Assembly-dependent Surface Targeting of the Heterodimeric GABA_B Receptor Is Controlled by COPI but Not 14-3-3

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Cell surface expression of transmembrane proteins is strictly regulated. Mutually exclusive interaction with COPI or 14-3-3 proteins has been proposed as a mechanism underlying such trafficking control of various proteins. In particular, 14-3-3 dimers have been proposed to “sense” correctly assembled oligomers, allowing their surface targeting by preventing COPI-mediated intracellular retention. Here we examined whether such a mechanism is involved in the quality control of the heterodimeric G protein-coupled GABA_B receptor. Its GB1 subunit, carrying the retention signal RSR, only reaches the cell surface when associated with the GB2 subunit. We show that COPI and 14-3-3 specifically bind to the GB1 RSR sequence and that COPI is involved in its intracellular retention. However, we demonstrate that the interaction with 14-3-3 is not required for proper function of the GABA_B receptor quality control. Accordingly, competition between 14-3-3 and COPI cannot be considered as a general trafficking control mechanism. A possible other role for competition between COPI and 14-3-3 binding is discussed.

INTRODUCTION

Cell surface targeting of transmembrane proteins is strictly regulated and often requires correct oligomeric assembly. The molecular mechanisms underlying such assembly-dependent surface expression are subject to intense investigations. Intracellular retention of unassembled subunits displaying dibasic retention signals appears to be a common feature of many different types of membrane proteins. Prototype of such a signal is the C-terminal di-lysine KKXX motif, which is recognized by the coat protein I complex (COPI) that mediates retrieval from the *cis*-Golgi to the ER in COPI-coated vesicles (Bonifacino and Lippincott-Schwartz, 2003; Lee *et al.*, 2004). More recently, the di-arginine RXR motif has been identified as an intracellular retention signal (Zerangue *et al.*, 1999) also recognized by COPI (Yuan *et al.*, 2003). Di-arginine retention signals are widely used, in particular in ion channels, including K_{ATP} and KCNK3 potassium channels (Zerangue *et al.*, 1999; O’Kelly *et al.*, 2002) or

NMDA and kainate type glutamate receptors (Wentholt *et al.*, 2003; Jaskolski *et al.*, 2005).

Oligomeric assembly may overcome such intracellular retention through steric hindrance of the interaction with COPI (or possibly other proteins involved in the retention), as has been demonstrated, e.g., for the FcεRI receptor (Lettourneur *et al.*, 1995). Alternatively, it has been proposed that 14-3-3 dimers may “sense” oligomeric protein assembly. Yuan *et al.* (2003) have demonstrated mutually exclusive binding of COPI and 14-3-3 proteins to the RKR retention signal of the Kir6.2 K_{ATP} channel subunit and proposed that oligomeric assembly may increase the affinity for 14-3-3 dimers. Thus, monomeric Kir6.2 subunits would be retained inside the cell because of their higher affinity for COPI, whereas assembled channels would be released from the retention because of their higher affinity for 14-3-3 dimers, competing COPI out. Mutually exclusive interaction with either COPI or 14-3-3 has also been proposed to play a role in the trafficking control of several other proteins (O’Kelly *et al.*, 2002), but in these cases the 14-3-3 binding was regulated rather through serine phosphorylation, with no obvious link to oligomeric assembly. Competition between 14-3-3 and COPI was therefore proposed to be a general mechanism of cell surface expression control.

In the present study, we examined the molecular mechanisms involved in the quality control system of the γ -aminobutyric acid (GABA) type B receptor. This G protein-coupled receptor (GPCR) is an obligate heterodimer (Marshall *et al.*, 1999). Its GB1 subunit carries the retention signal RSR in its cytosolic C-terminal tail and only reaches the cell surface when associated with the GB2 subunit (Margeta-Mitrovic *et al.*, 2000; Calver *et al.*, 2001; Pagano *et al.*,

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Abbreviations used: CC, coiled coil; COPI, coat protein I complex; GABA, γ -aminobutyric acid, GB1/2, GABA type B receptor 1/2; GPCR, G protein-coupled receptor; HEK, human embryonic kidney.

Figure 1. The GABA_B receptor quality control. HEK293 cells were transfected with the indicated plasmids and analyzed 1 d after transfection. (A) Confocal imaging of (N- or C-terminally) GFP-tagged GABA_B receptor subunits in live cells. Scale bars, 10 μ m. Because initial experiments revealed no differences between the two major GB1 splice variants, GB1a and GB1b (differing only in their N-terminal sequence), all experiments depicted in this article were done with GB1a. (B) ELISA. The HA tag fused to the N-termini of the GABA_B receptor subunits is only accessible for the antibody when the receptor is at the cell surface or when the cells are permeabilized. The ratio of the signals on non-permeabilized versus permeabilized cells (top panel) therefore indicates the % surface expression of the HA-tagged protein (bottom panel). Right panel: schematic representation of the ELISA results. PM, plasma membrane.

2001). However, the molecular mechanisms underlying the intracellular retention of GB1 and its assembly-dependent surface expression with GB2 were yet unknown. The similarity between the GB1 and the Kir6.2 RXR retention signals suggested that competition between COPI and 14-3-3 may also be involved here. In line with this, 14-3-3 proteins have previously been demonstrated to also interact with GB1, in a region encompassing its RSR retention signal (Couve *et al.*, 2001).

We now demonstrate that both COPI and 14-3-3 can indeed interact with the GB1 RSR sequence, but, surprisingly, the interaction with 14-3-3 is not required for proper function of the GABA_B receptor trafficking control. Thus, competition between 14-3-3 and COPI is not a general mechanism of cell surface expression control.

MATERIALS AND METHODS

Plasmids and Transfection

New plasmids were constructed by standard PCR and subcloning (Sambrook *et al.*, 1989) or site-directed mutagenesis by QuikChange (Stratagene, La Jolla, CA). Primers were synthesized by Eurogentec (Seraing, Belgium). Pfu Turbo Polymerase was from Stratagene, restriction enzymes from NEB (Beverly, MA), T4 DNA ligase from Fermentas (Hanover, MD), and DNA purification kits from Qiagen (Hilden, Germany). All PCR- or QuikChange-derived parts were verified by sequencing (Genome Express, Meylan, France). All constructs are in pRK, except for the 14-3-3 ζ in pcDNA3. HA-GB1, HA-GB1_{ASA}, HA-GB2, and cMyc-GB2 plasmids were described previously (Galvez *et al.*, 2001). GFP was fused to the C-terminal ends of GB1 or GB2, respectively, via

a linker reading AS, or introduced after residue 929 of GB1 via a linker reading LE (GB1_{AC}-GFP). In GB1_{KKXX} the KKTN was introduced after residue 921, in GB2_{KKXX} after residue 820. To generate constructs with cleavable N-terminal HA and fluorescent tags, GFP and a thrombin cleavage site (taken from a plasmid generously provided by V. Homburger) were introduced via the *Mlu*I site between the HA tag and the receptors. A FLAG-14-3-3 ζ plasmid was a generous gift from L. Limbird. An optimized ribosomal docking sequence (Kozak, 1987) was added in order to improve its expression.

Cell culture and transfection of HEK293 cells was performed as described (Galvez *et al.*, 2001).

Immunofluorescence and Confocal Imaging

PFA-fixed and Triton-permeabilized cells were incubated overnight with anti-Calreticulin (1:100, Alexis Biochemicals, Lausen, Switzerland) or anti-GM130 (1:150, BD Biosciences, Palo Alto, CA), then 45 min with anti-rabbit-Cy3 (1:4000, Jackson ImmunoResearch, West Grove, PA) or anti-mouse-Alexa594 (1:2000, Molecular Probes, Eugene, OR), respectively. Coverslips were mounted with Gel/Mount (Biomed, Foster City, CA).

Confocal imaging was performed on an LSM 510 Meta confocal microscope with a Plan-Apochromat 63 $\times$ /1.4 oil objective and Immersol 518F (Carl Zeiss, Jena, Germany). Live cell imaging was performed at 37°C in 138 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5.5 mM glucose, 10 mM HEPES, pH 7.5, and 2 g/l bovine serum albumin (BSA). GFP was excited at 488 nm and detected through a 505-nm long pass or a 505–530-nm band pass filter in absence or presence of Cy3 or Alexa594, respectively, which were excited at 543 nm and detected through a 585–615-nm bandpass filter. Pinholes were adjusted to yield optical slices of <0.8 μ m.

ELISA

PFA-fixed and Triton-permeabilized (or not) cells were blocked with phosphate-buffered saline + 1% fetal calf serum, incubated 30 min with 0.5 mg/l anti-HA (3F10; Roche, Penzberg, Germany), washed, incubated 30 min with 1 mg/l HRP-coupled anti-rat F(ab')₂ (Jackson), and washed again. Bound an-

Figure 2. Recognition and retrieval of GB1 by COPI. (A) Immobilized peptides containing the GB1 RSR retention signal, its inactive ASA mutant, or the KKXX COPI-binding sequence were incubated with HEK293 membrane extracts. Retained proteins were analyzed by immunoblot with antibodies against COPI subunits. (B) Colocalization of GB1-GFP with markers for ER (Calreticulin) and *cis*-Golgi (GM130). Scale bars, 10 μ m.

tibody was detected using a SuperSignal substrate (Pierce, Rockford, IL) and a Wallac Victor² counter (Molecular Devices, Sunnyvale, CA).

Pulldown and Immunoblot

Peptides were synthesized by Eurogentec and coupled to activated CH Sepharose 4B (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol. HEK293 membrane extracts were prepared as described (Bécamel *et al.*, 2002) in 50 mM Tris, pH 7.5, 1 mM EDTA, 10 mM CHAPS + protease inhibitors (Roche) and incubated with the extracts overnight. After washing, retained proteins were eluted with Laemmli buffer and analyzed by standard SDS-PAGE + immunoblotting (Sambrook *et al.*, 1989) with antibodies against α COP (Affinity BioReagents, Golden, CO), β COP (clone maD, Sigma, St. Louis, MO), 14-3-3 (clone H-8; Santa Cruz Biotechnology, Santa Cruz, CA), or 14-3-3 ζ (clone C-16; Santa Cruz), and peroxidase-coupled anti-mouse or -rabbit antibodies, respectively (Amersham).

Forward Transport Kinetics

Cells were incubated 2.5 h at 15°C, with 3 U/ml thrombin (Calbiochem, La Jolla, CA) during the last 1.5 h. After washing, cells were transferred to 37°C, fixed with PFA after different times, and analyzed by ELISA as described above.

Adenylate Cyclase Inhibition

Cells transfected with plasmids for both GABA_B receptor subunits and G α_{i1} were stimulated 5 min with various concentrations of GABA (Sigma) and then 15 min with 10 μ M forskolin (Sigma) in DMEM + 1% BSA and 0.1 mM of the phosphodiesterase inhibitor RO-20-1724 (Sigma). The reaction was stopped by addition of Triton X-100 to a final concentration of 0.5%. The amount of cAMP accumulated in the cells was quantified using the cAMP Dynamic kit (CIS Bio International, Bagnols sur Cèze, France) according to the manufacturer's instruction.

RESULTS

Intracellular Retention and Cell Surface Targeting of the GABA_B Receptor and Its Subunits

We first verified the subcellular distribution of GFP-tagged GB1 and GB2 by confocal microscopy in transiently transfected live human embryonic kidney (HEK) 293 cells. Though the GABA_B receptor is a neurotransmitter receptor,

Figure 3. Interaction of the GB1 RSR with 14-3-3. (A) Immunoblot analysis of the same samples as in Figure 2A, but with antibodies recognizing 14-3-3 ζ or all seven 14-3-3 isoforms. (B) Immobilized peptides containing the GB1 RSR retention signal, its inactive ASA mutant, as well as a phosphorylated RpSR and a nonphosphorylatable RAR variant were incubated with membrane extracts of FLAG-14-3-3 ζ -transfected cells. Retained proteins were analyzed by anti- β COP and anti-FLAG immunoblot.

its assembly-dependent surface expression has been shown to work in various cell types, suggesting a ubiquitous mechanism. In agreement with previously reported results on fixed cells (Margeta-Mitrovic *et al.*, 2000; Calver *et al.*, 2001; Pagano *et al.*, 2001), GB2 alone was targeted to the plasma membrane, whereas GB1 was retained inside the cell, unless its RSR retention sequence was mutated to ASA or unless it was coexpressed with GB2 (Figure 1A). Similar results were obtained whether the GFP was fused to the N- or C-terminus of the subunits and are further confirmed by ELISA (Figure 1B). Interestingly, the retention of GB1 was abolished when GFP was fused in close proximity (7 instead of 38 amino acids distance) to the RSR (GB1 $_{\Delta C}$ -GFP). This is in line with the hypothesis that the GB1 retention requires interaction of its RSR sequence with another, rather large protein or protein complex such as COPI, which can be sterically hindered by the bulky GFP close to the RSR.

Interaction of the GB1 RSR with COPI

To test whether the GB1 RSR sequence is recognized by COPI, we next incubated HEK293 membrane extracts with immobilized peptides containing the RSR or the mutant ASA sequence and surrounding amino acids of the GB1 C-terminal tail (SRQQLRSRRHPPT or SRQQLASARHPPT) and analyzed the retained proteins by immunoblot. Positive control was a peptide in which the RSR and the subsequent residues were replaced by KKTN, prototype of the C-terminal KKXX COPI-binding motif. Because the COPI complex easily dissociates into two subcomplexes (Fiedler *et al.*, 1996), we used antibodies against α COP and β COP, i.e., one subunit of either subcomplex. Indeed, we found both α - and β COP immunoreactivity in the eluates of the KKXX and the RSR, but not the ASA peptide (Figure 2A), demonstrating that indeed the COPI complex specifically interacts with the GB1 RSR.

COPI is well known to retrieve proteins from the Golgi to the ER in COPI-coated vesicles. The interaction of the GB1 RSR with COPI therefore strongly suggests that the same mechanism is responsible for the GB1 intracellular retention. If this is true, then GB1 should not, as generally thought, be retained in the ER (Couve *et al.*, 1998), but reach at least the *cis*-Golgi, where COPI-coated vesicles are formed. Indeed, immunofluorescence studies revealed that GB1 not only colocalized with the ER marker calreticulin, but also with the *cis*-Golgi marker GM130 (Figure 2B). Thus, GB1 can leave the ER and reach the *cis*-Golgi, in line with COPI mediating the GB1 intracellular retention by retrieving it from the *cis*-Golgi to the ER.

Figure 4. 14-3-3 is not required for the GABA_B receptor surface expression control. Retention and surface targeting of GB1 mutants not interacting with 14-3-3 was monitored as before by ELISA (top panel), or confocal imaging (bottom panel; scale bars, 10 μm).

Interaction of the GB1 RSR with 14-3-3

The findings that not only COPI but also 14-3-3ζ and ε specifically bind to the Kir6.2 RKR sequence (Yuan *et al.*, 2003), and that 14-3-3ζ and η interact with GB1 through a region encompassing its RSR (Couve *et al.*, 2001) suggested that the GB1 RSR may also interact with 14-3-3ζ and possibly other 14-3-3 isoforms. We therefore analyzed the eluates of our pulldown (see above) by immunoblot with antibodies recognizing 14-3-3ζ or all seven 14-3-3 isoforms. Both recognized a band in the eluates of the RSR, but not the ASA, nor the KKXX peptide (Figure 3A). Also when using extracts of cells expressing recombinant FLAG-tagged 14-3-3ζ, the latter was specifically retained by the RSR, but not the ASA peptide (Figure 3B). Thus indeed 14-3-3ζ and possibly other 14-3-3 isoforms with similar electrophoretic mobility interact with the GB1 RSR.

Interaction with 14-3-3 proteins is often regulated through serine phosphorylation (Fu *et al.*, 2000). However, 14-3-3 binding was not enhanced, but rather abolished by phosphorylation of the RSR serine (RpSR; Figure 3B), even in presence of the phosphatase inhibitor NaVO₃ (1 mM; unpublished data). In contrast, the COPI precipitation appeared slightly reduced, pointing to the possibility that the GB1 interaction with COPI might nevertheless be modulated by phosphorylation of the RSR serine, but not through competition with 14-3-3. Interestingly, the 14-3-3 binding was also markedly reduced by mutation of the RSR serine into alanine (RAR), whereas COPI binding was not or only marginally affected.

14-3-3 Binding Is Not Required for Surface Targeting of the GABA_B Receptor

14-3-3 proteins have been proposed to release proteins from intracellular retention through competition with COPI binding (O'Kelly *et al.*, 2002; Yuan *et al.*, 2003). In particular, COPI and 14-3-3 have been demonstrated to both interact directly with the Kir6.2 RXR in a mutually exclusive way (Yuan *et al.*, 2003), strongly suggesting the same for the GB1 RXR.

In contrast, 14-3-3 overexpression failed to increase surface expression of GB1 (unpublished data). Of note, also in the experiments of O'Kelly *et al.* (2002) 14-3-3 overexpression had no effect on KCNK3 trafficking. Both results might be

explained by a relatively low overexpression with respect to the high level of endogenous 14-3-3 (about fourfold in our experiment).

Moreover, a GB1^{KKXX} and a GB1^{RAR} mutant were both not only retained inside the cell when expressed alone, but also surface-targeted when coexpressed with GB2, exactly like the wild-type GB1 (Figure 4), although these mutations abolished or markedly reduced 14-3-3 binding in our pull-down assays (see Figure 3). Thus, the GB1 interaction with 14-3-3 is not required for proper function of the GABA_B receptor surface expression control system.

To test a possible role of the GB1 interaction with 14-3-3 in the kinetics of the GABA_B receptor trafficking to the cell surface, we next used an assay combining selective enzymatic removal of HA tags from receptors at the cell surface with reversible temperature-induced block of forward transport from the ER/ERGIC to the plasma membrane (Rosenberg *et al.*, 2001). Neither overexpression of 14-3-3, nor the RAR mutation affected the time course of the restoration of the cell surface expression of the HA-tagged receptor (Figure 5).

14-3-3 Does Not Affect the GABA_B Receptor Coupling to G_i Proteins

In search for the physiological role of the GABA_B receptor interaction with 14-3-3 we next examined receptor-mediated activation of G_i proteins, as measured by their inhibitory effect on adenylate cyclase-mediated cAMP production. Neither overexpression of 14-3-3, nor the RAR mutation affected the potency nor efficacy of the GABA_B receptor inhibition of forskolin-stimulated cAMP formation (Figure 6).

The GB2 Coiled-Coil Domain Is Sufficient to Release GB1 from Its Intracellular Retention

It has been previously shown that the GB2 C-terminal tail, when transferred to another transmembrane protein, contains all the molecular determinants necessary to release GB1 from its intracellular retention, and, moreover, that the GB2 coiled-coil (CC) domain is crucial for this (Margeta-Mitrovic *et al.*, 2000). We now tested whether the GB2 CC is also sufficient to bring GB1 to the cell surface and whether it requires to be attached to a transmembrane protein. As depicted in Figure 7, the GB1 retention was clearly overcome by coexpression of the

Figure 5. 14-3-3 does not affect the GABA_B receptor trafficking kinetics. Time course of the GABA_B receptor forward transport from the ER/ERGIC to the plasma membrane. GB1 (WT vs. RAR, as indicated) carrying an N-terminal HA tag followed by a thrombin cleavage site (Thr) was coexpressed with GB2 (and 14-3-3 ζ , as indicated). Cells were incubated at 15°C to block transport from the ER/ERGIC to the plasma membrane, and thrombin was added to remove HA tags from receptors already present at the cell surface. The intracellularly accumulated receptors still carrying their tags were then released by transfer to 37°C. Cells were fixed after different times, and the relative surface expression of the HA-tagged receptors was analyzed by ELISA.

GB2 CC domain as a soluble protein without any membrane attachment (only fused to GFP to verify its expression). Thus, no other parts of GB2 besides its CC domain are required to release GB1 from its intracellular retention.

GB2 Sterically Masks the GB1 Retention Signal

How does GB2 release GB1 from its COPI-mediated retention? The GB1 RSR being localized just adjacent to its CC domain, one may speculate that the interaction with the GB2 CC could sterically hinder the interaction of COPI with the RSR or the KKXX at the same position in our GB1_{KKXX}. If this is true, then the masking should also work in the other way round, i.e., GB1 should be able to mask a retention signal introduced adjacent to the GB2 CC. Indeed, a GB2_{KKXX} (with KKTN adjacent to its CC domain, equivalent to our GB1_{KKXX} construct) was retained inside the cell and brought to the cell surface by GB1, as well as by GB1_{ASA} or

Figure 6. 14-3-3 does not affect the GABA_B receptor signaling function. GB1 (WT vs. RAR, as indicated) was coexpressed with GB2 (and 14-3-3 ζ , as indicated). Cells were incubated with 10 μ M forskolin to directly stimulate adenylate cyclase activity (i.e., cAMP production), plus various amounts of GABA to inhibit adenylate cyclase activity through G_i proteins activated by the GABA_B receptor, and cAMP production was monitored. Presence of equivalent amounts of receptors at the cell surface was verified by ELISA on intact cells from the same transfections (unpublished data).

Figure 7. The GB2 coiled-coil (CC) domain is sufficient to overcome the GB1 retention. HA-GB1 was expressed alone or together with GB2, or a soluble protein containing the GB2 CC domain and GFP (to verify its expression), or GFP alone, and its surface expression was monitored by ELISA.

GB1_{KKXX}, which do not bind 14-3-3 (Figure 8). Conversely, GB2_{KKXX} was also capable of bringing GB1 to the surface, indicating that the two proteins mutually masked their retention signals.

DISCUSSION

Competition between COPI and 14-3-3 proteins has been proposed as a mechanism controlling the cell surface targeting of various proteins (O'Kelly *et al.*, 2002), in particular the assembly-dependent surface expression of oligomeric transmembrane proteins carrying RXR type retention signals (Yuan *et al.*, 2003). The aim of our study was to further examine the role of COPI and 14-3-3 in assembly-dependent surface expression of oligomeric transmembrane proteins using the heterodimeric G protein-coupled GABA_B receptor as a model system (Figure 1, A and B).

Intracellular Retention of GB1 by COPI

We demonstrate here that the RSR retention signal of the GABA_B receptor's GB1 subunit specifically interacts with COPI (Figure 2A). This complex is well known to retrieve transmembrane proteins carrying KKXX type retention signals from the *cis*-Golgi to the ER in COPI-coated vesicles (Bonifacino and Lippincott-Schwartz, 2003; Lee *et al.*, 2004), suggesting it may also mediate the intracellular retention of GB1 through the same mechanism. In agreement with this, the GB1 RSR can be replaced by KKXX without changing the trafficking properties of GB1 (Figure 4). Moreover, we show that GB1 is not, as previously thought, exclusively retained in the ER (Couve *et al.*, 1998), but reaches the *cis*-Golgi, in line with COPI being responsible for its intracellular retention (Figure 2B).

RXR type intracellular retention signals, first described by Zerangue *et al.* in 1999, have meanwhile been identified in a large number of different transmembrane proteins. However, it has been demonstrated only recently and only for one example, the Kir6.2 RKR, that RXR retention signals may be recognized by COPI (Yuan *et al.*, 2003). Our demonstration that the GB1 RSR also interacts with this complex now confirms that RXR type retention signals are generally recognized by COPI (Figure 2A). Interestingly, the COPI immunoreactive bands in our pulldown assays appeared stronger for the eluate of the RSR as compared with the KKXX peptide, suggesting that COPI may bind with higher affinity to RXR compared with KKXX retention signals.

Figure 8. Masking of a retention signal in GB2 by GB1. Cells were transfected with the indicated plasmids, and GB1/GB2 surface expression was monitored by ELISA (left) or confocal imaging (right; scale bars, 10 μ m).

However, the interaction may be influenced by the local sequence context (Zerangue *et al.*, 2001). Further, it remains to be elucidated whether RXR and KKXX motifs interact with the same or different sites of the COPI complex. Interestingly, the even more closely related KKXX and KKKXX retention signals have recently been shown to be recognized by different COPI subunits (Eugster *et al.*, 2004).

The finding that GB1 needs to associate with GB2 to reach the cell surface had been striking evidence that GPCRs, traditionally believed to be monomers, can form dimers (Marshall *et al.*, 1999). It has meanwhile become widely accepted that many (if not all) GPCRs can (if not must) exist and function as dimers, and it is emerging that dimerization may be a prerequisite for cell surface targeting also of other GPCRs (Bulenger *et al.*, 2005). Intracellular retention of un-assembled monomers by COPI may therefore apply to various GPCRs. In line with this, COPI has recently been demonstrated to also interact with the G protein-coupled V₂ vasopressin receptor (Hermosilla *et al.*, 2004). One may also speculate that COPI could recognize the arginine cluster in the C-terminal tail of the α_{1D} -adrenergic receptor, which is retained inside the cell unless associated with the α_{1B} -adrenergic receptor (Hague *et al.*, 2004).

14-3-3 Is Not Involved in Surface Targeting of the GABA_B Receptor

We demonstrate here that the GB1 RSR cannot only interact with COPI, but also with 14-3-3 ζ , and possibly other 14-3-3 isoforms (Figure 3), in line with the recently reported findings for the Kir6.2 RKR sequence (Yuan *et al.*, 2003). That study nicely demonstrated direct and mutually exclusive interaction of either COPI or 14-3-3 with the Kir6.2 RKR in vitro, strongly suggesting the same is true for their interaction with the GB1 RSR. This pointed to the possibility that 14-3-3 proteins, through competition with COPI, might be involved in the trafficking control of proteins with RXR type retention signals such as Kir6.2 or GB1. Accordingly, Yuan *et al.* (2003) have proposed that oligomeric assembly of Kir6.2 subunits may increase the affinity for 14-3-3 dimers, competing COPI out and releasing the oligomer from the retention. Though this is an elegant model, it did not seem to apply to the assembly-dependent surface targeting of the GABA_B receptor, because 14-3-3 has been reported to bind only to GB1, but not GB2 (Couve *et al.*, 2001); hence the GB1/GB2 heterodimerization is not expected to increase the number of 14-3-3 binding sites and thus the affinity for 14-3-3 dimers. Further, GB1 also forms homodimers (Maurel *et al.*, 2004), but these are still retained inside the cell (Villemure *et al.*, 2005), albeit the dimeric presentation of the RXR.

Moreover, using GB1 mutants that do not bind 14-3-3, but still COPI (Figure 3), we now present unequivocal evidence that the GB1 interaction with 14-3-3 is not involved in the GABA_B receptor trafficking control, because these mutants behave exactly like WT GB1 regarding their intracellular retention in absence and surface targeting in presence of GB2 (Figure 4). Of note, though both studies of O'Kelly *et al.* (2002) and Yuan *et al.* (2003) nicely demonstrate mutually exclusive binding of COPI and 14-3-3 to their proteins in vitro, neither study provides direct evidence that 14-3-3 proteins can indeed bring these proteins to the cell surface by preventing COPI binding in vivo.

We further demonstrate that the 14-3-3 interaction with the GABA_B receptor does not affect its trafficking kinetics (Figure 5), nor its signaling function (Figure 6). The GB1 interaction with 14-3-3 may serve other functions yet to be elucidated.

CC Domain Interaction Prevents Recognition of Adjacent Retention Signals by COPI

But how is GB1 released from its intracellular retention? We demonstrate here that the GB2 CC domain is sufficient to bring GB1 to the cell surface (Figure 7). Moreover, a retention signal introduced adjacent to the CC domain in GB2 is also masked by GB1 (Figure 8). These data strongly suggest that the interaction of the two CC domains prevents the recognition of an adjacent retention signal by COPI, most likely by steric hindrance. Alternatively, one might speculate that the CC domain interaction could induce a conformational change reducing the affinity of the adjacent retention signals for COPI. However, this appears unlikely to explain our results with the various combinations tested, i.e., with the RSR as well as the KKXX on GB1 (Figure 4) and even the KKXX on GB2 (Figure 8). Simple steric hindrance appears more likely to explain the retention signal masking in all three combinations. Of note, the data presented by Yuan *et al.* (2003) do not rule out the possibility that the oligomeric assembly of Kir6.2 may also overcome the intracellular retention through simple steric hindrance of COPI binding.

We have previously demonstrated that fusion with the GB1 and GB2 C-terminal tails can transfer the GABA_B receptor quality control system to other GPCRs, permitting to partly control the subunit composition of surface-expressed receptor dimers (Kniazef *et al.*, 2004; Goudet *et al.*, 2005; Hlavackova *et al.*, 2005). However, although indeed the subunit carrying the GB1 C-terminal tail reached the cell surface only when associated with the subunit carrying the GB2 C-terminal tail, the latter could still also reach the cell sur-

face as a monomer or homodimer, largely complicating the analysis. We now demonstrate that this system can be improved by introduction of an additional retention signal on the C-terminal tail of GB2. On coexpression of GB1 and GB2-KKXX, monomers or homodimers of either subunit are retained inside the cell, and only the heterodimers reach the cell surface, through mutual masking of their retention signals (Figure 8). This system may greatly facilitate the analysis of specific GPCR or other plasma membrane protein heterodimers.

Which Role for Competition between COPI and 14-3-3?

Taken together, our data demonstrate that although both COPI and 14-3-3 interact with the GABA_B receptor's RXR retention motif, the interaction with 14-3-3 is not required to release the receptor from its COPI-mediated intracellular retention. Therefore, competition between COPI and 14-3-3 cannot, as previously proposed (O'Kelly *et al.*, 2002; Yuan *et al.*, 2003), be considered as a general mechanism in trafficking control.

Conversely, one may speculate that competition between COPI and 14-3-3 could rather occur in the other way round: 14-3-3 proteins could bind to GB1 already at the ER level, and, because of their dimeric nature, possibly link GB1 with other proteins. Because GB1 is not functional without GB2, such association would be useless and might even hinder correct assembly with GB2. At the Golgi level, COPI could then remove such undesired complexes from GB1 by competing 14-3-3 out. COPI-mediated retrieval of transmembrane proteins may thus also serve to dissociate prematurely associated protein complexes.

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