Visualization of G Protein-coupled Receptor Trafficking with the Aid of the Green Fluorescent Protein

ENDOCYTOSIS AND RECYCLING OF CHOLECYSTOKININ RECEPTOR TYPE A*

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A chimeric protein consisting of the cholecystokinin receptor type A (CCKAR) and the green fluorescent protein (GFP) was used for studying receptor localization, internalization, and recycling in live cells in real time in four different cell lines. Fusion of the C' terminus of the CCKAR to the N terminus of the GFP did not alter receptor ligand binding affinity, signal transduction, or the pattern of receptor surface expression and receptor-mediated cholecystokinin (CCK) internalization. The use of a new GFP mutant with increased fluorescence allowed the continuous observation of CCKAR-GFP in stably expressing cell lines. Newly obtained biologically active fluorescent derivatives of CCK were used for simultaneous observation of receptor and ligand trafficking in CHO, NIH/3T3, and HeLa cells stably expressing the fluorescent CCKAR and in transiently transfected COS-1 cells. Receptor internalization was predominantly ligand dependent in HeLa, COS-1, and CHO cells, but was mostly constitutive in NIH/3T3 cells, suggesting the existence of cell-specific regulation of receptor internalization. The CCKAR antagonists, L-364,718 and CCK 27–32 amide potently inhibited spontaneous internalization of the receptor. The average sorting time of CCK and the receptor in the endosomes was about 25 min. The receptor recycled back to the cell membrane with an average time of 60 min. While the ligands sorted to lysosomes, no receptor molecules could be detected there, and no receptor degradation was observed during recycling. These results demonstrate the usefulness of GFP tagging for real time imaging of G protein-coupled receptor trafficking in living cells and suggest that this technique may be successfully applied to the study of the regulation and trafficking mechanisms of other receptors.

G protein-coupled receptors (GPCR)† are involved in numerous biological processes ranging from peptide hormone and neurotransmitter-regulated function to smell, taste, and light receptors and to viral entry into cells. This very broad spectrum of activities indicates that these receptors have a central role in cell biology and in many cases may be attractive targets for drug development. G protein-coupled receptor function is significantly regulated by the mechanisms that determine receptor trafficking within the cell. The molecular and cellular mechanisms involved in regulation of translocation, sequestration, recycling, and degradation of G protein-coupled receptors are not well understood, and the available data are largely controversial. The study of the trafficking by means of localization of the receptors with antibodies is laborious and does not allow for the efficient observation of receptor translocation in intracellular compartments. The green fluorescent protein (GFP) from jelly fish is becoming widely used as a molecular reporter to monitor gene expression, localization, and intracellular protein trafficking in living cells (1–3). Many GFP-lagged proteins retain their biological activity and have the same trafficking pattern as the native proteins (4–8). However, the majority of the proteins that have been labeled with GFP for microscopy studies are expressed at relatively high levels, which makes the detection of the fusion proteins significantly easier. Localization of GPCR is a technically challenging procedure because most of the receptors are expressed at very low levels. The approaches utilizing either cells that overexpress the receptors or the use of GFP mutants with much higher fluorescence than that of the wild type protein can be applied for the localization of labeled GPCR in living cells. In the present paper we have explored the possibilities of GFP labeling for the study of G protein-coupled receptor trafficking using the type A cholecystokinin receptor (CCKAR). Cholecystokinin, a well studied bioactive peptide, was initially discovered in the gut and subsequently in the central nervous system (9). It exerts numerous effects through its action as a hormone and a neurotransmitter. The CCKAR was well characterized with respect to the mechanisms of signal transduction (10) and phosphorylation upon ligand binding (11). It was shown to undergo sequestration upon binding of agonists (12–17) like many other GPCR. It was suggested that internalization of the CCKAR and many other GPCRs is ligand-dependent, but direct proof has been missing. It is also unclear whether only binding of agonists versus antagonists can trigger GPCR internalization, since it has been shown that signal transduction is not necessary for internalization.
ization (18). Furthermore, cell surface receptor trafficking has not been directly observed previously in real time in living cells. To address these questions, we have developed the tools that allowed for the direct observation of the CCKAR and its ligand in living cells in real time, and have applied them for the study of receptor and ligand trafficking in four different cell types. Attachment of GFP did not influence receptor expression, affinity, signal transduction, or internalization. The fluorescence of the newly created mutant of GFP was intense enough for observation of the CCKAR by confocal microscopy in different cell types that expressed the receptor at natural levels. Direct observation of the CCKAR-GFP fusion receptor proved to be a powerful tool for studying ligand-dependent receptor internalization and recycling.

**EXPERIMENTAL PROCEDURES**

**Materials**

Rhodamine Green trifuoroacetic acid, succinimidyl ester, rhodamine red concanavalin A (ConA), tetramethylrhodamine-transferrin, hexyl ester of rhodamine 6G, MitoTracker Red CMXROS, and Lysotracker Red from Molecular Probes Inc. (Eugene, OR). Cholecystokinin-8 was purchased from Research Plus, Inc. (Bayonne, NJ). Cys.29 N-hydroxysuccinimidyl esters was a kind gift from Dr. Brigitte Schmidt (Center for Light Microscope Imaging and Biotechnology, Carnegie Mellon University, Pittsburgh, PA).

NIH/3T3, CHO, HeLa, and COS-1 cells were obtained from the American Type Culture Collection (Rockville, MD).

**Methods**

**Construction of the CCKAR-GFP and GFP-CCKAR Expression Vectors**—The full open reading frame of the rat CCKAR (nucleotides 199–1485; Ref. 20) either with GFP-CCKAR or with GFP-CCKAR was amplified using appropriate primers containing EcoRI and XhoI (CCKAR-hGFP) or XbaI and BamHI along with a stop codon (hGFP-CCKAR) restriction sites at the 5′ and 3′ ends, respectively, using PCR. The humanized S6ST GFP, pHGF-S6ST (CLONTECH), was mutated to pGFP-PF64L, T65C, I167T by site-directed mutagenesis (MutaGene® phagemid in vitro mutagenesis kit, Bio-Rad). Either XhoI and BamHI or XbaI and EcoRI (hGFP-CCKAR) restriction sites were added to the 5′ and 3′ ends of the mutated hGFP using PCR. Either the 5′ or 3′ end of CCKAR-hGFP was ligated to the XbaI or the 5′ end of the mutant hGFP, respectively, with T4 DNA ligase (Life Technologies, Inc.) and the fused products were ligated into either pCDL-SRα or pCDL-SRαNeo at the EcoRI and BamHI sites. Alternatively, to generate pF25-CASKAR-GFP, the coding region of the rat CCKAR was amplified by PCR using appropriate primers containing Nhel restriction sites. The PCR product was digested with Nhel and cloned into the Nhel-cut vector pCMV-GFPsg25. Plasmid pCMV-GFPsg25 expresses a mutated GFP protein under the control of the early cytomegalovirus promoter.

**Transient Expression of the Wild Type Rat CCKAR, CCKAR-GFP, and GFP-CCKAR in COS-1 Cells**—Two micrograms of pCDL-SRα containing either the wild type rat CCKAR, CCKAR-hGFP, or hGFP-CCKAR cDNA insert subcloned at the EcoRI and BamHI sites in the sense orientation were transfected into near confluent COS-1 cells using the DEAE/dextran method as described (21). Approximately 24 h after transfection, cells were trypsinized and replated on glass coverslips for examination by confocal laser scanning microscopy at 48 h.

**Generation of Cell Lines StablyExpressing the CCKAR Receptors**—The wild type rat CCKAR and CCKAR-hGFP cDNAs were stably transfected by electroporation (500 millifarads, 0.25 kV, Bio-Rad Gene Pulser) of 2 × 105 CHO and NIH/3T3-cells/ml in a volume of 0.25 ml with 20 μg of the linearized recombinant pCDL-SRαNeo containing either the wild type rat CCKAR or CCKAR-hGFP cDNA subcloned at the EcoRI and BamHI sites in the sense orientation in the presence of 500 μg/ml salmon sperm DNA as a carrier. Cells stably expressing the receptors were then selected for G-418 resistance (250 μg/ml G-418/ml) and by fluorescence-activated cell sorting (Coulter, ECP-Elite, Miami, FL). NIH/3T3 cells were maintained in DMEM, 10% calf serum, 250 μg/ml G-418, and CHO cells were maintained in Ham’s F-12 medium, 10% fetal bovine serum, 250 μg/ml G-418 at 37 °C in a 6% CO2 atmosphere. To generate CCKAR-GFPsg25-expressing HeLa cell lines, 5 × 105 HeLa cells were transfected with 10 μg of pF25CCKAR-GFP by the calcium phosphate method. Two days after transfection, single cell fluorescence-activated cell sorting was performed into 96-well plates without prior selection. Sorting was performed on a FACStar Plus (Becton Dickinson, Mountain View, CA) using an argon ion laser at 488 nm was used to excite GFPsg25 (run at 200 milliwatts with a 500-nm long pass emission filter). Positive, CCKAR-GFPsg25-expressing single cell colonies were identified using an inverted fluorescence microscope and further expanded in DMEM, 10% calf serum, 50 μg/ml G-418.

**Radioligand Binding Displacement Studies**—Stably transfected CHO cells were plated at a density of 9.0 × 105 cells/ml in 24-well tissue culture plates and assayed the following day for radioligand binding. For binding displacement studies, cells were incubated for 90 min with 50 pm 125I-CCK-8 in the absence or presence of increasing concentrations of cold CCK-8 in 0.5 ml of DMEM, 0.1% BSA at 37 °C. Nonspecific binding was defined as total binding in the presence of 1 μM cold CCK-8 and was always less than 10% of total binding. After termination of the binding reaction by washing the cells two times with PBS, 0.1% BSA at 4 °C, cells were solubilized with 1 ml of 0.1 N KOH and radioactivity was detected in a Packard/Autogamma counter. Binding parameters, Bmax and KD, were determined with the nonlinear least squares curve fitting computer program, LIGAND (22).

**DNA Sequencing**—The wild type rat CCKAR and CCKAR-GFP cDNAs were sequenced using the Dye Terminator kit and a model 377 DNA Sequencer (Applied Biosystems).

**Inositol Phosphate Assay**—Stably transfected CHO cells were plated at a density of 2.0 × 105 cells/well on 24-well culture plates with DMEM, 10% calf serum in the presence of 100 μCi/ml myo-[2-3H]inositol and incubated overnight. The following day, the medium was aspirated and the cells were incubated with FI buffer (20 mM HEPES, 2 mM CaCl2, 1.2 mM MgSO4, 10 mM LiCl, 11.1 mM, glucose, 0.5% BSA) and exposed to the indicated concentrations of peptide. Total [3H]inositol phosphates were measured by strong anion exchange chromatography (Dowex AG 1 X8), using a modification of the method described by Berridge et al. (23). Eluates were assayed using a Beckman liquid scintillation counter.

**Synthesis and Characterization of RG-CCK-8 and Cy3.29-CCK-8**—Cholecystokinin octapeptide (6 mg, 5 μmol) was dissolved in a mixture of acetonitrile (50 μl) and aqueous solution of sodium bicarbonate (0.2 M, pH 9.0, 50 μl). A solution of either Rhodamine Green trifluoroacetic acid, succinimidyl ester (5 mg, 7.5 μmol) or Cy3.29 succinimidyl ester (5 mg, 7.5 μmol) in dimethylformamide (10 μl) was added under nitrogen in the dark. The reaction mixtures were stirred at room temperature over a silical gel filter, and the fresh aqueous solution of hydroxylamine (25 μl, 1.5 M, pH = 7.8) was added. After 2 h the reaction mixtures were dissolved in 30% aqueous acetonitrile (4 ml), and products were separated by HPLC under reverse-phase conditions using a semipreparative YMC-Pack ODS-AM column (10 mm × 300 mm), (eluent: acetonitrile/water/0.05% trifluoroacetic acid, gradient 30–70% of acetonitrile, 60 min). Yields were 30–50%. NMR spectroscopy was performed on a Varian VXR-500 spectrometer (500 MHz for H). The mass spectra were generated on a Bruker Reflex II time of flight mass spectrometer. For RG-CCK-8, mass spectra were as follows: C25H29N4O5S2Na, calculated 5212.16; found, m/z = 5212.19; C25H29N4O5S2Na, calculated 1441.5; found, m/z = 1441.7; C25H29N4O5S2Na, calculated 1419.5; found, m/z = 1420.2. For Cy3.29-CCK-8, mass spectra were as follows: C25H29N4O5S2, calculated, 1689.9; found, m/z = 1696.8. Double quantum tilted correlation NMR spectra of RG-CCK-8 and Cy3.29-CCK-8 (data not shown) had all expected cross-peaks for NH-α-H bond systems, and aliphatic and aromatic parts of the molecules.

**Confocal Laser Scanning Microscopy**—Cells were grown in Nunc cover glass chamber slides in medium without phenol red and observed on a Zeiss inverted LSM 410 laser scanning confocal microscope. Fluorescence of rhodamine green, Lysotracker Green, and GFP was excited using a 488-nm argon/krypton laser, and emitted fluorescence was detected with 515–540-nm band pass filter. For Lysotracker Red, rhodamine red, Cy3.29-CCK-8, and tetramethylrhodamine, a 568-nm helium/neon laser was used for excitation and fluorescence was detected with a 590-nm band pass filter.

**Cloning Study of the Concentration Dependence on RG-CCK-8 and Cy3.29-CCK-8 Binding to the Cells, Transfected with CCK-A Receptor cDNA**—The cells were incubated for 1 h with various concentrations of the compound in phenol red-free medium in a CO2 incubator. Cells were rinsed three times with medium and observed under the microscope using identical parameters for all concentrations. The images were stored on an optical disk and analyzed with Zeiss LSM software. A minimum of 10 images for each concentration were quantitated.

2 R. H. Stauber, P. Carney, G. A. Gaitanaris, K. Horie, N. I. Tarasova, E. A. Hudson, and G. N. Pavlakis, submitted for publication.

3 A. D. Pfeffer, J. B. Pfeffer, and A. D. Pfeffer, unpublished.
RESULTS

Construction and Characterization of CCKAR and GFP Fusion Proteins

To study the localization and trafficking of the CCKAR in live cells, we constructed a chimeric cDNA of the coding region of the rat CCKAR and fused its C terminus before the stop codon, to the full coding region of the mutant GFP cDNA. The C-terminus of GFP was also fused to the N terminus of the receptor resulting in GFP-CCKAR. The receptor fused to the T65S mutant of GFP (24) could be visualized by CLSM in transiently transfected COS-1 that overexpressed CCKAR-GFP, but the intensity of T65S GFP fluorescence was not sufficient for detection of receptor molecules in stably transfected NIH/3T3 cells that expressed natural levels of the receptor. The use of the GFP molecule with mutations that were found previously to increase the fluorescence approximately 150-fold (F64L, T65C, and I167T)2 allowed for direct observation of the fusion receptor in stably transfected cells. GFP-CCKAR, when expressed in COS-1 cells, showed no binding of 125I-CCK-8. Confocal microscopy revealed that GFP-CCKAR had an intracellular localization, suggesting that attachment of the GFP to the N terminus of the CCKAR abrogates the transport of the receptor to the cell membrane.

To determine the effect of fusing GFP to the intracellular C terminus of the CCKAR on receptor function, CHO cells expressing wild type CCKAR were compared with CHO cells expressing CCKAR-GFP for cell surface receptor density, CCK-8 affinity, and CCK-8-stimulated signal transduction. The CHO cells expressed CCKAR-GFP on their surface with a fluorescence provided the strongest fluorescence intensity, chemical and light stability and resistance to intracellular degradation within the cells necessary for the kinetic studies of ligand uptake. Cy3.29 derivatives have emission maximum at 570 nm and do not emit below 540 nm and thus allow for simultaneous detection of the GFP fluorescence. Rhodamine green, BODIPY, and Cy3.29-CCK-8 were obtained by the method used earlier for the synthesis of heptagastrin derivatives (25). The structures of the compounds were confirmed by NMR and mass spectroscopy. Both RG and Cy3.29-CCK-8 retained a high affinity toward the gastrin receptor (EC50 1.70 ± 0.29 × 10^6 receptors/cell and KD 3.78 ± 0.71 × 10^-9) compared with CHO cells expressing wild type CCKAR (EC50 1.73 ± 0.28 × 10^6 receptors/cell and KD 3.3 ± 3.3-fold increase and an EC50 of 4.38 ± 3.0 nM for CHO cells expressing the CCKAR-GFP (Fig. 2).

Fluorescent Derivatives of CCK-8

In a previous study we compared the properties of three fluorescent derivatives of gastrin, rhodamine green, BODIPY, and Cy3.29 heptagastrin (25). The rhodamine green (RG) moiety provided the strongest fluorescence intensity, chemical and light stability and resistance to intracellular degradation within the cells necessary for the kinetic studies of ligand uptake. Cy3.29 derivatives have emission maximum at 570 nm and do not emit below 540 nm and thus allow for simultaneous detection of the GFP fluorescence. Rhodamine green and Cy3.29-GFP-GFP were obtained by the method used earlier for the synthesis of heptagastrin derivatives (25). The structures of the compounds were confirmed by NMR and mass spectroscopy. Both RG and Cy3.29-CCK-8 retained a high affinity toward the gastrin receptor (Ka 8 ± 3.0 nM in displacement of 125I-labeled cholecystokinin-8 by RG-CCK-8) and showed specific binding to NIH/3T3, CHO, and HeLa cells stably transfected with rat CCKAR cDNA, but not to untransfected cells. The concentration dependence of binding of RG-CCK-8 and Cy3.29-GFP-GFP was determined using the method of Scatchard (36) and a linear regression program on a microcomputer. The resulting dissociation constants were 3.3 ± 3.3-fold increase and an EC50 of 4.38 ± 3.3 nM for the CHO cells expressing the CCKAR-GFP (Fig. 2).

150-fold (F64L, T65C, and I167T)2 allowed for direct observation of the fusion receptor in stably transfected cells. GFP-CCKAR, when expressed in COS-1 cells, showed no binding of 125I-CCK-8. Confocal microscopy revealed that GFP-CCKAR had an intracellular localization, suggesting that attachment of the GFP to the N terminus of the CCKAR abrogates the transport of the receptor to the cell membrane.

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CCK-8 to NIH/3T3 cells transfected with CCK-A receptor cDNA (Fig. 3A) showed a typical saturation curve, usually observed for radiolabeled hormones upon binding to their receptors. The $K_D$ for the binding was calculated from these data to be 0.7 ± 0.2 nM for RG-CCK-8 and 2.5 ± 0.5 nM for Cy3.29-CCK-8. Preincubation of the cells with 10 μM cholecystokinin for 10 min reduced fluorescence of bound fluorescent derivatives of CCK to background levels. Both compounds induced intracellular Ca$^{2+}$ release in NIH/3T3 cells transfected with CCKAR at concentrations as low as 10 nM (Fig. 3B), which was identical to that of the parent peptide, indicating that the derivatives retained the biological properties of CCK-8.

To estimate the time needed for internalization, the cells expressing the CCKAR or CCKAR-GFP were saturated with Cy3.29-CCK-8 at 4 °C for 30 min, rinsed with medium, and observed by confocal microscopy at 20 °C at 1-min intervals. No internalization was observed at 4 °C, but at 20 °C the bound ligand was clustered into aggregates on the cell surface with evidence of internalization as early as 4–7 min of incubation. The pattern of ligand binding and internalization time was identical in the case of the cells expressing wild type CCKAR and CCKAR-GFP (data not shown) and identical to cholecystokinin receptor type B, which had been characterized earlier (26).

Intracellular stability of rhodamine green dye allowed us to develop a technique for the study of accumulation of fluorescent peptides within cells (26), which was applied to RG-CCK-8. The intracellular localization of CCKAR-GFP in different cell types in the absence of ligands

The major part of the green fluorescence corresponding to CCKAR-GFP was observed on the cell membrane in CHO and HeLa cells stably transfected with CCKAR-GFP (Fig. 5, A and B). The receptor molecules appeared to be evenly distributed on the plasma membrane. A significant part of the fluorescence was on the cellular membrane in transiently transfected COS-1 cells (Fig. 5D). However, COS-1 and HeLa cells also had significant labeling of the endoplasmic reticulum (ER) (identified with the help of the red fluorescent ER marker, the hexyl ester of rhodamine 6G (27); Fig. 6, A and B, respectively). In CHO cells, a small part of the fluorescence was diffusely distributed throughout the cytoplasm (Fig. 5A). Surprisingly, in stably transfected NIH/3T3 cells, the fluorescent receptor had a predominantly intracellular localization (Fig. 5C). To determine the relative distribution of the fluorescent CCKAR-GFP, the cell surface was labeled by a brief exposure to rhodamine B concanavalin A. The green fluorescence of CCKAR-GFP that appeared to colocalize with the red fluorescence of ConA was considered to correspond to the receptor molecules residing on the plasma membrane. Total CCKAR-GFP fluorescence and the fluorescent component that colocalized with ConA were quantitated with the help of Zeiss LSM software. In the absence of the ligand, CHO cells had 75.8 ± 9.4% of the receptors on the cell surface, HeLa cells had 64 ± 9.1%, and NIH/3T3 had 25.6 ± 6.3%. In transiently transfected COS-1 cells that overexpressed CCKAR-GFP, part of the GFP fluorescence colocalized with the mitochondrial marker, MitoTracker Red CMXRos.
Endocytosis and Recycling of Cholecystokinin Receptor

Effect of Agonists—In all three stably transfected cell lines, addition of CCK-8 caused the green fluorescence of the receptor to disappear almost completely from the cell surface and to move into intracellular vesicles identified by the tetramethyl rhodamine derivative of transferrin, used as a marker of endosomal compartments. In the absence of the ligand, almost no receptor molecules could be detected in endosomes and lysosomes, indicating that overexpression of the receptor may change the pattern of receptor localization.

Ligand-induced Translocation of CCKAR-GFP

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Time Course of Receptor Recycling

The relative amount of CCKAR-GFP on the cell surface was determined after exposure to the agonist CCK-8. CCKAR-GFP-expressing CHO cells were pretreated with cycloheximide to inhibit de novo synthesis of receptor molecules, and incubated with CCK-8 for 10 min at 37 °C, a period previously shown to be sufficient to cause internalization of the receptor from the cell surface. The cells were then rinsed with a medium containing cycloheximide and left in the incubator for varying time intervals, and briefly exposed to ice-cold rhodamine B ConA and observed by CLSM. The number of receptor molecules on the cell surface increased gradually after 10 min exposure to CCK-8 and returned to the initial value of the untreated cells within 60 min (Fig. 11), consistent with the data from Fig. 4.

DISCUSSION

CCK acting at CCKARs present in the gastrointestinal tract and nervous systems regulates the physiological processes governing digestion and satiety (9, 30). At the cellular level, the
CCK mediates ligand-induced activation of phospholipase C, adenylyl cyclase, and phospholipase A$_2$ (10, 31). For several receptors, the cellular responses to effectors have been shown to be regulated in part by the movement and compartmentalization of the receptors and their effectors within the cell. Elucidation of the mechanisms underlying the trafficking of receptors and their effectors is therefore critical for understanding the physiological response to a variety of hormones such as CCK. Earlier studies have demonstrated sequestration of the CCKAR upon binding of agonists (12, 13) and internalization of CCK in pancreatic acinar cells and in CHO cells transfected with CCKAR cDNA (14–17). The present work describes the study of CCKAR trafficking upon binding of agonists and antagonists that was observed in real time in four different cell types. The convenience of labeling of the receptor with the highly fluorescent marker, GFP, in combination with the recent advances in the techniques of laser scanning confocal microscopy and the development of image processing software, allowed for not only qualitative, but also quantitative characterization of spontaneous and ligand-induced receptor trafficking. Direct observation of fluorescent receptor allowed the characterization of CCKAR-GFP localization and distribution inside the cells avoiding the artifacts of fixation.

Our previous studies have demonstrated that the C terminus of the CCKAR is totally dispensable for ligand binding, signal transduction, and internalization of the CCKAR (32). Consistent with these studies, the attachment of GFP to the C terminus of the receptor did not cause noticeable changes in receptor function demonstrated by radioligand displacement studies and CCK-stimulated increase in total [H]$^3$H]inositol phosphates in CHO cells stably expressing wild type CCKAR and CCKAR-GFP, nor were there noticeable changes in the pattern and rate of receptor-mediated ligand internalization. Fusion of the N terminus of the CCKAR to the C terminus of GFP (resulting in GFP-CCKAR) disrupted the transport of the receptor to the plasma membrane. In COS-1 cells expressing GFP-CCKAR, the fluorescent receptor was distributed throughout the cytoplasm and nucleus, and no binding of CCK could be detected. In contrast, when the C terminus of the receptor was fused to the C terminus of GFP, the resulting fusion receptor was correctly transported to the cell surface. The GFP-tagged receptor molecules on the plasma membrane were able to bind CCK and transduce a signal, and be internalized similar to the wild type receptor.

CCKAR-GFP was distributed evenly along the cell membrane in all studied cell lines, suggesting that it is not dissociation of the receptor molecules with certain parts of the membrane. In contrast, the endothelin receptor, which also belongs to the GPCR family, was found to reside in caveolae in COS-1 cells overexpressing the receptor, and immunofluorescence microscopy revealed a distribution of the receptor to many small micropatches in the periphery of the cells (33).

Thus, different GPCRs may differ in distribution along the membrane.

In all three cell lines stably expressing the fluorescent CCKAR, a significant fraction of the receptor molecules was found in the intracellular compartments even in the absence of the ligand. Intracellular receptor molecules were localized predominantly at the site of their synthesis, the endoplasmic reticulum. The receptor was found in the ER in all studied cell lines. CCKAR-GFP molecules in the ER do not appear to be short-lived, since incubation of the cells with the protein synthesis inhibitor cycloheximide did not lead to complete disappearance of CCKAR-GFP from the ER. The amount of the receptor in the ER differed from one cell line to another significantly, suggesting that the time needed to transport CCKAR from the ER is different in different cell types.

Overexpression of CCKAR-GFP led to saturation of all intracellular membranes with the receptor. In COS-1 cells that overexpressed the fluorescent receptor, fluorescence corresponding to CCKAR-GFP was detected not only in the ER, endosomes, and plasma membrane (as in the cells with natural levels of receptor expression), but also in lysosomes and mitochondria. Although COS-1 cells that were transiently transfected with CCKAR-GFP had normal binding of the radiolabeled hormone, microscopy studies have revealed that the majority of the receptor molecules on their cell surface was not able to be internalized even in the presence of micromolar concentrations of the ligand, suggesting that they were not functionally coupled for internalization. Overexpression of...
monitored by CLSM and quantitated with Zeiss LSM software.

cells were exposed to the ligand for 10 min at 37 °C. Colocalization was

take place. We speculate that different levels of arrestins or

if the mediators of internalization are present in certain
toward ligand-induced phosphorylated receptor is much higher

able to interact with both the resting receptor molecules and

increased number of receptor molecules on the cell surface. It
turning to the cell surface (36). Thyrotropin-releasing hormone
concludes the properties of the system and creating artifacts.

that this strategy should be used with caution, since unnatu-

GPCR and their mutants in COS-1 cells is frequently applied

Receptors are frequently categorized into two types, based on

their pattern of internalization, receptors that internalize and

recycle constitutively and receptors that internalize upon bind-

of receptor function. Antagonists may stabilize receptor mole-

cules in an inactive conformation that has lower affinity for an in-

teraction with an internalization factor, thus inhibiting spontane-

ous internalization. This property may not be universal for all anti-

agonists. Antagonists that behave as inverse agonists stabilize the in-

active conformation of the receptor and inhibit spontaneous sig-

nal transduction. The inverse agonism of L-364,718 and CCK

27–32 amide was not tested in signal transduction previously,

but in internalization of the CCKAR they have demonstrated

the properties of inverse agonists. Further studies of different
types of antagonists are needed to understand whether it is

possible to activate internalization of the receptor without ac-

tivating signal transduction, and GFP-tagged receptors can pro-

vide the tools for such studies.

The recycling time of the CCKAR estimated from the direct

observation of CCKAR-GFP after binding of the ligand (Fig. 11)
correlates with the kinetics of fluorescent ligand uptake (Fig. 4).
The recycling time of CCKAR-GFP was found to be within
the range of recycling times for the other GPCR, which are
between 20 and 60 min (26, 35–38). The time needed for intra-
cellular sorting of the ligand and receptor has not been previ-
ously estimated. It is remarkable that the sorting of CCK-8 and
the CCKAR is very efficient, since no receptor molecules could be
detected in the lysosomes and no degradation of the receptor
could be observed by measurement of CCKAR-GFP fluores-
cence after prolonged exposure to CCK in the presence of the
protein synthesis inhibitor, cycloheximide. Studying the time
dependence of RG-CCK-8 accumulation inside the cells con-

firmed the stability of the receptor molecules during recycling.

As demonstrated in Fig. 4, the uptake of the ligand during the
second and the third cycles is not much different from that of
the first cycle. However, such efficient sorting may not be a
common property of all GPCR. The hCG/LH receptor, for ex-
ample, is degraded in lysosomes after internalization and only
a very small portion of receptor molecules recycle back to the

cell surface (35). Most of the thrombin receptor molecules are
degraded after endocytosis, with only approximately 25% re-
turning to the cell surface (36). Thyrotropin-releasing hormone
receptor is recycled, but a fraction of the internalized receptor
is targeted to a degradative pathway and results in down-
regulation of the receptor following prolonged exposure to the
ligand (37). Application of GFP tagging may allow for a direct
visualization of the fate of other GPCRs after ligand binding
and internalization, thus contributing to a better understand-
ing of receptor function.
CONCLUSIONS

Fusion of the C terminus of GPCR to the N terminus of the GFP mutant was found to be a valuable tool in studying receptor localization and trafficking. CCKAR-GFP allowed for the direct observation of spontaneous and ligand-induced internalization of the receptor. Application of two-color microscopy to the receptor-GFP fusion protein and fluorescent ligands allowed for observation of receptor and ligand sorting inside the endosomes in living cells in real time. GPCR fusion to GFP has a great potential in studying the structural requirements to GPCRs and their agonists and antagonists in evoking receptor trafficking. It can also help elucidate the cellular mechanisms of receptor translocation to the plasma membrane after biosynthesis, molecular mechanisms of receptor sequestration upon binding of the ligand, characterization of cellular factors involved in receptor trafficking, and identification of structural elements of GPCRs that define receptor localization.

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Endocytosis and Recycling of Cholecystokinin Receptor

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