A new approach to analyze cell surface protein complexes reveals specific heterodimeric metabotropic glutamate receptors

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ABSTRACT **G-protein-coupled receptors (GPCRs) can form heteromeric complexes. Herein, we describe a new approach to test the heteromerization of 2 receptors, or 2 receptor subunits, and to study the stoichiometry of the resulting complexes. As a proof-of-concept study, we investigated whether metabotropic glutamate receptors (mGluRs), in addition to being well-known homodimers, can form heteromers. To that aim, we combine the benefits of time-resolved fluorescence resonance energy transfer (trFRET) with the specific, cell-surface labeling of SNAP- and CLIP-tagged rat mGluR subunits, expressed in a mammalian cell line. First, we show that mGlu2 and mGlu4 subunits (but not mGlu2 and mGlu1) can heteromerize. Moreover, our trFRET data are consistent with mGluR subunits forming strict homodimeric receptors on single expression, and a combination of strict heterodimeric and strict homodimeric receptors on coexpression. Second, a comprehensive analysis reveals that from the 21 possible pairs of 2 mGluR subunits out of 7 subtypes (mGlu1 to 8, but not 6), only 11 are able to form heterodimers. These findings were further validated by biochemical and functional complementation studies. In addition to describing a new method to analyze cell-surface receptor complexes, our data reveal a new level of complexity within the mGluR family.— Doumazane, E., Scholler, P., Zwier, J. M., Trinquet, E., Rondard, P., Pin, J.-P. A new approach to analyze cell surface protein complexes reveals specific heterodimeric metabotropic glutamate receptors. *FASEB J.* 25, 66–77 (2011). www.fasebj.org**

Key Words: GPCR · oligomerization · heteromerization · multilabeling · time-resolved fluorescence resonance energy transfer

Many cell surface receptors organize into multiprotein complexes (1–5), and among them, G-protein-coupled receptors (GPCRs) represent major targets for drug development (6, 7). Although GPCRs may activate heterotrimeric G proteins as monomers (8, 9), many have been shown to form homomeric or heteromeric complexes, offering multiple possibilities for their regulation (4, 10–18). However, only few studies (19–21) bring clear evidence for the existence of such complexes at the cell surface, and when heteromers are identified, it is often unknown whether they are limited to strict heterodimers or whether they correspond to the association of different homodimers or even higher-order homooligomers (4, 22).

The analysis of GPCR heteromers in living cells is made difficult by the lack of efficient methods to differentially label distinct populations of receptors at the cell surface. So far, the most powerful techniques use resonance energy transfer based on fluorescence or bioluminescence (FRET and BRET) and rely on the insertion of fluorescent or bioluminescent proteins into the receptors of interest. However, these techniques suffer from 2 major limitations. First, the signal-to-noise ratio is limited due to high background and to signal originating from the intracellular pool of proteins. Second, the absence of FRET does not imply the absence of a physical interaction, since unfavorable relative orientation of the fluorophores can prevent energy transfer (23, 24).

Time-resolved FRET (trFRET) appears as an interesting alternative to conventional RET approaches, first, because of a much better signal-to-noise ratio (25, 26). This is due to the large Stokes shift and the long lifetime of lanthanoid chelates and cryptates, which are used as trFRET donors (27). In addition, an absence of trFRET cannot be due to the unfavorable dipole orientation of the donor and acceptor molecules, since the efficiency is mostly independent of their relative orientation (27). When trFRET-compatible fluorophores are targeted to the extracellular surface of proteins, using antibodies (28, 29) or genetically encoded tags labeled with nonpermeant synthetic substrates (20), it is then possible to selectively analyze the proximity between cell-surface proteins. Such available tags include the

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SNAP, CLIP, HALO, and ACP tags (30–32), which can be easily, specifically, and covalently labeled using the adequate fluorescent substrate. We recently reported that the SNAP-tag technology offers the possibility to detect GPCR homomers at the cell surface (20); however, such a single labeling approach cannot be used to monitor the formation of heteromeric complexes, which prompted us to develop a multilabeling approach.

Among class C GPCRs, the 8 metabotropic glutamate receptors (mGluRs) play central roles in regulating synaptic transmission (33, 34) and represent relevant therapeutic targets for a number of neurological and psychiatric diseases (35–37). mGluRs are known to form homodimeric receptors stabilized by an intersubunit disulfide bridge (38, 39), an organization that appears mandatory for glutamate-mediated receptor activation (40–42). However, unlike some class A GPCRs (16, 19, 22, 43), and unlike the class C $GABA_B$ receptor, mGluRs appear to be limited to dimers and do not form higher-order oligomers (20).

Several observations prompted us to go beyond this classical view of homodimeric mGluRs and to explore the possible formation of heteromeric entities: some mGluR subtypes are colocalized (34); and closely related class C GPCRs, such as the $GABA_B$, umami taste, and sweet taste receptors are mandatory heterodimeric receptors (44–46). Thus, one might assume that mGluRs may also form heteromers.

Herein, we first report on the development of a new approach combining trFRET along with the labeling of SNAP and CLIP tags. Using this approach, we show it is possible to study stoichiometry of homo- and heterooligomers at the surface of living cells. We also show that some mGluRs can spontaneously form heteromers, yet are limited to strict dimers, that are still functional. These data reveal a new level of complexity in the mGlu receptor family.

MATERIALS AND METHODS

Reagents

LY354740, quisqualic acid, (2*S*)-2-amino-4-phosphobutanoic acid (L-AP4), and [^{3}H]-LY341495 were purchased from Tocris Bioscience (Ellisville, MO, USA), SNAP-Surface782 was from New England Biolabs (Ipswich, MA, USA), saponin was from Sigma-Aldrich (St. Louis, MO, USA), and Lipofectamine 2000 and Fluo4-AM were from Invitrogen (Carlsbad, CA, USA). SNAP-Lumi4, SNAP-Green, CLIP-Lumi4, CLIP-Green, and CLIP-Red were from Cisbio Bioassays (Bagnols-sur-Cèze, France).

Plasmids and transfection

The pRK5 plasmids encoding wild-type mGluR subunits and GABAB receptor subunits (GB1a and GB2) from rats, tagged with the hemagglutinin (HA), FLAG, or SNAP inserted just after the signal peptide, under the control of a cytomegalovirus promoter, were previously described (20, 47). CLIP (ACT10) coding sequence (gift from Kai Johnsson, Ecole Polytechnique Fédérale, Lausanne, Switzerland) was amplified by PCR and inserted into pRK5-FLAG-GB2 at the *Mlu*I site between the FLAG and the GB2 sequence. In both pRK5-HA-SNAP-GB1a and pRK5-FLAG-CLIP-GB2, the *Mlu*I site, in 5′ of the SNAP or the CLIP, was then removed by site-specific mutagenesis (ACGCGT mutated in ACGCGA), leaving the 3′ *Mlu*I site unique in both plasmids. The sequence encoding GB1a or GB2 between *Mlu*I and *Hind*III (or *Xba*I) was then replaced by the sequence coding mGlu1a, 2, 3, 4a, 5a, 7a, or 8a obtained by digestion or PCR from constructs described elsewhere (47). The final plasmids encode HA-SNAP-mGlu1a, 2, 3, 4a, 5a, 7a, or 8a, and FLAG-CLIP-mGlu1a, 2, 3, 4a, 5a, 7a, or 8a. The sequence coding EGFP and 6xHis was fused just before the stop codon of HA-SNAP-mGlu2 to obtain the plasmid encoding HA-SNAP-mGlu2-GFP. The sequence coding C1 (48), the 47-residue coiled-coil region of the C terminus of GB1a, or C2, the 49-residue coiled-coil region of GB2 (C2), and the endoplasmic reticulum retention signal KKTN were inserted just before the stop codon to obtain the plasmids encoding HA-SNAP-mGlu2-C1KKXX, HA-SNAP-mGlu2-C2KKXX, and HA-mGlu2-C2KKXX. The plasmid encoding FLAG-SNAP-D2 (human dopamine receptor D2, short isoform) was from Cisbio Bioassays.

HEK-293 and COS-7 cells were cultured in DMEM (Life Technologies, Invitrogen) supplemented with 10% fetal calf serum (Lonza, Basel, Switzerland) and nonessential amino acids, penicillin, and streptomycin (Life Technologies). Cells were transfected by electroporation (47) or by the reverse Lipofectamine 2000 protocol (49).

SNAP/CLIP multilabeling, fluorescence, and trFRET measurement

COS-7 cells transfected by lipofection with plasmid DNA encoding SNAP- and/or CLIP-tagged constructs (1–50 ng each, completed to 150 ng/well with noncoding DNA) were plated in polyornithine-coated, black-walled, dark-bottom, 96-well plates at 10^5 cells/well. At 30 to 40 h after transfection, adherent cells were washed and incubated with culture medium containing SNAP and/or CLIP substrates for 2 h at 37°C. To determine fluorescence and trFRET values (see **Table 1** for nomenclature), different concentrations of SNAP and CLIP substrates were used: 0.3 μM SNAP-Lumi4 for Exp_S; 1 μM CLIP-Green for Exp_C; 0.3 μM SNAP-Lumi4 and 1 μM CLIP-Green for $FRET_{SC}$; 0.1 μM SNAP-Lumi4 and 0.06 μM SNAP-Green for $FRET_{SS}$; and 1 μM CLIP-Lumi4 and 0.8 μM CLIP-Green for $FRET_{CC}$. Cells were then washed 3 times with warm Tris-Krebs buffer (20).

TABLE 1. *Nomenclature*

Name	Description	Analyzed population
Exp_S	Lumi4 fluorescence of cells labeled with SNAP-Lumi4	SNAP-tagged subunits
Exp_C	Green fluorescence of cells labeled with CLIP-Green	CLIP-tagged subunits
$FRET_{SC}$	trFRET signal of cells labeled with SNAP-Lumi4 and CLIP-Green	SNAP/CLIP dimers
$FRET_{SS}$	trFRET signal of cells labeled with SNAP-Lumi4 and SNAP-Green	SNAP/SNAP dimers
$FRET_{CC}$	trFRET signal of cells labeled with CLIP-Lumi4 and CLIP-Green	CLIP/CLIP dimers

Fluorescence and trFRET were read under 100 μl Tris-Krebs buffer/well using an Infinite F500 spectrofluorimeter (Tecan, Männedorf, Switzerland). Fluorescence of Lumi4 (excitation at 340 nm, emission at 620 nm, 50-μs delay, and 400-μs integration time), Green (485 nm, 520 nm, 0 μs, and 1000 μs), and trFRET (340 nm, 520 nm, 50 μs, and 400 μs) were measured. The unspecific signal, measured on cells transfected with empty plasmids, was subtracted to obtain the specific signal. As shown in **Fig. 2*C*, *D***, the fluorescence intensity of SNAP-Lumi4- and CLIP-Green-labeled cells were correlated with the number of SNAP- and CLIP-tagged receptors, respectively. However, the slopes (0.47 and 0.74) were different, due to the intrinsic properties of the fluorophores and the chosen settings of the fluorimeter. Thus, the Green fluorescence signal read for Exp_C was divided by a correcting factor, 1.58, so that it can be compared with Exp_S.

Determination of the oligomeric size of SNAP homomers

At 30 h after lipofection with SNAP-D2 (20 ng/well), SNAP-mGlu1 (20 ng/well), or SNAP-mGlu2 (50 ng/well), HEK-293 cells were labeled, as described before, with both SNAP-Lumi4 (constant, 100 nM) and SNAP-Green (variable, 1 nM to 1 μM). In such conditions, SNAP tags are all occupied by either Lumi4 or Green; thus, for each labeling condition, the Green specific fluorescence F_A of cells was measured as previously described, and the acceptor molar ratio x_A = [Green]/[Lumi4] was calculated as

$$x_A = \frac{F_A/F_{A,max}}{1 - F_A/F_{A,max}}$$

where $F_A/F_{A,max}$ is the Green specific fluorescence of cells labeled with SNAP-Green only (300 nM).

The fluorescence decay of Lumi4 at 620 nm from 100 to 2500 μs was also monitored using a PHERAstar microplate reader (BMG Labtech, Offenburg, Germany) equipped with a nitrogen laser, and a monoexponential function was fitted to the data in order to determine τ_{DA}, the apparent fluorescence lifetime of Lumi4 in presence of Green. Then, for each labeling condition, the apparent FRET efficiency was calculated as $E_{app} = 1 - \tau_{DA}/\tau_D$ (27), where τ_D is the fluorescence lifetime of Lumi4 in cells labeled with SNAP-Lumi4 only (300 nM).

The relationship among E_{app}, x_A, and the pairwise FRET efficiency E was modeled by Raicu (50) for any oligomeric size N, under the assumptions of both absence of free monomers and equal separation distance of all fluorophore pairs within an oligomer. In particular, using the notation $u = x_A/(1 + x_A)$,

$$E_{app} = \frac{3 \cdot E \cdot u^3}{1 + 2 \cdot E} + \frac{6 \cdot E \cdot (1 - u) \cdot u^2}{1 + E} + 3 \cdot E \cdot (1 - u)^2 \cdot u$$

when $N = 4$,

$$E_{app} = \frac{2 \cdot E \cdot u^2}{1 + E} + 2 \cdot E \cdot (1 - u) \cdot u$$

when $N = 3$, and $E_{app} = E \cdot u$ when $N = 2$, which were fitted to the experimental data.

Western blot analysis

At 24 h after electroporation, adherent COS-7 cells plated in 10-cm dishes were labeled with 1 μM SNAP-Surface782 and 1 μM CLIP-Red in culture medium for 2 h at 37°C. Cells were then washed with PBS, treated with 10 mM *N*-ethylmaleimide for 20 min at 4°C, washed, treated with lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS; and protease inhibitors) for 45 min at 4°C and harvested. After centrifugation for 30 min at 12,000 *g*, the supernatant was heated with loading buffer (NuPAGE LDS sample buffer 4×, Invitrogen) for 10 min at 37°C. Electrophoresis was performed using precast NuPAGE Novex 3–8% Tris-acetate gel (Invitrogen) and blotted onto nitrocellulose membrane. Membranes were imaged on an Odyssey infrared scanner (LI-COR Biosciences, Lincoln, NE, USA) at 800 nm for SNAP-Surface782 and at 700 nm for CLIP-Red.

Cytosolic calcium measurements

At 24 h after electroporation with plasmid DNA encoding receptor (1–3 μg/10^7 cells), glutamate transporter EAAC1 (1 μg), and chimeric G-protein α-subunit $G\alpha_{qi9}$ (1 μg, except for G_q-coupled mGlu1), HEK-293 cells were loaded with Fluo-4 AM, and drug-triggered cytosolic calcium release was monitored on a FlexStation (Molecular Devices, Sunnyvale, CA, USA) as described previously (51).

Ligand binding assay

COS-7 cells were transfected by electroporation with plasmid DNA encoding SNAP- or CLIP-mGlu2 (0.05–1 μg/10^7 cells) and plated in polyornithine-coated, 24-well plates at 7.5×10^5 cells/well. At 30 h after transfection, cells were incubated for 4 h at 4°C with 2 nM [^{3}H]-LY341495, washed 3 times with Tris-Krebs buffer, and lysed for 15 min in 0.1 M NaOH. Radioactivity was measured with a Wallac MicroBeta microplate liquid scintillation counter (Perkin Elmer, Boston, MA, USA). Unspecific binding was determined in presence of 1 mM glutamate.

RESULTS

Specific labeling of cell-surface SNAP- and CLIP-tagged mGluRs with trFRET fluorophores

We recently applied trFRET to the study of GPCR complexes at the surface of living cells using a europium cryptate and a red fluorophore, d2, as a FRET donor and acceptor (20). In an effort to obtain brighter and more chemically stable fluorophores, we used here a new terbium cryptate, Lumi4, which can transfer energy to different fluorophores emitting green, yellow, or red visible light (**Fig. 1*A***). We used "Green," a fluorescein-like acceptor developed by Cisbio Bioassays, that possesses multiple advantages when used in trFRET experiments with Lumi4 as a donor. First, at the Green peak emission wavelength, Lumi4 emission is very low. Second, the calculated Förster radius of the Lumi4/Green pair is 46 Å, a distance short enough (12, 39) to largely limit trFRET resulting from proximity between proteins not directly interacting with each other (Fig. 1*B*). We next developed an approach to specifically label mGluRs with Lumi4 and Green at the surface of living cells.

SNAP and CLIP tags represent a powerful strategy to specifically label proteins in living cells with 2 distinct classes of substrates, benzylguanines and benzylcytosines (30, 31). SNAP and CLIP are 19-kDa suicide enzymes that catalyze the irreversible transfer of a fluorescent label from a substrate to a unique cysteine

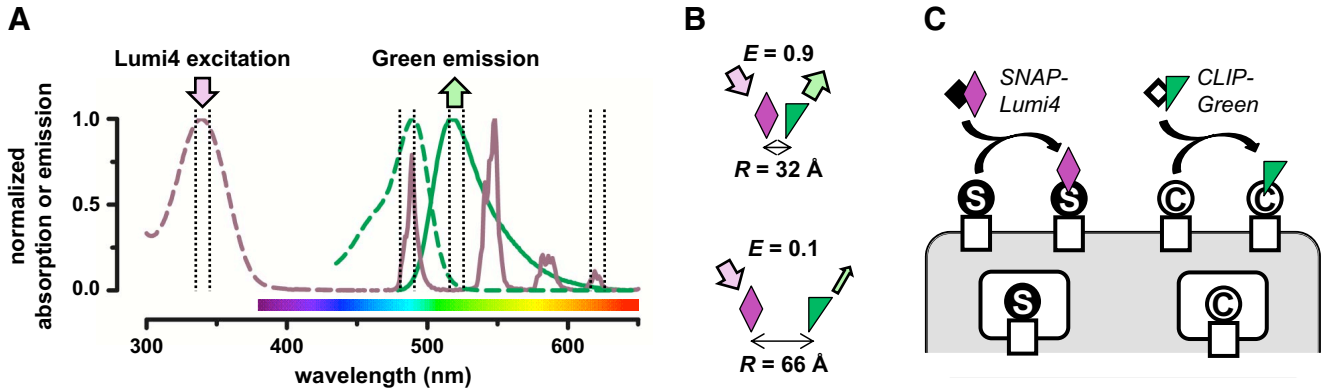

Figure 1. trFRET fluorophores and SNAP/CLIP multilabeling. *A*) Lumi4 (violet lines) and Green (green) absorption (dashed lines) and emission spectra (solid). Vertical dotted lines indicate center wavelength ± bandpass of the excitation and emission filters used to measure Lumi4-Tb (340 and 620 nm) and Green fluorescence (485 and 520 nm). Arrows indicate filters used to measure trFRET (340 and 520 nm). *B*) Influence of the separation distance *R* between trFRET donor Lumi4-Tb (diamond) and acceptor Green (right triangle) on FRET efficiency *E*. $E = R_0^6/(R^6 + R_0^6)$, where R_0 is the Förster radius, which is 46 Å for the Lumi4/Green pair. Note that GPCR transmembrane domains are ~40 Å in diameter. *C*) Schematics representing the effect of nonpermeant substrates on subunits (rectangles) fused to a SNAP (solid circle) or CLIP tag (open circle) at their N terminus, present both in intracellular compartments and at the plasma membrane. Represented substrates are SNAP-Lumi4, an O^6-benzylguanine (solid tilted square) derivatized with Lumi4, and CLIP-Green, an O^2-benzylcytosine (open tilted square) derivatized with Green.

of their own active site: SNAP becomes covalently labeled after incubation with fluorescent substrates that contain the O^6-benzylguanine backbone, whereas CLIP is a modified SNAP engineered to specifically react with distinct O^2-benzylcytosine-derived substrates. We envisioned this multilabeling strategy as an opportunity to selectively label 2 different receptors with our trFRET-compatible donor and acceptor fluorophores (Fig. 1*C*). We thus used 4 different substrates: benzylguanines conjugated with either Lumi4 or Green to label SNAP tags (SNAP-Lumi4 and SNAP-Green, respectively) and derivatives of benzylcytosine to label CLIP-tag fusion proteins (CLIP-Lumi4 and CLIP-Green).

We fused all mGluR subunits (except mGlu6) to either SNAP or CLIP tags at their extracellular amino-terminal end and transfected them in mammalian cells. These mutant receptors were found to have similar pharmacological properties as their respective wild type, as illustrated by the ability of tagged mGlu1, mGlu2, and mGlu4 receptors to stimulate second messenger responses similar to wild type (Supplemental Fig. 1). We next demonstrated that these constructs can be efficiently labeled with the 4 substrates mentioned above. With the use of either SNAP-mGlu2 or CLIP-mGlu2 (**Fig. 2*A*, *B***), the specific fluorescence reached a plateau as the concentration of substrate was increased, reflecting complete labeling. Incubating cells with the optimal concentration of substrate thus leads to the

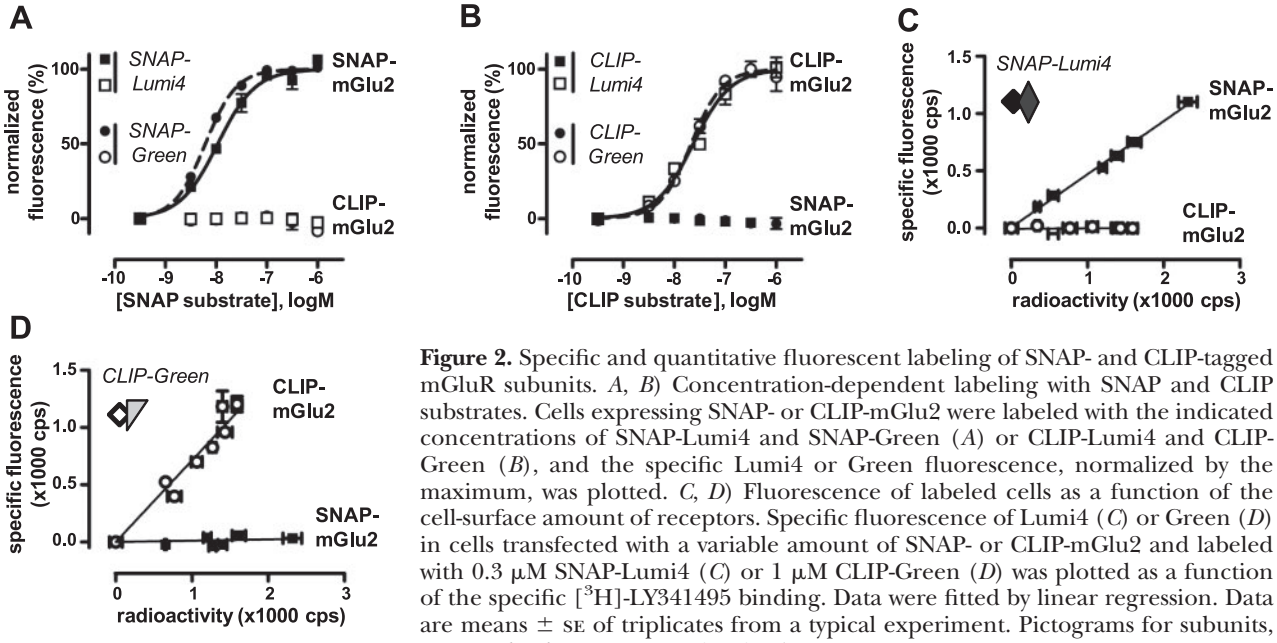

Figure 2. Specific and quantitative fluorescent labeling of SNAP- and CLIP-tagged mGluR subunits. *A*, *B*) Concentration-dependent labeling with SNAP and CLIP substrates. Cells expressing SNAP- or CLIP-mGlu2 were labeled with the indicated concentrations of SNAP-Lumi4 and SNAP-Green (*A*) or CLIP-Lumi4 and CLIP-Green (*B*), and the specific Lumi4 or Green fluorescence, normalized by the maximum, was plotted. *C*, *D*) Fluorescence of labeled cells as a function of the cell-surface amount of receptors. Specific fluorescence of Lumi4 (*C*) or Green (*D*) in cells transfected with a variable amount of SNAP- or CLIP-mGlu2 and labeled with 0.3 μM SNAP-Lumi4 (*C*) or 1 μM CLIP-Green (*D*) was plotted as a function of the specific [^{3}H]-LY341495 binding. Data were fitted by linear regression. Data are means ± SE of triplicates from a typical experiment. Pictograms for subunits, tags, and substrates are as in Fig. 1.

attachment of exactly 1 fluorophore/accessible tagged subunit (30–32). Furthermore, we measured no significant signal after incubating SNAP-mGlu2 with CLIP substrates or CLIP-mGlu2 receptors with SNAP substrates, demonstrating the specificity of the 4 substrates toward their respective tags (Fig. 2).

For studies of cell-surface oligomerization, it is crucial to label only cell-surface receptors. As negatively charged molecules, Lumi4 and Green are unlikely to permeate through the plasma membrane. To confirm this, we used a tagged GB1 subunit of the $GABA_B$ receptor, which is retained in intracellular compartments, except when coexpressed with its partner subunit GB2 (52, 53). While SNAP- or CLIP-tagged GB1 was labeled with SNAP or CLIP substrates in the presence of GB2, no labeling was obtained in the absence of GB2, indicating a lack of accessibility of the fluorescent substrates for intracellular SNAP and CLIP tags (Supplemental Fig. 2*A*, *B*). Taken together, these results demonstrate that SNAP and CLIP fluorescent labeling is specific, total, and restricted to cell-surface receptors.

In addition to being specific, the detection of SNAP and CLIP subunits is quantitative. Indeed, we showed (Fig. 2*C*) that the fluorescence of SNAP-Lumi4-labeled cells is proportional to the amount of SNAP-mGlu2 at the cell surface measured through binding of a non-cell-permeant radioligand (R^2=0.987). We thus define Exp_S as the expression of cell-surface SNAP subunits determined by fluorescent SNAP labeling. Similarly, Fig. 2*D* shows that fluorescence after CLIP-Green labeling is proportional to CLIP-mGlu2 expression (R^2=0.922), and we define Exp_C as the expression of cell-surface CLIP subunits determined by the specific fluorescence signal after CLIP-Green labeling, divided by a correcting factor in order to be equivalent to Exp_S (see Materials and Methods and Supplemental Fig. 2*C*, *D*).

Specific detection of 3 distinct dimer populations using trFRET

As mGlu2 is a homodimeric receptor, cells cotransfected with SNAP- and CLIP-mGlu2 are expected to express 3 populations of dimers: SNAP/CLIP dimers, SNAP/SNAP dimers, and CLIP/CLIP dimers. Our approach allows the analysis of any of these populations. In fact, if SNAP-Lumi4 and CLIP-Green are combined to label each subunit specifically, we expect a trFRET signal in cells expressing both SNAP- and CLIP-mGlu2 subunits but not in cells expressing only SNAP-mGlu2 or only CLIP-mGlu2. This scenario was indeed what we observed, as shown in **Fig. 3**, allowing us to define $FRET_{SC}$ as the trFRET signal specific of SNAP/CLIP dimers (Table 1). Using the method previously described by Maurel *et al.* (20), we then optimized the SNAP-Lumi4 and SNAP-Green concentrations to detect SNAP/SNAP dimers (Supplemental Fig. 2*E*) and observed that under these labeling conditions, trFRET ($FRET_{SS}$) is present in cells expressing SNAP-mGlu2 alone or in combination with CLIP-mGlu2 (Fig. 3). We also optimized the CLIP substrates concentrations to detect CLIP/CLIP dimers (Supplemental Fig. 2*F*) and detected trFRET signal ($FRET_{CC}$) only in cells expressing CLIP-mGlu2 (Fig. 3).

These data demonstrate that, using 3 separate labeling conditions, it is possible to discriminate 3 populations of oligomers: the SNAP/SNAP and CLIP/CLIP homomers, as well as the SNAP/CLIP heteromers.

mGlu2 subunits form strict dimers at the cell surface

mGlu2 subunits form dimers, but one cannot exclude that they may also form higher-order oligomers. We first investigated the strict dimeric architecture of the mGlu2 receptor by the method of Brock *et al.* (48). We engineered chimeric mGlu2 subunits containing complementary coiled-coil regions (C1 and C2) and intracellular retention signals (KKXX) in order to control the subunit composition of the receptors reaching the cell surface (Supplemental Fig. 3): mGlu2 dimers that carry 2 SNAP tags (SNAP-mGlu2) or only 1 (SNAP-mGlu2-C1-KKXX+mGlu2-C2-KKXX). As no $FRET_{SS}$ signal is observed in the latter case (**Fig. 4*A***), we conclude that there is no close interaction between such chimeric mGlu2 dimers, consistent with mGlu2 subunits assembling into strict dimers only.

However, whether mGlu2 subunits with their native C-terminal tail assemble into strict dimers remains unknown. We addressed this question by combining experimental FRET data and theoretical models. First, SNAP-tagged mGlu2 subunits were expressed and labeled with various ratios of SNAP-Lumi4 and SNAP-Green (Fig. 4*B*). The molar ratio [Green]/[Lumi4]

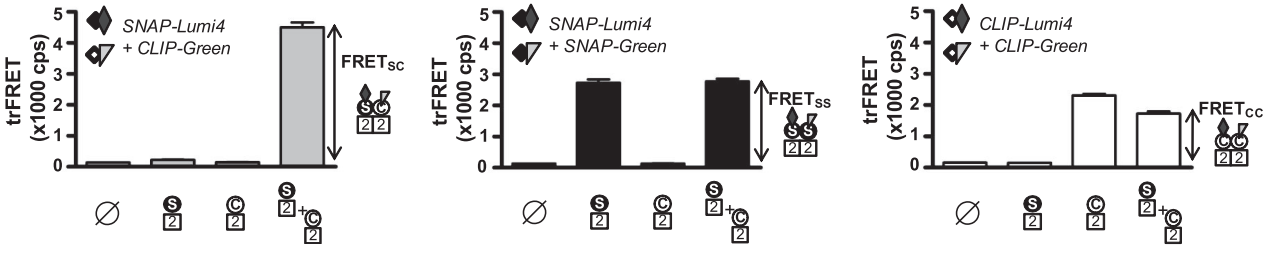

Figure 3. trFRET multilabeling reveals distinct populations of dimers: specificity of $FRET_{SC}$, $FRET_{SS}$, and $FRET_{CC}$. Cells expressing SNAP-mGlu2 and/or CLIP-mGlu2, as indicated at bottom, were labeled with the indicated SNAP and CLIP substrates. Bar plot shows the raw trFRET signal, *i.e.*, the intensity of Green emission after excitation of Lumi4; the nonspecific background signal was not subtracted. Population responsible for the specific signal in the last bar is sketched at right. Data are means ± SE of triplicates from a typical experiment. Pictograms for subunits, tags, and substrates are as in Fig. 1.

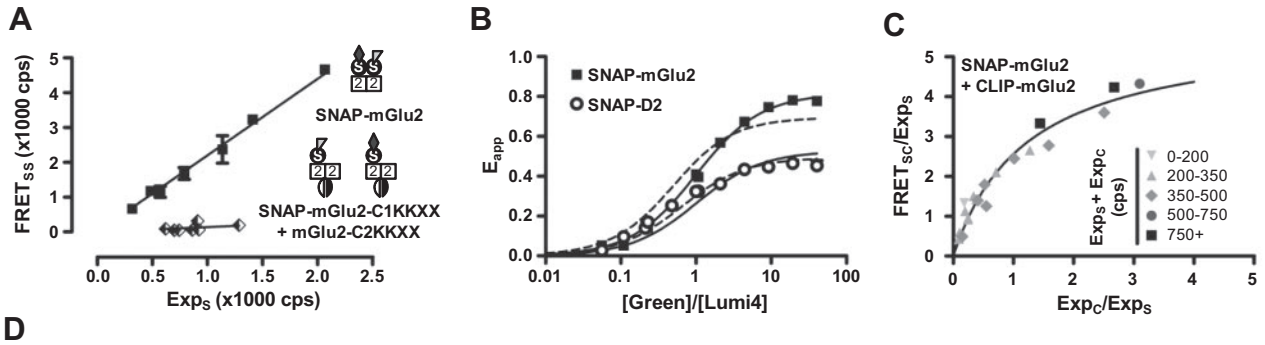

Figure 4. mGluR subunits form strict homodimers and not higher-order oligomers. *A*) FRET$_{SS}$ as a function of SNAP cell-surface expression in cells expressing at the cell-surface mGlu2 dimers with 2 SNAP-tags (SNAP-mGlu2) or mGlu2 dimers with only 1 SNAP-tag (SNAP-mGlu2-C1KKXX+mGlu2-C2KKXX). C1KKXX (open tail) and C2KKXX (solid tail) are complementary coiled-coil regions (C1 and C2) carrying an endoplasmic reticulum retention signal (KKXX) that are fused to the C terminus of mGlu2 subunits. Only the sketched receptors are targeted to the cell surface. *B*) Oligomeric size of SNAP-tagged receptors. Indicated receptors were expressed in cells and simultaneously labeled with 100 nM SNAP-Lumi4 and 1 nM to 1 μM SNAP-Green. Molar ratio of fixed acceptor was determined through Green fluorescence; apparent FRET efficiency E_{app} was estimated through Lumi4 emission decay. Models assuming a dimeric receptor (solid lines) or tetrameric receptor (dashed lines) were fitted to the experimental data. *C*) trFRET saturation curve of SNAP-mGlu2 with CLIP-mGlu2. Both constructs were transfected at various ratios and levels in cells, and Exp$_S$, Exp$_C$, and FRET$_{SC}$ were determined. FRET$_{SC}$/Exp$_S$ is shown as a function of the CLIP-to-SNAP expression ratio, Exp$_C$/Exp$_S$. Total expression of tagged subunits, Exp$_S$ + Exp$_C$, is coded by different shades of gray. *D*) trFRET saturation curves, as described in panel *C*, of the indicated couples of tagged subunits. Data are means ± SE of triplicates from a typical experiment.

attached to SNAP tags after labeling was determined using the Green direct fluorescence, while the apparent FRET efficiency was determined using the donor fluorescence decay. In Fig. 4*B*, the apparent FRET efficiency is shown as a function of [Green]/[Lumi4]. It is known that the shape of this curve is affected by the oligomeric size of the receptor *i.e.*, the number of subunits per oligomer (18, 54, 55). In a recent report by Raicu (50), this curve was modeled for any oligomeric size, under the assumption that the separation distance is the same between all pairs of fluorophores. We fitted Raicu's models for dimers, trimers, and tetramers to our experimental data and observed that the dimer model was the best. This was verified for SNAP-mGlu1 as well (data not shown). We used the dopamine D2 receptor as control, which has been shown to form tetramers in transfected cells (16). In contrast to SNAP-mGlu2 and SNAP-mGlu1, the data generated with SNAP-D2 were best described by the tetramer model (Fig. 4*B*). Although it is difficult to conclude on the exact stoichiometry of the D2 receptor, these data clearly exclude a strictly dimeric organization. These results support the view that mGlu2 and mGlu1 are not higher-order oligomers but are indeed strict homodimers.

We conclude from these data that mGluR subunits form strict homodimers at the surface of living cells.

trFRET saturation assay reveals heteromers of mGluR subunits

In contrast to the study of homooligomers, the use of a multilabeling strategy, such as SNAP/CLIP, is required to address heterooligomers. We developed a new SNAP/CLIP trFRET saturation assay, which is comparable to the FRET and BRET saturation assays but allows a very good quantification of the parameters.

First, we validated the trFRET saturation assay on mGlu2 homodimers. SNAP- and CLIP-mGlu2 were expressed at various levels, and 3 parameters were determined: Exp$_S$, Exp$_C$, and FRET$_{SC}$, which reflect the amounts of SNAP subunits, CLIP subunits, and SNAP/CLIP dimers at the cell surface (Table 1). In Fig. 4*C*, we plotted the FRET$_{SC}$/Exp$_S$ ratio as a function of the CLIP-to-SNAP expression ratio Exp$_C$/Exp$_S$. As expected, this curve saturates and is fitted (R^2=0.95) with the function: FRET$_{SC}$/Exp$_S$ = FRET$_{MAX}$/[1 + (Exp$_C$/Exp$_S$)/FRET$_{50}$], with FRET$_{MAX}$ = 5.72 ± 0.47 and FRET$_{50}$ = 1.23 ± 0.21 as best-fit parameters. Interestingly, the curve remained unchanged despite strong variations in the total subunit expression (Fig. 4*C*), consistent with trFRET that does not result from random collision (54). Notably, when analyzed with Raicu's models for homooligomers, our experimental data are more consistent with the model for strict dimers rather than those for trimers or tetramers (data not shown).

SNAP/CLIP trFRET saturation assays were also performed for other mGlu receptors (Fig. 4*D*). We observed a saturation for SNAP-mGlu1/CLIP-mGlu1 (group I) and for SNAP-mGlu4/CLIP-mGlu4 (group III), with FRET$_{50}$ values that are 107 ± 7 and 111 ± 14% (n=3) of the trFRET$_{50}$ for SNAP-mGlu2/CLIP-mGlu2 (group II), respectively, suggesting that these mGluRs are also strict dimers. Interestingly, whereas mGlu4 had a FRET$_{MAX}$ similar to mGlu2 (108±5%), we

observed a significantly lower $FRET_{MAX}$ for mGlu1 (60±1%). One possible explanation is a difference in the maximal FRET efficiency, originating from a longer distance between SNAP and CLIP tags in mGlu1 compared with mGlu2 or mGlu4.

The previous experiments validated the use of trFRET saturation assay to reveal the specific interaction between a SNAP- and a CLIP-tagged subunit. We then addressed the question of mGluR subunits forming heteromeric entities. To that aim, we tested the possible heteromerization of mGlu2 with mGlu1 and mGlu4, taken as representatives of each group of mGluRs, through a trFRET saturation assay (**Fig. 5*A***). A very weak trFRET signal was detected between SNAP-mGlu2 and CLIP-mGlu1 and between SNAP-mGlu1 and CLIP-mGlu4 (Fig. 5*A*), as well as between SNAP-mGlu1 and CLIP-mGlu2 and between SNAP-mGlu4 and CLIP-mGlu1 (data not shown). In contrast, we found a strong and saturating trFRET signal between SNAP-mGlu2 and CLIP-mGlu4 that is similar to the curve obtained with SNAP-mGlu4 and CLIP-mGlu2. Interestingly, the $trFRET_{50}$ for mGlu2/mGlu4 was similar to the $trFRET_{50}$ for mGlu2 alone (95±12% for SNAP-mGlu2/CLIP-mGlu4 and 124±18% for SNAP-mGlu4/CLIP-mGlu2, n=3).

Taken together, these data reveal that some pairs of mGluR subunits, but not all, are capable of assembling into heterooligomers of unknown stoichiometry.

trFRET competition assay reveals that mGlu2/mGlu4 are heterodimers, not dimers of homodimers

The trFRET signal between mGlu2 and mGlu4 can originate from the formation of a strict heterodimeric receptor formed by 2 subunits of distinct subtypes but might as well result from proximity between homodimeric mGlu2 and mGlu4 receptors. Indeed, GPCR tetramers were shown to exist (16, 19, 43), including the closely related $GABA_B$ receptor (20). In Fig. 5*B*, we show 4 possible configurations of mGlu2/mGlu4 heteromers. (*a*–*c*). It is not possible to discriminate them through $FRET_{SC}$ analysis. However, it is possible to discriminate them by analysis of $FRET_{SS}$ and $FRET_{CC}$, which are specific of SNAP/SNAP dimers and CLIP/CLIP dimers. Indeed, SNAP-mGlu2 dimers are disrupted by CLIP-mGlu4 expression in configurations *a*, *c* but not in *b*, *d*; similarly, CLIP-mGlu4 dimers are disrupted by SNAP-mGlu2 expression in configurations *a*, *d* but not in *b*, *c* (Fig. 5*B*).

First, we verified that the $FRET_{SS}/Exp_S$ ratio is constant over a wide range of SNAP-mGlu2 expression (Supplemental Fig. 4*A*) and that the $FRET_{CC}/Exp_C$ ratio is constant over a wide range of CLIP-mGlu2 expression (Supplemental Fig. 4*B*). Thus, we can interpret a decrease of these values by a disruption of SNAP/SNAP and CLIP/CLIP dimerization. Then, we transfected cells with various ratios of SNAP-mGlu2 and either CLIP-mGlu2, CLIP-mGlu4, or CLIP-mGlu1. We separately labeled the cells to determine Exp_S, Exp_C, $FRET_{SS}$, and $FRET_{CC}$. Both CLIP-mGlu2 and CLIP-mGlu4 decreased $FRET_{SS}/Exp_S$ (Fig. 5*C*). In contrast, CLIP-mGlu1, which does not associate with SNAP-mGlu2, did not affect $FRET_{SS}/Exp_S$. These results show that CLIP-mGlu4 interacts with SNAP-mGlu2 in a competitive way, excluding the configurations in Fig. 5*Bb, d*. Conversely, in the same transfections, SNAP-mGlu2 decreased $FRET_{CC}/Exp_C$ when CLIP-mGlu2 or CLIP-mGlu4 was coexpressed but not when CLIP-mGlu1 was

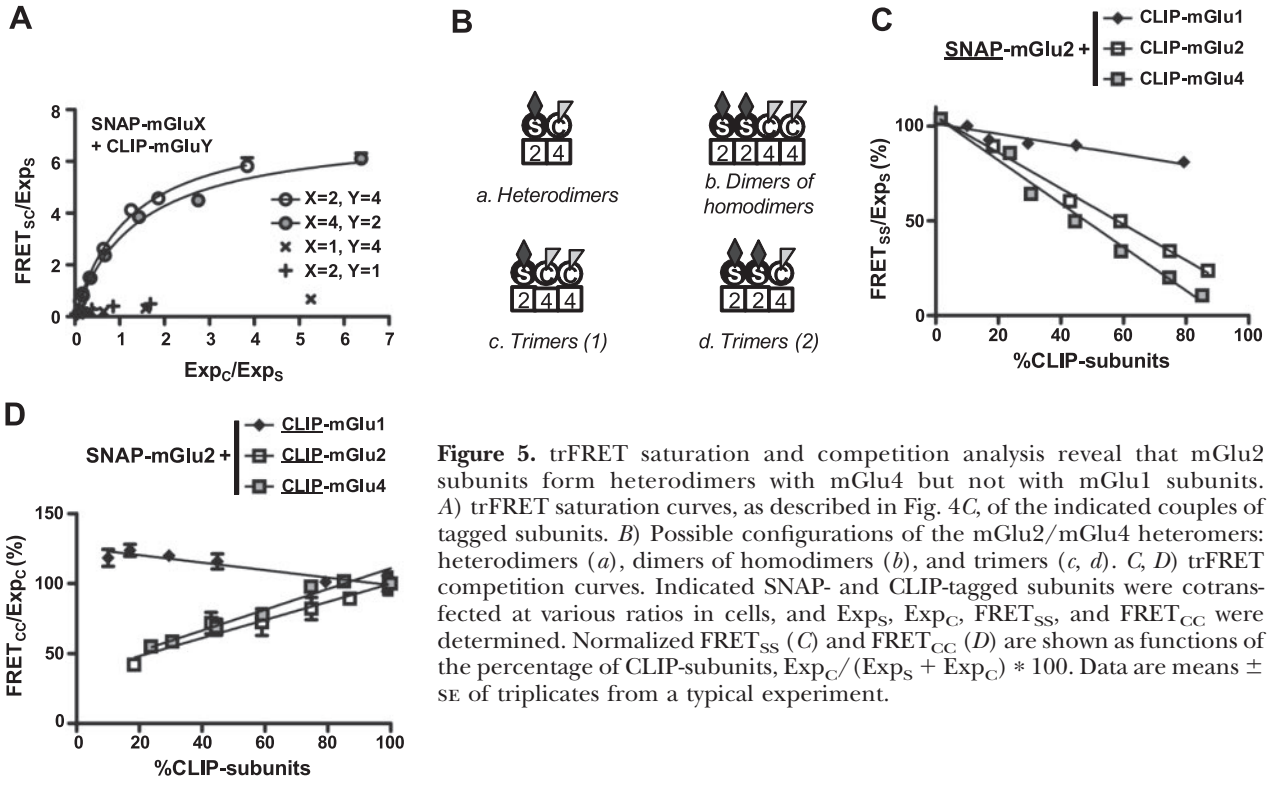

Figure 5. trFRET saturation and competition analysis reveal that mGlu2 subunits form heterodimers with mGlu4 but not with mGlu1 subunits. *A*) trFRET saturation curves, as described in Fig. 4*C*, of the indicated couples of tagged subunits. *B*) Possible configurations of the mGlu2/mGlu4 heteromers: heterodimers (*a*), dimers of homodimers (*b*), and trimers (*c*, *d*). *C*, *D*) trFRET competition curves. Indicated SNAP- and CLIP-tagged subunits were cotransfected at various ratios in cells, and Exp_S, Exp_C, $FRET_{SS}$, and $FRET_{CC}$ were determined. Normalized $FRET_{SS}$ (*C*) and $FRET_{CC}$ (*D*) are shown as functions of the percentage of CLIP-subunits, $Exp_C/(Exp_S + Exp_C) * 100$. Data are means ± SE of triplicates from a typical experiment.

coexpressed (Fig. 5*D*). This further excludes the configurations in Fig. 5*Bb, c*. We conclude that mGlu2/mGlu4 heteromers are strict heterodimers (Fig. 5*Ba*).

The fact that mGlu2 and mGlu4, but not mGlu1, can compete with mGlu2 for the formation of dimers is also well represented by the graphs shown in Supplemental Fig. 5, where $FRET_{SS}$, $FRET_{CC}$, and $FRET_{SC}$ are normalized by the total expression of SNAP- and CLIP-tagged subunits (Exp_S+Exp_C) and shown as functions of the percentage of CLIP subunits. This analysis further supports the heterodimerization of mGlu2 and mGlu4 subunits.

Biochemical identification of mGlu2/mGlu4 heterodimeric receptors

We next examined the stability of the mGlu2/mGlu4 heterodimers observed in our trFRET experiments. Homodimeric mGluRs are covalent dimers that can be solved by PAGE in nonreducing conditions. We thus labeled intact cells expressing heterodimeric mGluRs formed by SNAP- and CLIP-tagged subunits with fluorescent substrates and then analyzed the membrane proteins by PAGE. We used a SNAP-mGlu2 fused to GFP at its C terminus so that SNAP-mGlu2-GFP dimers migrate at an apparent molecular mass (290 kDa) greater than CLIP-mGlu4 dimers (240 kDa). As shown in **Fig. 6*A***, on coexpression of SNAP-mGlu2-GFP and CLIP-mGlu4, we detected a protein migrating at an apparent molecular mass less than SNAP-mGlu2-GFP dimers but greater than CLIP-mGlu4 dimers. This protein is labeled with both SNAP and CLIP substrates and is absent in single transfections. These characteristics clearly identify it as SNAP-mGlu2-GFP/CLIP-mGlu4 heterodimers. In contrast, we could not detect SNAP-mGlu1/CLIP-mGlu2 heterodimers in cells coexpressing SNAP-mGlu1 and CLIP-mGlu2 (Fig. 6*A*). These results demonstrate the presence and stability of mGlu2/mGlu4 heterodimeric receptors at the surface of transfected cells.

mGlu2/mGlu4 heterodimeric receptors are functional

It is not known, so far, whether mGlu2/mGlu4 heterodimeric receptors are functional. mGluRs are multidomain allosteric dimeric receptors in which the two subunits communicate with each other (40–42). In homodimeric mGluRs, one subunit can directly transactivate the partner subunit, as shown by coupling-deficient mutants that functionally complement binding-deficient mutants (48, 56, 57). Here, we demonstrate that intersubunit transactivation (Fig. 6*B*) is retained in mGlu2/mGlu4 heterodimers. Indeed, whereas the mGlu2/3-specific agonist LY354740 could not elicit a functional response in cells that express either mGlu4 or the coupling-deficient mutant mGlu2(F756P), a signal was obtained in cells coexpressing both of them. Notably, the decrease in agonist potency and efficacy at mGlu4/mGlu2(F756P) heterodimers compared with wild-type mGlu2 mGluRs composed of 1 binding-deficient and 1 coupling-deficient subunit is in agreement with previous reports of a partially impaired function for (48, 56). In conclusion, this functional complementation experiment demonstrates that allosteric communication between the two different subunits of mGlu2/mGlu4 heterodimeric receptors is conserved.

Inter- and intrasubgroup heteromerization in the mGluR family

The finding that mGlu2 can heterodimerize with mGlu4 but not with mGlu1 raises the question of the specificity of interaction between the various types of mGluR subunits. To determine the capacity of mGluR subunits to heteromerize with each other, we con-

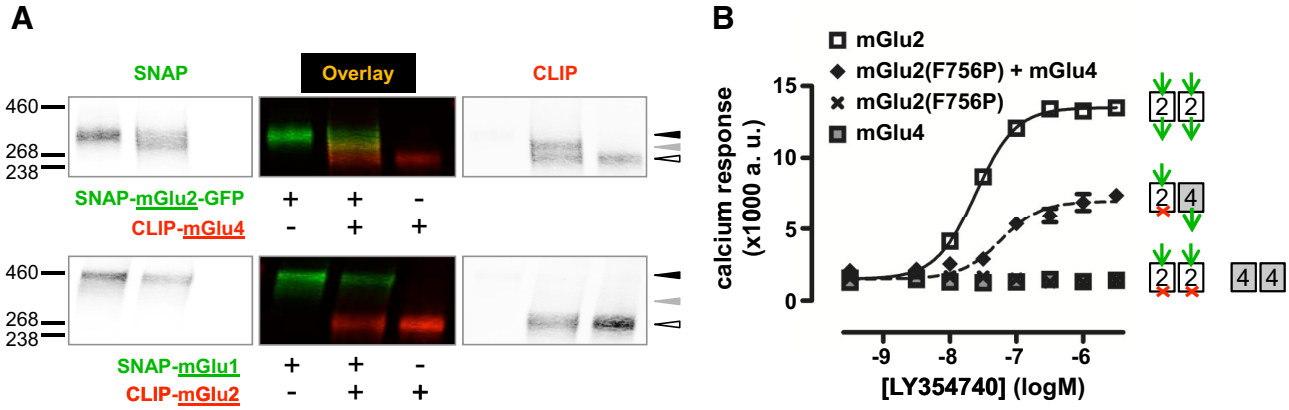

Figure 6. mGlu2/mGlu4 heteromers are covalent and functional heterodimeric receptors. *A*) Western blot analysis of cell-surface dimeric receptors. Cells expressing indicated tagged subunits were labeled with SNAP-Surface782 and CLIP-Red before lysis, gel electrophoresis in nonreducing conditions, and acquisition of the fluorescence of both SNAP and CLIP labels. Standard molecular mass (kDa) is at left; symbols at right indicate expected size (from top to bottom) for SNAP/SNAP dimers, SNAP/CLIP heterodimers, and CLIP/CLIP dimers. *B*) Calcium response mediated by indicated constructs coexpressed with chimeric G-protein α-subunit G_{qi9} on stimulation by mGlu2 agonist LY354740. Data are means ± SE of triplicates from a typical experiment. Schematics at right illustrate signal transduction in wild-type and mutant dimeric receptors. mGlu2, but not mGlu4, subunits can bind LY354740 (inward arrow). Wild-type mGlu2 and mGlu4 subunits, but not mGlu2(F756P), which is mutated in its third intracellular loop (cross), can couple to G protein (outward arrow).

ducted a large heteromerization assay for all subunits in the mGluR family (except mGlu6). We expressed each of the 49 possible combinations of 1 SNAP- and 1 CLIP-tagged mGluR subunit and determined the $FRET_{SC}/(Exp_S+Exp_C)$ ratio in these cells (**Fig. 7*A***). We verified that the observed difference was not due to poor expression of one construct relative to the other (data not shown). Interestingly, we found 2 distinct groups of association compatibility: group I mGluRs (subtypes 1 and 5) on the one hand and group II (subtypes 2 and 3) + group III (subtypes 4, 7, and 8) on the other: group I mGluR subunits interact together, but do not associate with others while group II and III mGluR subunits preferentially associate with each other but not with those from group I (Fig. 7*B*).

DISCUSSION

Despite a widespread interest in the homo- and heteromerization of GPCRs over the past decade, there is no easy way to analyze the stoichiometry of these complexes at the surface of living cells. In the present study, we propose a new approach that provides information about the organization of GPCR complexes. It consists in the specific labeling of 2 cell surface proteins with 2 distinct fluorophores compatible with trFRET using the SNAP- and CLIP-tag labeling strategy. We first showed that mGlu receptors expressed in transfected cells are restricted to homodimers and do not form higher-order oligomers. Then, we showed that subunits from distinct mGlu subtypes can form heteromers, exemplified by the mGlu2/mGlu4 pair. Other pairs, such as mGlu2/mGlu1, in contrast, cannot heteromerize. We further investigated the organization of the mGlu2/mGlu4 heteromers and showed that they are indeed heterodimers, not dimers of homodimers. Moreover, this result was confirmed by a Western blot analysis that reveals that mGlu2/mGlu4 heterodimers are covalently linked, and by a functional complementation between a mutant mGlu2 subunit that can bind an agonist but cannot couple to a G protein and an mGlu4 subunit that cannot bind the agonist but can couple to a G protein. Finally, we tested the ability of all pairs of subunits from 2 subtypes out of 7 (all mGluRs except mGlu6) to heteromerize and described the specificity of interaction in the mGluR family.

We previously reported that the covalent labeling of cell surface proteins with trFRET compatible fluorophores using the suicide enzyme SNAP tag allows an easy analysis of their oligomeric assembly (20). This technique presents many advantages over conventional FRET or BRET techniques: only cell-surface receptors are labeled and detected; SNAP-tag labeling is highly flexible, as for instance the [acceptor]/[donor] ratio can be easily varied; and photophysical properties of the lanthanide cryptates, combined with a time-resolved acquisition of the FRET signal, avoid all contamination of the sensitized acceptor emission by the donor emission and by the direct excitation of the acceptor. Data are thus more reliable, and experiments can be performed in microplates, so that many transfection and labeling conditions are tested in parallel. Moreover, we used here Lumi4, a new terbium cryptate that is exceptionally bright, 10–20 times brighter than the europium cryptate previously used (20), significantly increasing the detection sensitivity in a number of assays (26). In association with the Green acceptor, a trFRET signal can be obtained only if the 2 fluorophores are distant by <66 Å, thus increasing the probability of measuring a FRET signal only between 2 directly interacting proteins. Interestingly, recent reports have shown that trFRET measurements can be further implemented on a microscope (58) and that oligomerization can be studied directly in native tissues thanks to specific receptor ligands labeled with trFRET fluorophores (17), opening new avenues for trFRET studies.

Here we showed that the question of the size of homooligomers can be addressed with trFRET combined with SNAP-tag labeling. We generated trFRET data and compared them with a model recently described by Raicu (50); in contrast to models adapted from Veatch and Stryer (59), this model does not make the assumption that there is exchange between subunits and takes into account multiple possible pathways for energy transfer in oligomers larger than dimers. Our data then clearly show that trFRET and SNAP-tag labeling are perfectly adapted to this strategy.

However, the analysis of heteromer formation required another, orthogonal labeling method. We chose to take advantage of the CLIP tag, a mutant SNAP-tag enzyme that specifically reacts with benzylcytosine derivatives. For this purpose, we developed specific SNAP and CLIP substrates carrying either a trFRET donor or an acceptor, and showed that a perfect orthogonal labeling of SNAP and CLIP-tagged proteins is obtained. Then, depending on the combination of SNAP and

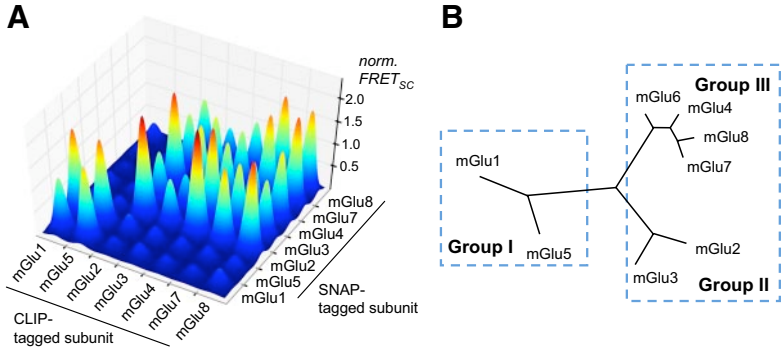

Figure 7. Intra- and intergroup heterodimerization properties of members of the mGluR family. *A*) Three-dimensional plot of $FRET_{SC}$, normalized by Exp_S + Exp_C in cells expressing indicated constructs. *B*) Tree showing the phylogenetic distance between rat mGluR subtypes. The 3 groups are indicated; the 2 boxes indicate the 2 proposed heterodimerization compatibility groups.

CLIP substrates, we can separately detect the cell-surface populations of SNAP/SNAP homomers, CLIP/CLIP homomers, and SNAP/CLIP heteromers formed by 2 proteins, one carrying the SNAP tag and the other the CLIP tag. It is also possible to quantify the cell surface expression of SNAP and CLIP subunits. Notably, all these parameters could be determined simultaneously in a microplate format, allowing several different transfection conditions to be tested in parallel. In saturation assays, the trFRET between SNAP and CLIP subunits could reveal the formation of heteromers. Moreover, in competition assays, the trFRET between 2 SNAP subunits, or between 2 CLIP subunits, could precise the organization of the SNAP/CLIP heteromers, *i.e.*, determine whether they were heterodimers or dimers of homodimers.

While mGluRs were first described as homodimeric (38–42), other class C GPCRs, such as the GABAB receptor and the sweet and umami taste receptors, were shown to function as heterodimers (44–46). Surprisingly, the hypothesis that mGluR subunits could also form heteromeric entities has never been thoroughly investigated, except in the original study (38) reporting the dimeric nature of mGlu5 receptors, where no coimmunoprecipitation could be detected between mGlu1 and mGlu5. Unexpectedly, we discovered that specific combinations of mGluR subunits have the propensity to form heteromers. The reasons for this divergence with the data reported by Romano *et al.* (38) remain unclear, but our data are well supported by both biochemical and functional complementation evidence. In addition, we show that heteromers of mGluR subunits form strict heterodimeric receptors and that mGlu2 has the same capacity to form homodimers as to form heterodimers with mGlu4. Accordingly, such heterodimers may form in cells coexpressing the 2 receptors, pending spatiotemporally identical synthesis.

Interestingly, our systematic trFRET association assay revealed specificity in the heteromerization process. Intragroup heteromeric mGluR receptors can form within the 3 groups. Moreover, group II and III mGluR subunits can also produce intergroup heteromeric receptors that are functional, as exemplified by mGlu2/mGlu4 heterodimers in our trans-complementation experiment. In contrast, neither group II nor III subunits group I subunits. Of note, such specificity prevents the formation of heterodimeric mGluRs composed of subunits coupled to different G proteins. Indeed, whereas both group I mGluRs are coupled to G_q, all group II and III mGluRs are coupled to $G_{i/o}$ proteins. This illustrates some specificity in this process, consistent with the known general location of these receptors in neurons: group I mGluRs are almost exclusively postsynaptic, while group II and III mGluRs are mostly found in presynaptic elements (34).

Although our data show that heterodimeric mGluRs can form in transfected cells, whether such heterodimers exist *in vivo* remains unclear. Each mGluR has a specific expression pattern in the brain (34), but in a number of cases, 2 different mGluRs are coexpressed in the same neurons and also colocalized. For instance, mGlu7 and mGlu8 are colocalized in the presynaptic active zone of GABAergic interneurons in the hippocampus (60). The mGlu1 and mGlu5 subunits were shown to be coexpressed in striatal neurons (61), the dentritic tip of rod bipolar cells (62), and interneurons (63). Eventually, retinal amacrine cells coexpress most mGluRs except 3 and 6 (64) and neuronal stem cells coexpress mGlu3, mGlu4, and mGlu5 (65).

Our current findings and hypotheses may offer a hitherto unappreciated explanation for the existence of functional heteromers *in vivo*, as determined previously. For example, the blockade of *both* mGlu1 and mGlu5 with specific antagonists is required to block chemically induced hippocampal long-term depression, while genetic deletion of mGlu5 is sufficient (66). These data are consistent with the existence of mGlu1/mGlu5 heterodimeric receptors, since both mGlu1 and mGlu5 antagonists are needed to block such a receptor (67). Others speculated about the existence of a mGlu2/mGlu3 heterodimeric receptor to explain the desensitization of hippocampal LY379268-induced cAMP response in wild-type mice, which is absent in mGlu3-knockout mice. However, further studies are necessary to demonstrate the existence of such heterodimers *in vivo*. In contrast, our results can rule out the existence of other heterodimeric mGluRs *in vivo*: for example, even though mGlu3 and mGlu5 were found to be simultaneously up-regulated in reactive astrocytes (69), our results suggest that they do not form heterodimeric receptors.

Besides possible heterodimerization processes within their own family, mGluRs have also been shown to associate with other class C GPCRs or with GPCRs from other classes. For example, mGlu1a and another class C receptor, the calcium sensing receptor, were shown to heterodimerize *in vitro* by disulfide linkage and also to colocalize in rat hippocampus and cerebellum (70). Gonzalez-Maeso *et al.* (4) showed that mGlu2 directly interacts with the serotonin 5-HT2A receptor and that this complex triggers unique cellular responses when targeted by hallucinogenic drugs. Moreover, mGlu5 can assemble with the class A adenosine A_{2A} receptor and dopamine D_2 receptor, as shown by sequential resonance energy transfer, and they colocalize in specific postsynaptic structures in GABAergic striatopallidal neurons (71). These few examples of GPCR complexes illustrate the increasing possible roles played by receptor dimerization and oligomerization and the important pharmacological implications they can trigger. Thus it will become increasingly important in drug development to analyze and characterize these heteromers. Of note, the approach we propose is versatile, given it may be used to detect the homo- and heteromeric populations of virtually any cell surface membrane proteins as long as the extracellular domain of the proteins of interest can be labeled without impairing their function. FJ

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