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Assembly-dependent Surface Targeting of the Heterodimeric GABA<sub>B</sub> 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<sub>B</sub> 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<sub>B</sub> 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<sub>ATP</sub> and KCNK3 potassium channels (Zerangue et al., 1999; O’Kelly et al., 2002) or NMDA and kainate type glutamate receptors (Wenthold 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 FceRI receptor (Le-tourneur 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<sub>ATP</sub> 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.,
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 GABAB 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 14-3-3Δ in pcdNA3. HA-GB1, HA-GB1ASA, 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/H9004 C-GFP). In GB1KKXX 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 MluI site between the HA tag and the receptors. A FLAG-14-3-3/HA9256 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 (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-rabbit F(ab’2) (Jackson), and washed again. Bound an-

a linker reading AS, or introduced after residue 929 of GB1 via a linker reading LE (GB1/A-S-GFP). In GB1^K/KKK, the KKTN was introduced after residue 921, in GB2^K/KKK, 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 MluI site between the HA tag and the receptors. A FLAG-14-3-3 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 (Biomeda, Foster City, CA).

Confocal imaging was performed on an LSM 510 Meta confocal microscope with a Plan-Apochromat 63×/1.10 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 μm.

**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-
Though the GABAB receptor is a neurotransmitter receptor, we first verified the subcellular distribution of GFP-tagged GB1-GFP with markers for ER (Calreticulin) and cis-Golgi (GM130). Scale bars, 10 μm.

**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 μm.

**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 14-3-3ζ 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-14-3-3ζ-transfected cells. Retained proteins were analyzed by anti-βCOPI and anti-FLAG immunoblot.
Interaction of the GB1 RSR with 14-3-3

The findings that not only COPI but also 14-3-3/H9256 and H9280 specifically bind to the Kir6.2 RKR sequence (Yuan et al., 2003), and that 14-3-3/H9256 and H9257 interact with GB1 through a region encompassing its RSR (Couve et al., 2001) suggested that the GB1 RSR may also interact with 14-3-3/H9256 and possibly other 14-3-3 isoforms. We therefore analyzed the eluates of our pulldown (see above) by immunoblot with antibodies recognizing 14-3-3/H9256 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 14-3-3/H9256, the latter was specifically retained by the RSR, but not the ASA peptide (Figure 3B). Thus indeed 14-3-3/H9256 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 (RpSR; Figure 3B), even in presence of the phosphatase inhibitor NaVO3 (1 mM; unpublished data). 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 (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 (RpSR; Figure 3B), even in presence of the phosphatase inhibitor NaVO3 (1 mM; unpublished data).

14-3-3 Binding Is Not Required for Surface Targeting of the GABAB 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 GB1KKXX and a GB1RAR 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 GABAB receptor surface expression control system.

To test a possible role of the GB1 interaction with 14-3-3 in the kinetics of the GABAB 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 GABAB Receptor Coupling to G proteins

In search for the physiological role of the GABAB receptor interaction with 14-3-3 we next examined receptor-mediated activation of G 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 GABAB 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
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 GB1KKXX. 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 GB2KKXX (with KKTN adjacent to its CC domain, equivalent to our GB1KKXX construct) was retained inside the cell and brought to the cell surface by GB1, as well as by GB1ASA or GB1CC, which do not bind 14-3-3 (Figure 8). Conversely, GB2KKXX 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).

RXX 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 immuno-reactive 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.
However, the interaction may be influenced by the local sequence context (Zerangue et al., 2001). Further, it remains to be elucidated whether RXXR and KKXX motifs interact with the same or different sites of the COPI complex. Interestingly, the even more closely related KKXX and KXXX 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 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 α1D-adrenergic receptor, which is retained inside the cell unless associated with the α1D-adrenergic receptor (Hague et al., 2004).

**14-3-3 Is Not Involved in Surface Targeting of the GABA<sub>B</sub> Receptor**

We demonstrate here that the GB1 RSR cannot only interact with COPI, but also with 14-3-3<sub>α</sub>, 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 RXXR 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<sub>B</sub> 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 RXXR.

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<sub>B</sub> 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<sub>B</sub> 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 sterical 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 KXXX on GB1 (Figure 4) and even the KXXK on GB2 (Figure 8). Simple sterical 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 sterical hindrance of COPI binding.

We have previously demonstrated that fusion with the GB1 and GB2 C-terminal tails can transfer the GABA<sub>B</sub> receptor quality control system to other GPCRs, permitting to partly control the subunit composition of surface-expressed receptor dimers (Kniazeff 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-KXXX, 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, while 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, while COPI and 14-3-3 interact with the GABA_B receptor’s RXR motifs. Mol. Biol. Cell 24, 499–509.


