Functional Calcitonin Gene-related Peptide Receptors Are Formed by the Asymmetric Assembly of a Calcitonin Receptor-like Receptor Homo-oligomer and a Monomer of Receptor Activity-modifying Protein-1*

Received for publication, March 1, 2007, and in revised form, August 24, 2007 Published, JBC Papers in Press, September 4, 2007 DOI 10.1074/jbc.M701790200

Madeleine Héroux1‡, Mireille Hogue1, Sébastien Lemieux§, and Michel Bouvier1,2

From the 1Department of Biochemistry and Groupe de Recherche Universitaire sur le Médicament and 2Department of Computer Science and Operations Research, Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, Québec H3C 3J7, Canada

In addition to their interactions with hetero-trimeric G proteins, seven-transmembrane domain receptors are now known to form multimeric complexes that can include receptor homo- or hetero-oligomers and/or accessory proteins that modulate their activity. The calcitonin gene-related peptide (CGRP) receptor requires the assembly of the seven-transmembrane domain calcitonin receptor-like receptor with the single-transmembrane domain receptor activity-modifying protein-1 to reach the cell surface and be active. However, the relative stoichiometric arrangement of these two proteins within a receptor complex remains unknown. Despite recent advances in the development of protein-protein interactions assays, determining the composition and stoichiometric arrangements of such signaling complexes in living cells remains a challenging task. In the present study, we combined bimolecular fluorescence complementation (BiFC) with bioluminescence resonance energy transfer (BRET) to probe the stoichiometric arrangement of the CGRP receptor complex. Together with BRET competition assays, co-immunoprecipitation experiments, and BiFC imaging, dual BRET/BiFC revealed that functional CGRP receptors result from the association of a homo-oligomer of the calcitonin receptor-like receptor with a monomer of the accessory protein receptor activity-modifying protein-1. In addition to revealing the existence of an unexpected asymmetric oligomeric organization for a G protein-coupled receptor, our study illustrates the usefulness of dual BRET/BiFC as a powerful tool for analyzing constitutive and dynamically regulated multiprotein complexes.

Increasing evidence indicates that “signalosomes,” regrouping different components of a signaling pathway into multiprotein complexes with well-defined stoichiometries, play crucial roles in defining the specificity and efficacy of signal transduction (1, 2). For instance, the calcitonin gene-related peptide (CGRP) receptor results from the association of the calcitonin receptor-like receptor (CRLR), a seven-transmembrane domain (7TM) receptor belonging to the superfamily of G protein-coupled receptors, with a one-transmembrane domain protein, the receptor activity-modifying protein-1 (RAMP1), which is part of a three member family (RAMP1, -2, and -3). When expressed individually, both CRLR and RAMP1 are retained intracellularly in the endoplasmic reticulum (ER), whereas their association permits their common plasma membrane targeting and determines the pharmacological properties of the receptor (3–5). From these data, it was proposed that the formation of a functional CGRP receptor requires a 1:1 stoichiometric association of CRLR and RAMP1. Consistent with this idea, cross-linking experiments, using bivalent reagents, captured complexes consisting of one CRLR and one RAMP1 molecule (6). However, the observation that both CRLR and RAMP1 can form homo-oligomers (often refer to as dimers) when expressed alone (3, 6–8) adds a new level of complexity that reactualizes the question of the number of CRLR and RAMP1 molecules forming the receptor complex in living cells. The present study was therefore undertaken to probe the composition and relative stoichiometric arrangement of the CGRP receptor.

Despite the emergence of several cell-based techniques to detect protein-protein interactions (9–11), the lack of assays monitoring interactions between more than two proteins simultaneously makes stoichiometric analysis of protein complexes in living cells a challenging endeavor. Among the techniques used to study protein-protein interactions in living cells, resonance energy transfer (RET) and protein fragment complementation assays are gaining in popularity. RET approaches rely on the nonradiative transfer of energy between luminescence and/or fluorescent donor and acceptor molecules that are

* This work was supported by a grant from the Canadian Institute for Health Research (to M.B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Recipient of a studentship from the Canadian Institute for Health Research.
2 Recipient of a research Chair in Signal Transduction and Molecular Pharmacology. To whom correspondence should be addressed: Institute for Research in Immunology and Cancer, Université de Montréal, P.O. Box 6128, Downtown Station, Montréal, PQ H3C 3J7, Canada. Tel.: 514-343-6319; Fax: 514-343-6843; E-mail: michel.bouvier@umontreal.ca.

3 The abbreviations used are: CGRP, calcitonin gene-related peptide; 7TM, seven-transmembrane domain; BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; COS7, simian kidney fibroblasts; CRLR, calcitonin receptor-like receptor; ER, endoplasmic reticulum; Fz1, Frizzled 1; Fz4m, Frizzled 4 mutant; HEK, human embryonic kidney; RAMP1, receptor activity-modifying protein-1; RET, resonance energy transfer; RLuc, Renilla luciferase; YFP, yellow fluorescent protein; YC, C-terminal fragment of YFP; YN, N-terminal fragment of YFP; PBS, phosphate-buffered saline; HA, hemagglutinin.
attached to potential protein partners (12). For protein complementation assay, the reconstitution of a reporter protein (typically an enzyme) from its two fragments attached to the potential protein partners under study is taken as evidence for interactions between the partners (9). The development of a protein complementation assay known as bimolecular fluorescence complementation (BiFC), based on the reconstitution of a yellow fluorescent protein (YFP) upon reassociation of its two fragments (13), opens the possibility of combining this technology with resonance energy transfer approaches to probe interactions between at least three partners at the same time in living cells (14, 15). Taking advantage of this possibility, we combined BiFC with a bioluminescence resonance energy transfer assay (BRET) that uses Renilla luciferase (RLuc) and YFP as the energy donor and acceptor, respectively. Using this dual BRET/BiFC approach in combination with BRET competition, co-immunoprecipitation, and subcellular distribution microscopy studies, we found that at least two molecules of CRLR associate with a single molecule of RAMP1 to generate an asymmetrical oligomeric complex that can reach the cell surface as a functional entity. In addition to shedding new light on the CGRP receptor-gomeric complex that can reach the cell surface as a functional molecule of RAMP1 to generate an asymmetrical oligomeric complex, we found that at least two molecules of CRLR associate with a single molecule of RAMP1 to generate an asymmetrical oligomeric complex that can reach the cell surface as a functional entity. In addition to shedding new light on the CGRP receptor-gomeric complex that can reach the cell surface as a functional entity.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—Plasmids encoding CRLR, RAMP1, Myc-CRLR, and Myc-RAMP1 were generously provided by S. M. Foord (GlaxoSmithKline), CRLR-YFP was provided by P. M. Sexton (Monash University, Clayton, Australia), Fz1-RLuc and Fz4m were provided by R. T. Moon (University of Washington School of Medicine, Seattle, WA), CD8-C1 was provided by R. C. Malenka (Stanford University School of Medicine, Palo Alto, CA), and YFP BiFC fragments were provided by T. K. Kerppola (University of Michigan Medical School, Ann Arbor, MI). GABA<sub>α</sub>R1-RLuc and β-arrestin2-RLuc were previously described (16). Myc-CRLR-RLuc, Myc-RAMP1-RLuc, and HA-RAMP1-RLuc (hereafter called CRLR-RLuc, RAMP1-RLuc, and HA-RAMP1, respectively) have also been previously described (8). The RAMP1-RLuc was generated by subcloning the coding sequence of enhanced YFP at the C terminus of Myc-RAMP1, yielding a five-amino acid linker encoding VPVAT between the two proteins. To obtain the CRLR, RAMP1, and CD8-C1 BiFC constructs, the cDNAs encoding amino acids residues 1–154 (YN) and 145–238 (YC) of enhanced YFP were fused at the C terminus of Myc-CRLR, Myc-RAMP1, and CD8-C1, creating a 10-amino acid linker encoding VPVNSGGGGS between the CRLR, RAMP1, or CD8-C1 and the YFP fragment. The two YFP fragments provide a 10-amino acid overlapping region (residues 145–154) that facilitates complementation (17).

**Cell Culture and Transfections**—Human embryonic kidney 293T cells (HEK293T) and simian kidney fibroblasts (COS7) were maintained as described previously (8). Transfections were performed using the calcium phosphate precipitation method for HEK293T cells (18) or the FuGENE 6 transfection reagent (Roche Applied Science) for COS7 cells, according to the manufacturer’s protocol. Transfected cells were incubated at 37 °C for 24 h and switched at 30 °C for another 24 h to promote maturation of the YFP fluorophore in BiFC experiments (17). For other experiments, transfected cells were incubated at 37 °C for 48 h.

**Radioligand Binding**—Transfected HEK293T cells were washed three times with cold PBS and incubated on ice for 3 h in binding buffer (Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin and 20 mM HEPES, pH 7.4) with 200 pM [<sup>125</sup>I]CGRP (GE Healthcare Life Sciences) in the absence (total binding) or presence of increasing concentrations of unlabeled CGRP (Bachem AG). Nonspecific binding was defined as binding in the presence of 1 μM unlabeled CGRP. After incubation, the cells were washed once with binding buffer, washed twice with PBS, and then solubilized with 0.5 M NaOH. The associated cellular radioactivity was measured in a γ-counter.

**BRET Titration Curves and BRET/BiFC Assays**—Transfected HEK293T cells were washed twice with PBS, detached, and resuspended in PBS containing 0.1% glucose. The cells were then distributed in 96-well plates (100,000 cells/well) (white plates from Corning), and coelenterazine H (Molecular Probes) was added at a final concentration of 5 μM. The readings were collected immediately following coelenterazine H addition using a Mithras LB 940 instrument (Berthold Technologies). The BRET signal corresponds to the ratio of the light emitted by the YFP (510–550 nm) over the light emitted by the RLuc (460–500 nm). The values were corrected by subtracting the background BRET signal detected when RLuc constructs were expressed alone. For BRET titration curve experiments, the cells were transfected with a constant amount of RLuc construct and increasing quantities of YFP construct. For competition assays, a constant amount of the competitor construct was also co-transfected. Total luminescence and fluorescence were measured (see below) to control for relative expression levels of donor and acceptor proteins, respectively. BRET values were then plotted as a function of the total fluorescence/luminescence ratios (YFP/RLuc) (19). For BRET/BiFC experiments, the cells were transfected with the different RLuc, YN, and YC constructs. Total luminescence and fluorescence were measured to control for relative expression level of RLuc fusion proteins and reconstitution of YFP from YN- and YC-fused proteins, respectively. For β-arrestin2 recruitment experiments, the cells distributed in 96-well plates were first treated with αCGRP (Bachem AG) for 10 min before the addition of coelenterazine H and BRET reading.

**Total Fluorescence and Luminescence Measurements**—Transfected HEK293T cells resuspended in PBS 0.1% glucose were distributed in 96-well plates (100,000 cells/well) (white plates with clear bottoms from Corning), and the total fluorescence, emitted by YFP constructs or reconstituted YFP from YN and YC constructs, was measured using a FluoroCount (Packard Bioscience) with excitation and emission filters set at 485 and 530 nm, respectively. The values were corrected by subtracting the background signal detected in cells that were not transfected with YFP or BiFC constructs. The cells were then incubated for 10 min with coelenterazine H at a final concentration of 5 μM, and total luminescence was determined using a LumiCount (Packard Bioscience).
**Asymmetrical Assembly of the CRLR-RAMP1 Complex**

*Fluorescence Imaging—* COS7 cells expressing the different fusion proteins were fixed and analyzed by confocal laser-scanning microscopy using a LSM 510 META Zeiss. YFP fluorescence was measured after excitation at 488 nm with a 505–530-nm emission filter, whereas DsRed fluorescence was detected after excitation at 543 nm with a 560-nm long pass emission filter.

*Co-immunoprecipitation—* Transfected HEK293T cells were lysed for 1 h at 4 °C in solubilization buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM iodoacetamide, 10 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor, and 5 μg/ml leupeptin) and centrifuged at 150,000 × g for 1 h at 4 °C to remove unsolubilized material. Protein concentration of the lysates was determined, and the total fluorescence emitted by reconstituted YFP was measured for each condition by distributing 130 μg of lysate in 96-well plates (see above). For each condition, the same quantity of lysate was then incubated overnight at 4 °C with immobilized anti-HA rat antibodies (Roche Molecular Biochemicals). Antibody-antigen complexes were washed four times with cold solubilization buffer containing 150, 250, 350, and finally 150 mM NaCl, respectively, and transferred to 96-well plates to measure the total fluorescence emitted by reconstituted YFP associated with the immunoprecipitated antigen complexes. To control for the amount of HA-RAMP1 immunoprecipitated in each condition, antibody-antigen complexes were then resuspended in sample buffer (60 mM Tris, pH 6.8, 2% SDS, 4.5 M urea, and 100 mM dithiothreitol), resolved onto 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and subjected to immunoblotting using rabbit anti-HA antibodies (1/10 000; Santa Cruz Biotechnology).

*Statistical Analysis—* In Fig. 1, multiple nonlinear regression analysis were performed using a bootstrap strategy to determine whether the BRET<sub>max</sub> and BRET<sub>50</sub> values obtained for each curves were significantly different. Nonlinear regressions were generated using Equation 1, where BRET<sub>max</sub> and BRET<sub>50</sub> parameters were optimized using the Levenberg-Marquardt method (20) following the implementation proposed by Press et al. (21). Using resampling with replacement (22), 100,000 bootstrap repetitions of this procedure were executed. The generated curves correspond to the optimal fit obtained from the data, whereas the gray zones represent 0.05–0.95 percentiles of the 100,000 curves, calculated for each YFP/Rluc data points. Significance of the difference in BRET<sub>max</sub> and BRET<sub>50</sub> is quantified by p values that are determined by a one-sided resampling-based test carried out on 1,000,000 repetitions. The p values were obtained by counting the proportion of fitted BRET<sub>max</sub> or BRET<sub>50</sub> values that were not greater for the experimental conditions (i.e. in the presence of RAMP1, CD8-C1, CRLR, or Fz4m) than for the control data set (i.e. in the presence of empty vector). A p value below 0.05 was considered significant.

\[
BRET = \frac{BRET_{max} \cdot x}{BRET_{50} + x} \quad (Eq. 1)
\]

where x is the YFP/Rluc ratio.

In Figs. 2, 4, 5, 6, and 8 the data represent the means ± S.E. calculated from three to four independent experiments. Statistical significance of the difference was assessed using analysis of variance followed by Dunnett’s test (Figs. 2 and 5) or Student’s t test (Figs. 4, inset, and 6).

**RESULTS**

*Probing CRLR and RAMP1 Interactions Using BRET Competition Assays—* Previous studies have indicated that both CRLR and RAMP1 can form homo-oligomers when expressed alone (3, 6–8), raising questions about the oligomeric composition of the CRLR-RAMP1 complex. To examine whether the association of CRLR with RAMP1 competes with the homo-oligomerization of each of the partners or results from the association between homo-oligomers, we performed BRET competition assays that assess the effect of CRLR and RAMP1 co-expression on their homo-oligomerization status. For this purpose, CRLR and RAMP1 were genetically fused at their C termini to either the energy donor (Rluc) or acceptor (YFP). Such fusion did not significantly affect the normal subcellular distribution of CRLR or RAMP1 nor their ability to confer CGRP binding upon co-expression (Ref. 8 and data not shown). BRET titration curves were generated by expressing a constant level of the donor fusion protein and increasing amounts of the protein attached to the acceptor. As can be seen in Fig. 1, for both the CRLR-Rluc-CRLR-YFP and RAMP1-Rluc-RAMP1-YFP pairs, BRET signals increased as an hyperbolic function of the acceptor concentration (expressed as YFP/Rluc), representing the progressive engagements of the energy donors by the acceptor. Whereas the value of the curve asymptotes (BRET<sub>max</sub>) reflects the orientation and distance between the energy donor and acceptor within the dimer, the concentration of acceptor/donor (YFP/Rluc) yielding 50% of the BRET<sub>max</sub> (BRET<sub>50</sub>) indicates the relative amount of YFP-fused partner needed to saturate the energy donor (19). It should therefore be emphasized that the lower BRET<sub>max</sub> value observed for CRLR-Rluc-CRLR-YFP compared with RAMP1-Rluc-RAMP1-YFP is not an indication of a lower propensity to form oligomers but most likely reflects distinct relative position of the energy donor and acceptor within the oligomers.

To assess the influence of CRLR-RAMP1 complex formation on the homo-oligomerization profiles of both CRLR and RAMP1, the BRET titration curves were carried out in the presence of their untagged reciprocal partner (RAMP1 in the case of the CRLR-Rluc-CRLR-YFP and CRLR for the RAMP1-Rluc-RAMP1-YFP). If the association of CRLR with RAMP1 occurs at the expense of homo-oligomerization, the addition of a constant amount of untagged reciprocal partner would be predicted to cause a rightward shift in the BRET titration curve (increase in BRET<sub>50</sub>) resulting from the scavenging of the YFP and Rluc fusion proteins by the untagged partner. Indeed, the reduction in the proportion of fusion proteins available for RET would lead to an increase in the YFP/Rluc ratio needed to reach BRET<sub>max</sub> values. In contrast, a change in BRET<sub>max</sub> in the absence of BRET<sub>50</sub> modification would suggest a conformational rearrangement of the complex resulting from the association of the homo-oligomers with the untagged reciprocal partner leading to a change in the orientation and/or the distance...
Asymmetrical Assembly of the CRLR-RAMP1 Complex

Figure 1. CRLR and RAMP1 homo-oligomers BRET titration curves in the presence and absence of competitors. BRET signal, total fluorescence, and luminescence were measured in HEK293T cells transfected with the indicated plasmids. The data points obtained in three independent experiments were pooled and used to generate the curves. The gray zones represent the confidence intervals generated from bootstrapping analysis derived from 100,000 random resampling of data sets. In parallel, the expression of the negative controls (CD8-C1 and Fz4m) was verified by Western blot (CD8-C1) and immunofluorescence (Fz4m) (data not shown).

Between YFP and RLuc within the oligomeric complex. To determine whether the BRET\textsubscript{max} and BRET\textsubscript{50} value obtained for each curve was significantly affected by the addition of the untagged reciprocal partners, multiple repeated nonlinear regression analyses of the BRET titration curves were performed using a bootstrap strategy described under “Experimental Procedures.”

Co-expression of RAMP1 in CRLR-RLuc-CRLR-YFP-expressing cells led to a significant increase in BRET\textsubscript{max} without significantly affecting the BRET\textsubscript{50} (Fig. 1A), indicating that RAMP1 did not compete for CRLR homo-oligomerization but promoted a conformational change leading to a better orientation for RET or a smaller distance between YFP and RLuc within the CRLR-CRLR oligomer. To verify the selectivity of action of RAMP1, we assessed the effect of another single transmembrane protein, CD8, which, similarly to RAMP1 and CRLR expressed alone, is retained in the ER as a result of its fusion to the C1 cassette of the N-methyl-D-aspartate receptor NR1 subunit (CD8-C1 (23)). Although properly expressed, as assessed by Western blot analysis (data not shown) and fluorescence microscopy (see Fig. 3), CD8-C1 was without effect on the CRLR-RLuc-CRLR-YFP BRET titration curve, confirming that overexpressing a protein in the ER is not sufficient to affect the BRET signals.

When considering the RAMP1-RLuc-RAMP1-YFP pair, cotransfection of CRLR promoted a significant rightward shift in the titration curve (increase in BRET\textsubscript{50}) with no change in the BRET\textsubscript{max} (Fig. 1B), indicating that the presence of CRLR reduced the fraction of RAMP1 partners available for RET, thus suggesting that CRLR competes for RAMP1 homo-oligomerization. Although the change in BRET\textsubscript{50} is relatively modest (2.3-fold), bootstrapping analysis of the curves, determined by a one-sided resampling-based test, indicated that the difference between the curves carried out in the presence and absence of CRLR was highly significant ($p = 3 \times 10^{-6}$). The selectivity of RAMP1 homo-oligomerization competition by CRLR was assessed by evaluating the effect of an unrelated 7TM receptor, Frizzled 4, which was truncated from its last four amino acids (Fz4m) to mimic the intracellular localization of RAMP1 and CRLR expressed alone (24). As can be seen in Fig. 1B, the expression of Fz4m, which was confirmed by immunofluorescence microscopy (data not shown), did not significantly affect the RAMP1-RLuc-RAMP1-YFP BRET titration curve.

Taken together, the BRET competition assays indicate that RAMP1 association to the complex can occur without dissociation of the CRLR homo-oligomer, whereas CRLR interaction with RAMP1 appears to interfere with the formation of RAMP1 homo-oligomers. These data are compatible with an asymmetrical complex model whereby one molecule of RAMP1 associates with a CRLR homo-oligomer.

Establishing the BiFC Assay for Detection of the CRLR and RAMP1 Homo- and Hetero-oligomers—Although the BRET competition assays described above suggest that the CGRP receptor is formed by a CRLR homo-oligomer associated to a RAMP1 monomer, it does not provide a direct proof of the existence of such an asymmetric complex. To directly test this model, we combined the use of BRET and BiFC to probe the simultaneous interaction between three proteins. The dual BRET/BiFC approach is based on the transfer of energy between the luminescent enzyme RLuc and YFP reconstituted as a result of BiFC. In these experiments, the YFP is split in two parts (designated YN and YC fragments) that are each expressed as fusion proteins to the C termini of CRLR and RAMP1. To verify the functionality of the fusion proteins, we assessed the ability of the different CRLR-RAMP1 BiFC combinations to confer radioligand binding. As can be seen in Fig. 2, fusion of YN and YC fragments did not prevent CRLR or RAMP1 from associating into a ligand binding-competent CGRP receptor. Indeed, co-expression of all possible BiFC combinations (CRLR-YN-RAMP1-YC, CRLR-YC-RAMP1-YN, CRLR-YN-CRLR-YC + RAMP1, and RAMP1-YN-RAMP1-YC + CRLR) conferred $[^{125}\text{I}]$CGRP binding (Fig. 2A), with affinities comparable with that obtained upon co-expression of wild-type CRLR and RAMP1 (Fig. 2B). However, the maximal binding conferred by the co-expression of RAMP1-YN-RAMP1-YC with CRLR was significantly lower than that conferred by the other combinations. This difference most likely reflects the fact that, as will be demonstrated later (see Figs. 5–7), RAMP1 homo-oligomers are unable to interact with CRLR and thus cannot be part of an active CGRP receptor.
Asymmetrical Assembly of the CRLR-RAMP1 Complex

Given that the interactions between protein partners are presumably stabilized by the reconstitution of YFP in a BiFC experiment (13), the amount of monomeric RAMP1 available for CGRP receptor formation should be lower in the RAMP1-RAMP1-YC configuration, thus leading to lower maximal [125I]CGRP binding.

To determine whether BRET/BiFC can be used to probe the CRLR-RAMP1 complex, we first needed to establish whether the interactions between CRLR and RAMP1 can be detected using YFP BiFC. For this purpose, the cells were transfected with either CRLR-YN-CRLR-YC, RAMP1-YN-RAMP1-YC, or CRLR-YN-RAMP1-YC pairs. As shown in Fig. 3A, the fluorescence resulting from the reconstitution of YFP could be detected by confocal microscopy for each of the three pairs, whereas no fluorescence was detected in cells expressing each of the constructs individually (data not shown). Only marginal fluorescence was observed upon co-expression of any of the CRLR or RAMP1 BiFC constructs with the CD8-C1-YN or -YC fusion proteins (CD8-C1-YN-CRLR-YC, CD8-C1-YN-RAMP1-YC, CRLR-YN-CD8-C1-YC, and RAMP1-YN-CD8-C1-YC) used as negative controls (Fig. 3B). In contrast, a strong signal, expected for the CD8 constitutive dimer, was observed in cells co-transfected with CD8-C1-YN-CD8-C1-YC (Fig. 3A, bottom panel), indicating that the lack of fluorescence reconstitution upon co-expression with CRLR or RAMP1 BiFC partners did not result from inappropriate expression or folding of the CD8-C1-YN and -YC fusion proteins, thus confirming the selectivity of the BiFC signals observed. Taken together, these results demonstrate that BiFC allows the detection of both homo- and hetero-oligomers between CRLR and RAMP1. Higher magnification images were then used to assess the subcellular distribution of the BiFC signal observed (Fig. 3C). The YFP fluorescence resulting from co-expression of CRLR-YN with CRLR-YC or of RAMP1-YN with RAMP1-YC co-localized extensively with the ER marker DsRed-ER, confirming that neither CRLR nor RAMP1 can efficiently exit the ER when expressed individually (3, 6) and that the presence of the YFP fragments or their complementation did not affect their cellular trafficking. This result also clearly demonstrates that homooligomerization of each protein occurs in the ER. Notably, fluorescence reconstituted by the homo-oligomerization of CD8-C1-YN and -YC was also found to localize to the ER, confirming that this protein has a subcellular distribution identical to that of CRLR and RAMP1 expressed alone and thus represents an appropriate control. In contrast, co-transfection of CRLR-YN and RAMP1-YC led to YFP fluorescence detected throughout the secretory pathway and at the plasma membrane (Fig. 3C), demonstrating that the BiFC did not interfere with the documented ability of the CRLR-RAMP1 complex to exit the ER and reach the cell surface (3, 6). Taken together, these results validate the usefulness of BiFC to monitor CRLR and RAMP1 homo- and hetero-oligomerization and to assess the subcellular distribution of these complexes.

Validation of the Dual BRET/BiFC Approach to Detect Ternary Complexes in Living Cells—To validate that BiFC can be combined with BRET to detect a ternary complex involving CRLR and RAMP1, we took advantage of the known complex formed between the CGRP receptor and β-arrestin2 upon agonist stimulation (6). As illustrated in Fig. 4, CGRP promoted a dose-dependent increase in BRET between β-arrestin2-RLuc and CRLR-YN-RAMP1-YC, reflecting the recruitment of β-arrestin2-RLuc to the YFP reconstituted by the formation of the CRLR-RAMP1 complex. Interestingly, the EC50 for the CGRP-promoted recruitment of β-arrestin2-RLuc to the CRLR-YN-RAMP1-YC and to the CRLR-YFP/RAMP1 complexes were very similar (72.4 ± 8.2 versus 11.8 ± 2.8 nM), confirming normal functionality of the CRLR-YN-RAMP1-YC BiFC pair. The significantly smaller maximal BRET signal observed for β-arrestin2-RLuc recruitment to CRLR-YN-RAMP1-YC versus CRLR-YFP/RAMP1 (Fig. 4, inset) is expected given the intrinsically lower level of fluorescence obtained by BiFC compared with that obtained for full-length YFP (25 data not shown). BRET signal being dependent on the relative expression levels of the energy donor (RLuc) and acceptor (YFP), the partial YFP reconstitution achieved in BiFC is deemed to generate lower maximal BRET levels. No transfer of energy was detected after agonist stimulation of cells expressing β-arrestin2-RLuc with either the CRLR-YN and untagged RAMP1 or the RAMP1-YC and untagged CRLR (Fig. 4, inset), consistent with our previous observation that neither YN nor YC fragments alone generate a
fluorescence signal. In addition to validating the use of dual BRET/BiFC to monitor dynamic interactions between three partners in living cells, these results confirm that YFP reconstitution at the C terminus of CRLR and RAMP1 did not interfere with the functionality of the receptor.

Probing the Arrangement of the CRLR-RAMP1 Complex Using BRET/BiFC—We next used the BRET/BiFC approach to directly test the asymmetrical organization of the CGRP receptor (one molecule of RAMP1 associated with a homo-oligomer of CRLR) proposed by the BRET competition assays described above. For this purpose, different BRET/BiFC combinations were tested.

First, the CRLR-YN-RAMP1-YC pair was used. As shown in Fig. 5A, co-expression of CRLR-YN and RAMP1-YC with CRLR-luc yielded a significant BRET signal, indicative of the presence of a CRLR oligomer within the CGRP receptor complex. To verify the selectivity of the signal, we used an unrelated 7TM receptor, Fz1, which similarly to the CRLR/H18528 RAMP1 complex is readily targeted at the cell surface. As expected, co-expression of the negative control Fz1-Rluc with CRLR-YN-RAMP1-YC only led to a marginal BRET, confirming that the signal observed between CRLR-YN-RAMP1-YC and CRLR-Rluc resulted from a true oligomeric interaction. When the CRLR-YN-RAMP1-YC BiFC pair was co-expressed with RAMP1-Rluc, a negligible BRET signal, not different from that observed with the negative control Fz1, was detected, suggesting that the CRLR-RAMP1 complex could...
Asymmetrical Assembly of the CRLR-RAMP1 Complex

For the CRLR-YN-CRLR-YC BiFC pair (Fig. 5B), co-expression with RAMP1-RLuc led to a robust BRET signal, further supporting the possibility that RAMP1 can associate with a CRLR homo-oligomer. In this case, because CRLR-YN-CRLR-YC is retained intracellularly (Fig. 3C), the GABA_A_R1 7TM receptor, which is also retained in the ER, was used as an appropriate control. Only a marginal signal was observed upon expression of CRLR-YN-CRLR-YP with GABA_A_R1-RLuc, confirming the selectivity of the signal detected with RAMP1-RLuc. Interestingly, co-expression of CRLR-RLuc with CRLR-YN-CRLR-YC did not generate a BRET signal significantly different from that obtained with GABA_A_R1-RLuc, suggesting that CRLR cannot form complexes larger than dimers.

Finally, when the RAMP1-YN-RAMP1-YP pair (Fig. 6C) was co-expressed with CRLR-RLuc, the weak BRET signal observed was not significantly different from that obtained with the negative control GABA_A_R1-RLuc, consistent with the notion that RAMP1 homo-oligomers cannot interact with CRLR. The lack of significant BRET signal did not result from the inability of the RAMP1-YN-RAMP1-YP pair to engage into protein complexes, because a significant BRET was observed upon co-expression with RAMP1-RLuc.

Assessment of CRLR-RAMP1 Complexes by Co-immunoprecipitation—The results obtained using BRET/BiFC are consistent with the notion that the CGRP receptor is formed by a CRLR homo-oligomer associated to a single RAMP1 molecule. However, these are based in part on the absence of BRET signal between two RAMP1 molecules and CRLR (Fig. 5, A and C) that could result either from a lack of interaction or from a three-dimensional configuration of the complex that is not permissive to BRET, because of an inadequate orientation or an excessive distance between the fluorophores. To confirm that the lack of BRET truly resulted from a lack of interaction, we applied a more direct biochemical approach involving the immunoprecipitation of the CRLR-RAMP1 complex. Classical co-immunoprecipitation does not allow monitoring the presence of three proteins in the same complex. Thus, to directly assess the existence of a ternary complex, we monitored the reconstitution of a fluorescent signal from the YFP fragments attached to two of the three potential partners, following the immunoprecipitation of the third one. More specifically, HA-RAMP1 was co-expressed with either CRLR-YN and CRLR-YC or CRLR-YN and RAMP1-YC, and HA-RAMP1 was immunoprecipitated. As shown in Fig. 6B, immunoprecipitation using an anti-HA antibody resulted in the co-sedimentation of a CRLR-YN-CRLR-YC complex, as indicated by the significant fluorescence signal detected in the immunoprecipitate of cells co-expressing HA-RAMP1, CRLR-YN, and CRLR-YC. In contrast, only marginal fluorescence was observed upon anti-HA immunoprecipitation in cells co-expressing HA-RAMP1, CRLR-YN, and RAMP1-YC, indicating that no CRLR-YN-RAMP1-YP complex could be co-immunoprecipitated with HA-RAMP1. The difference in the co-immunoprecipitation results obtained for CRLR-YN-CRLR-YC and CRLR-YN-RAMP1-YP cannot be attributed to variations in the expression levels or in the ability of the constructs to reconstitute YFP, because equivalent levels of fluorescence were detected before immunoprecipitation (Fig. 6A), and similar amounts of HA-RAMP1 were immunoprecipitated for both conditions (Fig. 6B, inset). Thus, in agreement with the BRET studies, co-immunoprecipitation experiments indicate that, whereas RAMP1 can interact with a CRLR homo-oligomer, CRLR can only associate with a single RAMP1 molecule.
Subcellular Distribution of CRLR and RAMP1 Complexes Using BiFC Fluorescence Microscopy—Because the interaction between CRLR and RAMP1 is absolutely required for their cell surface targeting (3, 6), we assessed the subcellular distribution of CRLR and RAMP1 homo-oligomers as a means to further explore the arrangement of the CRLR-RAMP1 complex. The presence of either CRLR or RAMP1 homo-oligomers within the CGRP receptor complex at the cell surface was assessed by determining the subcellular distribution of CRLR-YN and RAMP1-YC in the presence of RAMP1 and CRLR, respectively. As seen in Fig. 7A, and previously illustrated in Fig. 3C, expression of the CRLR-YN-CRLR-YC pair in the absence of RAMP1 led to the reconstitution of intracellular YFP fluorescence that is entirely co-localized with the DsRed-ER marker. Co-expression of RAMP1 promoted the apparition of cell surface YFP fluorescence, thus confirming the presence of a CGRP receptor complex composed of a CRLR homo-oligomer and RAMP1 at the plasma membrane. These fluorescence patterns in the presence and absence of RAMP1 are identical to those observed for CRLR-YFP (Fig. 7B), indicating that CRLR oligomerization did not affect the ability of RAMP1 to promote CRLR plasma membrane targeting. When considering the RAMP1-YN-RAMP1-YC BiFC pair, the reconstituted YFP fluorescence was restricted to the ER, whether CRLR was expressed or not, indicating that RAMP1 homo-oligomers do not reach the plasma membrane even in the presence of CRLR (Fig. 7C). This contrasts with the cell surface fluorescence observed when co-expressing RAMP1-
YFP with CRLR (Fig. 7D), thus suggesting that RAMP1 can traffic to the plasma membrane only as a monomer associated to CRLR. Altogether, the BRET/BiFC, co-immunoprecipitation and confocal microscopy data demonstrate the presence of a complex formed by at least two CRLR and one RAMP1 molecule at the plasma membrane.

**Functionality of the Asymmetrical CRLR-RAMP1 Complex—**

To determine whether the asymmetrical complex formed by a CRLR homo-oligomer and a RAMP1 monomer represents a functional CGRP receptor, we assessed the ability of this complex to recruit β-arrestin2 upon receptor stimulation. For this purpose, we monitored agonist-promoted BRET between β-arrestin2-Rluc and the YFP reconstituted by the assembly of CRLR-YN and CRLR-YC in the presence and absence of RAMP1. As shown in Fig. 8, in the absence of co-expressed RAMP1, only a marginal agonist-stimulated BRET could be detected (most likely reflecting the low level of endogenous RAMP1 expressed in these cells (3)). Co-transfection of RAMP1 led to a robust increase of this dose-dependent BRET signal between β-arrestin2-Rluc and CRLR-YN-CRLR-YC, indicating that β-arrestin2 is recruited to a CRLR homo-oligomer and that RAMP1 is needed for this interaction. The presence of RAMP1 in the functional receptor complex is further supported by the CGRP-promoted BRET observed between β-arrestin2-Rluc and CRLR-YN-RAMP1-YC presented in Fig. 4. Taken with the previous results indicating that only RAMP1 molecule can associate with a CRLR homo-oligomer, these observations demonstrate that asymmetrical complexes formed by at least two CRLR and one RAMP1 represent genuine CGRP receptors.

**DISCUSSION**

In the present study, the combination of BRET competition, co-immunoprecipitation, BiFC microscopy, and dual BRET/BiFC assays allow proposal of a new protein composition for the CGRP receptor. Several lines of evidence converge to suggest that the receptor complex is composed of a CRLR homo-oligomer and a single RAMP1 molecule. Arguments supporting the presence of at least two CRLR molecules within the CGRP receptor include: 1) the observation that co-expression of RAMP1 did not compete with the formation of CRLR homo-oligomer but rather induced a conformational rearrangement of the oligomer as assessed by BRET competition assays (Fig. 1A); 2) the robust BRET signal obtained between CRLR-YN-RAMP1-YC and CRLR-Rluc (Fig. 5A) as well as between CRLR-YN-CRLR-YC and RAMP-Rluc (Fig. 5B), and the co-immunoprecipitation of CRLR-YN-CRLR-YC with HA-RAMP1 (Fig. 6), directly demonstrating the association of a CRLR homo-oligomer with RAMP1; 3) the detection of YFP fluorescence reconstituted from the CRLR-YN-CRLR-YC pair at the plasma membrane in the presence of RAMP1 (Fig. 7A), indicating that CRLR homo-oligomer can be trafficked to the cell surface; and 4) the agonist-promoted recruitment of β-arrestin2-Rluc to the CRLR-YN-CRLR-YC-RAMP1 complex detected by BRET (Fig. 8), illustrating that the CRLR homo-oligomer is part of an active CGRP receptor. The same set of experiments revealed that only one molecule of RAMP1 contributes to the receptor complex. Indeed, 1) in BRET competition assays, CRLR was found to compete with the assembly of RAMP1 homo-oligomer (Fig. 1B); 2) no BRET could be detected between CRLR-YN-RAMP1-YC and RAMP1-Rluc (Fig. 5A) nor between RAMP1-YN-RAMP1-YC and CRLR-Rluc (Fig. 5C), suggesting that RAMP1 homo-oligomers cannot interact with CRLR; 3) CRLR-YN-RAMP1-YC could not be co-immunoprecipitated with HA-RAMP1, confirming that two RAMP1 molecules cannot be associated to CRLR; and 4) the RAMP1-YN-RAMP1-YC reconstituted fluorescence was restricted to the ER even in the presence of CRLR (Fig. 7C), confirming that RAMP1 homo-oligomer cannot associate with CRLR and be trafficked to the plasma membrane. Taken together, the data indicate that although both CRLR and RAMP1 can form homo-oligomers in the ER, only RAMP1 monomer can associate with CRLR homo-oligomer to form an asymmetrical CGRP receptor complex that can reach the cell surface.

However, the evidence indicating that RAMP1 homo-oligomers cannot interact with CRLR is partly based on the absence of BRET, co-immunoprecipitation, and the lack of cell surface trafficking observed with certain BiFC pairs. It could be argued that YFP reconstitution at the C terminus of CRLR and RAMP1 could force conformational rearrangements that would artifically prevent interactions between RAMP1 homo-oligomers and CRLR. It should however be emphasized that the lack of interaction between CRLR and a RAMP1 homo-oligomer in these different experimental settings was observed whether the BiFC pairs used were formed by the CRLR-RAMP1 or the RAMP1-RAMP1 complexes. Yet these two BiFC pairs had normal interaction patterns with their other partners (i.e. CRLR-Rluc and β-arrestin2-Rluc with CRLR-YN-RAMP1-YC, and RAMP1-Rluc with RAMP1-YN-RAMP1-YC; Figs. 4 and 5, A and C). It therefore seems highly unlikely that the YFP reconstitution by the two distinct BiFC pairs only aberrantly affected the complexes involving two RAMP1 and one CRLR. It seems more reasonable that this convergence reflects an intrinsic inability of the native RAMP1 homo-oligomer to associate with CRLR. The latter interpretation is also supported by the results of the BRET competition assays (Fig. 1) that do not rely on the
reconstitution of YFP. Indeed, these experiments showed that the interaction of CRLR with RAMP1 competed with the RAMP1 homo-oligomerization, indicating that only monomeric RAMP1 could associate with CRLR. This contrasted with the lack of competition of RAMP1 on the CRLR homo-oligomerization, which is consistent with the overall model that a single RAMP1 interacts with a CRLR homo-oligomer.

In addition to provide new insights on the CRLR-RAMP1 complex, our study represents a proof of principle that BRET can be combined with BiFC and fluorescence microscopy to probe stoichiometry of both constitutive and dynamically regulated complexes in living cells. Combination between BRET and BiFC has previously been used to monitor the interaction between the constitutive G_{\beta y} dimer, used as the BiFC pair and either G_{o} or G protein effectors (14, 15). However, to our knowledge, this is the first use of BRET/BiFC to probe the stoichiometric arrangement of a protein complex. Changes in FRET signals observed upon co-expression of potential partners have previously been used to infer stoichiometries of protein complexes (26, 27). However, as is the case for the BRET competition assays presented in Fig. 1, the interpretations drawn form these experiments are based on several assumptions that make definitive conclusions more difficult. In contrast, dual BRET/BiFC assays directly monitor interactions between three proteins and thus represent a direct stoichiometric readout. However, because it is still a matter of debate whether or not BiFC fragments can dissociate after reconstitution (25), the YFP assembly could stabilize complexes between proteins that normally associate only transiently. This is particularly important when studying dynamically regulated interactions. As much as possible, proteins that form a stable complex should be fused to the BiFC pair, whereas the regulated partner of the complex should be fused to Rluc. This strategy is well illustrated by our experiments monitoring the CGRP-promoted recruitment of \( \beta \)-arrestin2 to the CRLR-RAMP1 complexes (Figs. 4 and 8), where the dynamically regulated partner, \( \beta \)-arrestin2, was fused to Rluc, whereas CRLR and RAMP1 that form a constitutive complex were used as BiFC pairs.

The notion that the functional CGRP receptor is composed of a CRLR oligomer associated to a single RAMP1 molecule introduces an unanticipated level of complexity in the oligomeric assembly of this receptor. Indeed, our previous study using a membrane-impermeable cross-linker failed to detect molecular species including more than one CRLR and one RAMP1 (6). The lack of stabilization of CRLR homo-oligomer or CRLR oligomer associated to RAMP1 by the bivalent cross-linker used in that previous study most likely reflects the absence of available neighboring lysines allowing the capture of these complexes. However, the detection of a CRLR oligomer-RAMP1 monomer complex in the present study is consistent with the previous observation that the amount of RAMP1 homo-dimers detected by Western blot analysis following immunoprecipitation decreased upon co-transfection of CRLR that suggested a competition between the CRLR-RAMP1 complex and RAMP1 homo-oligomer formation (6, 28). Such competition is also seen in living cells, as indicated by the inhibitory effect of CRLR expression on the BRET signal generated by the RAMP1 homo-oligomer observed in the present study (Fig. 1).

The asymmetry of the CRLR-RAMP1 complex raises intriguing questions concerning its tri-dimensional arrangement and the contribution of each subunit in agonist binding and subsequent G protein activation. Although the currently available data do not allow conclusion regarding the binding and activation mode of the complex, the fact that [\( ^{125} \)I]CGRP can be cross-linked to both CRLR and RAMP1 (3, 5) suggests that domains from the two proteins participate or are in close proximity to the binding pocket. Given that only one RAMP1 molecule is present in the complex, these data indicate either that RAMP1 possesses multiple CGRP interaction sites, allowing simultaneous hormone binding to two or more CRLR molecules, or that only one CRLR unit per receptor complex can bind a CGRP molecule at a time.

Our finding indicating that only one molecule of RAMP1 contributes to the CGRP receptor complex also raises questions concerning the potential role of RAMP1 homo-oligomerization. Given that it competes with the formation of the CRLR-RAMP1 complex, the homotypic RAMP1 interaction may have a regulatory influence on the formation of the CGRP receptor by titrating the available RAMP1 able to interact with CRLR. In addition to this potential regulatory role, the homo-oligomer could also have intrinsic functions, independent of the role of RAMP1 as co-receptor. Given that RAMP1 homo-oligomer subcellular distribution is limited to the ER, a role in protein folding, maturation, and/or cargo export could be envisaged for substrates other than CRLR. Consistent with this notion, RAMP1 has a membrane topological organization similar to that of ER export proteins, such as VIP36 and ERGIC-53, that exert their cargo trafficking function as oligomers (29, 30).

In conclusion, in addition to demonstrating the usefulness of the dual BRET/BiFC approach to investigate multiprotein complexes organization, our study revealed an unexpected arrangement for a G protein-coupled receptor signaling module formed between a 7TM receptor oligomer and a one-transmembrane domain accessory protein monomer. Considering the increasing number of partners that were shown to interact with 7TM receptors (31), dual BRET/BiFC assays should prove to be useful tools to monitor both the composition and the stoichiometry of newly identified signaling modules. The asymmetrical nature of the complex formed by the association between a CRLR homo-oligomer and a single RAMP1 molecule raises several intriguing questions concerning the three-dimensional organization as well as the binding and signaling modes of the CGRP receptor. Given the importance of the CGRP receptor as a potential target for the development of new antimigraine therapeutics, the structural and functional consequences of the receptor asymmetrical oligomeric assembly will undoubtedly be investigated in future studies.

Acknowledgments—We are grateful to S. M. Foord (GlaxoSmithKline) for the generous gift of CRLR, RAMP1, Myc-CRLR, and Myc-RAMP1; R. T. Moon for Fz4m and Fz1-Rluc; P. M. Sexton for CRLR-YFP; R. C. Malenka for CD8-C1; and T. K. Kerppola for YFP BiFC fragments cDNAs. We acknowledge the contribution of V. Percherancier and F. F. Hamdan in the conception of the BiFC partners used in this study. We also thank P. René and M. Lagacé for critical reading of the manuscript and C. Charbonneau for help with confocal microscopy.
REFERENCES

1. Hoeller, D., Volarevic, S., and Dikic, I. (2005) Curr. Opin. Cell Biol. 17, 107–111
2. Luttrell, L. M. (2005) J. Mol. Neurosci. 26, 253–264
3. McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M. G., and Foord, S. M. (1998) Nature 393, 333–339
4. Leuthauser, K., Guger, R., Aldecoa, A., McKinney, R. A., Muff, R., Fischer, J. A., and Born, W. (2000) Biochem. J. 351, 347–351
5. Hilairet, S., Foord, S. M., Marshall, F. H., and Bouvier, M. (2001) J. Biol. Chem. 276, 29575–29581
6. Hilairet, S., Belanger, C., Bertrand, J., Laperrriere, A., Foord, S. M., and Bouvier, M. (2001) J. Biol. Chem. 276, 42182–42190
7. Sexton, P. M., Albiston, A., Morfis, M., and Tilakaratne, N. (2001) Cell Signal. 13, 73–83
8. Heroux, M., Breton, B., Hogue, M., and Bouvier, M. (2007) Biochemistry 46, 7022–7033
9. Michnick, S. W. (2001) Curr. Opin. Struct. Biol. 11, 472–477
10. Wallrabe, H., and Periasamy, A. (2005) Curr. Opin. Biotechnol. 16, 19–27
11. Pfleger, K. D., and Eidne, K. A. (2006) Nat. Methods 3, 165–174
12. Truong, K., and Ikura, M. (2001) Curr. Opin. Struct. Biol. 11, 573–578
13. Hu, C. D., Chineno, Y., and Kerppola, T. K. (2002) Mol. Cell 9, 789–798
14. Rebois, R. V., Robitaille, M., Gales, C., Dupre, D. J., Baragli, A., Trieu, P., Ethier, N., Bouvier, M., and Hebert, T. E. (2006) J. Cell Sci. 119, 2807–2818
15. Dupre, D. J., Robitaille, M., Ethier, N., Villeneuve, L. R., Mamarbachi, A. M., and Hebert, T. E. (2006) J. Biol. Chem. 281, 34561–34573
16. Perroy, J., Adam, L., Qanbar, R., Chenier, S., and Bouvier, M. (2003) EMBO J. 22, 3816–3824
17. Hu, C. D., and Kerppola, T. K. (2003) Nat. Biotechnol. 21, 539–545
18. Mellon, P., Parker, V., Gluzman, Y., and Maniatis, T. (1981) Cell 27, 279–288
19. Mercier, J. F., Salahpour, A., Angers, S., Breit, A., and Bouvier, M. (2002) J. Biol. Chem. 277, 44925–44931
20. Marquardt, D. W. (1963) J. Soc. Indust. Appl. Math. 11, 431–441
21. Press, W. H., Teukolsky, S. A., Vetterling, W. T., and Flannery, B. P. (2002) Numerical Recipes in C: The Art of Scientific Computing, Cambridge University Press, New York
22. Efron, B., and Tibshirani, R. J. (1994) An Introduction to the Bootstrap, Chapman & Hall/CRC, New York
23. Xia, H., Hornby, Z. D., and Malenka, R. C. (2001) Neuropharmacology 41, 714–723
24. Kaykas, A., Yang-Snyder, J., Heroux, M., Shah, K. V., Bouvier, M., and Moon, R. T. (2004) Nat. Cell Biol. 6, 52–58
25. Kerppola, T. K. (2006) Nat. Rev. Mol. Cell. Biol. 7, 449–456
26. Zheng, J., and Zagotta, W. N. (2004) Neuron 42, 411–421
27. Kerschensteiner, D., Soto, F., and Stocker, M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 6160–6165
28. Udawela, M., Hay, D. L., and Sexton, P. M. (2004) Semin. Cell Dev. Biol. 15, 299–308
29. Appenzeller, C., Andersson, H., Kappeler, F., and Hauri, H. P. (1999) Nat. Cell Biol. 1, 330–334
30. Fiedler, K., Parton, R. G., Kellner, R., Etzold, T., and Simons, K. (1994) EMBO J. 13, 1729–1740
31. Bockaert, J., Fagni, L., Dumuis, A., and Marin, P. (2004) Pharmacol. Ther. 103, 203–221