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The RXR-Type ER-Retention/Retrieval Signal of $GABA_{B1}$ Requires Distant Spacing from the Membrane to Function

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Running title: ER retention of $GABA_{B1}$

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ABBREVIATIONS: GABA, γ-aminobutyric acid; ER, endoplasmic reticulum

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Abstract

Functional γ-aminobutyric acid type B ($GABA_B$) receptors are normally only observed upon co-expression of $GABA_{B1}$ with $GABA_{B2}$ subunits. A C-terminal arginine-based endoplasmic reticulum (ER) retention/retrieval signal, RSRR, prevents escape of unassembled $GABA_{B1}$ subunits from the ER and restricts surface expression to correctly assembled heteromeric receptors. The RSRR signal in $GABA_{B1}$ is proposed to be shielded by C-terminal coiled-coil interaction of the $GABA_{B1}$ with the $GABA_{B2}$ subunit. Here, we investigated whether the RSRR motif in $GABA_{B1}$ remains functional when grafted to ectopic sites. We found that the RSRR signal in $GABA_{B1}$ is inactive in any of the three intracellular loops but remains functional when moved within the distal zone of the C-terminal tail. C-terminal deletions that position the RSRR signal closer to the plasma membrane drastically reduce its effectiveness, supporting that proximity to the membrane restricts access to the RSRR motif. Functional ectopic RSRR signals in $GABA_{B1}$ are efficiently inactivated by the $GABA_{B2}$ subunit in the absence of coiled-coil dimerization, supporting that coiled-coil interaction is not critical for release of the receptor complex from the ER. The data are consistent with a model in which removal of RSRR from its active zone rather than its direct shielding by coiled-coil dimerization triggers forward trafficking. Since arginine-based intracellular retention signals of the type RXR, where X represents any amino acid, are used to regulate assembly and surface transport of several multimeric complexes, such a mechanism may apply to other proteins as well.

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$GABA_B$ receptors are the G-protein coupled receptors for GABA, the predominant inhibitory neurotransmitter in the mammalian central nervous system. $GABA_B$ receptors modulate synaptic transmission by controlling neurotransmitter release and by causing postsynaptic hyperpolarization (Bettler et al., 2004; Bowery et al., 2002; Calver et al., 2002). They are broadly expressed in the nervous system and have been implicated in a variety of neurological and psychiatric conditions. In heterologous cells, functional $GABA_B$ receptors are usually only observed upon co-expression of $GABA_{B1}$ with $GABA_{B2}$ subunits, which provided compelling evidence for heteromerization among G-protein coupled receptors (Jones et al., 1998; Kaupmann et al., 1997; Kaupmann et al., 1998; Kuner et al., 1999; Marshall et al., 1999; Ng et al., 1999; White et al., 1998). Two $GABA_{B1}$ subunit isoforms, $GABA_{B1a}$ and $GABA_{B1b}$, arise from the *$GABA_{B1}$* gene by differential promoter usage (Bettler et al., 2004; Kaupmann et al., 1997). The data therefore support the existence of two predominant $GABA_B$ receptors in the nervous system, the heteromeric $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors. However, knock-out studies also suggest that $GABA_{B1a}$ and $GABA_{B1b}$ could be functional in neurons that naturally lack $GABA_{B2}$ expression (Gassmann et al., 2004).

In the $GABA_B$ heteromer, the $GABA_{B1}$ subunit binds GABA and all competitive $GABA_B$ ligands (Kaupmann et al., 1998), while the $GABA_{B2}$ subunit is predominantly responsible for activating the G-protein (Galvez et al., 2001; Grunewald et al., 2002; Havlickova et al., 2002; Margeta-Mitrovic et al., 2001; Robbins et al., 2001). Trafficking of unassembled $GABA_{B1}$ subunits to the plasma membrane is prevented by an arginine-based ER-retention/retrieval signal, the four amino-acids RSRR, in the cytoplasmic tail of $GABA_{B1}$ (Couve et al., 1998; Margeta-Mitrovic et al., 2000; Pagano et al., 2001). This ER-retention/retrieval signal is proposed to be shielded by C-terminal coiled-coil interaction of the $GABA_{B1}$ with the $GABA_{B2}$ subunit. Within the RSRR motif the serine residue and the third arginine are not absolutely critical for function,

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as they can be substituted by other amino acids (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). More recently, it was shown that the sequence context of the RSRR signal in $GABA_{B1}$ influences its function (Grunewald et al., 2002). Thus, the full ER-retention/retrieval motif in $GABA_{B1}$ was extended to the sequence QLQSRQQLRSRR, which includes part of the coiled-coil domain. Arginine-based ER-retention/retrieval signals were observed in a number of other multi-subunit proteins, e.g. the K_{ATP} channels (Zerangue et al., 1999) and NMDA receptors (Scott et al., 2001), where they control stoichiometry and surface expression of the channel complex. From the available data it emerges that the core ER-retention/retrieval motif is RXR, consisting of two arginines that are separated by any amino acid (X).

Di-lysine ER-retention/retrieval signals require a strict spacing relative to the C-terminus. In contrast to KK-signals, functional RXR signals are found in a variety of cytosolic positions, including intracellular loops and the N- and C-termini in Type II and Type I membrane proteins, respectively (Schutze et al., 1994; Zerangue et al., 1999). This broad distribution initially suggested that many proteins that harbor the consensus sequence RXR are retained in the ER. This was recently challenged in a study that showed that the RXR-dependent ER-retention/retrieval machinery is sensitive to the length of the spacer that separates the RXR motif and the receptor-anchored membrane (Shikano and Li, 2003). Here, we studied whether the RSRR signal in $GABA_{B1}$ can still function when grafted to ectopic cytoplasmic positions and whether it can be masked by $GABA_{B2}$ regardless of its position. The data let us propose a new mechanism to explain RSRR inactivation upon $GABA_B$ subunit dimerization.

Materials and Methods

Generation of mutant expression plasmids. All constructs were subcloned into the cytomegalovirus-based eukaryotic expression vector pCI (Promega, Madison, WI). Overlap

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extension PCR (Horton et al., 1990) was used to introduce ectopic RSRR and LRSRR motifs into a $GABA_{B1a}$ mutant (R1[ASAA]) where the endogenous RSRR was inactivated by substitution of arginine with alanine residues (Pagano et al., 2001). Overlap extension PCR was also used to construct $GABA_{B1a}$ deletion mutants, leaving the wild-type RSRR unchanged.

Cell surface labeling. HEK293 cells for transient transfection of expression constructs were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's MEM (Life Technologies, Basel, Switzerland) supplemented with 10% fetal calf serum and 2 mM L-glutamine. The photoaffinity ligand [^{125}I]CGP71872 specifically binds to the GABA-binding site of $GABA_{B1}$ subunits and does not permeate the plasma membrane (Pagano et al., 2001). [^{125}I]CGP71872 labeling of intact cells therefore reveals $GABA_{B1}$ protein at the cell surface, while labeling of lysed cells will reveal total $GABA_{B1}$ protein, independent of where in the biosynthetic pathway it is present. 6 hrs after transfection of expression plasmids using Lipofecatmine™ 2000 Transfection Reagent (Invitrogen AG, Basel, Switzerland), HEK293 cells were transferred to 6-well plates. After an additional 24 hr incubation cells were washed twice with ice-cold HEPES, pH 7.6. Half of the cells were then used for photoaffinity labeling of surface receptors (S in Fig. 2, 6 and 7), the other half for labeling of total receptors in the cell homogenates (H in Fig. 2, 6 and 7). For surface labeling, intact cells were incubated in the dark for 1hr at room temperature with 0.8 nM [^{125}I]CGP71872. Subsequently, cells were washed twice with ice-cold Krebs-Tris buffer (118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose, 20 mM Tris-Cl, pH 7.4) to remove unbound ligand. Bound [^{125}I]CGP71872 was crosslinked to the receptor using UV light (Kaupmann et al., 1997). Photoaffinity labeled cells were then harvested and the radioactivity was determined in a gamma counter (Packard, Zurich, Switzerland). For [^{125}I]CGP71872 labeling of total $GABA_{B1}$ protein,

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cells were harvested and lysed prior to incubation with the photoaffinity ligand. Preparation of lysates and [^{125}I]CGP71872 binding was as described (Kaupmann et al., 1997). For 10% SDS-PAGE cell pellets and homogenates were resuspended in Krebs-Tris buffer containing 0.1% SDS. An aliquot was used for determination of protein concentration (Micro Protein Assay, BioRad, Munich, Germany). Equal amounts of total protein were used when comparing surface receptors (S) and total receptors in cell homogenates (H). We normalized the input of radiolabeled protein in the SDS-PAGE by using equal counts of the homogenate samples (H) for each set of transfections (expression with and without $GABA_{B2}$). Photoaffinity-labeled protein was detected using autoradiography. The S/H ratio of the radioactivity incorporated into the cell surface and the homogenate fraction was determined from the autoradiograms. Because of the differences in the radiolabeling procedure for surface and homogenate receptors, the percentage S/H sometimes exceeds the theoretical value of 100%. Loading was controlled for by Western blot analysis with the polyclonal $GABA_{B1}$ antibody Ab174.1 that is directed against the C-terminal tail of $GABA_{B1}$ (Malitschek et al., 1998). Surface labeling with [^{125}I]CGP71872 was compared with surface biotinylation (Fig. 3). For the biotinylation experiments we used membrane impermeable EZ-link Sulo-NHS-SS-biotin (Pierce, Rockford IL). 48 hrs after transfection HEK293 cells were washed three times in PBS and then incubated with 1 mg/ml Sulfo-NHS-SS-biotin for 30 min at 4°C on a rocking table. To quench the biotinylation reaction the cells were then washed in PBS and incubated in 50 mM glycine in PBS for 5 min. After three washes in PBS the cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl pH 7.5). The lysates were cleared by centrifugation at 10'000 g for 10 min. Aliquots were taken and mixed with 2xSDS loading buffer to detect total $GABA_{B1}$ protein expressed. The remaining cleared lysates were incubated with avidin beads (Pierce) at 4°C overnight. Following 5 washes in RIPA buffer biotinylated proteins were eluted

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from the avidin beads using SDS loading-buffer. Finally, total and eluted $GABA_{B1}$ proteins were separated on SDS-PAGE and analyzed on Western blots.

Western blots. Following SDS-PAGE, proteins were blotted onto a nitrocellulose membrane (Immobilon-P, Millipore, Bedford, MA) by standard electrophoretic transfer. After blotting, the membrane was blocked with 5% non-fat milk powder in PBS for 1 hr at room temperature. Rabbit antiserum Ab174.1 (1:2500, (Malitschek et al., 1998)), the monoclonal anti-β–tubulin antibody MAB3408 (1:500, Chemicon, Temecula, CA), and peroxidase-coupled secondary antibodies (donkey anti-rabbit or anti-mouse conjugates, 1:2500, Amersham Biosciences, Little Chalfont, UK) were incubated in PBS containing 2.5% non-fat milk powder and 0.1% Tween-20 for 1 hr at room temperature. After antibody incubation three wash steps with PBS containing 0.1% Tween-20 were carried out for 10 min. The blots were developed using the ECL chemiluminescent detection system (Amersham Biosciences) and exposed to Kodak BioMax maximum resolution X-ray films (Sigma-Aldrich, St. Louis, MO).

Fluorimetric measurement of changes in the intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]i$). For measurement of $\Delta[Ca^{2+}]i$ all transfections included $G\alpha qz_{IC}$ to artificially couple $GABA_B$ receptors to PLC (Franek et al., 1999). Transfected HEK293 cells were plated into poly-D-Lysine coated 96-well plates (BD Biosciences, Erembodegem, Belgium). After transfection (48-72 hr), cells were loaded for 45 min with 2 µM fluo-4 AM (Molecular Probes, Leiden, NL) in Hank's balanced salt solution (Life Technologies, Basel, Switzerland) supplemented with 20 mM HEPES buffer and 50 µM probenecid (Sigma, Buchs, Switzerland). Plates were washed and transferred to a fluorimetric image plate reader (FLIPR) (Molecular Devices, Crawley, UK).

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Fluorescence changes ΔF upon addition of GABA (final concentration of 0.1 mM) were recorded as a function of time, as described (Pagano et al., 2001). No quantitative comparison between experiments was made, because the signal amplitude depends on the transfection efficiency.

Results

Generation and characterization of GABA$_{B1}$ mutants with ectopic RSRR signals

To study whether ectopic RSRR motifs are functional in GABA$_{B1}$, we introduced the RSRR motif into a GABA$_{B1}$ protein where the endogenous RSRR motif is inactivated by substitution of arginine with alanine residues. This protein, R1[ASAA], is efficiently transported to the cell surface in the absence of GABA$_{B2}$ (Pagano et al., 2001). Whenever possible, we inserted the RSRR motif at positions that already harbored an arginine or a serine residue, which is expected to minimize interference with the wild-type amino-acid sequence. A scheme depicting the insertion sites of ectopic RSRR motifs in R1[ASAA] is shown in Fig. 1A. The positions of the ectopic RSRR motifs in the primary sequence of GABA$_{B1a}$ are listed in Fig. 1B. We confirmed expression of mutant GABA$_{B1}$ proteins in transiently transfected HEK293 cells by Western blot analysis, using an antibody directed against a C-terminal epitope (Fig. 1C). In general, the expression levels of mutant GABA$_{B1}$ proteins are comparable to those of the wild-type GABA$_{B1a}$ (R1) and R1[ASAA] proteins (Fig. 1C, upper panel). The only exception is R1[R862SRR], which harbors the ectopic RSRR motif in the C-terminal tail and for unknown reasons is poorly expressed. Alternatively, it is also possible that some of the C-terminal epitopes in R1[R862SRR] are affected by the mutation and are no longer recognized by the antibody. Equal loading was controlled for by Western blot analysis with a β-tubulin antibody (Fig. 1C, lower panel).

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RSRR remains functional at the C-terminus but not in any of the intracellular loops

To examine the functionality of ectopic RSRR motifs, we expressed $GABA_{B1}$ mutants either in isolation or together with $GABA_{B2}$. We determined the ratio of surface and total $GABA_{B1}$ protein levels by photoaffinity labeling of intact and lysed cells, respectively, with the membrane impermeable antagonist [^{125}I]CGP71872. Following SDS-PAGE, labeled proteins were visualized by autoradiography. We consistently observed that wild-type and mutant $GABA_{B1}$ proteins bind significantly more [^{125}I]CGP71872 when co-expressed with $GABA_{B2}$, suggesting that $GABA_{B2}$ assists $GABA_{B1}$ in reaching a binding-competent conformation. To correct for this as well as variability in transfection efficiency, the amount of protein sample subjected to gel electrophoresis was normalized to the respective amount of radioactivity incorporated into the cell homogenates (H). Consequently, for the reason mentioned above, substantially less immunostained $GABA_{B1}$ protein is seen on all Western blots of samples where $GABA_{B2}$ was coexpressed (Fig. 2). For each transfection photoaffinity-labeled $GABA_{B1}$ protein at the cell surface (S) was compared to total $GABA_{B1}$ protein labeled in the cell homogenates (H). We investigated whether the binding-incompetent form of $GABA_{B1}$, which is more abundant in the absence of $GABA_{B2}$, is able to reach the cell surface or not. We used biotinylation of intact cells and precipitation with avidin-sepharose as an alternative method to [^{125}I]CGP71872 labeling to detect proteins expressed at the cell surface (Fig. 3). We failed to detect significant amounts of $GABA_{B1}$ protein expressed at the cell surface of HEK293 cells transfected with $GABA_{B1}$ alone (R1), indicating that the binding-incompetent form of $GABA_{B1}$ fails to reach the cell surface in the absence of $GABA_{B2}$. This is also supported by recent studies that show that ligand binding is a critical requirement for plasma membrane expression (Mah et al., 2005; Valluru et al., 2005). In all our experiments we therefore used photoaffinity labeling with [^{125}I]CGP71872 to quantify $GABA_{B1}$ protein at cell surface.

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As shown in Fig. 2, wild-type $GABA_{B1}$ (R1) is retained in the ER and therefore does not bind the photoaffinity ligand at the cell surface. However, upon coexpression with $GABA_{B2}$ (R1 + R2), or inactivation of the RSRR motif (R1[ASAA]), $GABA_{B1}$ is released to the cell surface, in agreement with previous reports (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). Insertion of RSRR motifs into any of the three intracellular loops (mutant proteins R1[RS616RR], R1[RS624RR], R1[RV690RSRR], R1[E699RSRR] and R1[E796RSRR]) failed to confer detectable intracellular retention in our assay. Similarly, mutants with an ectopic RSRR motif in the C-terminal tail at positions R862 (R1[R862SRR]), S877 (R1[RS877RR]) or S917 (R1[RS917RR]) were efficiently transported to the cell surface, no matter whether they were expressed alone or in combination with $GABA_{B2}$. In contrast, insertion of an ectopic RSRR motif in the C-terminal tail at positions S887 (R1[RS887RR]) and R939 (R1[R939SRR]) resulted in partial intracellular retention. In summary, transposing the RSRR ER-retention/retrieval motif of $GABA_{B1}$ to ectopic positions indicates that it can be functional in preventing transport to the cell surface in the cytoplasmic tail but not in any of the intracellular loops. Functional RSRR signals are efficiently masked at ectopic sites by heterodimerization with $GABA_{B2}$, as shown by the release of the R1[RS887RR] and R1[R939SRR] proteins to the cell surface in the presence of $GABA_{B2}$.

Ectopic RSRR motifs do not interfere with receptor function

The experiments described above show that all $GABA_{B1}$ subunits with ectopic RSRR motifs can reach the cell surface when co-expressed with $GABA_{B2}$. This suggests that the mutated $GABA_{B1}$ proteins fold correctly and assemble into heterodimers. When expressed in heterologous cells, $GABA_{B1}$ is not functional by itself, even when artificially targeted to the cell surface by inactivation of the RSRR signal or by shielding it with a C-terminal $GABA_{B2}$ peptide (Margeta-

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Mitrovic et al., 2000; Pagano et al., 2001). To confirm heteromeric assembly between mutated $GABA_{B1}$ and wild-type $GABA_{B2}$ subunits we examined whether co-expression of the subunits yielded functional receptors. Upon transient co-expression of the subunits with a chimeric Gα subunit, $G\alpha qz_{IC}$ (Franek et al., 1999), in HEK293 cells we measured GABA-induced increases in intracellular Ca^{2+} levels by fluorimetry. As illustrated in Fig. 4, all $GABA_{B1}$ mutants can be activated with 0.1 mM of GABA upon co-expression with $GABA_{B2}$, similarly to wild-type $GABA_{B1}$ (R1 + R2). Hence, insertion of ectopic RSRR motifs does not interfere with G-protein coupling of the mutant proteins.

Appropriate spacing to the plasma membrane is required for ER-retention/retrieval of $GABA_{B1}$

RXR-type motifs were proposed to have an operating range and to be sensitive with regard to their spacing from the plasma membrane (Shikano and Li, 2003). This could explain why in $GABA_{B1}$ ectopic RSRR motifs are only functional when located within the distal C-terminal tail (Fig. 2). Conflicting with this explanation, the ectopic RSRR motif at S917, in between the functional motifs at S887 and R939, is unable to confer intracellular retention (Fig. 2, construct R1[RS917RR]). Small changes in the local sequence context can alter the signal strength of arginine-based ER-retention motifs (Zerangue et al., 2001). For example, the functionality of RXR signals is described to improve when a hydrophobic amino acid, in particular leucine, precedes the arginine cluster. We therefore investigated whether insertion of a leucine preceding the RSRR in R1[RS917RR] rescues intracellular retention. We additionally tested whether including a leucine in the R1[RS887RR] and R1[R939SRR] proteins, which are less well retained than R1[ASAA], improves retention. Indeed, insertion of a leucine preceding the RSRR at

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position S917 renders the otherwise non-functional ectopic motif functional (Fig. 5, R1[LRS917RR] versus R1[RS917RR]). In contrast, insertion of leucine in R1[RS887RR] or R1[R939SRR] does not improve intracellular retention of these proteins. Intracellular retention of the R1[LRS917RR] protein further supports that the distal cytoplasmic tail has the potential to harbor functional RSRR signals.

We next tested whether the spacing to the plasma membrane affects the functionality of the ER-retention/retrieval motif in $GABA_{B1}$. To that aim, we constructed three deletion mutants that gradually move the endogenous RSRR motif closer to the plasma membrane (Fig. 6). Deletion of 9 amino acid residues has no effect on the functionality of the RSRR motif, while deletion of 30 or 52 amino acids increasingly boosts cell surface expression of $GABA_{B1}$. This gradual increase in surface expression clearly shows that the spacing to the plasma membrane is critical for RSRR function.

Masking of ectopic RSRR signals in $GABA_{B1}$ does not involve C-terminal coiled-coil domain interaction

Two reports indicate that surface trafficking is not entirely dependent on coiled-coil domain interaction between the $GABA_{B1}$ and $GABA_{B2}$ subunits (Grunewald et al., 2002; Pagano et al., 2001). For example, $GABA_{B2}$ mutants lacking the C-terminal coiled-coil domain (R2ΔLZ2) are able to traffic $GABA_{B1}$ to the cell surface. We therefore investigated whether coiled-coil interaction is necessary for masking the functional ectopic RSRR motifs in R1[RS887RR] and R1[R939SRR] proteins by co-transfecting them with R2ΔLZ2 (Pagano et al., 2001). As shown in Fig. 7 and in agreement with earlier reports, R2ΔLZ2 is able to traffic wild-type $GABA_{B1}$ (R1) to the cell surface, but to a smaller extent than wild-type $GABA_{B2}$ (R2). Both wild-type $GABA_{B2}$ and R2ΔLZ2 are able to traffic the R1[RS887RR] and R1[R939SRR] proteins with functional

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ectopic RSRR motifs to the cell surface. In addition R1[LRS917RR], which is efficiently retained in the absence of $GABA_{B2}$ (Fig. 5) is trans-located to the cell surface by co-expression with R2ΔLZ2 (S/H ratio = 69%; not shown). This indicates that coiled-coil domain interaction between the cytoplasmic tails of $GABA_{B1}$ and $GABA_{B2}$ is not crucial for masking the ectopic RSRR motifs in the mutant $GABA_{B1}$ subunits. Obviously, additional interaction sites between $GABA_{B1}$ and $GABA_{B2}$ mediate heterodimerization and compensate for the lack of coiled-coil domain interaction, thereby presumably preventing the ectopic RSRR motifs from binding to protein(s) that localize it in the ER.

Discussion

The generic membrane trafficking signals RXR and KK are part of quality control mechanisms that prevent incorrectly folded and/or assembled membrane proteins from reaching the cell surface. Signals of the RXR-type are generally used to control assembly of multimeric protein complexes. It is assumed that the RXR motif is masked upon association with an appropriate partner subunit and consequently only correctly assembled complexes are able to exit the ER. In contrast to the carboxy-terminal di-lysine signal KK, which exhibits a strict spacing relative to the C-terminus, RXR-type signals are found in a variety of sequence positions. In octameric K_{ATP} channels they are localized in the cytoplasmic tail of the pore forming α subunit (Kir6.1/2) as well as in a cytoplasmic loop of the regulatory β subunit SUR1 (Zerangue et al., 1999). In addition, the related ER-retention/retrieval motif RR was identified in the cytosolic N-terminus of the MHC class II invariant chain isoform lip33, a Type II membrane protein (Schutze et al., 1994). In the experiments presented herein, we transposed the RSRR ER-retention/retrieval signal of $GABA_{B1}$ from its normal position adjacent of the coiled-coil domain to ectopic

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positions within the cytoplasmic tail or within the three intracellular loops. We show that the RSRR motif is not functional in any of the intracellular loops, but that it is partially functional at two ectopic positions within the cytoplasmic tail (Fig. 2). A previous study suggested that the functionality of the RSRR motif of $GABA_{B1}$ depends on surrounding sequences (Grunewald et al., 2002). In particular, amino-acid residues that are part of the coiled-coil domain and neighbor the RSRR motif N-terminally were proposed to be important for recognition of the RSRR motif. From these earlier experiments it was concluded that the minimal ER retention sequence in $GABA_{B1}$ is comprised of the amino acids QLQXRQQLRSRR, where X can be either S or D (Grunewald et al., 2002). Our data demonstrate that there is not a strict requirement for the RSRR motif to be in its normal sequence context to be functional, since the motif mediates retention when moved N-terminally of QLQXRQQLRSRR to position S887 (R1[RS887RR]) or C-terminally to position R939 (R1[R939SRR]) (Fig. 2). However, the R1[RS917RR] protein, harboring a RSRR motif positioned in between the motifs in R1[RS887RR] and R1[R939SRR], is not retained. This suggests that the sequence environment and/or the secondary structure of the area where the ectpoic RSRR motif has been inserted are nevertheless of some influence. It was proposed that small changes in the local sequence context can alter the signal strength of arginine-based ER-retention motifs and that it is favorable when a hydrophobic amino acid, in particular leucine, precedes the arginine cluster (Zerangue et al., 2001). This sequence configuration is also observed for the ER-retention/retrieval signal in wild-type $GABA_{B1}$. R1[RS917RR] and the partly retained R1[RS887RR] and R1[R939SRR] proteins violate this rule. Insertion of a leucine preceding the RSRR rescues intracellular retention of R1[RS917RR] but does not increase retention of R1[RS887RR] and R1[RS939RR] (Fig. 5). This reinforces that the local sequence context can influence RSRR functionality and supports that the distal cytoplasmic tail is accessible for intracellular retention at various sites.

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It is emerging that different types of ER-retention/retrieval motifs have characteristic operating ranges with respect to the distance to the plasma membrane. Whereas carboxy-terminal KK motifs are operational proximal to the membrane, RXR-type motifs are most effective at a certain distance away from the intracellular plasma membrane (Shikano and Li, 2003). In our experiments the ectopic RSRR motifs in the intracellular loops may therefore be positioned too close to the plasma membrane to be in the active zone. It is also conceivable that the binding of a putative RSRR-interacting protein involved in ER retention depends on additional sequence elements within $GABA_{B1}$. Appropriate spacing between the RSRR motif and such additional sequence elements may be lost in $GABA_{B1}$ proteins with mutations in the intracellular loops. Alternatively, in certain ectopic positions the RSRR motif might be inaccessible due to simple sterical hindrance. We show that C-terminal deletions that progressively move the wild-type RSRR motif closer to the membrane gradually reduce its signal strength, favoring that primarily the spacing to the plasma membrane is important for RSRR function (Fig. 6).

Functional ectopic RSRR signals in $GABA_{B1}$ are efficiently masked by the $GABA_{B2}$ subunit in the absence of coiled-coil dimerization (Fig. 7). This agrees with earlier findings that coiled-coil interaction is not absolutely necessary for shielding (Pagano et al., 2001). The mechanism by which $GABA_{B2}$ prevents intracellular retention of $GABA_{B1}$ therefore remains unclear. The data presented herein suggest a model in which global conformational changes associated with heteromeric assembly remove the RSRR signal from the active zone, thereby restricting its access and triggering surface delivery of the complex. COPI and 14-3-3 are prime candidates for regulating aspects of $GABA_B$ receptor trafficking. COPI components can interact with arginine-based motifs and compete for binding with proteins of the 14-3-3 family (Yuan et al., 2003). It is thought that 14-3-3 binding overcomes ER-retention by preventing recycling of correctly assembled proteins from the ER-Golgi intermediate compartment to the ER via COP1

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vesicles (Nufer and Hauri, 2003; O'Kelly et al., 2002). 14-3-3 proteins are known to associate with the C-terminus of $GABA_{B1}$ through a domain partially overlapping with the coiled-coil domain (Couve et al., 2001). It is conceivable that COP1 components bind to RSRR when $GABA_{B1}$ in unassembled, which recycles $GABA_{B1}$ back to the ER. Following heteromeric assembly and removal of the RSRR motif from its active zone, COP1 could then be replaced by 14-3-3, which avoids recycling and allows for surface trafficking.

In conclusion, our results support that the RSRR ER-retention/retrieval signal of $GABA_{B1}$ is only functional within the distal C-terminal tail. Moreover, coiled-coil interaction is not crucial for inactivation of wild-type (Pagano et al., 2001) and ectopic RSRR motifs. In the light of these data, we propose that removal of the RSRR motif from its active zone rather than direct coiled-coil shielding may trigger surface delivery of the receptor complex. On a broader scope, the data suggest that many proteins featuring the RXR consensus sequence in proximity of the membrane escape intracellular retention because the motif does not reach into its operational zone.

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Footnotes

a)

This work was supported by the Swiss Science Foundation (Grant 3100-067100.01 to BB) and the Désirée and Niels Yde Foundation.

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Legends to figures

Fig. 1. Description of $GABA_{B1}$ mutants with ectopic RSRR motifs. A, Schematic diagram depicting the insertion sites of the ectopic RSRR motifs in a $GABA_{B1a}$ mutant protein where the endogenous RSRR sequence C-terminal to the coiled-coil domain was mutated to ASAA. The seven transmembrane helices are shown as black boxes. B, Nomenclature of the $GABA_{B1a}$ constructs used in this study. Residues that were inserted to generate the ectopic RSRR motif are underscored. The positions of the ectopic RSRR motifs are numbered. Residue numbering refers to wild-type $GABA_{B1a}$ protein (GenBank accession number Y10369). C, Western blot analysis confirming expression of the mutant $GABA_{B1a}$ proteins in HEK293 cells. An antibody specific for the $GABA_{B1}$ C-terminus detects a band of approximately 100kD (top panel). A second band is sometimes visible, presumably representing incompletely processed intracellular $GABA_{B1}$ protein. R1[R862SRR], which harbours the ectopic RSRR motif in the C-terminus proximal to the coiled-coil domain, is poorly expressed or inefficiently recognized by the antibody. Equal loading of HEK293 cell lysates was controlled for by Western blot analysis with a β-tubulin antibody (bottom panel).

Fig. 2. Surface targeting of wild-type (R1) and mutant $GABA_{B1a}$ proteins expressed individually and in combination with $GABA_{B2}$ (R2). Cell homogenates (H) and intact cells (S) were photoaffinity-labeled with the membrane-impermeable $GABA_{B1}$-specific antagonist [^{125}I]CGP71872 and subjected to SDS-PAGE. To correct for the variability in transfection efficiency, the amount of protein sample subjected to gel electrophoresis was normalized to the respective amount of radioactivity incorporated into the cell homogenates (H) for each set of transfection (expression with and without $GABA_{B2}$). Labeled proteins were then visualized by

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autoradiography (photoaffinity labeling PAL). Loading was controlled for by Western blot analysis with a polyclonal antibody raised against $GABA_{B1}$ (WB). It is evident that a larger fraction of immunolabeled $GABA_{B1}$ protein binds the photoaffinity ligand when $GABA_{B2}$ is co-expressed (lanes 1,2 versus 3,4). For each transfection we compared photoaffinity-labeled $GABA_{B1}$ protein at the cell surface (S) to total $GABA_{B1}$ protein labeled in the cell homogenates (H) (% S/H). Insertion of an ectopic RSRR motif at positions S887 (R1[RS887RR]) and R939 (R1[R939SRR]) results in partial intracellular retention (lane 2 versus 1), which is overcome by coexpression with $GABA_{B2}$ (lane 4 versus 2) . The % S/H values indicated represent the experiment shown in the figure. One-way analysis of variance (ANOVA) followed by a pair-wise comparison via Tukey HSD test confirmed that the % S/H values (mean ± SD) for R1[RS887RR] (17.0 ± 10.1, n=3) as well as R1[R939SRR] (26,7 ± 11.9, n=3) differ significantly from the one for R1[ASAA] (57.8 ± 8.7, n=4) ($p < 0.05$ in both cases).

Fig. 3. Surface biotinylation of $GABA_{B1}$ (R1) and R1[ASAA] in the presence and absence of $GABA_{B2}$. The fraction of $GABA_{B1}$ protein at the cell surface is similar when measured with surface biotinylation or with the $GABA_{B1}$-specific antagonist [^{125}I]CGP71872 (Fig. 2), supporting that binding-incompetent $GABA_{B1}$ protein is not delivered to the cell surface.

Fig. 4. Functional analysis in HEK293 cells of $GABA_B$ receptors with ectopic RSRR motifs in the $GABA_{B1}$ subunit. Artificial coupling of $GABA_B$ receptors to PLC upon co-expression with a chimeric Gα subunit, $G\alpha qz_{IC}$ (Franek et al., 1999) results in an intracellular Ca^{2+} transient that is measured by changes in fluo-4 AM fluorescence intensity. All $GABA_{B1}$ mutants can be activated by GABA (0.1 mM) upon co-expression with $GABA_{B2}$, similarly to wild-type $GABA_{B1}$ (R1 +

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R2). Representative Ca^{2+} transients of 12 wells are shown. Bars below traces indicate application of GABA.

Fig. 5. Intracellular retention of $GABA_{B1}$ protein after insertion of a leucine residue preceding the ectopic RSRR motif at S917. Following transfection with the indicated $GABA_{B1}$ expression constructs, intact HEK293 cells were photoaffinity-labeled with the membrane-impermeable $GABA_{B1}$-specific antagonist [^{125}I]CGP71872 and subjected to SDS-PAGE. Labeled proteins were then visualized by autoradiography (PAL). Loading was controlled for by Western blot analysis with a polyclonal antibody raised against $GABA_{B1}$ (WB). Upon insertion of a leucine preceding the ectopic RSRR at S917 (R1[LRS917RR]) no labeled protein is detected indicating that the expressed $GABA_{B1}$ protein fails to be transported to the cell surface. No increased retention is observed with R1[LRS887RR] and R1[LR939SRR] following insertion of a leucine residue.

Fig. 6. The RSRR motif is gradually inactivated when positioned closer to the membrane. A, Schematic diagram showing the coding regions of different $GABA_{B1}$ expression constructs with deletions of 9 amino acids (R1ΔR862-Q870), 30 amino acids (R1ΔR862-E891) or 52 amino acids (R1ΔR862-H913) between transmembrane domain 7 and the extended ER-retention/retrieval motif QLQSRQQLRSRR. The number of residues that separate the extended ER-retention/retrieval motif from the transmembrane domain is indicated below each construct. B, Cell surface targeting of wild-type (R1), R1[ASAA] and deletion mutants in HEK293 cells in the absence of $GABA_{B2}$. Cell homogenates (H) and intact cells (S) were photoaffinity-labeled with the membrane-impermeable $GABA_{B1}$-specific antagonist [^{125}I]CGP71872 and subjected to SDS-

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PAGE. Labeled proteins were then visualized by autoradiography (PAL). Loading was controlled for by Western blot analysis with a polyclonal antibody raised against $GABA_{B1}$ (WB). For each transfection photoaffinity-labeled $GABA_{B1}$ protein at the cell surface (S) was then compared to total $GABA_{B1}$ protein labeled in the cell homogenates (H) (% S/H). A deletion of 9 amino acids (R1ΔR862-Q870) has no effect on the functionality of the endogenous ER-retention-retrieval motif. However, deletion of 30 amino acids (R1ΔR862-E891) and 52 amino acids (R1ΔR862-H913) gradually increases cell surface expression of $GABA_{B1}$.

Fig. 7. Coiled-coil domain interaction is not necessary for masking functional ectopic RSRR motifs. Wild-type $GABA_{B1}$ (R1) and the $GABA_{B1}$ mutants with functional ectopic RSRR (constructs R1[RS887RR] and R1[R939SRR]) were expressed alone (lanes 1 and 2) or in combination with wild-type $GABA_{B2}$ (R2) (lanes 3 and 4) or a $GABA_{B2}$ mutant lacking the coiled-coil domain (R2ΔLZ2) (lanes 5 and 6). Cell homogenates (H) and intact cells (S) were photoaffinity-labeled with the membrane-impermeable $GABA_{B1}$-specific antagonist $[^{125}I]$CGP71872 and subjected to SDS-PAGE. Labeled proteins were then visualized by autoradiography (PAL). Loading was controlled for by Western blot analysis with a polyclonal antibody raised against $GABA_{B1}$ (WB). R2ΔLZ2 is able to traffic wild-type $GABA_{B1}$ to the cell surface, but to a smaller extent than wild-type $GABA_{B2}$ (left panel, for each transfection compare photoaffinity-labeled $GABA_{B1}$ protein at the cell surface (S) to total $GABA_{B1}$ protein labeled in the cell homogenates (H)). Both wild-type $GABA_{B2}$ and R2ΔLZ2 are able to traffic the $GABA_{B1}$ mutants R1[RS887RR] and R1[R939SRR] to the cell surface to the same extent (middle and right panel).

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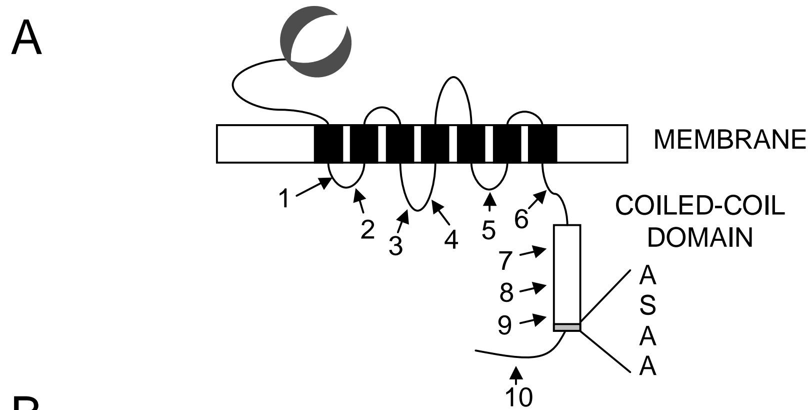
Figure 1

A



B

Construct	Name	Insertion site of ectopic RSRR	Position
1	R1[RS616RR]	internal loop 1	Ser 616
2	R1[RS624RR]	internal loop 1	Ser 624
3	R1[V690RSRR]	internal loop 2	Val 690 (after)
4	R1[E699RSRR]	internal loop 2	Glu 699 (after)
5	R1[E796RSRR]	internal loop 3	Glu 796 (after)
6	R1[R862SRR]	c-term (proximal of coiled-coil)	Arg 862
7	R1[RS877RR]	c-term (within coiled-coil)	Ser 877
8	R1[RS887RR]	c-term (within coiled-coil)	Ser 887
9	R1[RS917RR]	c-term (within coiled-coil)	Ser 917
10	R1[R939SRR]	c-term (distal of coiled-coil)	Arg 939

C

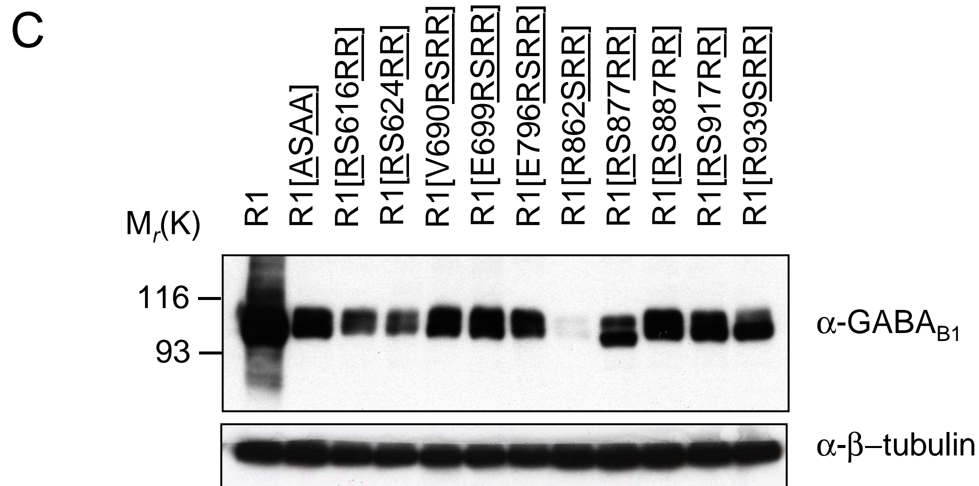


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Figure 2

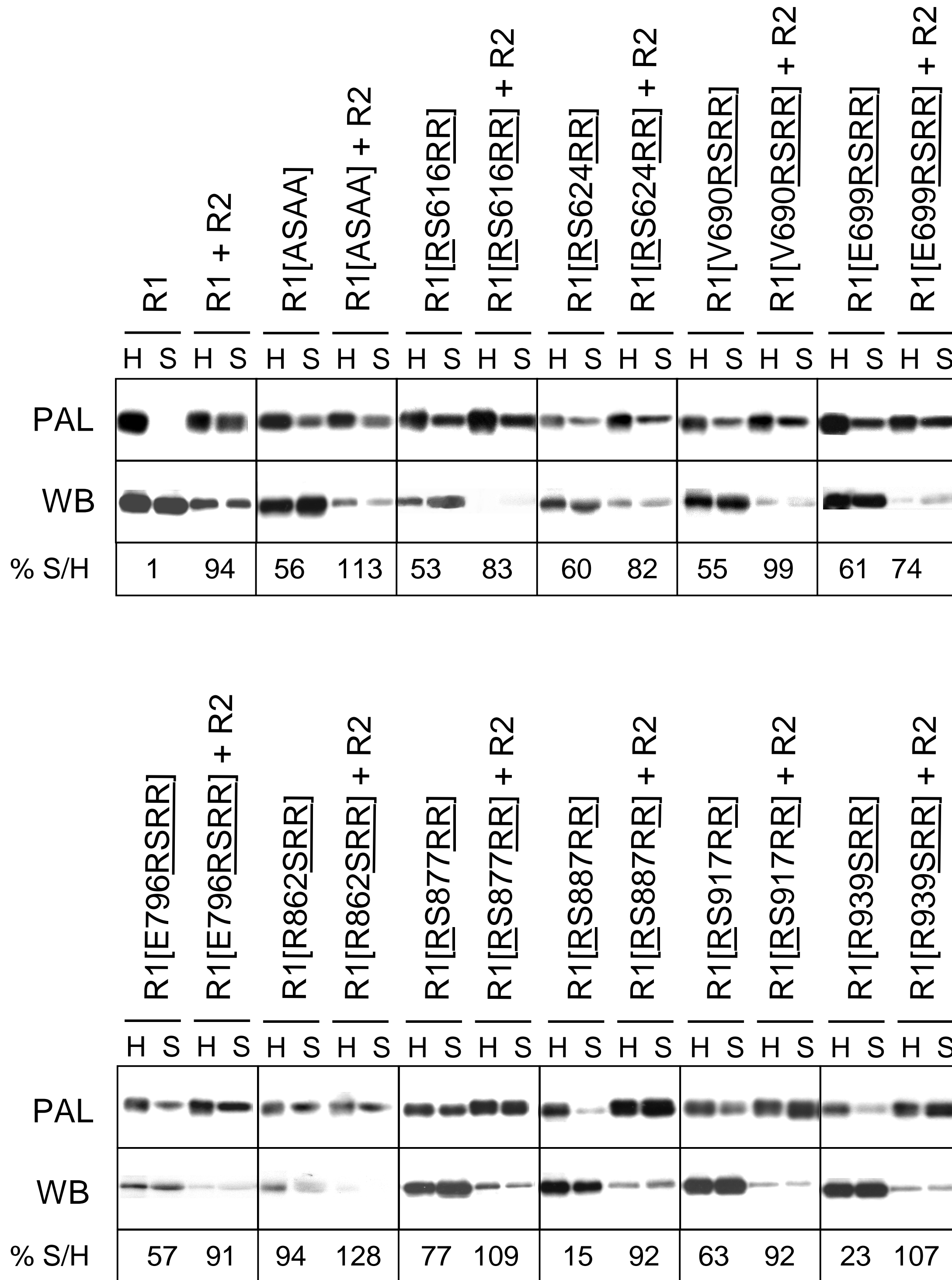

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Figure 3

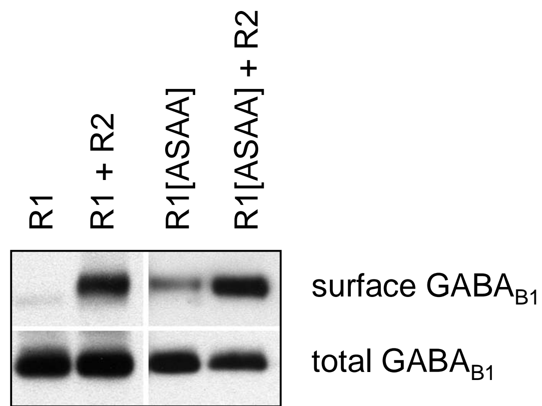

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Figure 4

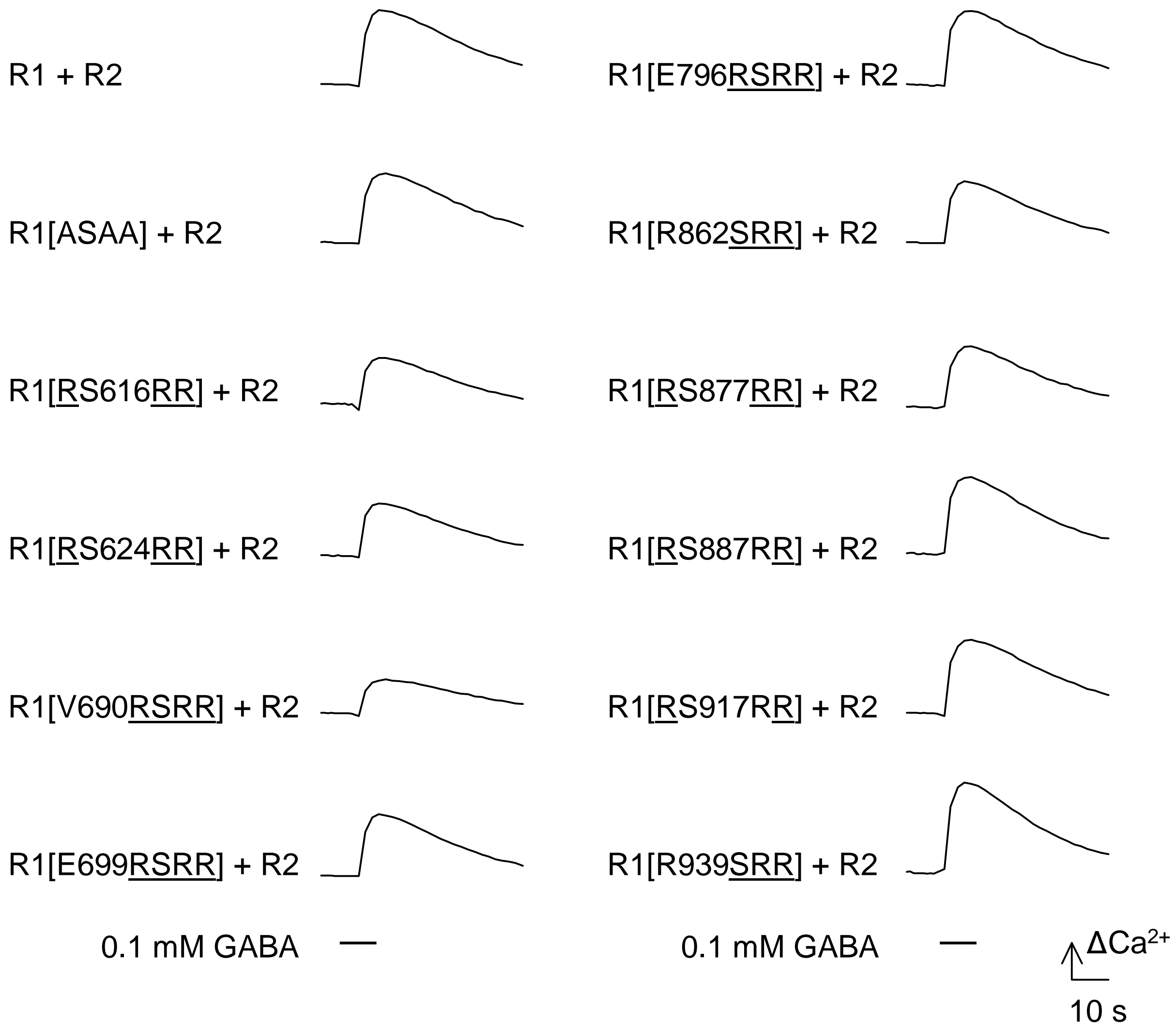

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Figure 5

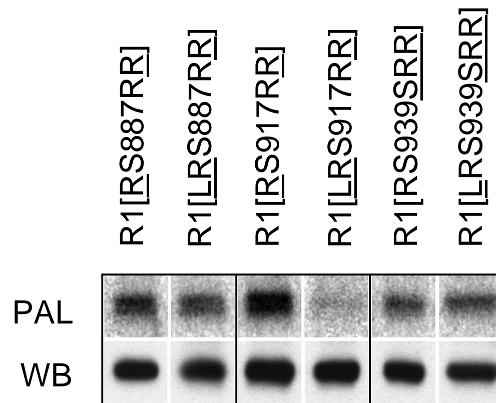

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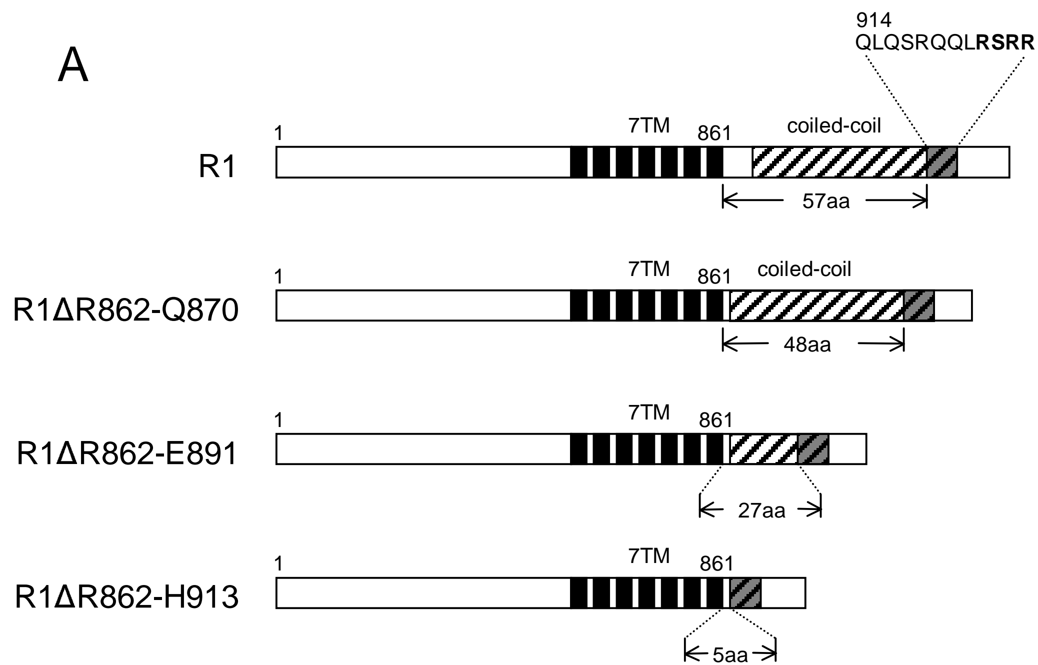
Figure 6



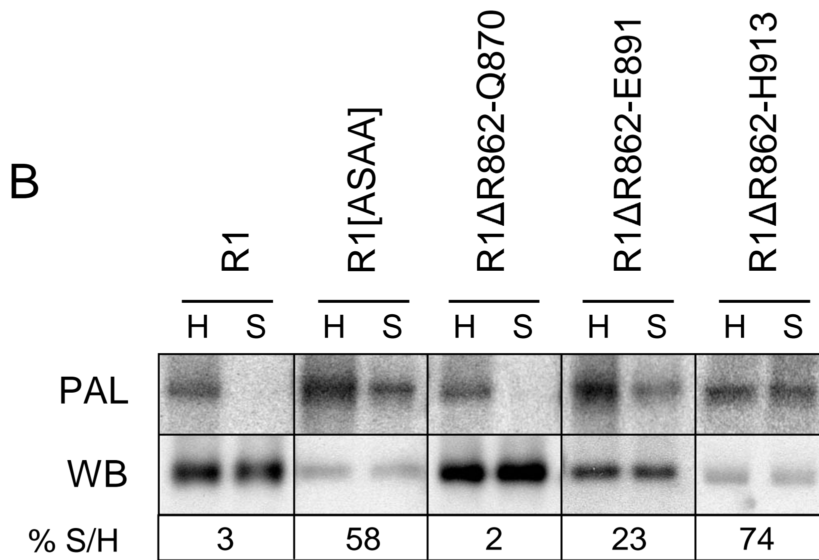


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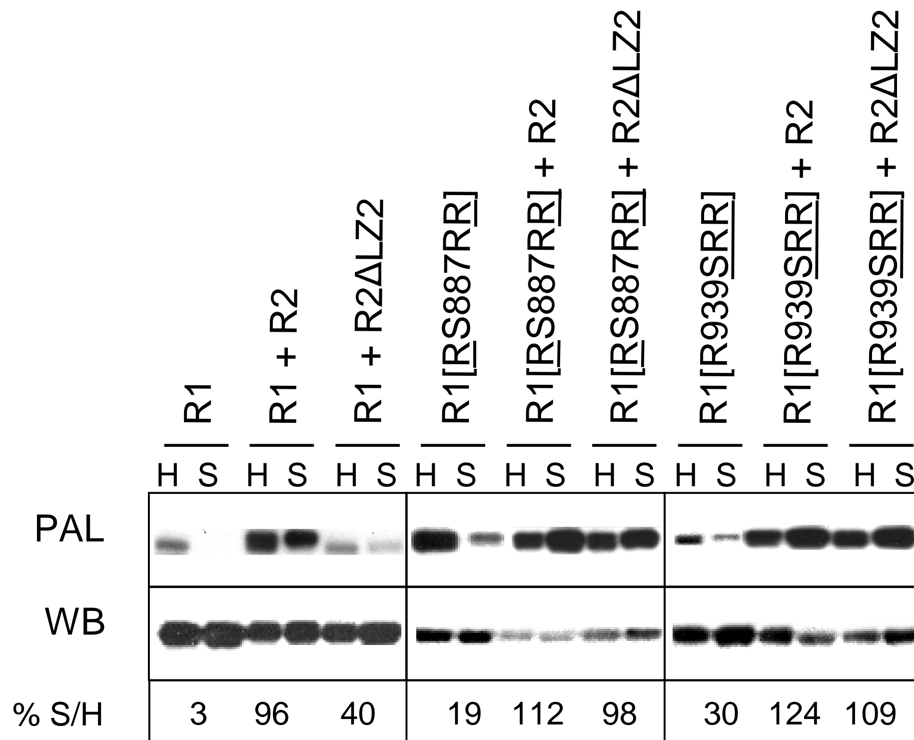
Figure 7



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