP kinase and total p44/42 MAP kinase were detected with simultaneous immunoblotting using polyclonal and monoclonal antibodies against each of them, followed by IRDye 680RD and IRDye 800 CW secondary antibodies, respectively. Images were captured with the Odyssey infrared imaging system.

Fluorescence microscopy*

Neuro2A cells stably expressing mKOPR-tdT or mKOPR-eGFP were plated onto poly-D-lysine coated 8-well Lab-Tek II chambered coverglass. About 40 hours later, cells were treated with or without 1 or 10 μ M U50,488H at 37°C for 30 min, and live cells were observed under a Nikon Eclipse TE300 microscope fitted with a 40 $\times$ objective lens. For neuro2A cells stably expressing FLAG-mKOPR or FLAG-tagged human KOPR, cells were plated onto poly-D-lysine coated coverglass seated in 24-well plates and treated similarly. FLAG-mKOPR was stained with anti-FLAG M1 and a secondary antibody conjugated with Alexa Fluo594 and visualized under the same fluorescence microscope. For rat primary cortical neurons expressing mKOPR-tdT, 8–12 days after transfection, neurons were incubated with U50,488H of 10 μ M at 37°C for 30 min, and same live neurons were sequentially visualized by fluorescence microscopy at time points of –5 and 30 min.

Live cell imaging with a confocal microscope

Cells were grown on poly-D-lysine coated 8-well Lab-Tek II Chambered Coverglass (No. 1.5). Live cell imaging was conducted in Confocal Imaging Core Facility of Temple University School of Medicine by using a Leica DM IRE2 microscope with a TCS SL confocal system and a 63 $\times$, 1.40 numerical aperture oil immersion objective in a CO₂- and 37°C temperature-controlled incubator. A DPSS561 laser was used as the light source for imaging tdT. Time-lapse images were acquired by line sequential scanning using 3 $\times$ line averaging and 3 $\times$ frame averaging at 400 Hz scan speed.

Results

In addition to mKOPR-tdT, all the experiments, except live cell imaging, were also performed on FLAG-mKOPR and mKOPR-eGFP for comparison. Several groups have shown that the N-terminal FLAG tag (DYKDDDDK epitope tag) and C-terminal eGFP tag did not affect KOPR properties (e.g. Chu et al., 1997; Zhang et al., 2002; Schulz et al., 2002; McLaughlin et al., 2003). The fusion proteins facilitate KOPR studies by enabling immunoblotting or immunostaining using anti-FLAG antibodies (Chu et al., 1997; Zhang et al., 2002; Li et al., 2012) and fluorescence microscopy of eGFP (Schulz et al., 2002; McLaughlin et al., 2003).

KOPR-tdT retained ligand binding affinities

Membranes of Neuro2A cells transiently transfected with each construct were used for experiments. mKOPR-tdT, mKOPR-eGFP and FLAG-mKOPR had similar affinities for the selective agonist U50,488H (8–10 nM) and the endogenous peptide ligand dynorphin A (1–17) (~0.1 nM), indicating that fusion with tdT does not change the affinities of the receptor for these ligands (Fig. 1).

KOPR-tdT was mostly located on cell surface, similar to FLAG-KOPR and KOPR-eGFP

To determine % of the KOPR on cell surface, we performed [³H]diprenorphine binding (~1 nM) to the KOPR using intact cells (Li et al., 1999). Percentages of the KOPR on cell surface were determined to be 75 $\pm$ 5%, 72 $\pm$ 5% and 65 $\pm$ 3% (mean $\pm$ sem, n=3 each) for mKOPR-tdT, mKOPR-eGFP and FLAG-mKOPR, respectively. There is no significant difference among the three by one-way ANOVA in % of the KOPR on cell surface,

indicating that tdT fusion does not change KOPR targeting to plasma membrane. The results here are in accord with the results of imaging studies shown in Fig. 3.

KOPR-tdT retained the KOPR signal properties

Incubation of Neuro2A cells transiently-transfected with mKOPR-tdT, mKOPR-eGFP or FLAG-mKOPR for 5 minutes with U50,488H increased p44/42 MAPK phosphorylation in a dose-dependent manner (Fig. 2). More importantly, activation of mKOPR-tdT enhanced p44/42 MAPK phosphorylation to similar extents as stimulation of mKOPR-eGFP or FLAG-mKOPR. The results indicate that tdT fused to the C-terminus of KOPR has little effects on the KOPR agonist-stimulated signal transduction along the p44/42 MAPK pathway.

KOPR-tdT underwent U50,488H-induced internalization, similar to FLAG-KOPR and KOPR-eGFP

Neuro2A cells stably transfected with mKOPR-tdT or mKOPR-eGFP were established. Cell viability or morphology appeared unaffected by transfection. Under the fluorescence microscope, cells expressing mKOPR-tdT were much brighter than those expressing mKOPR-eGFP at comparable expression levels (data not shown), in accord with the higher brightness of tdT than eGFP. Cells were treated with vehicle or 10 μ M U50,488H for 30 minutes at 37°C, and live cells were observed under a fluorescence microscope. In vehicle-treated cells, most of mKOPR-tdT and mKOPR-eGFP were located on cell surface (Fig. 3), consistent with the data above on % of the KOPR on cell surface determined by radioligand binding. In contrast, in U50,488H-treated cells most of the mKOPR-eGFP and mKOPR-tdT were located inside cells as punctates (Fig. 3), indicating that U50,488H causes internalization. Similarly 10 μ M U50,488H promoted internalization of FLAG-mKOPR (Fig. 3) or FLAG-tagged human KOPR (data not shown) stably expressed in Neuro2A cells, which was detected by immunocytochemistry using an anti-FLAG antibody M1 followed by Alexa 594-conjugated secondary antibodies. Pretreatment with norbinaltorphimine, a KOPR antagonist, completely blocked KOPR internalization caused by U50,488H and norbinaltorphimine itself did not induce changes in KOPR location (data not shown). In addition, 1 μ M U50,488H caused internalization of each of the four KOPRs in Neuro2A cells to similar extents as 10 μ M U50,488H (data not shown). These results indicate that fusing KOPR to tdT or eGFP does not change U50,488H-induced internalization of KOPR.

Dynamics of U50,488H-induced internalization of KOPR-tdT in live cells

Cells were grown on poly-D-lysine coated 8-well Lab-Tek II Chambered Coverglass and observed under a confocal microscope in real time. After bath application of U50,488H, internalization of KOPR-tdT stably expressed in Neuro2A cells occurred as early as 4 min, reached a plateau at about 30 min and lasted at least 1 h (Fig. 4(A)). KOPR-tdT signals were so bright that only 6% of laser strength was used and no photobleaching was detected after almost one hour of recording.

Live cell imaging of KOPR-tdT in cultured primary neurons

Rat primary cortical neurons were prepared and transfected with KOPR-tdT upon plating. Under fluorescence microscopy, neurons expressing KOPR-tdT were evident 24 hours after transfection and no noticeable neuronal toxicity associated with the expression of KOPR-tdT was observed during the culture period of 8–12 days. Transfection efficiency was less than 5%. Between days 8 and 12, KOPR-tdT was expressed on both neuronal cell bodies and processes, and U50,488H induced significantly more punctates in neuronal cell bodies and processes 30 min after addition of the agonist, compared to the same neuron 5 min before the agonist treatment (Fig. 4(B)).

Discussion

To the best of our knowledge, this is the first report of the following observations: **(1)** A GPCR-tdT fusion protein retained the GPCR properties, in terms of cell surface expression, ligand binding and agonist-induced trafficking and signaling; **(2)** A GPCR-tdT fusion protein allowed visualization of agonist-promoted internalization in real time; **(3)** Live cell imaging of KOPR-tdT enables visualization of time course of agonist-induced internalization of KOPR in real time for up to 1 h, and U50,488H-induced KOPR internalization occurred as early as 4 min; **(4)** An agonist induced KOPR trafficking in primary neurons. It is noteworthy that the molecular weight of tdT (476 amino acids) is almost twice those of most of other fluorescent proteins (about 240 amino acids).

There are few studies on the GPCR-RFP fusion (Teichmann et al., 2012), likely due to following factors. Oligomerization of the earlier versions of RFPs (e.g., DsRed) resulted in formation of intracellular aggregates of RFPs or RFP-tagged non-GPCR proteins, which are toxic to cells (Campbell et al., 2002; Shaner et al., 2004; Shaner et al., 2005). When dsRed was fused with KOPR, the fusion proteins formed intracellular aggregates and failed to transport to cell surface in PC12 cells (personal communications, Dr. Vladana Vukojević of Karolinska Institute, Stockholm, Sweden). In addition, the first generation of monomeric RFP showed low brightness and low photostability (Campbell et al., 2002; Shaner et al., 2004).

Advantages of GPCR-tdT over GPCR-GFPs

Green fluorescent protein and its spectral variants (GFPs), especially the enhanced green fluorescent protein (eGFP), have been widely used to fuse with GPCRs, with slight or no changes in receptor properties. The GPCR-GFPs have been very useful for examining GPCR trafficking and subcellular localization in live and fixed cultured cells (see (Milligan, 2000; Kallal & Benovic, 2000; Wiesner et al., 2005; Arun et al., 2005; Teichmann et al., 2012) for reviews) and even in specific neurons in knockin mice in vivo (e.g., Chan et al., 2004; Scherrer et al., 2006). However, lack of spectral separation of GFPs from tissue autofluorescence and low abundance of GPCRs in vivo may pose challenges for imaging of GPCR-GFPs in tissues from knockin mice. In contrast, tdT, a photostable RFP, has advantages associated with its long-wavelength emission, such as low autofluorescence, spectral separation from GFPs, great tissue penetration, and reduced cell toxicity produced by laser. Moreover, tdT has higher brightness and thus better resolution and less photobleaching problems than GFPs. These advantages of tdT over GFPs are critical for use of GPCR-fluorescence protein fusion strategy for visualizing GPCRs in vivo and real-time imaging studies of GPCRs in vitro.

Species differences in the U50,488H-induced KOPR trafficking

In the current study, mKOPRs were internalized by U50,488H in Neuro2A cells and rat primary cortical neurons. The time-course of U50,488H-induced internalization of mKOPR-tdT in Neuro2A cells is reminiscent of our previous studies on human KOPR internalization in CHO cells as determined by radioligand binding (Li et al., 1999). However, we previously reported that, when expressed in CHO cells, the human, but not rat, KOPR (untagged or FLAG-tagged) underwent internalization following treatment with U50,488H and the differential regulation was attributed to differences in the C-terminal domain sequences (Li et al., 1999; Zhang et al., 2002). Since the rat and mouse KOPRs have identical amino acid sequence in the C-terminal domains and share 99.5% overall amino acid sequence identity (Zhu et al., 1995), the differences between FLAG-rat KOPR expressed in CHO cells and mKOPRs expressed in Neuro2A cells and primary neurons may be due to differences in cellular milieu. Results from in vitro studies by other groups also

varied regarding species differences in U50,488H-induced KOPR trafficking in other cell lines, including AtT20, HEK293 and NG108-15 cells (Schulz et al., 2002; McLaughlin et al., 2003), further suggesting cell type-dependent effects may play some roles. Generating mKOPR-tdT knockin mice will allow us to examine whether agonist-induced mKOPR internalization occurs *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Dynorphine A (1–17)	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
eGFP	enhanced green fluorescent protein
FLAG tag	DYKDDDDK epitope tag
GFPs	green fluorescent protein and its spectral variants
GPCR	G protein-coupled receptor
KOPR	kappa opioid receptor
MAPK	mitogen-activated protein kinase
mKOPR	mouse kappa opioid receptor
RFP	red fluorescent protein
norbinaltorphimine	17,17'-(dicyclopropylmethyl)-6,6',7,7'-6,6'-imino-7,7'-bimorphinan-3,4',14,14'-tetrol
tdT	tdTomato
U50,488H	<i>trans</i> -(±)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate salt

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Figure 1. mKOPR-tdT, mKOPR-eGFP and FLAG-mKOPR displayed similar affinities for U50,488H and dynorphin A (1-17)

Neuro2A cells were transiently transfected with each construct. About 48 hours later, cells were harvested and membranes were prepared. Competitive inhibition of [³H]diprenorphine binding to the KOPRs in membranes by U50,488H and dynorphin A (1-17) was performed. The K_i values (nM) were determined to be 8.0, 9.2 and 9.4 for U50,488H, and 0.11, 0.094 and 0.095 for dynorphin A (1-17), for the three receptors, respectively. This figure represents one of the two experiments performed.

Figure 2. U50,488H activated mKOPR-eGFP, mKOPR-tdT and FLAG-mKOPR to enhance p44/42 MAPK phosphorylation to similar extents

Neuro2A cells transiently transfected with each construct were incubated in Opti-MEM medium for 1 h. Vehicle or U50,488H was added to the final concentrations from 10^{-8} M to 10^{-6} M. Five min later, Laemmli loading buffer was added and SDS-PAGE and immunoblotting were performed with antibodies against phospho-specific MAPK and total MAPK. The figures represent one of the two experiments with similar results.

Figure 3. Similar U50,488H-promoted internalization of mKOPR-tdT, mKOPR-eGFP, and FLAG-mKOPR stably expressed in Neuro2A cells
Cells were treated with vehicle or 10 μ M U50,488H for 30 minutes at 37°C and observed using live cells (mKOPR-tdT or mKOPR-eGFP) or after fixation and immunostaining (FLAG-mKOPR) under a fluorescence microscope. The figures represent one of the three experiments with similar results using distinct stable clones.

Figure 4. Real-time imaging of mKOPR-tdT in neuronal cells

(A) Live cell confocal imaging of mKOPR-tdT stably expressed in Neuro2A cells. Cells were grown on the chambered coverglass (No. 1.5) and placed in a CO₂- and 37°C temperature-controlled incubator on a confocal microscope stage. U50,488H was added to the medium to a final concentration of 10 μM. Time-lapse images were captured before and after the bath application of U50,488H. The number in the upper right corner of each image indicates the time lapsed in minutes. The 1st and 2nd images were taken 5 min before and 1 min after the treatment, respectively, and the cells were then imaged every 3 min for about 1 hour. The figures represent one of the three experiments with similar results, including more than 30 individual cells. **(B) Live cell imaging of mKOPR-tdT transfected into rat primary cortical neurons.** Primary neurons were cultured for 8–12 days after transfection. Images were captured under a fluorescence microscope 5 min before and 30 min after the bath application of U50,488H at 10 μM, respectively. The figures represent one of the 8 neurons with similar results.