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Gatekeepers controlling GPCR export and function

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Abstract

Regulated export of G protein-coupled receptors (GPCRs) from intracellular stores involves chaperones and escort proteins, which promote their progression to the cell surface, and gatekeepers, which retain them in intracellular compartments. Functional GABA_B receptors, the paradigm of this phenomenon, are composed of GB1 and GB2 subunits forming a hetero-dimer. GB1 is retained in the endoplasmic reticulum (ER) in the absence of GB2. A specific ER-resident gatekeeper, PRAF2, is involved in GB1 retention and prevents its progression into the biosynthetic pathway. GB1 can be released from PRAF2 only upon competitive interaction with GB2. PRAF2 is ubiquitous and belongs to a subgroup of the mammalian Ypt-interacting protein (Yip) family. Several other GPCRs are likely regulated by Yip proteins, which might be involved in the pathophysiology of human diseases that are associated with impaired receptor targeting to the cell surface.

Molecular mechanisms regulating receptor cell surface density

The number of receptors present at the cell surface that can be reached and activated by cognate ligands is evidently essential in terms of downstream signaling outputs. Most studies addressing regulation of G protein coupled receptors (GPCRs) have focused on desensitization, the termination of activated receptor signaling, on GPCR endocytosis and recycling, and on the transcriptional control of GPCR-coding genes. Indeed, for a long time it has been assumed that, apart from translation, the only level of regulation of native receptor proteins engaged in the secretory pathway was to successfully pass through quality-control check points, which prevent unfolded proteins to reach the cell surface, redirecting them to the degradation pathway. The regulated export of nascent GPCRs from intracellular stores is a concept that has emerged recently. It is based on the observation that in primary cells several GPCRs are only marginally expressed at the cell surface, whereas abundant stores exist both in the endoplasmic reticulum (ER) and in the Golgi apparatus. One of the pioneering observations of this phenomenon was that in both primary and transfected cells, an important proportion of the PAR1 thrombin receptor was intracellular and co-localized with Golgi markers. Whereas surface PAR1 was internalized and subsequently targeted to lysosomes upon thrombin activation, the intracellular pool was translocated to the plasma membrane, leading to the recovery of thrombin responsiveness [1]. Subsequently, a series of independent observations coincided to suggest that the regulated export of GPCRs from the Golgi apparatus and the ER was actually a much wider phenomenon than previously anticipated (see [2, 3] for an historical overview). It also appeared that receptors are released from intracellular compartments upon appropriate extracellular signals and/or association with a variety of chaperones or escort proteins (reviewed in [2, 3]). However, some

key issues of this novel paradigm of regulation, such as the identification of the molecular tethers that retain GPCRs in the intracellular compartments or the characterization of the mechanisms of their release from these compartments remained poorly understood.

The GABA_B receptor paradigm

The metabotropic GABA_B receptor has been one of the most intensively investigated GPCRs over the past three decades, because of the very unusual regulation of its cell surface export. It belongs to the class-C family of GPCRs, also comprising the metabotropic glutamate receptor, the calcium sensing receptor and the taste T1R, which is characterized by a large extracellular Venus flytrap (VFT) domain involved in ligand recognition. Together with the ionotropic GABA_A receptor, GABA_B responds to the γ -aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system (CNS). Presynaptic GABA_B receptor reduces neurotransmitter release, whereas postsynaptic receptor causes hyperpolarization of neurons [4]. By inhibiting synaptic transmission, GABA_B plays a key role in modulating neuronal activity and impaired function of GABA_B has been associated with multiple neurological and psychiatric diseases [5-7]. Peripheral GABA_B receptors have also been identified, particularly in the gastrointestinal tract [8]. Baclofen, a GABA_B agonist, is currently used to treat spasticity of various pathophysiological origins and alcoholism, whereas peripherally-restricted GABA_B agonists devoid of CNS side effects are under investigation to treat gastroesophageal reflux disease [9]. In addition, recent studies indicate that peripheral GABA_B receptors might represent a pharmacological target for tumor therapy [10] and for improving pancreatic β -cell survival [11].

In addition to its functional roles, the GABA_B receptor occupies a special position among GPCRs, since several paradigms established for the GPCR family are based on pioneering studies based on GABA_B as receptor model. Unlike the other class-C GPCRs, which form homo-dimers, functional GABA_B receptors are obligate hetero-dimers made of one GB1 and one GB2 subunit [12-14]. Mice lacking GB1 do not exhibit any detectable electrophysiological, biochemical, or behavioral response to agonists [15, 16]. Similarly, GB2^{-/-} mice display spontaneous seizures, hyperalgesia, hyperlocomotor activity, and severe memory impairment, analogous to GB1^{-/-} mice, although atypical electrophysiological GABA_B responses are present in a few areas of the brain [17]. The loss of normal GABA_B function *in vivo* and *in vitro* when either GB1 or GB2 is missing is explained by the particular distribution of key functional tasks between receptor subunits. Indeed, GB1 is responsible for ligand recognition through its VFT domain, whereas GB2 does not bind to any known GABA_B ligand [18]; its VFT domain only behaves like an allosteric modulator of the GB1 VFT, enhancing its agonist affinity [19]. The transmembrane region of GB2, instead, is responsible for G-protein coupling [20, 21] and also facilitates cell surface expression of GB1 [22].

A missing mechanistic aspect in the regulated delivery of GABA_B to the cell surface

The cell surface density of functional hetero-dimeric GABA_B receptors is controlled by a unique mechanism of delivery from the biosynthetic compartments to the plasma membrane. Early studies reported that recombinant GB1 subunits fail to reach the cell surface when expressed in heterologous systems or overexpressed in ganglion neurons [23]. The explanation of this phenomenon is that GB1 is retained in the ER and does not reach the cell surface in the absence of GB2 [12-14].

Subsequent investigations identified an arginine based ER retention/retrieval “RSRR” signal in the carboxyterminal tail of GB1 [22]. This signal is a variant of a more general RXR motif (two arginine residues separated by any aminoacid) found in a variety of ER-retained subunits of multimeric protein complexes, such as the octameric ATP-sensitive K⁺ channels [24] or the N-methyl-D-aspartate (NMDA) receptors [25]. The alanine substitution of the arginine residues of the RXR motif allowed the corresponding GB1-AXA subunit to reach the cell surface even in the absence of GB2. It was then proposed that only the shielding of this retention signal via a coiled-coil interaction with the C-terminus of GB2 might allow the GB1-GB2 hetero-dimer to progress along the secretory pathway delivering functional GABA_B receptors to the cell surface [22]. A second key di-leucine motif was subsequently identified upstream of the RXR motif in the carboxyterminal tail of GB1, which also controls the cell surface export of the receptor in hippocampal neurons [26]. It was observed that in the absence of co-expressed GB2 the GB1-AA-ASA mutant, encompassing the mutation of both the RXR and the di-leucine motifs, was much better exported to the cell surface than the wild type GB1 or the single GB1-ASA mutant. Because the GB1-ASA mutant was partially accumulated within the trans-Golgi network (TGN) and because this accumulation was rescued by the double mutation, a two-step sequential mechanism regulating GABA_B export at distinct checkpoints in the ER and the Golgi was postulated [26]. However, a missing mechanistic aspect of the complex regulation of the GABA_B hetero-dimer delivery to the cell surface has remained for long time: the nature of the tether(s) that in the absence of association with GB2 retain(s) GB1 in the ER and in the TGN. This tether, which is responsible for the predominant ER localization of the GB1 subunit in primary neurons [27], might play an essential role in GABA_B receptor physiology.

PRAF2, the missing ER gatekeeper controlling GABA_B delivery to the cell surface

The coiled-coil domain of the carboxyterminal tail of the GB1, containing both RXR and LL motifs, is presumably involved in the interaction with a putative ER-resident “gatekeeper”, which regulates the delivery of the GABA_B heterodimer to the cell surface.

During the past ten years, multiple GB1 interacting proteins have been reported, which regulate GABA_B expression in some way, often interacting with the GB1 coiled-coil domain. However, under physiological conditions, none of these proteins appears to retain GB1 in the ER via a direct interaction. The cytoplasmic scaffolding protein 14-3-3 $\square$ can dissociate GABA_B receptor hetero-dimers present at the plasma membrane [28] [29] and does not interact with GB1 in the ER [29, 30]. The cytoplasmic coatomer COP1 might rather be involved in the retrieval of monomeric GB1 from the cis-Golgi to the ER [30]. Msec-7, an ARF1, 3,6 guanine-nucleotide exchange factor, which specifically interacts with the GB1 tail di-leucine motif, enhances GABA_B trafficking from the Golgi to the cell surface, after its exiting from the ER. GISP, another scaffolding protein of the AKAP family, was reported to enhance GABA_B receptor function possibly by slowing down receptor desensitization and lysosomal sorting [31]. The transcription factor CCAAT/enhancer-binding protein homologous protein CHOP is markedly overexpressed upon ER stress, for example during cerebral ischemia. In this context, CHOP interacts with both GB1 and GB2 in the ER preventing or disrupting hetero-dimerization, and forward trafficking of the receptors to the plasma membrane [32]. Because of its marginal level of expression under physiological conditions and the fact that it is not an ER resident protein, it is unlikely that CHOP is responsible for GB1 retention in this compartment. In conclusion, none of the GB1-interacting partners identified so far displayed the

expected biochemical and/or functional properties for a GB1 gatekeeper in the ER.

Ultimately, Prenylated Rab Acceptor Family 2 (PRAF2), an ER-resident 4-transmembrane domain protein, was identified recently as a major GABA_B gatekeeper [27]. PRAF2 interacts with the GB1 subunit of GABA_B but not with the GB2 subunit, this interaction involving both RXR and LL motifs of the carboxy-terminal tail of GB1. PRAF2-bound GB1 remains trapped in the ER and its release only occurs upon competitive displacement of PRAF2 by GB2. Thus, the local concentration of PRAF2, relative to that of GB1 and GB2, tightly controls cell surface receptor density and thus impacts on GABA_B function in neurons or in transfected cells (Figure 1). Interestingly, minor (less than two-fold) modifications of PRAF2 concentration in a localized region of mouse brain, the ventral tegmental area (VTA), caused major behavioral effects in animal models (Figure 2). These effects could be attributed to the resulting local changes in cell surface GABA_B receptor expression [27].

Although identified ten years ago as an interacting partner of the CCR5 chemokine receptor in a two-hybrid screen [33], PRAF2 had no clearly identified functions so far; it was found enriched in human brain [34], overexpressed in multiple cancers [35, 36] being correlated with poor prognosis [37]. PRAF2 belongs to a family of three ubiquitous 4-transmembrane domain proteins [35], localized either in the ER (PRAF2 and 3) or in the Golgi apparatus (PRAF1) (Table 1). Similarly to the function of PRAF2 on GABA_B export, PRAF3 was reported to delay the ER exit of the Na⁺-dependent glutamate transporter Excitatory Amino-Acid Carrier 1 (EAAC1) [38, 39]. PRAF1, the first identified member of the PRAF family, was proposed to activate the dissociation of prenylated Rab proteins from the GDP dissociation inhibitor GDI, thus facilitating the association of prenylated Rabs with target Golgi membranes [40].

The property of PRAF1 (also known as PRA1) to interact with numerous prenylated Rab GTPases [41] led to classify PRAF family members among mammalian Yips (Ypt-interacting proteins, Ypts being the yeast Rab homologues) [42]. Mammalian Yips (Table 1) also comprise 5-transmembrane proteins of the YIPF (YIP1 domain family, 1 to 7) and YIF1 (Yip1 interacting factor homolog A and B) groups, as well as the 2-transmembrane protein family REEP (receptor expression enhancing proteins, 1 to 6). Collectively, Yips are involved in the control of cellular vesicle trafficking between the ER and the Golgi network, REEPs displaying the additional function of adapter proteins for cargo receptors [43].

PRAF gatekeepers might regulate other GPCRs in various tissues

Although the mechanistic aspects of PRAF2-dependent regulation have only been elucidated for the GABA_B receptor, it is quite plausible that PRAFs might collectively regulate several other GPCRs. Indeed, as indicated above, PRAF2 was originally identified in two-hybrid screens as an interaction partner of the chemokine CCR5 receptor, regulating in some way its membrane localization [33]. PRAF3, on the other hand, was reported to restrict the subcellular localization of the β 2-adrenoceptor and the D2-dopaminergic receptor to the ER [39]. In preliminary studies, we found that PRAF gatekeepers associate (in BRET and co-IP experiments) with other chemokine receptors, such as CCR2 and CCR7, and with 5-HT₂ serotonergic receptors (unpublished data). Finally, both PRAF2 [35] and PRAF3 [38, 44] are expressed in most tissues, where they coexist with multiple GPCRs.

In addition to the GABA_B receptor, several other GPCRs undergo regulated translocation to the plasma membrane from ER or Golgi stores [2], where they might

possibly be retained by PRAFs or PRAF-like gatekeepers. In tubular renal cells, intracellular dopamine D1 receptors are recruited to the plasma membrane by agonist activation of cell surface receptors [45] or via atrial natriuretic peptide-dependent heterologous activation [46]. Similarly, the sustained stimulation of μ -opioid receptors can redistribute δ -opioid receptors (DOP) to the plasma membrane *in vivo*, improving DOP-dependent anti-nociceptive effects [47]. Several other GPCRs poorly expressed at the plasma membrane, such as the odorant receptors [48], the human GnRH receptor [49], the α_{1D} -adrenoceptor [50], the LH receptor [51], and the CCR5 chemokine receptor [52] represent other candidates for gatekeeper-controlled translocation to the cell surface.

Although the functional organization of the GABA_B receptor as an obligatory hetero-dimer is quite unique among GPCRs, it is currently admitted that most if not all the receptors of this family are expressed at the cell surface as homo-dimers [53]. Several observations indicate that, among other functions, homo-dimerization might also contribute to quality control of newly synthesized receptors [54]. An interesting hypothesis in this context is that the homo-dimerization of two correctly folded receptors might displace the interaction with resident gatekeepers similarly to hetero-dimerization. The GABA_B model might thus represent an atypical example of a more general phenomenon.

Potential involvement of PRAFs in human disease

Similar or even larger changes of PRAF2 than those we observed inducing phenotypic effects in animals can either be detected physiologically, during neuronal differentiation for example, or in pathological samples, such as human cancer tissues [36] and brain areas of persons deceased after excessive alcohol intake [55]. These

findings indicate a potential causative connection between the concentration of PRAF gatekeepers and pathological changes in humans. In addition, although not functionally investigated so far, several variants of PRAF2 have been reported (see the link www.nextprot.org/db/entry/NX_O60831/sequence), which are located in various domains of the protein, including within the regions that are likely at the interface with GB1 [27]. Therefore, both qualitative and quantitative changes of these gatekeepers might have an impact on GPCR receptivity and consequently be involved in human disease.

Finally, the new concept of GPCR gatekeepers might lead to revisit already known pathophysiological mechanisms. For example, in the kidney, where both PRAF2 and PRAF3 are present, variants of the V2 vasopressin receptor (V2R) are poorly expressed at the cell surface of tubular cells, impairing water reabsorption and causing nephrogenic diabetes insipidus [56]. Membrane-permeable pharmacological chaperones have been reported, which bind to intracellular receptors and facilitate their cell surface export, with a partial rescue of receptor function and of excessive diuresis [57]. It would be interesting in this context to examine the association of wild-type and variant V2Rs with PRAFs, as an increased affinity of the variant receptor for its cognate gatekeeper, might well explain its retention in the ER.

Concluding remarks

The identification of the resident gatekeeper, which retains the GB1 subunit of the GABA_B receptor in the ER upon interaction with both the di-leucine and RXR motifs of the carboxyterminal tail of GB1 until it is displaced by the GB2 subunit, completes the picture of the complex mechanism regulating the delivery of functional GABA_B receptors to the cell surface. PRAFs were already reported to interact with and/or

regulate several other GPCRs and plasma membrane transporters. In addition, among mammalian Yips, REEP family proteins, which were initially considered as GPCR escorts, might also behave like gatekeepers regulating the ER exit of some receptors [43]. Therefore, for many GPCRs and more generally for multi-span plasma membrane proteins, the gatekeeper-regulated release from intracellular stores might become a new parameter to be considered when analyzing the effectiveness of receptor response in physiological conditions, during agonist treatment or in the case of pathological export defects. These considerations raise a number of new questions* in the field, such as how gatekeepers are themselves regulated, how many other GPCRs are potentially regulated by these types of gatekeepers, in which organs and for which functions, and are changes in gatekeeper concentration or point mutations at the interface with receptors involved in human disease. We anticipate that these questions are sufficiently important to promote additional investigations in the years to come.

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Figure Legends

Figure 1. Retention and release of GB1 is governed by the relative concentrations of GB1, GB2 and PRAF2. (A) In neurons GB2 and PRAF2 compete for binding to GB1. Hetero-dimerization of GB1 with GB2 allows the release from the ER and the export of the functional GABA_B receptor to the cell surface. Association with PRAF2 maintains the GB1 subunit in the ER. (B) The heterologous expression of GB1 in transfected cells, which contains endogenous PRAF2 and no GB2, reproduces the conditions existing in neurons of GB2-KO mice. GB1 is retained in the ER and no GABA binding sites can be measured at the cell surface. (C) If PRAF2 concentration is decreased (i.e. siRNA treatment) in transfected cells expressing exogenous GB1, or GB1 markedly overexpressed, the available PRAF2 molecules are completely saturated by GB1. Unbound GB1 is not retained and can reach the cell surface. Surface GABA binding sites can be measured in these conditions corresponding to surface GB1. However, because of the absence of GB2 subunits, functional GABA_B receptors capable of transducing a signal are lacking.

Figure 2. Locomotor activity in mice expressing exogenous PRAF2 in the VTA.

To examine whether perturbing the stoichiometry of GB1, GB2 and PRAF2 might affect GABA_B-dependent regulations *in vivo*, PRAF2 concentration was enhanced (2-3 folds) in the ventral tegmental area (VTA), a brain area containing GABA_B-expressing neuron, by stereotaxic injection of an adenovirus driving the local expression of the exogenous gatekeeper. Activation of the dopaminergic circuits from the VTA to the nucleus accumbens (NAcc) produces locomotor activity, which is inhibited by GABA_B activation (blue-boxed panels). In case of loss of surface GABA_B

477 receptors in VTA dopaminergic neurons, caused by PRAF2-mediated GB1 retention,
478 dopamine release is increased and locomotor activity enhanced (red-boxed panels).
479 The unilateral change of PRAF2 concentration in the VTA led to a remarkable
480 contralateral turning phenotype (see supplemental videos in [26]).
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Name	Other Names	Yeast homologue	Gene ID	AA number	TM	Localization / Functions	Regulated Receptors/Transporters
YIPF5 (Yip1 domain family, member 5)	Yip1; YPT-interacting protein 1A; Yip1A; FinGER5; SMAP5; SB140	Yip1p	81555	257	5	ER exit sites, ER to Golgi Transport [58, 59]	
REEP1 (receptor expression- enhancing protein1)	Yip2a	Yip2, Yop1	65055	201	2	ER morphology and dynamics[60]	Odorant receptors [48]; bitter taste receptor [61]; $\alpha 2_c$ AR [43]
REEP2	Yip2d		51308	252	2	ER resident [43]	Odorant receptors [48]; sweet receptor [62]; $\alpha 2_c$ AR [43]
REEP3	Yip2b		221035	255	2	clearing ER from metaphase chromatin [63]	Odorant receptors [48]
REEP4	Yip2c		80346	257	2	clearing ER from metaphase chromatin [63]	
REEP5	Yip2e		7905	189	2		
REEP6	Yip2f		92840	184	2	ER resident [43]	$\alpha 2_c$ AR [43]
RABAC1,	Yip3; PRA1, PRAF-1	Yip3	10567	185	4	Golgi [44]; Prenylated Rab acceptor [40]	
PRAF-2	Yip6a; JM4		11230	178	4	ER [35]	CCR5 [33], GABA _B [27]
ARL6IP5	JWA; PRA2; jmx; hp22; PRAF-3 ; DERP11; HSPC127; addicisin; GTRAP3- 18; Yip6b		10550	188	4	ER [44]; control of ER exit [39]	EAAC1 [38]; EAAT1,2,4; β_2 AR; $\alpha 2_b$ AR; D ₂ R [39]; DOR [64]
YIPF6	FinGER6; MGC21416; Yip4	Yip4	286451	237	5	Golgi [65]	
YIPF1	FinGER1; DJ167A19.1, Yip5a	Yip5	54432	306	5	Golgi [65]	
YIPF3	KLIP1; FinGER3; C6orf109; dJ337H4.3; Yip5b		25844	350	5	Cis-Golgi [66] [65]	
YIPF2	FinGER2; Yip5c		78992	316	5	Golgi [65]	
YIF1A (Yip1 interacting factor homolog A)	54TM; YIF1; YIF1P; FinGER7	Yif1	10897	293	5	ER to Golgi transport; delivery into dendrites [67]	
YIF1B	FinGER8		90522	314	5	Golgi [65]; ER- Golgi trafficking [68]	5-HT1AR [68]
YIPF7	FinGER9, Yip1B		285525	280	5	ERGIC; cisGolgi; skeletal muscle [69]	
YIPF4	FinGER4; Nbla11189		84272	244	5	Cis-Golgi [66]	

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483 **Table 1. Classification and nomenclature of PRAFs and mammalian Yips**

*Outstanding Questions Box

- How many GPCRs undergo a similar regulation of their cell surface export by PRAFs, other Yips or functionally equivalent additional ER and Golgi gatekeepers?
- What is the molecular basis of gatekeeper selectivity for cargo receptors?
- How are ER and Golgi gatekeepers coordinated to regulate the export of a specific receptor?
- Is GPCR oligomerization a more general mechanism controlling receptor export to the cell surface?
- How are PRAFs and more generally Yips themselves regulated? How this type of regulation might be connected with receptor function and regulation?
- Are there pathological conditions associated with mutations or quantitative changes of receptor gatekeepers?
- Can diseases associated with impaired cell surface targeting of GPCR variants be explained by enhanced interaction between receptors and ER or Golgi gatekeepers?
- Is it possible to target receptor – gatekeeper interfaces with chemical compounds to enhance receptor export and treat diseases?

Highlights

Functional GABA_B receptors are obligate hetero-dimers of the GB1 and GB2 subunits

The agonist binding GB1 is retained inside the cells in the absence of association with GB2

GB2 competitively releases GB1 from PRAF2, a specific, endoplasmic reticulum-resident gatekeeper

The stoichiometry of PRAF2 relatively to GB1 and GB2 is a key parameter for GABA_B function *in vivo*

Figure 1

Figure 1

Figure 2

Figure 2