THE IMPORTANCE OF TM3-4 LOOP SUBDOMAINS FOR FUNCTIONAL RECONSTITUTION OF GLYCINE RECEPTORS BY INDEPENDENT DOMAINS

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Running title: Independently folding domains of the glycine receptor

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Background: Truncated non-functional GlyRs are reconstituted in channel function by co-expression with a C-terminal domain.
Results: TM3-4loop residues are expressed independently of TM4 and the truncated GlyR receptor.
Conclusion: TM4 and the C-terminus are not sufficient to rescue GlyR function rather residues of the TM3-4loop are required.
Significance: TM3-4loop residues trigger directly or indirectly conformational changes necessary for GlyR functionality.

SUMMARY

Truncated glycine receptors that have been found in human patients suffering from the neuromotor disorder hyperekplexia or in spontaneous mouse models resulted in non-functional ion channels. Rescue of function experiments with the lacking protein proportion expressed as a separate independent domain demonstrated restoration of glycine receptor functionality in vitro. This construct harbored most of the TM3-4loop, TM4, and the C-terminus and was required for concomitant transport of the truncated α1 and the complementation domain from the endoplasmic reticulum towards the cell surface, thereby enabling complex formation of functional glycine receptors. Here, the complementation domain was stepwise truncated from its N-terminus in the TM3-4loop. Truncation of more than 49 amino acids led again to loss of functionality in the receptor complex expressed from two independent domain constructs. We identified residues 357-418 in the intracellular TM3-4loop as being required for reconstitution of functional glycine-gated channels. All complementation constructs showed cell surface protein expression and correct orientation according to glycine receptor topology. Moreover, we demonstrated that the truncations did not result in a decreased protein-protein interaction between both glycine receptor domains. Rather, deletions of more than 49 amino acids abolished conformational changes necessary for ion channel opening. When the TM3-4loop subdomain harboring residues 357 to 418 was expressed as a third independent construct together with the truncated N-terminal and C-terminal glycine receptor domains, functionality of the glycine receptor was again restored. Thus, residues 357-418 represent an important determinant in the process of conformational rearrangements following ligand binding resulting into channel opening.
INTRODUCTION

Glycine is the major inhibitory neurotransmitter in brainstem and spinal cord in human and rodents. Following binding to its postsynaptic receptor, an intrinsic chloride channel will be opened. The glycine receptor (GlyR) undergoes a developmental shift after birth, with a replacement of homomeric α2 receptors representing the embryonic GlyR isoform (GlyR_N) by the adult heteromeric α1β or α3β configuration (GlyR_A) (1,2). The adult pentameric receptor complex is composed of two α and three β subunits (2α1/3β or 2α3/3β) (3,4) and anchored via gephyrin to the cytoskeleton. GlyRs belong to the superfamily of Cys-loop receptors as well as GABA<sub>A/C</sub> receptors, nicotinic acetylcholine receptors (nAChR), and the 5HT<sub>3</sub> receptor. The Cys-loop in the extracellular N-terminus of the receptor proteins is conserved among all family members, while GlyRs harbor an additional disulfide bond in the extracellular loop C (5). Each subunit exhibits a large extracellular N-terminus, four transmembrane domains (TM) connected by intra- or extracellular loop structures with TM2 facing the ion channel pore, and an extracellular C-terminus. The X-ray structure analysis of homologous proteins as the acetylcholine-binding protein (AChBP) and the prokaryotic ligand-gated ion channels ELIC (cloned from Erwinia chrysanthemi) and GLIC (from Gloeobacter violaceus) provided lots of structural information on the N-terminus, the TMs, and the short loops between TM1-2 and TM2-3, except for the large intracellular loop between TM3 and TM4 (6,7). This intracellular domain (iD) shows the highest variability among all Cys-loop receptors and seems to have developed late in evolution as the prokaryotic isoforms ELIC and GLIC lack this domain. Previous reports have shown that this domain is involved in receptor trafficking and anchoring. Anchoring is facilitated via interaction of the GlyRβ TM3–4 loop with E-domain monomers of gephyrin which forms a hexagonal lattice structure at the postsynaptic density (8,9). Two basic motifs near TM3 and TM4 in GlyRα1 were found to interact with Gβγ subunits which enhances the apparent glycine affinity of the receptor and results in an increase in ion channel open probability (10). Recently, pharmacological modulation of the GlyRα1 via the TM3-4loop has been reported for ethanol as well as propofol (11,12). In contrast, the TM3-4loop seems not to be important for signal transduction in general, as a replacement of this large domain in GABA<sub>C</sub> and the 5HT<sub>3</sub> receptor by the short linker present in GLIC still resulted in functional ion channels (13). A lack of the TM3-4loop, TM4, and the C-terminus as present in the mouse model oscillator, however, results in non-functionality of the GlyR complex. A coexpression of those truncated N-terminal receptor domains (α1-trc) together with the lacking domain as a separate construct resulted in rescue of ion channel functionality, thus demonstrating that GlyRs are composed of independently folding domains (14). Rescue efficiency was largely dependent on the presence of the basic motif RRKRRH in the α1-trc at its C-terminal end. This motif has previously been shown to be important for surface integration, and subunit-specific sorting (15,16).

In the present study, we have investigated protein-protein interactions within GlyRα1 protein when coexpressed from two independently folding domains. Truncated GlyRs result in non-functionality. Coexpression with the lacking protein domain regained functional channels. We truncated the complementation construct harboring parts of the TM3-4loop, TM4, and the C-terminus from its N-terminal end. With a deletion of more than 49 residues functionality was again almost abolished. Truncation of 62 amino acids never resulted in a functional ion channel configuration. Therefore, residues S<sup>406</sup> to E<sup>411</sup> localized at the interface between the last variable (V2) and constant (C3) region before TM4 (Fig.1) are critical for ion channel reconstitution from two independent domains. Cell surface expression and correct membrane orientation corresponding to GlyR topology was demonstrated for all GlyR domain constructs. Finally, protein-protein interactions of the N-terminal truncated α1-trc together with the complementation constructs were not disturbed. Moreover, the constituted two-domain receptor seemed to fail at the transduction of ligand binding into channel opening. This
disability was conquered by coexpression with the 62 amino acids of TM3-4loop357-418 as a third independent construct. Thus, functional GlyRs can be rebuilt from three small independent domains substantiating the mosaic structure of Cys-loop receptors.

EXPERIMENTAL PROCEDURES

Transfection of HEK293 cells - HEK293 (human embryonic kidney) cells were grown in Earl’s MEM medium (PAA), supplemented with 10% fetal calf serum, L-glutamine (200 mM) and 50 U/ml penicillin and streptomycin at 37°C and 5% CO2. 24 h after splitting (2 x 10^6 cells for a 10 cm dish; 0,15 x 10^6 cells for a 3 cm dish), the cells were transiently transfected using the calcium-phosphate precipitation method (17). Cell culture medium was replaced 24 h after transfection.

Preparation of whole-cell lysates - Whole cell lysates were prepared 48 h post-transfection adding 500 µl of preheated 2 x SDS sample buffer onto a 10 cm dish of transfected HEK293 cells. The samples were heated for 10 min at 95°C before application to SDS-PAGE.

Membrane protein preparation - The preparation was performed 48 h post-transfection with HEK293 cells grown in 10 cm dishes as described above. After detaching the cells with cold 1x PBS (PAA) and centrifugation (10 min, 1000 x g), the cell pellet was resuspended in 3 ml buffer H (10 mM potassium phosphate buffer pH 7.4, 250 mM EDTA, 250 mM EGTA and protease inhibitor cocktail tablets, Roche). The probes were homogenized in a tapered glass tissue grinder and with an ultra turrax, followed by centrifugation for 10 min at 16.000 x g. Afterwards, the pellets were resolved in buffer H and the homogenization and centrifugation step was repeated once. The pellets were resolved in buffer B (25 mM potassium phosphate buffer pH 7.4, 200 mM KCl, 250 mM EDTA, 250 mM EGTA and protease inhibitor cocktail tablets, Roche).

Biotinylation of cell surface proteins - The biotinylation assay was performed 48 h post-transfection with HEK293 cells grown in 10 cm dishes as described above. After removing the cell culture medium and three washing steps with cold 1x PBS (PAA) the surface proteins were labeled with EZ-link Sulfo-NHS-SS-Biotin (Pierce) for 30 min, followed by washing with quenching buffer (192 mM glycine, 25 mM Tris in PBS pH 8.0) for 10 min. The cells were detached with ice-cold 1x PBS and centrifugated for 10 min at 1000 x g, followed by cell lysis with TBS (Tris-buffered saline with 1% Triton X-100 and protease inhibitor cocktail tablet, Roche) and a 1 min centrifugation step at 13.000 x g. The supernatant (whole protein fraction) was incubated with 50 µl of streptavidin-agarose beads (Pierce) for 2 h at 4°C while rotating. After removing the supernatant and washing the beads three times in TBS, the biotinylated proteins were eluted by boiling with 50 µl 2 x SDS sample buffer for 5 min.

Co-immunoprecipitation - Transfected HEK293 cells were grown on 10 cm dishes as described above and used 48 h after transfection. After washing the cells on ice with cold 1x PBS 3 times, 600 µl lysis-buffer (20 mM Tris-HCl, 160 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% Triton X-100, protease inhibitor cocktail tablets, pH 7.4) was added at each plate to scrape off the cells. After centrifugation (10 min, 4°C and 15000 rpm) the supernatant (lysate) was used as Input for co-immunoprecipitation experiments. To 600 µl of the respective solution, 2 µl MAb2b antibody (1:200, Synaptic Systems) and 60 µl protein A-sepharose beads (1:1 slurry, Sigma) were added and agitated over night at 4°C. Then, the supernatant was removed and the beads were washed three times (50 mM Tris-HCl, 160 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 8.0). Proteins were eluted from the beads by incubating in 30 µl 2 x SDS sample buffer at 95°C for 7 min.

Western blot analysis - For SDS-PAGE, 11% or 15% polyacrylamide gels were used followed by a Western blot on nitrocellulose membranes. Membranes were blocked for 1 h with TBB (TBS + 5% BSA) and incubated for 1 h or over night (4°C) with primary antibodies. The secondary antibody was horseradish peroxidase-coupled (incubation 1 h) for detection with the ECLplus system (GE Healthcare).

Immunocytochemistry - Transfected HEK293 cells were grown on polylysine-coated cover slips,
fixed with 4% paraformaldehyde and 4% sucrose in PBS (10 min), washed three times with PBS, and blocked as well as permeabilized with 0.1% Triton X-100, 5% donkey serum in PBS (30 min) at room temperature. Cells were incubated with primary antibodies for 1 h at room temperature and washed three times with PBS. This procedure was repeated with secondary antibodies. Finally, cells were embedded in Mowiol and subjected to confocal microscopy on a DMIRE2 confocal microscope (Leica). For live stain, transfected cells were incubated with primary antibody for 1 h at 4°C before fixation without permeabilization. All experiments were performed 24 h post-transfection.

**Antibodies used** - For different stainings, monoclonal mouse-anti-GlyRα (1:200, MAb4a, Synaptic Systems, recognized epitope harbors amino acids 96 - 105 of mature glycine receptor α1-subunit.), polyclonal rabbit-anti-myc (1:200, C-19 or A-14, Santa Cruz), monoclonal mouse-anti-myc (1:200, 9E10, Santa Cruz) or monoclonal mouse-anti-GFP (1:500, Roche), rabbit anti-Na+,K+ ATPase polyclonal antibody (Chemicon), and polyclonal goat-anti-GlyRα1 TM3-4loop (1:50, H18, Santa Cruz) were used as primary antibodies. Secondary antibodies were goat-anti-mouse-Alexa488 (1:400, Invitrogen), goat-anti-rabbit-Cy3 and donkey-anti-goat-Cy5 (both 1:250, Dianova). For live stain monoclonal mouse-anti-GlyRα1 (1:100, MAb2b, Synaptic Systems, antibody raised against amino acids 1-10 of the mature glycine receptor α1-subunit,) and polyclonal rabbit-anti-myc (1:50, Santa Cruz) were used as primary antibodies. For detection with enhanced chemiluminescence, a goat-anti-mouse-hrp (1:10.000, Dianova) antibody was utilized as secondary antibody.

**Alignment** - For multiple sequence alignments, the sequences and boundaries of the TM3-4loops were taken from the Uniprot data base annotation (18). GlyR subunit variants of the TM3-4loops from either human (Homo sapiens, hs), or mouse (Mus musculus, mm) were subjected to a multiple sequence alignment using the T-COFFEE algorithm (19).

**Electrophysiological recordings** - Maximal current amplitudes (I_{max}) were measured by patch-clamp technique in a whole-cell configuration mode. Current signals were amplified with an EPC-9 amplifier (HEKA). 24 h after transfection whole-cell recordings from HEK293 cells were performed by application of ligand (glycine 1 mM) using a U-tube system. The extracellular buffer consisted of 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2, 1 mM MgCl_2, 5 mM HEPES, pH adjusted to 7.2 with NaOH. The internal buffer was 120 mM CsCl, 20 mM N(Et)_4Cl, 1 mM CaCl_2, 2 mM MgCl_2, 11 mM EGTA, 10 mM HEPES, pH adjusted to 7.2 with CsOH. Recording pipettes were fabricated from borosilicate capillaries with an open resistance of about 4 MΩ. Current responses were measured at a holding potential of -60 mV. All experiments were carried out at room temperatures. Bar diagrams show the average value X of n measurements and the error bars are the standard error of the mean (SEM).

**RESULTS**

*Generation of successive N-terminal truncations of myc-iD-TM4 of the GlyR.* Truncated variants of the GlyR α1 subunit, which have been identified in a family with hyperekplexia (20) and are present in the mouse model oscillator (21) lacking most of the intracellular TM3-4loop, the TM4, and the C-terminus (α1-trc representing residues 1-357), are not able to gate Cl⁻ currents. Coexpressions with a corresponding complementation construct iD-TM4 (iD = intracellular domain), led to reconstitution of functional ion channels (14). In this study, we aimed at mapping the interaction site between GlyRα1-trc and the C-terminal complementation construct, myc-iD-TM4 (Fig. 2A). The iD of the GlyR can be subdivided into constant (C1-3) regions, which are highly conserved among species, and variable (V1-2) regions (Fig.1). We generated successive N-terminal truncations of the complementation construct (Fig. 2B) leading to constructs myc-ΔA22-TM4 (lack of V1, subdivision of C2), myc-ΔA27-TM4 (subdivision of C2), myc-ΔA43-TM4 (subdivision of V2), myc-ΔA49-TM4
(division between V2 and C3), myc-iΔA55-TM4 (split C3), myc-iΔA62-TM4 (lack of V1 to half C3), and myc-iΔA67-TM4 (lack of V1 to almost C3) with Δx representing the number of deleted amino acids. For GlyR reconstitution from three independent domains α1-trc, iΔ-Δ62-TM4, and the TM3-4loop were coexpressed (Fig. 2A, right panel). The construct 3-4loop harbors amino acid residues 357-418 (TM3-4loop 55-57 refers to TM3-4loop further on) (Fig. 2B, bottom). An N-terminal myc epitope (9E10) was added for easy immunodetection to either constructs iD-Δx-TM4 or the TM3-4loop (myc-TM3-4loop).

Protein expression of complementation constructs. The expression of the generated constructs (myc-iD-Δx-TM4) together with α1-trc was confirmed using crude membrane protein preparations of transfected HEK293 cells. The α1-trc was detected using MAb4a, a monoclonal antibody recognizing an N-terminal epitope of the GlyRα polypeptides. Expression of the complementation constructs was verified with an α-myc antibody. No significant differences in membrane expression were observed between myc-iD-Δx-TM4 constructs and α1-trc (Fig. 3A). A slight decrease for myc-iΔA62-TM4, and myc-iΔA67-TM4 was determined concomitantly with less coexpressed α1-trc but similar Na⁺,K⁺ ATPase expression compared with other preparations, e.g. with iD-Δ27-TM4. The expression of the Na⁺,K⁺ ATPase is much higher in untransfected cells, thus the overexpression of other membrane proteins following transfection seems to reduce the endogenous expression of the Na⁺,K⁺ ATPase. The surface expression was tested with biotinylation assays of cotransfected HEK293 cells (Fig. 3B). In addition to a signal in the whole cell pool and the intracellular/unbound protein fraction, all complementation constructs (iD-Δx-TM4 with x = number of amino acids deleted, Δ22-Δ67) showed a strong signal at the outer surface of transfected HEK293 cells when coexpressed with α1-trc. Note, complementation constructs harboring TM4 expressed in the absence of α1-trc get transported towards the cell surface only to some extent (14).

Formation of functional chloride channels. The assembly of functional GlyR complexes was analyzed using electrophysiological recordings in a whole-cell configuration of transfected HEK293 cells. The different complementation constructs with N-terminal truncations of up to 67 amino acids were not able to gate any Cl⁻ currents when expressed alone (data not shown). Isolated expression of α1-trc did not lead to functional ion channels, either. Coexpression of α1-trc together with myc-iD-TM4, however, rescued functional Cl⁻ channels (Fig. 4) (14). The ion channel complementation with myc-iΔA22-TM4 rescued the Cl⁻ influx with efficiency comparable to the full-length myc-iD-TM4 variant. The rescues with the truncated variants myc-iΔA27-TM4, myc-iΔA43-TM4, and myc-iΔA49-TM4 showed decreased I_{max} values. Maximal currents of α1-trc with myc-iΔA27-TM4 reached 79% (1,9 ± 0,34 nA) and with myc-iΔA43-TM4 75% (1,8 ± 0,15 nA) of the rescue efficiency compared to the full-length myc-iD-TM4 variant. The observed GlyR rescue efficiency for myc-iΔA49-TM4 was further decreased to 58% (1,4 ± 0,027 nA, Table 1). All glycine-gated currents in a two-domain approach were non-desensitizing as observed for full-length GlyRα1. Therefore, truncations up to 49 amino acids did not change GlyR channel properties such as desensitization behavior (Fig. 4A). Larger truncations of the complementation construct lead to an almost complete loss of rescue efficiency. With the myc-iΔA55-TM4 variant, responses were only observed in two out of 10 cells recorded. The two most extensive truncations (myc-iΔA62-TM4 and myc-iΔA67-TM4) led to a complete disappearance of glycine-evoked currents in at least three different batches of cells (Fig. 4B). Constructs iΔA55-TM4, iΔA62-TM4, and iΔA67-TM4 generated positively charged N-terminals with ‘RK’ at the beginning of iΔA55-TM4, ‘RAK’ for iΔA62-TM4, and ‘KISR’ for iΔA67-TM4. This could hinder an interaction with the intracellular C-terminus of α1-trc, which is also positively charged. Previously it was shown that an addition of the positively charged motif ‘RRKRRH’ to the N-terminus of the full-length iD-TM4 abolished ion channel function when coexpressed with α1-
trc harboring the same motif at its C-terminal end (14). When the truncated α1 variant present in the mouse mutant oscillator was used harboring ‘GDIT’ at its C-terminus instead of ‘RRHRRH’ functional rescue was still observed. Thus, interactions by charged motifs seem to play a role within the TM3-4loop of the GlyRα1 to permit conformational changes, respectively.

Mapping the interaction site. Loss of functionality can result from (i) loss of expression, or if expressed from (ii) loss of an intracellular interaction between the N-terminal truncated α1 variant and the complementation constructs, or (iii) wrong orientation of the complementation construct within the plasma membrane. As (i) protein expression as well as cell surface localization has been shown for all complementation constructs and α1-trc (Fig. 3A, B), lack of expression can be excluded as a reason for non-functionality of the GlyR channels. To test whether an intracellular protein-protein interaction is required for formation of functional ion channels, we performed co-immunoprecipitation assays. The α1-trc construct was cotransfected with one complementation variant (myc-iD-TM4, myc-iDΔ22TM4, myc-iDΔ27, myc-iDΔ43TM4, TM4 myc-iDΔ49TM4, myc-iDΔ55TM4, myc-iDΔ62TM4 or myc-iDΔ67-TM4) in HEK293 cells. The input was verified for α1-trc stained with Mab4a and iD-Δx-TM4 detected with α-myc (Fig. 5A). Following lysis of transfected HEK293 cells, α1-trc was precipitated using the GlyRα1 specific monoclonal antibody MAb2b. More binding capacity of antibody (lower concentration 1:150) resulted in more precipitated iD-Δx-TM4 protein (Fig. 5B, lower left panels). The co-precipitated myc-iDΔx-TM4 variants were detected with the α-myc antibody (9E10). A strong interaction was observed for α1-trc coexpressed with the full-length complementation construct myc-iD-TM4. In contrast to the observed decrease for lmax values, protein-protein interaction was not affected with increasing the truncated proportion of the complementation constructs at their N-terminal ends (Fig. 5B).

Membrane orientation of the myc-iDΔ62-TM4 domain. To exclude that non-functionality of α1-trc together with shortened complementation constructs (e.g. myc-iDΔ62-TM4) arose from a wrong orientation of the complementation constructs within the plasma membrane, live cell stainings were performed. It has been shown for membrane proteins that there exists a positive inside rule arguing that intracellular domains contain more positive-charged amino acid residues than extracellular domains (22,23). The myc-iDΔ62-TM4 construct fulfilled the criterion of more positively charged amino acid residues inside than at the extracellular side flanking the transmembrane helix 4. Live staining allows the detection of extracellular epitopes in a native protein configuration before fixation. HEK293 cells cotransfected with α1-trc and myc-iD-TM4 or α1-trc and myc-iDΔ62-TM4 were used. The extracellular N-terminal GlyRα1 epitope of α1-trc was stained with MAb2b (left columns Fig. 6). Detection of the complementation constructs was performed with an α-myc antibody (Fig. 6, middle and right columns). As the MAb2b epitope is localized extracellular, the α1-construct served also as a control for transfection. In both coexpressions, a myc-staining was neither observed for myc-iD-TM4 nor for myc-iDΔ62-TM4 (Fig. 6, middle panels in upper and middle line). Thus, the orientation of both complementation constructs was correct and in agreement with GlyR topology, respectively. These results were corroborated in permeabilized cells where the myc epitope localized at the intracellular N-terminus of the complementation constructs was recognized (Fig. 6, right panels in upper and middle line). Additionally, cells transfected with α1-trc and a complementation construct without the myc epitope (iDΔ62-TM4) showed no staining at all even not in permeabilized cells (Fig. 6, middle panel in lower line). As a positive control, N-terminally myc-tagged GlyRβ coexpressed with gephyrin, and GlyRα1 confirmed that the myc-antibody was able to recognize a native myc-epitope (Fig. 6, right panel in lower line).
Three domain configuration of the glycine receptor α1 subunit. The coexpression of α1-trc with myc-iDA62-TM4 generated no functional ion channels. In contrast, in transfections of α1-trc with full-length myc-iD-TM4, glycine-induced currents were observed. Non-functionality cannot be due to lack of expression (Fig. 3A, and 3B) or a wrong topology of the myc-iDA62-TM4 domain as live cell staining showed correct integration into the plasma membrane (Fig. 6). As protein-protein interaction was not disturbed (Fig. 5A), we wanted to analyze whether a three-domain expression of α1-trc, myc-iDA62-TM4, and the TM3-4loop domain would overcome the non-functionality of the two-domain configuration. Two additional constructs of the TM3-4loop were used which start shortly after the basic motif of the cytoplasmic TM3-4loop and end before TM4, one of them with a myc epitope (TM3-4loop and myc-TM3-4loop with residues 357-418). To control for expression of these GlyR variants, lysates of transfected HEK293 cells with α1-trc, iD and the TM3-4loop (with and without myc-epitope) were prepared. After Western blot analysis, the staining with an anti-myc antibody revealed the expression of the TM3-4loop constructs (Fig. 7). Next, the intracellular localization of α1-trc, myc-iD62-TM4 and TM3-4loop was investigated with immunocytochemical stainings with and without permeabilization of transfected HEK293 cells (Fig. 7A). A pDsRed-ER vector expressing a fusion protein of the ER targeting sequence of calreticulin fused to DsRed, or pDsRed-Monomer-Mem encoding GAP-43, a membrane protein, fused to DsRed were co-transfected. All constructs (α1-trc, the TM3-4loop, and the myc-iD62-TM4) were detectable in the ER (Fig. 7A, middle pictures). Additionally, all three domains were present at or near the plasma membrane and colocalized with each other, and with the membrane marker GAP-43 (Fig. 7A, lower panels). Thus, the prerequisites for a functional rescue – expression, co-localization and membrane localization of all three domains – were fulfilled. The functionality of the three coexpressed domains was investigated using whole-cell recordings of transfected HEK293 cells following glycine application (Fig. 7C). No currents were observed with two domains neither with α1-trc plus the TM3-4loop nor with α1-trc together with myc-iDA62-TM4 (Fig. 7C, upper two traces). In contrast, the receptor complex formation in a three-domain configuration using α1-trc together with the TM3-4loop, and the myc-iDA62-TM4 was able to gate Cl⁻ currents (Fig. 7C, lowest trace). The efficiency of rescue was 20% of the configuration α1-trc with the full-length complementation tail (Fig. 4B and 7C, Table 1). Accordingly, the 62 amino acids of the TM3-4loop are required for functionality of the ion channel. In conclusion, all three independent domains were concomitantly targeted to the plasma membrane and formed a functional GlyR configuration.

Protein-protein interaction is abolished with the intracellular TM3-4loop peptide. To analyze whether the lacking TM3-4loop proportion is also able to form a protein-protein interaction with α1-trc, we used a three-domain coexpression approach of α1-trc together with myc-iDA62-TM4 and the TM3-4loop construct (with and without myc-epitope). Both GlyR domains, the TM3-4loop and the myc-iDA62-TM4, differ only slightly in their molecular weight. Therefore, we coexpressed α1-trc together with the myc-TM3-4loop alone to detect a protein-protein interaction between these two constructs. Following input control, α1-trc was precipitated with the antibody Mab2b and the myc-TM3-4loop was detected with the α-myc antibody. The construct α1-trc failed to interact with myc-TM3-4loop. Next, the α1-trc was coexpressed with the TM3-4loop (with or without the myc-epitope) together with myc-iD62-TM4. As shown before, protein-protein interaction was observed for α1-trc and myc-iDA62-TM4 (Fig. 8B). The myc-TM3-4loop failed again to interact with α1-trc (Fig. 8B) although expression of this construct was shown and this configuration was able to generate functional Cl⁻ channels (Fig. 8A, lower panel). The analysis of the protein-protein interactions between the three GlyR domains demonstrated that α1-trc and myc-iD-A62-TM4 have to be inserted in close proximity in the plasma membrane, which in turn allowed interaction and therefore co-precipitation. Instead, the TM3-4loop construct is a soluble intracellular protein also localized near the surface shown with
immunocytochemical stainings. This intracellular peptide maybe moves back and forth thereby allowing the required movement for channel opening of α1-trc and myc-iD∆6-2-TM4 for at least some channels as the observed rescue efficiency was about 20% of α1-trc plus full-length myc-iD-TM4. However, we could not exclude that the intracellular TM3-4loop domain interacts with other intracellular proteins and/or recruits them to the plasma membrane and that this interaction then triggers conformational changes.

**DISCUSSION**

GlyRs are composed of subdomains which, when expressed independently, are able to restore ion channel functionality (14). The strategy to reassemble functional receptor proteins from non-functional modules takes advantage of the domain architecture of ion channels. Domain swapping experiments between different ion channel subunits have been shown to preserve receptor function in various receptor families, such as the glycine receptor with the prokaryotic GLIC or different glutamate receptors (24,25). For the GlyR nonsense and frameshift mutations have been described in patients suffering from hyperekplexia, a rare neuromotor disorder (20,26). A similar hyperekplexia-like phenotype was observed in the GlyR mouse mutant oscillator resulting from lack of GlyR expression due to introduction of a premature stop codon at amino acid position 355 (21). Loss of receptor proteins has also been found in other channelopathies, such as generalized epilepsy with febrile seizures plus (GEFS+) associated with truncated GABA_\text{A} receptor variants, (27).

Functionality of the truncated non-functional GlyRα1 variant from the mouse mutant oscillator was restored in vitro in HEK293 cells and primary spinal cord neurons by coexpression with an independent complementation domain (28). The restoration of GlyR functionality in the two-domain approach exhibited 50% of wild type activity. Here, large truncations of the TM3-4loop of the complementation domain resulted in loss of functionality. We set out to identify crucial subdomains of the TM3-4loop that enable an assembly from independent domains. Sequence alignments of all GlyR subunits showed that the TM3-4loop is of highest variability among GlyR members. The constant region C1 harbors a multifunctional basic motif, which has previously been shown to be important for cell surface expression, subunit-specific sorting, and binding to Gephy proteins (10,15,16). This motif, RRKRRH, is present at the C-terminal end of truncated GlyRα1 (C1 domain) and enhances the efficiency of ion channel rescue by an increase in plasma membrane integration not only of α1-trc but also of the complementation construct (14). In this study we used the truncated α1 with the RRKRRH motif present to ensure cell surface expression. Stepwise deletion of the complementation construct up to almost C3 (iD∆62-TM4 to iD∆67-TM4) resulted in a slight decrease in overall membrane expression. This decrease was coexistent with lower membrane expression of α1-trc, again corroborating the concomitant transport of receptor domains from the ER towards the plasma membrane (14,28).

Interestingly, the amount of cell surface protein observed for iD∆67-TM4 was indistinguishable from the other complementation constructs. Although protein biogenesis seemed unaffected, the rescue efficiency as a determinant for ion channel functionality in a two-domain receptor configuration dropped severely between truncations of 49 to 62 amino acids of the N-terminal part of the TM3-4loop (between V2 and C3). Our data clearly show that the variable region V2 and the conserved region C2 were not responsible for this effect. The V2 region consists of two motifs, one poly-asparagine motif (NNNN394-397) and a poly-proline motif (PPPAP401-405). The importance of NNNN394-397 in GlyRs is so far unknown, however, protein aggregation studies on proteins such as huntingtin in yeast cells have demonstrated that poly-asparagine stretches tend to aggregate and form polar zippers (29,30). Lack of PPPAP as in the construct iD∆49 decreased GlyR rescue efficiency to 58%, suggesting that, this motif may be of some importance for the correct GlyR configuration, yet the lower efficiency of ion channel rescue could also be a
consequence of continuous protein truncation and therefore loss of steric contact surfaces. Constructs iDΔ55-TM4, iDΔ62-TM4, and iDΔ67-TM4, comprise positively charged N-termini. Coexpression of truncated GlyRα1 with these variants lacking parts of the conserved region C3 resulted in almost non-functional ion channels. This observation might be explained by the positively charged N-termini of both constructs. The α1-trc domain harbors the RRKRRH motif at its C-terminus corresponding to the C1 region. Previous data have demonstrated that a coexpression of truncated α1 together with a complementation domain, which harbors positively charged residues at its N-terminal proportion abolished ion channel function. Thus, GlyR complex stability necessary for rescue of ion channel function was impaired when both intracellular ends were positively charged (14). With iDΔ55-TM4, iDΔ62-TM4, and iDΔ67-TM4 a similar overall configuration was created.

Orientation of proteins with only one TM depends on the strength of topogenic signals formed by positive charged residues next to transmembrane helices (31). Although positively charged residues are important topological determinant, a local effect of distance from a charged residue to the next TM is still not completely understood (23). Besides the disability of functional reconstitution with iDΔ62-TM4, the number of positively charged amino acids at the intracellular N-terminal part was sufficient to allow a correct orientation of TM4 according to GlyR topology.

We hypothesized that a protein-protein interaction within subdomains of the α1 TM3-4loop might be required for ion channel opening. Protein-protein interaction of the TM3-4loop of GlyRβ with the synaptic scaffold protein gephyrin has been shown to be critical for the equilibrium between synaptic and extrasynaptic GlyRs (32). Studies on movement and interaction of numerous residues in the large extracellular domain of various Cys-loop receptors showed up with a detailed knowledge of the ligand-binding pocket for several members of this superfamily (7,33). If GlyRs are expressed in a two-domain configuration, protein interactions were still detectable independent from the length of the deletion at the N-terminus of the complementation constructs. Thus, a lack of an intermolecular protein-protein interaction can be excluded as the reason for the decrease in ion channel rescue efficiency. TM4 most probably serve as a structural component for oligomerization of the receptor complex (34). However, all complementation constructs still contain TM4 and therefore, oligomerization is enabled. Although both GlyR domains are present at the plasma membrane, the lacking subdomain of the TM3-4loop seems to comprise a critical determinant required to allow conformational changes and thereby the transduction of ligand binding into channel opening.

As proposed by Bocquet et al., a movement following binding of ligand is passed through the whole protein (35). Rearrangements of several loop structures in the N-terminal part after ligand binding, as well as drug-bound or drug-free conformations of other Cys-loop receptors have been investigated with primary focus on the N-terminal and the transmembrane domains (36). Due to lack of crystal structures of the large intracellular domain for any kind of Cys-loop receptors, the structural clues for the TM3-4loop function are scarce. Recently, Breitinger et al. reported a correlation of differences in channel gating with the distribution of charged residues in a splice region of the GlyRα3 subunit (37). Here, we identified the subdomain L357-Q418 (V1-C2-V2-halfC3) in the TM3-4loop of GlyRα1 which enables both, the α1-trc and the complementation domain to procure in a distinct configuration, accompanied by a passing movement from TM3 to TM4 and thereby ion channel opening. Nevertheless, we could not exclude that this subdomain of the TM3-4loop interacts with another, so far not identified intracellular protein. In turn, such interaction could enable a recruitment of the TM3-4loop subdomain together with the unknown partner to the plasma membrane and concomitantly to an interaction with the other independent GlyR domains by chance resulting in some GlyR functionality. The functional three-domain architecture of GlyRs is consistent with the modular architecture of Cys-loop receptors composed of independently folding domains. Our data on GlyR domain complementation further
support a model system for novel gene therapeutic strategies concerning restoration of ion channel dysfunction.

REFERENCES

ACKNOWLEDGEMENTS

We thank Dr. Martin Eberhardt for critical reading of the manuscript and helpful comments. Especially acknowledged are Marina Wenzel and Rosa Weber for excellent technical assistance. Dr. Nima Melzer is gratefully acknowledged for providing the initial sequence alignment of various GlyR subunits.

FOOTNOTES

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1 Institute of Biochemistry, Emil Fischer Center, FAU Erlangen-Nuernberg, Fahrstr. 17, 91054 Erlangen, Germany
2 Institute for Clinical Neurobiology, University of Wuerzburg, Versbacherstr. 5, 97078 Wuerzburg, Germany
3 The abbreviations used are: GlyR, glycine receptor; TM, transmembrane region; iD, intracellular domain; trc, truncated; HEK, human embryonic kidney
FIGURE LEGENDS

**Fig. 1** Sequence alignment of various GlyR intracellular TM3-4loop sequences between amino acids 337 and 433. The amino acid numbering refers to the immature polypeptide; modified from Melzer et al. (15). The length of this loop differs between GlyRα-variants due to alternative splicing within the sequence. The sequences of the human (*Homo sapiens, hs*) and mouse (*Mus musculus, mm*) TM3-4loops of the GlyR α1, α2 and α3 subunits (including different splice variants) are shown. The alignment is arbitrarily subdivided into conserved (C1-3) and variable regions (V1 and V2, shaded in grey). Most charged amino acids (‘+’ positively; ‘-’ negatively) are conserved between all variants.

**Fig. 2** Glycine receptor α1 complementation constructs. A. Schematic representation of a single GlyRα1 wt (wildtype) subunit (left) the two-domain configuration (middle), and the functional three-domain complementation (right). The localization of the myc-epitope for detection of the construct is marked by a star (middle). Each GlyR subunit consists of a long extracellular N-terminus, four transmembrane domains connected by intra- (TM1-2; TM3-4) or extracellular (TM2-3) loop structures and a short extracellular C-terminus. The sequence of the long intracellular TM3-4loop begins with a basic motif ‘RRKRR’ marked by ‘++’. Middle panel, the α1-trc (trc = truncated) domain includes an artificial stop codon at position 356 (ends with residues SPM, see B). The iD-TM4 domain consists of the remaining part of the cytoplasmic TM3-4loop, the TM4 and the C-terminus. Right panel, shows the three-domain configuration with α1-trc, a truncated complementation construct lacking 62 residues at its N-terminus iDΔ62-TM4, and the TM3-4loop (TM3-4loop357-418 representing the lacking 62 residues). B. Truncated variants, myc-iDΔx-TM4, lack amino acids at the construct’s N-terminus. The numbering is relative to the first amino acid of the immature polypeptide. The TM3-4loop was subdivided into constant (C1-3) and variable (V1-2) regions. Positive (+) as well as negative (-) residue charge is indicated above the sequence. The α1-trc and all complementation constructs are depicted by bars according to their size with the first three amino acids of the appropriate construct included (e.g. iD22-TM4 starts with the amino acid residues LNL at the N-terminal end). The TM3-4loop (TM3-4loop357-418) begins with 357LNL and ends with residues ISR318).

**Fig. 3** Protein expression of GlyR domains. A. Membrane protein expression is shown for α1-trc and the myc-iDΔx-TM4 constructs. α1-trc exhibits an apparent molecular weight of 41 kDa and was stained with the pan-α antibody MAb4a recognizing an epitope in the N-terminal proportion of all GlyRα proteins. Myc-iDΔx-TM4 constructs (Δ22, Δ27, Δ43, Δ47, Δ55, Δ62, and Δ67) were detected with the α-myc antibody binding to 9E10 epitope at their N-terminal ends (see location in Fig. 2B). Na⁺,K⁺-ATPase (ATPase) was used as a loading control for membrane proteins (100 kD) as well as untransfected cells (UT) as negative control. Note, the Na⁺,K⁺-ATPase band was not present in membrane preparations from α1-trc + iD-Δ22-TM4. This does not inevitably mean that iD-Δ22-TM4 was less expressed. Here only half of the protein amount was loaded. The expression of iD-Δ22-TM4 was indistinguishable from other iD-Δx-TM4 constructs in whole cell protein amount, intracellular protein, and cell surface protein; see 3B. B. Biotinylated protein distribution within transfected HEK293 cells expressing α1-trc in combination with the indicated complementation construct. The upper blots depict the myc-iDΔx-TM4 variants (same used as in A) in the whole-cell protein fraction. Middle panels illustrate intracellular myc-iDΔx-TM4, due to continuous overexpression in transfected HEK293 cells and a determined binding capacity of the streptavidin beads used. Myc-iDΔx-TM4 proteins, which are located at the cell surface were detected with the lower blot (samples were applied onto two gels; lanes for Δ49, Δ55, Δ62 come from a different gel). The negative control for surface expression, GFP was detectable in the whole-cell protein sample
and in the intracellular fraction, but not in the surface pool. All blots were stained with either the pan-α GlyR antibody MAb4a or a mouse-anti-GFP antibody. A.+B. Myc-iΔx-TM4 variants differ in their molecular weights according to the amino acids truncated beginning with 20 kDa for myc-iD-TM4, detected using an α-myc (9E10) antibody. With the * reproducible construct-dependent unspecific bands using the α-myc antibody are marked. These bands maybe result from incomplete signal peptide cleavage.

Fig. 4 Rescue of function experiments of truncated GlyRα1 variants. A. Representative traces of cells transfected with α1-trc and truncated tail variants (myc-iΔx-TM4). Maximal glycine-gated currents were determined following 1 mM glycine (Gly) application in a whole-cell configuration of double-transfected HEK293 cells. Recordings were carried out at -60 mV, [Cl]- in = [Cl]- out. Agonists were applied via a U-tube for 1 s. Arrows point towards the appropriate rescue combinations of α1-trc together with different complementation constructs B. Bar diagram of the rescue efficiencies determined by the mean I max values with error bars representing SEM (standard error of the mean). The rescue efficiency in a three-domain GlyR configuration is emphasized with a black frame. When the TM3-4loop357-418 (3-4loop) was added as a separate expression construct to a nonfunctional two-domain configuration (α1-trc and myc-iΔ62-TM4), receptor function was again restored, although with low efficiency (compare α1-trc + myc-iΔ62-TM4 with α1-trc + myc-iΔ62-TM4 + TM3-4loop357-418).

Fig. 5 Protein-protein interactions of GlyRα1-trc with various complementation constructs. A.+B. Following cotransfection of α1-trc with one complementation construct, HEK293 cells were lysed (Input) and used for immunoprecipitation of α1-trc with the α1-specific GlyR antibody MAb2b binding to a native epitope in the far N-terminal proportion of the α1 protein. The complementation constructs myc-iΔx-TM4s were co-immunoprecipitated (co-IP) and detected with a monoclonal anti-myc (9E10) antibody. The presence of α1-trc and myc-iD-TM4 is indicated by a (+). A. Shows the input before Co-immunoprecipitation of α1-trc (stained with MAb4a) and the complementation constructs myc-iΔx-TM4 with x for Δ22, Δ27, Δ43, Δ49, Δ55, and Δ62. B. Co-IP in a two-domain receptor configuration. The negative control without any MAb2b showed no unspecific binding (first lane, lower panel). Increasing the amount of MAb2b (1:300 or 1:150 dilution) during the IP raised the specific signal for the co-precipitated myc-iD-TM4. All complementation constructs (myc-iΔx-TM4) showed a strong protein-protein interaction with α1-trc. The construct myc-iΔ55 runs reproducible at a higher apparent molecular weight. The IgG signals (50 kD and 25 kD) became visible due to use of two mouse monoclonal antibodies, one for precipitation (MAb2B) and the other for detection (MAb4a) of the GlyR proteins. Boxed gel lanes indicate that samples were run on different gels.

Fig. 6 Orientation of myc-iD-TM4 and myc-iΔ62-TM4 within the plasma membrane. Confocal images of live stained cells are shown. HEK293 cells were transfected with either α1-trc + myc-iD-TM4 or α1-trc + myc-iΔ62-TM4. The α1-trc was detected using MAb2b and the myc-iD-TM4 (myc-epitope is fused to the intracellular N-terminus) was stained using a α-myc antibody. As control, fixed and Triton X-100 treated cells showed intracellular protein protein (last picture in upper and middle row). As negative control, cells transfected with α1-trc + iΔ62-TM4 (without any tag) were stained with MAB2a for α1-trc and α-myc for the iΔ62-TM4 (last in permeabilized cells, + Triton-X100). Transfected cells with GlyRα1 + GlyRβ + gephyrin were used as positive control for the α-myc-antibody as the GlyRβ was tagged with a myc epitope at the N-terminal extracellular part. The scale bars represent 20 μm.

Fig. 7 Subcellular localization of independent GlyRα1 domains. A. GlyRα1 domains used in a three-domain GlyR configuration (α1-trc + myc-iΔ62-TM4 + TM3-4loop ) were cotransfected into HEK293 cells together with either pDsRed-ER, a vector expressing a fusion protein of the ER targeting sequence of calreticulin fused to DsRed, or pDsRed-Monomer-Mem encoding GAP-43. Overlays of two constructs
are shown, with the color of each construct indicated by its letter color. Cells were fixed and permeabilized prior to staining with MAb4a to recognize the α1-trc variant (shown in green) and α-myc antibody to detect the myc-iDΔ62-TM4 (depicted in cyan) as well as the H18 antibody detecting an epitope within the GlyRa1 TM3-4loop ε357-α438 (cyan, TM3-4loop). Calreticulin as well as GAP-43 are shown in red. The immunoreactivity was visualized by confocal microscopy. The scale bars represent 20 μm. B. Whole-cell lysates prepared from HEK293 cells transfected with combinations of α1-trc, (myc)-iDΔ62-TM4, and (myc)-TM3-4loop presence indicated by (+) below the blot. Both domains, the myc-TM3-4loop and myc-iDΔ62-TM4, were detected via their myc-epitopes. Arrows point towards the observed molecular weight of 16 kD for the TM3-4loop as well as to 13 kD for the myc-iDΔ62-TM4. C. Functionality of the three-domain receptor configuration. Following glycine application, no functional ion channels have been observed using two domains, α1-trc + myc-iDΔ62-TM4 or α1-trc + TM3-4loop (upper two traces). In the three domain configuration α1-trc + myc-iDΔ62-TM4 + TM3-4loop (middle trace), however, receptor function was rescued (see lowest trace with α1-trc + full length complementation construct myc-iD-TM4 for comparison of rescue efficiency). Whole cell recordings of transfected HEK293 cells were used, maximal glycine (Gly) - gated currents were determined with 1 mM Gly. Recordings were carried out at -60 mV, [Cl-]in = [Cl-]out. Agonists were applied via U-tube for 1 s. 9 or 10 cells of each combination were measured in at least three different batches of transfected cells.

Fig. 8 Protein-protein interaction in a three-domain conformation. The presence of constructs α1-trc and myc-iDΔ62-TM4 are indicated by a (+). Furthermore, either the TM3-4loop construct without a N-terminal myc-epitope was present or the myc-TM3-4loop harboring the myc-epitope. A. Input of α1-trc, myc-iDΔ62-TM4, and the myc-TM3-4loop detected with MAb4a or α-myc (9E10) antibody. The lower protein band in the input shows myc-iDΔ62-TM4; the upper band refers to the myc-TM3-4loop. Note, the small difference in molecular weight both polypeptides (myc-iDΔ62-TM4, 13 kD; myc-TM3-4loop, 16 kD). B. Co-IP of the three-domain receptor. Following immunoprecipitation, the TM3-4loop was never detectable. The lower panels show myc-iDΔ62-TM4 detected with an α-myc antibody. The α1-trc was stained concurrently using the pan-α antibody MAb4a.
## TABLES

**Table 1:** Whole-cell maximal currents [I\(_{\text{max}}\)] of various coexpressed GlyR domains

<table>
<thead>
<tr>
<th>expressed domains</th>
<th>number of cells</th>
<th>I(_{\text{max}}) [nA] 1 mM gly</th>
<th>normalized I(_{\text{max}}) [% of α1-trc + iD-TM4]</th>
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<tr>
<td>wt α1</td>
<td>9</td>
<td>6.8 ± 0.31</td>
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<tr>
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<td>9</td>
<td>2.4 ± 0.20</td>
<td>100%</td>
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<td>α1-trc + myc-iD(\Delta)22-TM4</td>
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<td>2.6 ± 0.34</td>
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<td>1.8 ± 0.15</td>
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<td>1.4 ± 0.24</td>
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<td>α1-trc + myc-iD(\Delta)55-TM4</td>
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<td>9</td>
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<td>α1-trc + 3-4loop</td>
<td>10</td>
<td>0 ± 0</td>
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HEK293 cells expressing different GlyR variants were patched 24 h post-transfection. Maximal currents (I\(_{\text{max}}\)) ± SEM (= standard error of the mean) gated with 1 mM glycine were recorded in 9-10 cells out of at least three batches of transfected HEK293 cells. * Only 2 out of 10 cells responded to glycine application, I\(_{\text{max}}\) value in this case includes also non-responding cells.
## FIGURES

### Figure 1

**Fig. 1**

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Figure 2

Fig. 2

A

wt GlyR α1  

two domain configuration  

three domain configuration

B

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|    | PAC |    |    |    |    |      |            |
|    | AXD |    |    |    |    |      |            |
|    | RPP |    |    |    |    |      |            |
|    | ARI |    |    |    |    |      |            |

|    | LNL |    |    |    |    |      |            |

337 HKETLQNYYKQLLDKLIRKLFYLFQDIELGNYK 457
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447 DDDEEEDSETRLNYKYYKQLLDKLIRKLFYLFQDIELGNYK
457 DDDEEEDSETRLNYKYYKQLLDKLIRKLFYLFQDIELGNYK
Figure 3

Fig. 3

A.

B.
Figure 4

**A**

Gly

α1-trc + myc-iDΔ67-TM4
α1-trc + myc-iDΔ62-TM4
α1-trc + myc-iDΔ55-TM4
α1-trc + myc-iDΔ49-TM4
α1-trc + myc-iDΔ43-TM4
α1-trc + myc-iDΔ27-TM4
α1-trc + myc-iDΔ22-TM4
α1-trc + myc-iD-TM4

1 nA

2 s

**B**

![Bar graph showing normalized current (I_{norm})](image)

Examples:

- α1-trc + i
- myc-iDΔ67-TM4
- myc-iDΔ62-TM4
- myc-iDΔ55-TM4
- myc-iDΔ49-TM4
- myc-iDΔ43-TM4
- myc-iDΔ27-TM4
- myc-iDΔ22-TM4
- myc-iD-TM4

Legend:

- TM3-4loop
- TM2-4loop
- TM2-4loop +
Figure 5

**Fig. 5**

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<td>+</td>
<td>+</td>
<td>α1-trc</td>
</tr>
</tbody>
</table>

**A**

- 46kDa
- 23kDa
- 7kDa

Input

- myc-iD-TM4
- α1-trc

**B**

- 46kDa
- 23kDa
- 7kDa

IP

- IgG
- α1-trc
- IgG
- myc-iD-TM4

α-myc / MAb4a
Fig. 6

\[ \text{a1-trc + myc-iD-TM4} \]

\[ \text{MAb2b} \quad \alpha\text{-myc} \quad +\text{Triton} \quad \alpha\text{-myc} \]

\[ \text{a1-trc + myc-iDΔ62-TM4} \]

\[ \text{MAb2b} \quad \alpha\text{-myc} \quad +\text{Triton} \quad \alpha\text{-myc} \]

\[ \text{controls: a1-trc+iDΔ62-TM4} \quad \alpha1+\text{myc-β} \]

\[ \text{MAb2b} \quad +\text{Triton} \quad \alpha\text{-myc} \]
Fig. 7

A  α1-trc + myc-iDΔ62-TM4 + TM3-4loop

<table>
<thead>
<tr>
<th></th>
<th>α1-trc</th>
<th>TM3-4loop</th>
<th>myc-iDΔ62-TM4</th>
</tr>
</thead>
<tbody>
<tr>
<td>myc-iDΔ62-TM4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α1-trc</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TM3-4loop</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B

17 kD
myc-TM3-4loop →
myc-iDΔ62-TM4

7 kD

<table>
<thead>
<tr>
<th></th>
<th>α1-trc</th>
<th>iDΔ62-TM4</th>
<th>myc-iDΔ62-TM4</th>
<th>myc-TM3-4loop</th>
<th>TM3-4loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-trc</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>iDΔ62-TM4</td>
<td>+</td>
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<td></td>
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</tr>
<tr>
<td>myc-iDΔ62-TM4</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>myc-TM3-4loop</td>
<td>+</td>
<td>+</td>
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<td></td>
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<tr>
<td>TM3-4loop</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

Gly

α1-trc + TM3-4loop
α1-trc + myc-iDΔ62-TM4

0.5 nA

2s

α1-trc + myc-iD-TM4
Fig. 8

A. Input

B. IP

+ + +

+ + +

46kDa

23kDa

7kDa

α-myc / MAb4a

+ +

+ +

+ +

myc-iΔ62-TM4

+ + +

+ + +

α1-trc

IgG

α1-trc

IgG

myc-TM3-4loop

myc-iΔ62-TM4
The importance of TM3-4 loop subdomains for functional reconstitution of glycine receptors by independent domains
Bea Unterer, Cord-Michael Becker and Carmen Villmann

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