Visualization of the Calcitonin Receptor-like Receptor and Its Receptor Activity-modifying Proteins during Internalization and Recycling*

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Expression of the calcitonin receptor-like receptor (CRLR) and its receptor activity modifying proteins (RAMPs) can produce calcitonin gene-related peptide (CGRP) receptors (CRLR/RAMP1) and adrenomedullin (AM) receptors (CRLR/RAMP2 or -3). A chimera of the CRLR and green fluorescent protein (CRLR-GFP) was used to study receptor localization and trafficking in stably transfected HEK 293 cells, with or without co-transfection of RAMPs. CRLR-GFP failed to generate responses to CGRP or AM without RAMPs. Furthermore, CRLR-GFP was not found in the plasma membrane and its localization was unchanged after agonist exposure. When stably coexpressed with RAMPs, CRLR-GFP appeared on the cell surface and was fully active in intracellular cAMP production and calcium mobilization. Agonist-mediated internalization of CRLR-GFP was observed in RAMP1/CGRP or AM, RAMP2/AM, and RAMP3/AM, which occurred with similar kinetics, indicating the existence of ligand-specific regulation of CRLR internalization by RAMPs. This internalization was strongly inhibited by hypertonic medium (0.45 M sucrose) and paralleled localization of rhodamine-labeled transferrin, suggesting that CRLR endocytosis occurred predominantly through a clathrin-dependent pathway. A significant proportion of CRLR was targeted to lysosomes upon binding of the ligands, and recycling of the internalized CRLR was not efficient. In HEK 293 cells stably expressing CRLR-GFP and Myc-RAMPs, these rhodamine-labeled RAMPs were co-localized with CRLR-GFP in the presence and absence of the ligands. Thus, the CRLR is endocytosed together with RAMPs via clathrin-coated vesicles, and both the internalized molecules are targeted to the degradative pathway.

Calcitonin gene-related peptide (CGRP)1 and adrenomedullin (AM) belong to the calcitonin family of regulatory peptides, both of which are potent vasodilators (1, 2). CGRP is a 38-amino acid neuropeptide produced by tissue-specific alternative splicing of the primary transcript of the calcitonin gene (3). CGRP immunoreactivity is present throughout the central and peripheral nervous system, and release of CGRP from nervous tissue has been demonstrated (4–6). Human AM, which consists of 52 amino acids, exhibits diverse biological activities consistent with its wide tissue distribution (2, 7, 8). It mainly exerts powerful cardiovascular effects as an autocrine/paracrine regulator. Both peptides have been reported to activate adenyl cyclase and phospholipase C, and to increase the intracellular calcium concentration in various cell types (9, 10). This suggests that their receptors activate different G proteins, Gαi and Gq. Specific or common binding sites for these peptides have been suggested to be present in both central and peripheral tissues; however, their formal cell receptors have not been identified.

Recently, human receptor activity-modifying protein 1 (RAMP1) was identified as an accessory protein that enhances the activity of endogenous CGRP receptors in Xenopus oocytes (11). This protein consisted of 148 amino acids including an N-terminal signal sequence and putative single transmembrane region, and was shown to induce trafficking of the seven-transmembrane domain calcitonin receptor-like receptor (CRLR) to the cell surface by fluorescence-activated cell sorting analysis. RAMP2 and -3 cDNAs (175 and 148 amino acids, respectively) were then cloned by expressed sequence tag analysis (11). When co-transfected with RAMP2 or -3 in mammalian cells the CRLR became a functional AM receptor (11–13). Although RAMP2 and -3 share only 30% homology and they show different tissue distributions, the CRLR/RAMP2 and CRLR/RAMP3 receptors in HEK 293T cells have been reported to be indistinguishable by radioligand binding, functional assay, and biochemical analysis (13).

Exposure of cells to agonists often leads to a rapid internalization of cell surface G protein-coupled receptors (GPCRs). This agonist-promoted phenomenon is common to a large number of GPCRs (14, 15). Internalization is believed to involve clathrin-coated vesicles and/or caveolin-rich vesicles. The internalized GPCRs may be recycled back to the plasma membrane rather than being trafficked to lysosomes where they are degraded (14, 15). These processes have been best studied and characterized for the β2-adrenergic receptor (14, 15). On the other hand, there have been no reports concerning the trafficking of RAMP molecules after agonist exposure.
The recent development of GPCR conjugated with green fluorescent protein (GFP) has provided the opportunity for a more extensive optical analysis of receptor trafficking events in individual cells (16–19). In this study, we characterized the biochemical properties of the functional CRLR chimera with GFP fused to the C terminus of the receptor, CRLR-GFP, with or without co-transfection of RAMPs. Moreover, direct visualization of the localization of CRLR-GFP and rhodamine-labeled RAMPs provided evidence that, when stably coexpressed, agonist-induced endocytosis of both molecules occurred through the same pathways and this was irreversible.

**EXPERIMENTAL PROCEDURES**

**Materials**

A plasmid containing human CRLR cDNA (20) was a kind gift from Dr. Kenji Kangawa (National Cardiovascular Research Institute, Osaka, Japan). \(^{125}\)I-Labeled human CGRP (specific activity 2177 Ci/mmol) and \(^{125}\)I-labeled human adrenomedullin (specific activity 1260 Ci/mmol) were from Peninsula Laboratories. Human CGRP and adrenomedullin were from Peptide Institute (Osaka, Japan). Fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate (TRITC) were from Dako. Tetramethylrhodamine-concanavalin A (ConA), tetramethylrhodamine-transferrin, and Lysotracker Red were purchased from Molecular Probes Inc. All other reagents were of analytical grade and were obtained from various suppliers.

**Methods**

Construction of the CRLR-GFP and Myc Epitope-tagged CRLR or RAMP Expression Vectors—A bright green mutant of GFP, enhanced GFP (CLONTECH), was attached to the C terminus of human CRLR by standard recombinant techniques. Briefly, the full open reading frame of the human CRLR, modified to provide a consensus Kozak sequence (21), was amplified using appropriate primers containing SauI and XbaI restriction sites at the 5' and 3' ends, respectively. The 5' and 3' ends of CRLR were ligated into the SauI and XbaI sites of modified pEGFP-N1 (CLONTECH), SnaiI → XbaI at its multilinking site. The fused product was then cloned into pCAGGS-Neo, modified to provide a consensus Kozak sequence, were obtained from a human embryonic cDNA library (CLONTECH). Each plasmid was then introduced into pIRESHyg (CLONTECH). The Myc epitope tag (EQKLISEEDL) was used in-frame to the 5' 'end coding CRLR by permission of the publisher. The 5' and 3' sequences were removed and replaced with KMTILASTYIFCLVFA (22). Myc-CRLR and Myc-RAMPs were cloned into pCAGGS/Neo and pIRESHyg, respectively. CRLR-GFP, Myc-CRLR, RAMPs, and Myc-RAMPs cDNAs were all sequenced using an Applied Biosystems 310 Genetic Analyzer.

**Cell Cultures and Stable Expression in HEK 293 Cells**—Human embryonic kidney cells (HEK 293) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin G, 100 units/ml streptomycin, and 50 μg/ml amphotericin B at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. The recent development of GPCR conjugated with green fluorescent protein (GFP) has provided the opportunity for a more extensive optical analysis of receptor trafficking events in individual cells (16–19). In this study, we characterized the biochemical properties of the functional CRLR chimera with GFP fused to the C terminus of the receptor, CRLR-GFP, with or without co-transfection of RAMPs. Moreover, direct visualization of the localization of CRLR-GFP and rhodamine-labeled RAMPs provided evidence that, when stably coexpressed, agonist-induced endocytosis of both molecules occurred through the same pathways and this was irreversible.

**Flow Cytometry**—Flow cytometry was performed to assess expression of constructs of CRLR-GFP in the absence and presence of RAMPs. Cells were dissociated, washed twice with fluorescence-activated cell sorting buffer (1% FBS, 0.02% sodium azide in phosphate-buffered saline (PBS)), and adjusted to 2 x 10⁶ per tube in the same buffer with 5 μg/ml propidium iodide. Samples were subjected to flow cytometry on an EPICS XL flow cytometer (Beckman Coulter) and analyzed using the EXPO 2 software (Beckman Coulter). Fluorophores (Sigma) for 15 min at 37 °C. The reactions were terminated by addition of lysis buffer (Amersham Pharmacia Biotech) followed by centrifugation at 2000 rpm for 10 min at 4 °C. Apliques of the supernatants were removed and cAMP content determined using a commercial enzyme immunoassay kit, according to the manufacturer's instructions for the non-acetylation protocol (Amersham Pharmacia Biotech).

**Measurement of Intracellular Ca²⁺**—Stably transfected HEK 293 cells (1 x 10⁶/ml) were loaded with 2 μM fura-2/AM (Molecular Probes Inc.) in Hanks' buffered salt solution containing 20 mM HEPES and 0.1% BSA for 30 min at 37 °C in the dark. After two washes, cells were resuspended at 1 x 10⁶ cells/ml. Cells (2 ml) were prewarmed to 37 °C and stimulated by 100 nM agonists in a quartz cuvette with a continuously stirring magnetic bar using a Perkin-Elmer LS 50B spectrofluorometer. Data were recorded as the relative ratio of fluorescence emitted at 510 nm after excitation at 340 and 380 nm (y axis) over time (x axis).

**Localization of CRLR-GFP, Rhodamine-labeled CRLR and RAMPs, and Tetramethylrhodamine-transferrin—HEK 293 cells stably expressing CRLR-GFP, CRLR-GFP/RAMPs, CRLR-GFP/Myc-RAMPs, or Myc-CRLR/RAMPs were plated onto 35-mm dishes containing a centered 22-mm well formed from a glass coverslip seated in a hole in the plastic. After rinsing with PBS, human CGRP or adrenomedullin was added to the dishes at a concentration of 100 nM in prewarmed serum-free DMEM containing 20 mM HEPES and 0.1% BSA for the indicated times at 37 °C. For receptor recycling studies, following 30-min agonist treatment, cells were washed three times with prewarmed PBS and medium was replaced with fresh DMEM for 1 h at 37 °C. Internalization and recycling were stopped with ice-cold PBS. Cells were then fixed with 3.7% formaldehyde/PBS for 15 min at room temperature and washed three times with PBS. The coverslips were mounted using Slow-Fade mounting medium (Molecular Probes Inc.). To examine whether Myc-RAMPs localize with CRLR-GFP during internalization and recycling, after the indicated incubation period, cells were fixed as described above, and binding CRLR or the corresponding secondary antibody. Following a 30-min incubation, cells were washed three times with PBS, counterstained using Slow-Fade. To simultaneously observe endocytosis of CRLR-GFP and transferrin, cells were incubated with 100 nM human CGRP or adrenomedullin and 100 μM tetramethylrhodamine-transferrin for 15 min at 37 °C, fixed, washed, and mounted. Events following agonist treatment were observed by confocal microscopy.

**Radioligand Binding Assays for Internalization and Recycling of CRLR-GFP and Myc-CRLR—Whole cell radioligand binding assays were performed as follows. Cells were seeded in 24-well culture plates (3 x 10⁴ cells/well). After 3 days, cells were pretreated with or without the inhibitor (0.45 μM sucrose, 15 min) and the incubated with serum-free DMEM containing 20 mM HEPES and 0.1% BSA for the indicated periods (up to 3 h) at 37 °C in the presence or absence of 100 nM agonists. Following agonist exposure, cells were washed twice with ice-cold PBS and then incubated with either \(^{125}\)I-labeled human CGRP (10 pM) or \(^{125}\)I-labeled human adrenomedullin (20 pM) in modified Krebs-Ringers-HEPES medium (20 mM HEPES, pH 7.4, 110 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 25 mM d-glucose, 1% BSA) for 3 h at 4 °C. Cells were harvested with 0.5 M NaOH, and associated radioactivity was counted in a γ-counter. For receptor recycling studies, following 30-min ligand occupancy, cells were washed three times with prewarmed PBS and then incubated with fresh DMEM for 60 min at 37 °C. After incubation, the medium was replaced with ice-cold Krebs-Ringers-HEPES medium. Following the same radioligand treatment for 3 h at 4 °C, cells were harvested and then counted in a γ-counter. Non-specific binding was measured in the presence of 1 μM unlabeled CGRP or AM.
RESULTS

Construction and Characterization of CRLR-GFP—For microscopic visualization of CRLR molecules, we constructed a chimera consisting of the human CRLR fused to mutant GFP (EGFP) at its C terminus. To examine the functionality of the CRLR-GFP fusion protein, intracellular cAMP concentrations were measured. The intact HEK 293 cells lacked functional CGRP and AM receptors, because they showed little cAMP response to agonist stimulation (Fig. 1A, left). In contrast to other RAMPs, when stably expressed alone in HEK 293 cells, RAMP1 induced cAMP responses to CGRP (maximal cAMP reached approximately 8-fold basal), but not to AM (data not shown), suggesting that RAMP1 and endogenous calcitonin receptor may function as CGRP receptors (23). In HEK 293 cells stably expressing CRLR-GFP alone, AM, but not CGRP, slightly increased cAMP contents; maximal cAMP level reached approximately 2.5-fold higher than the basal level (Fig. 1A, right). This was consistent with the finding that HEK 293 cells express endogenous RAMP2 (data not shown). After stable co-transfection of individual RAMPs, expression of constructs of the CRLR-GFP was assessed using flow cytometry. The fluorescence of the CRLR-GFP in the cells expressing RAMP1, -2, or -3 together yielded at least 100-fold greater intensity than HEK 293 control cells, with individual single peaks, indicating that the three types of cells expressed CRLR-GFP at almost 100% (Fig. 1B). Furthermore, RAMPs increased the fluorescence intensity of CRLR-GFP; 11.5% for RAMP1, 6.8% for RAMP2, and 6.7% for RAMP3 (Fig. 1C). When expressed with RAMPs, CRLR-GFP had significant ability to produce cAMP responses to CGRP or AM that were concentration-dependent (Fig. 2A). Expression of CRLR-GFP and RAMP1 (CRLR/RAMP1) induced cAMP responses to CGRP or AM (EC50 of 0.34 or 1.1 nM, respectively), which were 100-fold more potent than that in CRLR-GFP-expressing cells. In contrast, CRLR/RAMP2 or -3 specifically responded to AM, with EC50 of 0.39 or 0.86 nM, respectively. Such ligand specificity of CRLR by RAMPs was also observed in intracellular calcium mobilization (Fig. 2B). These [Ca2+]i increases by CGRP for CRLR/RAMP1 receptor and by AM for CRLR/RAMP2 or -3 receptor were receptor-mediated, because subsequent rechallenge with the same ligand failed to evoke significant responses, probably due to receptor desensitization. In CRLR/RAMP1 stable transfectants, following exposure to AM or CGRP elicited a small [Ca2+]i increase similar to that in HEK 293 cells stably expressing CRLR-GFP alone (data not shown), suggesting the existence of another CGRP receptor, calcitonin receptor/RAMP1, as mentioned above. These observations suggested that the fusion of the 238-amino acid GFP protein to the C terminus of the CRLR shows the full functional properties of the receptor in cells coexpressing RAMPs.

Localization of CRLR-GFP without Co-transfection of RAMPs—To determine the distribution of the fluorescent CRLR-GFP without co-transfection of RAMPs, the cell surface was labeled by brief exposure to tetramethylrhodamine-ConA (50 μg/ml in PBS). The green fluorescence corresponding to CRLR showed little co-localization with the red fluorescence of ConA, and was distributed throughout the cytoplasm. The staining pattern suggested that CRLR-GFP was probably in the endoplasmic reticulum, representing the pool of newly synthesized molecules not yet transported to the plasma mem-

brane (Fig. 3A). After 30-min exposure to 100 nM CGRP or AM, the distribution pattern of the CRLR-GFP was almost unchanged (Fig. 3, B and C).

Localization of CRLR-GFP with Co-transfection of RAMPs in the Absence of Ligands—A large proportion of the green fluorescence corresponding to CRLR-GFP was observed on the cell membrane in cells stably co-transfected with RAMPs, whereas a small number of receptors was diffusely distributed throughout the cytoplasm (Fig. 4).

Ligand-induced Internalization of CRLR-GFP with Co-transfection of RAMPs—Measurement of radioligand binding revealed that exposure of cells coexpressing RAMPs to 100 nM
CGRP or AM for up to 120 min produced rapid internalization of 50–70% of the cell surface CRLR-GFP, with similar kinetics (Fig. 5A). In all three stably transfected cell lines, addition of ligands caused the green fluorescence of the CRLR to disappear almost completely from the cell surface, depending on the ligand specificity; RAMP1/CGRP or AM, RAMP2/AM, and RAMP3/AM (Fig. 5B). Pretreating cells with a hypertonic sucrose solution has been demonstrated to be effective in blocking receptor endocytosis via clathrin-coated pits (24–26). Therefore, we preincubated our transfected cells with 0.45M sucrose for 15 min, followed by exposure to 100 nM ligands for various periods. Even after 120 min of agonist incubation, almost no receptor sequestration was observed (Fig. 6, A and B). Sucrose pretreatment did not affect intracellular cAMP production by ligands (data not shown). The CRLR-GFP fluorescence moved into intracellular vesicles identified by the tetramethylrhodamine derivative of transferrin, used as a marker of the endosomal compartment (27) (Fig. 7). Then, the majority of receptor molecules showed significant co-localization with the lysosomal marker, LysoTracker Red (28) (Fig. 8).
incubated for various periods. After removal of the ligands, cell surface CRLR-GFP increased less than 10% in all three stably transfected cell lines (Fig. 9, A and B).

Localization and Agonist-mediated Internalization of Myc-CRLR with Co-transfection of RAMPs—To investigate whether fusing the C terminus of GFP affects ligand-induced trafficking of CRLR, we have further constructed a chimeric molecule consisting of the CRLR fused to the Myc epitope tag at its N terminus (Myc-CRLR). Examination of numerous HEK293 cells stably co-transfected with RAMPs suggests that the rhodamine-labeled Myc-CRLR was diffusely distributed on the cell surface before agonist treatment (Fig. 10). Radioligand binding experiments in three stably transfected cell lines indicate that exposure of 100 nM CGRP or AM caused rapid internalization of the cell surface Myc-CRLR, with similar kinetics (Fig. 11A). In all three transfectants, addition of ligands caused the rhodamine fluorescence of the CRLR to disappear almost completely from the cell surface, depending on the ligand specificity; RAMP1/CGRP or AM, RAMP2/AM, and RAMP3/AM (Fig. 11B). Cells expressing Myc-CRLR and RAMPs were pretreated with cycloheximide (25 μg/ml) and incubated with the ligands (100 nM CGRP or AM) for 30 min at 37 °C. After agonist removal, cell surface Myc-CRLR increased less than 10–20% in the three stable transfectants (Fig. 12, A and B), consistent with that of the CRLR-GFP. Thus, recycling of the CRLR was highly inefficient, and the majority of receptor molecules were targeted to the degradative pathway.

Localization of Myc-RAMPs without Co-transfection of

**FIG. 5.** Agonist-promoted internalization of CRLR-GFP with coexpression of RAMPs. A, cells were treated for the indicated times with 100 nM CGRP for CRLR/RAMP1 receptor and with 100 nM AM for CRLR/RAMP2 and -3 receptors. Then, binding to 125I-CGRP for the former receptor and to 125I-AM for the latter was assessed. Binding at various time points was compared with the unstimulated cells. Non-specific/total binding ratios in CRLR/RAMP1, CRLR/RAMP2, and CRLR/RAMP3 receptors were 4.5 ± 0.7%, 36.9 ± 0.5%, and 35.1 ± 0.6%, respectively. Results represent the mean ± S.E. of three experiments.

**FIG. 6.** Inhibition of ligand-induced CRLR-GFP internalization by hypertonic medium. A, cells were pretreated with 0.45 M sucrose for 15 min, and then exposed to 100 nM ligand for different periods. Next, binding to 125I-CGRP for CRLR/RAMP1 receptor and to 125I-AM for CRLR/RAMP2 or -3 receptor was assessed. Binding at various time points was compared with the unstimulated cells. Data are the mean ± S.E. of three experiments. B, CRLR-GFP/RAMPs double transfectants were pretreated with 0.45 M sucrose for 15 min, and then exposed to 100 nM ligand for 30 min. CGRP for CRLR/RAMP1 (top); AM for CRLR/RAMP2 (middle); AM for CRLR/RAMP3 (bottom).

**FIG. 7.** Colocalization of CRLR-GFP (green) with the endosomal marker tetramethylrhodamine transferrin (red) after ligand exposure. Cells stably expressing CRLR-GFP and RAMPs were exposed to the marker and 100 nM ligand for 15 min. A, CGRP for CRLR/RAMP1; B and C, AM for CRLR/RAMP2 and -3, respectively.

**FIG. 8.** Colocalization of CRLR-GFP with the lysosomal marker LysoTracker Red after addition of the ligand. CRLR-GFP/RAMPs stable transfectants were treated with the marker and 100 nM ligand for 30 min. A, CGRP for CRLR/RAMP1; B and C, AM for CRLR/RAMP2 and -3, respectively.

**FIG. 9.** Recycling of CRLR-GFP in the cells stably expressing CRLR-GFP and RAMPs. A, cells were incubated for various periods after 30 min of exposure to 100 nM ligand (CGRP for CRLR/RAMP1; AM for CRLR/RAMP2 and -3, respectively) plus cycloheximide at 25 μg/ml. Thereafter, binding to 125I-CGRP for the CRLR/RAMP1 receptor and to 125I-AM for CRLR/RAMP2 and -3 receptors was assessed. Binding at various time points was compared with the unstimulated cells. Results represent the mean ± S.E. of three experiments. B, CRLR-GFP/RAMPs stable transfectants were incubated in medium containing cycloheximide at 25 μg/ml for 60 min after 30 min of exposure to 100 nM ligand plus cycloheximide. CGRP for CRLR/RAMP1 (top); AM for CRLR/RAMP2 (middle); AM for CRLR/RAMP3 (bottom).
CRLR—Prior to the evaluations of trafficking of both CRLR and RAMPs, we examined the localization patterns of RAMPs without co-transfection of CRLR using HEK 293 cell lines stably expressing Myc-RAMPs (Fig. 13). No rhodamine fluorescence of Myc-RAMP1 could be detected in the cells, regardless of no error in its sequence (data not shown). Rhodamine-labeled Myc-RAMP2 was distributed throughout the cytoplasm, but not on the cell surface. The fluorescent distribution did not change after addition of the ligands. The cells little showed cAMP response to agonist stimulation (data not shown), although they express endogenous CRLR. In contrast, the number of Myc-RAMP3 molecules was observed on the cell surface in the absence of ligands. The cell surface Myc-RAMP3 was found to undergo rapid internalization after exposure of CGRP or AM. The transfectant induced cAMP responses to CGRP and AM (maximal cAMP reached approximately 5- and 4-fold basal, respectively), suggesting that RAMP3 and endogenous calcitonin receptor may function as CGRP and AM receptors (23).

Colocalization of Rhodamine-labeled RAMPs and CRLR-GFP—To explore whether ligand-induced translocation of accessory proteins, RAMPs, occurs together with CRLR-GFP, we established HEK 293 cell lines stably expressing Myc-RAMPs and CRLR-GFP. AM was also significantly potent in stimulating cAMP production for CRLR/Myc-RAMP2 or -3 receptor to a level comparable to that observed for the CRLR/RAMP2 or -3 receptor (Fig. 14). Coexpression of Myc-RAMP1 induced only a small cAMP response to CGRP (data not shown). Prior to agonist treatment, a significant proportion of rhodamine-labeled RAMP2 and -3 molecules were co-localized with CRLR-GFP diffusely distributed in the plasma membrane (Fig. 15A). Addition of AM caused translocation of these RAMPs from the cell surface into intracellular vesicles, together with CRLR-GFP (Fig. 15B). After removal of the ligands, a large proportion of RAMPs remained in the cells and were co-localized with CRLR-GFP (Fig. 15C). Thus, RAMPs, which are co-localized with CRLR-GFP on the cell surface, undergo translocation with CRLR-GFP through the same pathway after ligand stimulation.

**DISCUSSION**

The development of proteins conjugated with GFP has contributed significantly to direct visualization of protein trafficking in individual cells avoiding the artifacts of fixation. The cytoplasmic C-terminal domain of GPCRs is well known to play a key role in regulation of several kinase activities and receptor interactions with other protein molecules. On the other hand, attachment of the GFP tag at the N terminus may hinder the translocation and processing of newly synthesized receptors and ligand binding. In fact, fusing the C terminus of GFP was found previously to produce fully functional cholecystokinin receptor (CCKAR) (16), β2-adrenergic receptor (β2-AR) (17), CXC-chemokine receptor 4 (CXCR4) (18), and epidermal growth factor receptor (19). Fusion of the N terminus of the CCKAR to the C terminus of GFP, however, disrupted transport of the receptor to the plasma membrane (16). Considering these results, we fused the GFP at the C terminus of human CRLR (CRLR-GFP).

CRLR-GFP failed to appear on the cell membrane and to generate responses to CGRP and AM without co-transfection of accessory proteins, RAMPs. When coexpressed with RAMPs, the CRLR-GFP was localized on the plasma membrane probably from the endoplasmic reticulum, and was fully functional in intracellular cAMP production and calcium mobilization depending on the ligand specificity of the RAMPs. This is the first report that the CRLR-RAMP system can also activate phospholipase C. Human SK-N-MC and vascular endothelial cells express endogenous and specific CGRP and AM receptors, respectively (9, 29). In our transfectants, the EC50 values in stimulating cAMP production for CGRP and AM receptors were
comparable to those observed in these native cells. Collectively, these observations suggested that fusion of the GFP protein to the C terminus of the CRLR shows full functional properties of the receptor when coexpressed with RAMPs.

Similarly to many other GPCRs, surface CRLR molecules in HEK 293 cells coexpressed with RAMPs internalized upon binding of the agonist, apparently different from spontaneous internalization of CCKAR and CXCR4 in NIH/3T3 cells (16, 18). Our experiments demonstrated that the internalization of CGRP receptors (CRLR/RAMP1) and AM receptors (CRLR/RAMP2 or -3) occurs rapidly and extensively with similar kinetics. This endocytosis is dependent on the agonist specificity of RAMPs consistent with the results of functional analysis. We have also demonstrated that RAMPs undergo endocytosis together with CRLR molecules after ligand exposure. From the available data, we cannot determine how the internalized proteins interact in the vesicles. The cytoplasmic C-terminal domains of RAMPs are too short compared with that of CRLR. It is therefore unlikely that RAMPs will regulate CRLR internalization.

Receptor internalization is believed to involve clathrin-coated vesicles and/or caveolin-rich vesicles (14, 15). The predominant pathway is via coated vesicles. Two independent
experimental approaches suggested that endocytosis of the CRLR involves coated pits: (i) localization of the fluorescent CRLR and transferrin receptors overlapped substantially; and (ii) internalization of the CRLR-GFP was strongly inhibited by hypertonic medium, which is known to selectively inhibit formation of coated pits and vesicles (24–26). β₂-AR (30), neurokinin-1 (31), luteinizing hormone (32), thrombin (33), and gastrin-releasing peptide (34) receptors are also believed to be internalized into coated vesicles.

Many receptors in the G protein-coupled superfamily have an NPXY motif in their seventh transmembrane domain, and this has been implicated in internalization of β₂-AR (35). However, mutations of this region in some GPCRs do not affect internalization, suggesting that this motif might not be sufficient for internalization in these receptors; for example, mutation of the Tyr in this motif in gastrin-releasing peptide receptor or angiotensin II receptor does not alter the extent of internalization (36, 37). Such a motif is absent in the CRLR molecule. Future experiments will focus on identifying the site(s) responsible for CRLR internalization.

Once receptors have undergone endocytosis, they can be recycled to the plasma membrane or targeted to lysosomes, where they undergo degradation. Many internalized receptors return to the cell surface without targeting to the degradative pathway in the presence of the protein synthesis inhibitor cycloheximide (14, 15). Here, we demonstrated that recycling of CRLR-GFP and rhodamine-labeled RAMPs was not efficient in HEK 293 cells, and a significant proportion of CRLR molecules, which co-localize with RAMPs, could be observed in lysosomes after addition of the ligands. Recently, addition of various substances to the transferrin receptors did not affect their recycling (38). In contrast, extended cytoplasmic tail of the transferrin receptors did not affect their recycling (38).

In this study the return of the Msc-CRLR to the cell surface after agonist removal was found to be quantitatively similar to that of the CRLR-GFP, suggesting that fusion of the 238-amino acid GFP protein to the C terminus of the CRLR does not alter trafficking behavior of the receptor. Receptors may be differentially regulated in different cell lines, which may be related to variability in the cellular component acting on the receptor. However, variation in the degree of inefficient recycling of CXCR4-GFP in different cell lines was slight (18). Several other GPCRs also were reported to undergo partial recycling; luteinizing-hormone/chorionic-gonadotropin (32), thrombin (33), thyrotropin-releasing hormone (39), secretin (40), and V2 vasopressin (41) receptors. Among these, a cluster of three serines was suggested to be a key element in the intracellular retention of the V2 vasopressin receptor. Such a motif is also present in the CXCR4, but not in the CRLR. The molecular motifs that specify the different trafficking of internalized receptors have not been identified. CRLR belongs to the secretin receptor family, and it is therefore likely that some common motif(s) will be responsible for the intracellular retention of both receptor molecules. Whether RAMPs are involved in the degradative pathway remains to be determined.

In summary, we utilized CRLR-GFP to visualize cellular localization and trafficking of CRLR in HEK 293 cells responding to agonists with or without coexpression of its accessory proteins, RAMPs. We demonstrated the rapid co-localization of the CRLR in transferrin-containing endosomes and then in lysosomes following ligand exposure, when only coexpressed with RAMPs. Moreover, the CRLR was internalized together with these RAMPs, both of which appeared to be targeted to the degradative pathway.

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