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Probing the Topology of the Glutamate Receptor GluR1 Subunit Using Epitope-Tag Insertions

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At least two different models for the transmembrane topology of the glutamate receptor subunits have been proposed. We investigated some features of these two models for the GluR1 subunit by inserting epitope tags between residues Lys^{502}-Pro^{503}, Ala^{632}-Glu^{633}, Lys^{712}-Pro^{713}, or after the C-terminal residue Leu^{889}. The accessibility of the tags then was detected using a tag-specific antibody before and after detergent-permeabilizing oocytes expressing the tagged subunits. The epitope tag inserted between residues Lys^{712}-Pro^{713} is extracellular and after Leu^{889} intracellular. Epitope tags inserted between residues Lys^{502}-Pro^{503} and residues Ala^{632}-Glu^{633} were not detectable. Collectively, these results provide supporting evidence for a previously proposed topological model of GluR subunits containing an N-terminal extracellular domain, three transmembrane domains, the first two of which are bridged by a reentrant membrane pore-lining loop, and an intracellular C-terminal domain. © 2000 Academic Press

Key Words: **epitope tag; oocyte; glutamate receptor; GluR1; topology; transmembrane domain.**

Hydropathic analysis of the first cloned glutamate receptor subunit GluR1 (1) suggested that it had a topology similar to that of nicotinic acetylcholine receptor (AChR) subunits with an extracellular N-terminal domain followed by three transmembrane domains, a large cytoplasmic intracellular domain, a fourth transmembrane domain, and an extracellular C-terminus. However, in recent years several investigators have examined the transmembrane topology of GluR subunits (2–4). One strategy involved introducing N-linked glycosylation sites at various positions within the GluR subunits and examining if these sites were glycosylated. A second strategy involved monitoring the ability of microsomal membranes to protect epitope tags engineered within different truncated GluR subunits from externally added proteases after they were expressed *in vitro* using reticulocyte cell-free translation systems supplemented with pancreatic microsomes (2). Both these strategies gave results that were consistent with the GluR1 subunit having an N-terminal extracellular domain, three transmembrane domains, the first two of which are bridged by a reentrant membrane pore-lining loop, and an intracellular C-terminal domain (2, 3).

The above-mentioned elegant experimental strategies provided the first solid experimental evidence for the current topological model for GluR subunits. However, because N-linked glycosylation of a cytoplasmic regions of the Na/K ATPase has been reported (5, 6) and because a membrane domain can insert into the lipid bilayer following full assembly of subunits or have a dynamic topology (7), inferring the GluR subunit topology solely based on indirect methods that involved either truncated subunits that were incapable of assembling into functional receptors or that relied on glycosylation of subunits at engineered ectopic N-linked glycosylation sites may not be completely reliable. Moreover, these methods have suggested a GluR topology that is still in conflict with earlier experimental findings that suggested that a region of the GluR6 subunit now considered to be the second discontinuous extracellular domain was intracellular because of the apparent phosphorylation of serine residues within this domain (8, 9).

In this paper, the topology of the GluR1 subunit was examined using a method that directly measures the location of inserted epitope tags. Epitope tags were engineered at four sites within the GluR1 subunit and the tagged subunits expressed in *Xenopus* oocytes. The accessibility of the epitope tags was then assessed with a tag-specific radiolabeled antibody, before and after permeabilizing the oocyte membrane with Triton X-100. The results obtained are consistent with the new model proposed for the topology of GluR subunits (Fig. 1). The results obtained also support the use of protease-protection assays or ectopic N-linked glyco-

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sylation sites as suitable alternate probes of membrane protein topology.

MATERIAL AND METHODS

Constructs. All site-directed mutagenesis was performed using the Altered Sites II *in vitro* Mutagenesis System (Promega, Madison, WI). *Xba*I restriction enzyme sites were introduced into the GluR1 subunit clone by site-directed mutagenesis and then double-stranded synthetic DNA casettes encoding the epitope tags were ligated into the engineered *Xba*I sites. The amino acid sequence SSQVTGEVIFQTPLIKNPSS corresponding to the epitope tag was inserted between residues Lys^{502}-Pro^{503} ($GluR1^{t503}$), Ala^{632}-Glu^{633} ($GluR1^{t632}$), Lys^{712}-Pro^{713} ($GluR1^{t712}$), or after the C-terminal residue Leu^{889} ($GluR1^{t889}$) of the GluR1 subunit. The DNA sequence integrity of the constructs was verified by standard dideoxy sequencing.

Expression of epitope-tagged GluR1 subunits in oocytes. cRNAs from linearized cDNA templates were synthesized *in vitro* using SP6 RNA polymerase in conjunction with reagents from the mMessage mMachine Kit (Ambion, Austin, TX). Oocytes were prepared for injection as previously described (10). Oocytes were injected with 50–100 ng of RNA per oocyte and incubated at 18°C in ND-96 solution (in mM): 96 NaCl; 2 KCl; 1 $MgCl_2$; 1.8 $CaCl_2$; 5 *N*-[2-hydroxyethyl]piperazine-N′-2-ethane sulfonic acid (HEPES); pH 7.6.

Solubilization of GluR proteins. Oocytes were homogenized using a microfuge tube plastic pestle in buffer A (in mM): 50 Na_2HPO_4-NaH_2PO_4, pH 7.5, 50 NaCl, 5 EDTA, 5 EGTA, 5 benzamidine, 15 iodoacetamide, 2 phenylmethylsulfonyl fluoride. The homogenized membranes were collected by centrifugation in a microfuge at 15, 000 rpm for 25 min. Receptors were solubilized by gentle agitation of oocyte membranes in buffer A containing 2% Triton X-100 at 4°C for 1 h. After removing cellular debris by centrifugation at 15, 000 rpm for 25 min, the cleared extracts were used in all experiments.

Immunopurifications. Triton X-100-solubilized oocyte membrane extracts (50 μl) were treated with protein sample buffer and fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were electroblotted onto polyvinylidene difluoride membrane (IMMUN-BLOT; Bio-Rad Laboratories, Hercules, CA).

Immunoblotting. Direct detection of tagged GluR1 proteins was performed by incubating the membranes with 20 nM ^{125}I-mAb 142 in phosphate-buffered saline solution containing 0.1% Tween and 5% non-fat milk powder. Following three successive washings of the membranes in PBS-0.1% Tween, the membranes were exposed to X-ray film.

Surface binding assays. Oocytes (10 oocytes/200 ml) were incubated with 20 nM ^{125}I-mAb 142 in ND-96 containing 10% heat inactivated horse serum, washed five times with 1 ml ND-96 and counted in a γ counter.

Solubilized receptor assays. Triton X-100 solubilized receptors were incubated (100 μl/well) overnight on Immulon 4 (Dynatech) wells coated with mAb 142 as previously described (11). The wells were then washed three times with PBS containing 0.05% Triton and then incubated with 20 nM [^{3}H]α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (Amersham, 49.3 Ci/mmol) for 1 h at 4°C in the presence of 100 mM NaSCN. The wells were then washed with ice-cold PBS-0.05% Triton solution and the bound radioactivity measured by scintillation counting.

Oocyte electrophysiology. Currents were measured using a standard two-microelectrode voltage clamp amplifier (oocyte clamp OC-725; Warner Instrument Corp., Hamden, CT) as previously described (12). Electrodes were filled with 3 M KCl and had resistances of 0.5–1.0 MΩ for the current electrode and 1–2 MΩ for the voltage electrode. All records were digitized (MacLab/2e interface; AD Instruments) and analyzed using KALEIDAGRAPH (Synergy Software, Reading, PA). The dose-response curves were fit to the Hill equation.

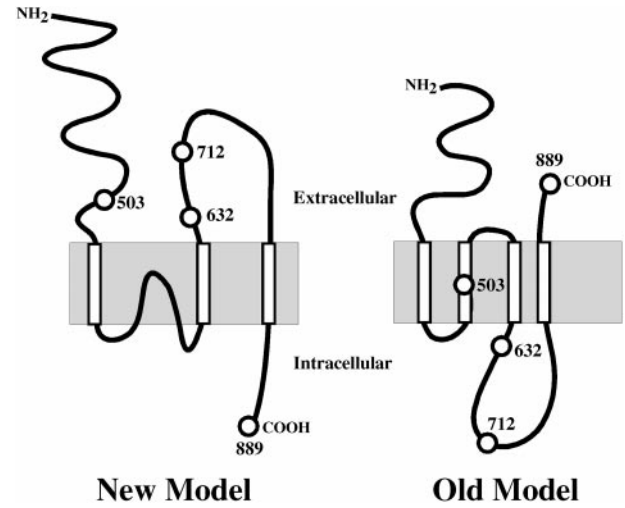

FIG. 1. Topological model of GluR1 subunit with epitope insertion sites. Shown are the proposed "new" and "old" models for the topology of the GluR1 subunit. The open circles indicate the predicted intracellular or extracellular location of the inserted epitope with respect to the membrane in the two models. The numbers correspond to the amino acid after which the epitope was inserted.

RESULTS

Expression of Epitope-Tagged GluR1 in Xenopus Oocytes

GluR1 subunits containing a 20 amino acid epitope tag for mAb 142 were inserted between residues Lys^{502}-Pro^{503}($GluR1^{t502}$), Ala^{632}-Glu^{633}($GluR1^{t632}$), Lys^{712}-Pro^{713} ($GluR1^{t712}$), or after residue Leu^{889} ($GluR1^{t889}$), three insertion sites of which would be extracellular and one intracellular based on the new topological model proposed for the GluR1 subunit (Fig. 1). These subunits were expressed from *in vitro* transcribed cRNAs microinjected into oocytes. Triton X-100 solubilized membrane proteins isolated from oocytes expressing the wild-type GluR1 ($GluR1^{wt}$), $GluR1^{t503}$, $GluR1^{t632}$, $GluR1^{t712}$, and $GluR1^{t889}$ were fractionated by SDS–PAGE and membranes immunoblotted with ^{125}I-mAb 142 against the epitope tag. All subunits were expressed in oocytes (Fig. 2) indicating that the inserted epitope tag did not grossly affect the expression of the engineered subunit. Additionally, the blot also indicated that mAb 142 did not cross-react with either the $GluR1^{wt}$ subunit or with other oocyte proteins and thus was suitable for further studies.

Accessibility of Epitope Tags Inserted within the GluR1 Subunit

To determine whether the inserted epitope tags were accessible from either side of the membrane and

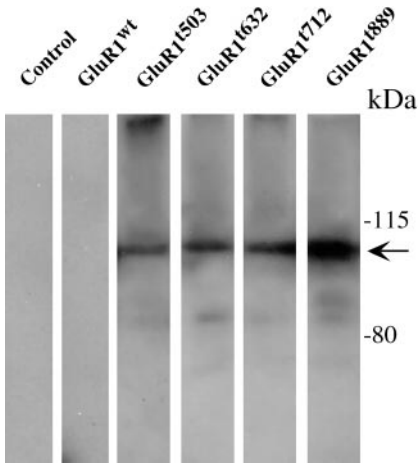

FIG. 2. Expression of epitope-tagged GluR1 subunits in oocytes. Triton X-100 solubilized membrane proteins isolated from oocytes expressing GluR1, GluR1^{t503}, GluR1^{t632}, GluR1^{t712}, and GluR1^{t889} were fractionated by SDS–PAGE and membranes immunoblotted with ^{125}I-mAb 142 against the epitope tag.

whether they interfered with ligand-binding, binding of mAb 142 to Triton X-100 solubilized GluR1 receptors expressed in oocytes was detected by monitoring [^{3}H]AMPA binding to receptors tethered through their tags to plastic wells coated with mAb 142. Extracts from oocytes expressing GluR1^{t502}, GluR1^{t632}, GluR1^{t712}, GluR1^{t889}, and wild-type GluR1 subunits (GluR1^{wt}) were tested. [^{3}H]AMPA binding to GluR1^{t889} receptors was detected (Fig. 3). No [^{3}H]AMPA binding was observed to the GluR1^{wt} receptor in the control experiment, because it lacked the epitope tag needed to immobilize it on mAb 142 wells. No [^{3}H]AMPA binding to GluR1^{t502} or GluR1^{t632} was detected indicating that the inserted epitopes either were inaccessible, or had interfered with assembly of the subunits or AMPA binding.

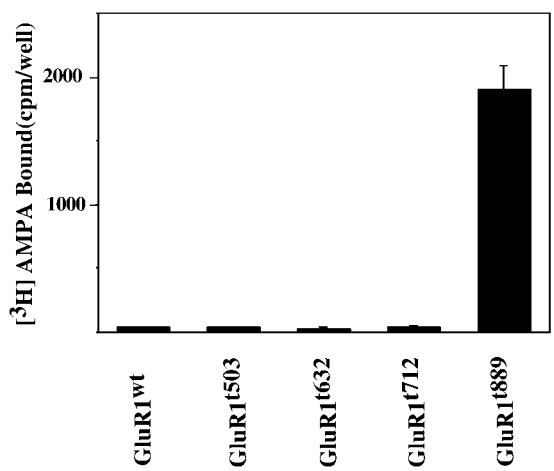

FIG. 3. Binding of [^{3}H] AMPA to detergent-solubilized epitope-tagged GluRs. Triton X-100 solubilized receptors were incubated (100 μl/well) overnight on Immulon 4 wells coated with mAb 142. Binding of [^{3}H] AMPA (20 nM) for 1 h at 4°C in the presence of 100 mM NaSCN to tethered detergent-solubilized epitope-tagged GluRs was measured in duplicate. Non-specific binding corresponds to the binding observed in wells containing extracts from oocytes expressing the GluR1^{wt} subunit. Error bars correspond to the standard error from duplicate measurements.

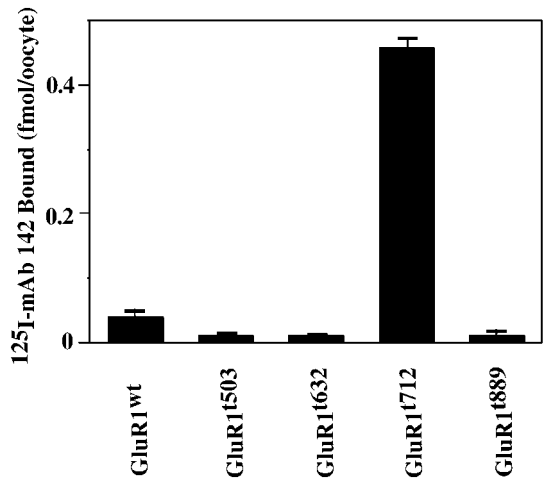

FIG. 4. Binding of ^{125}I-mAb 142 to oocytes expressing epitope-tagged GluR1 subunits. Binding of ^{125}I-mAb 142 (20 nM) to whole oocytes (10 oocytes/200 ml) was measured after a 2 h incubation. Non-specific binding corresponds to the binding observed to oocytes expressing the GluR^{wt} subunit. Error bars correspond to the standard error of measurements made from 10 oocytes.

Extracellular Location of Epitope Tag Inserted after Residue 712 of the GluR1 Subunit

To determine whether the inserted epitope tags were accessible from the extracellular side of the plasmalemma membrane, binding of ^{125}I-mAb 142 to pools of whole oocytes expressing GluR1^{t502}, GluR1^{t632}, GluR1^{t712}, GluR1^{t889}, and GluR1^{wt} subunits (as a control), was measured. ^{125}I-mAb 142 binding was detected only to oocytes expressing the GluR1^{t712} subunit (Fig. 4). This result demonstrates that the site of insertion of the tag after residue 712 is located on the extracellular surface of the plasmalemma membrane. Since cell surface receptors were detected with GluR1^{t712} receptors, the loss of [^{3}H]AMPA binding was most likely because the epitope tag was located within the ligand-binding site. The failure to detect binding on oocytes expressing the other GluR1 subunits suggested that the epitope tags were either inaccessible, intracellular, or had interfered with assembly or transport of the subunits to the cell surface membrane.

Functional Characterization of Epitope-Tagged GluR1 Homomers

To assess the ability of GluR1 subunits containing inserted epitope tags to form functional ion channels,

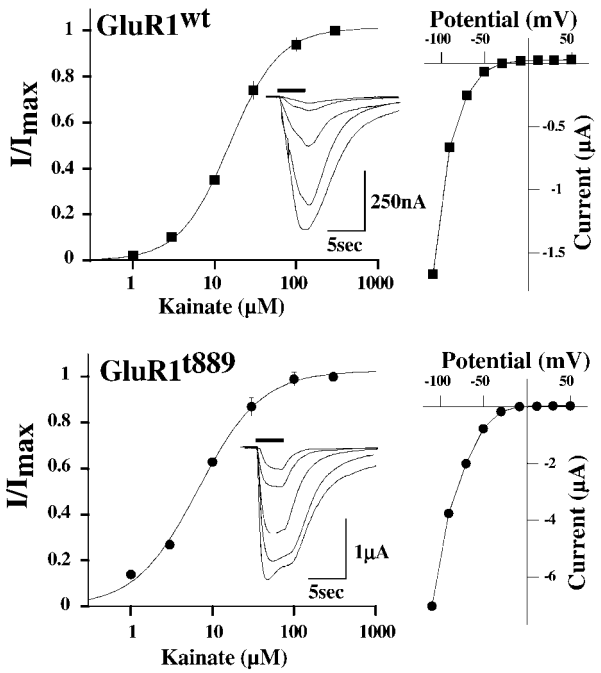

FIG. 5. Functional properties of epitope-tagged GluR receptors in oocytes. The kainate-induced currents traces obtained by 2 s application of different concentrations of the agonist are shown and were measured from oocytes clamped at a holding potential of −70 mV using a standard two-microelectrode voltage clamp set up. The maximum currents induced by 2 s application of 100 μM kainate at different holding potentials from −100 mV to +50 mV were also measured. The dose-response curves and the current-voltage relationship from oocytes expressing the GluR1^{wt} and GluR1^{t889} subunits are shown. The dose-response curve was fit to a Hill equation. The error bars on the dose-response curve corresponds to the standard error of measurements made from 2–3 oocytes.

currents elicited by application of varying concentrations of kainate and their current-voltage relationship at different holding potentials were measured by standard voltage-clamp experiments from oocytes expressing GluRs. Both the GluR1^{wt} and GluR1^{t889} subunits formed fully functional channels (Fig. 5). However, no currents were detected from oocytes expressing GluR1^{t502}, GluR1^{t632}, or GluR1^{t712} (data not shown). The loss of function for surface receptors formed from the GluR1^{t712} subunit is consistent with the interpretation that the inserted epitope had interfered with agonist binding rather than with subunit assembly or transport of the receptor to the cell surface membrane.

DISCUSSION

The topology originally proposed for the GluR1 subunit (1) on the basis of hydropathic analysis of its amino acid sequence was very similar to that of AChR subunits. However, glutamate receptor subunits show little sequence homology to subunits of the superfamily of ion channels gated by acetylcholine, glycine, GABA, or serotonin (13). Thus, the proposed topology reflected the undue influence of AChRs models on the topology of the GluR1 subunit. Since then, experimental evidence suggests that the GluR receptor subunits probably evolved from bacterial glutamate-binding proteins (14) with a topology that is very different from that of AChR subunits (2–4). While the bulk of the experimental evidence supports a different topological model for the GluR1 subunit from that of AChR subunit, at least one experimental finding remains controversial (8, 9). Hence, an independent strategy using detection of inserted epitope tags was used to test some aspects of this new model.

Insertion of epitope tags at all five positions did not appear to interfere with the synthesis of the GluR1 subunits as judged by immunoblots. Both GluR1^{t889} and GluR1^{t712} formed surface receptors and the epitope tag inserted after residue 712 was accessible from the extracellular surface of the oocyte. The fact that the GluR1^{t712} subunit was detected on the surface membrane indicated that the epitope at this locations did not interfere with transport of the GluR1 subunit. This result provides supportive evidence for an extracellular location of residues surrounding Lys^{712}. This result also lends further support to earlier explanations by other investigators that this region is extracellular and that the phosphorylation of serine residues within this region (8, 9) may possibly represent novel extracellular phosphorylation by an ectokinase. However, GluR1^{t712} receptors failed to bind [^{3}H]AMPA suggesting that residue 712 was located within the agonist-binding site. This interpretation is consistent with the findings of other investigators (15, 16) and the high resolution crystal structure of a monomeric S1-S2 fusion protein derived from the GluR4 (17). The detection of the epitope inserted in GluR1^{t889} only after Triton X-100 solubilization is consistent with the suggested intracellular location of its C-terminus. The tolerance of the epitope at this location suggests that the epitope did not interfere with folding, assembly, or transport of GluR1 subunits. The recent identification of several proteins that interact with the C-terminus of GluR subunits suggests that it helps anchor GluRs to scaffolding proteins (18). Both GluR1^{t502} and GluR1^{t632} subunits did not yield useful topological information probably because the sequences surrounding the sites of insertions either occluded mAb access to the inserted epitope, or the epitope interfered with proper subunit folding, assembly, or ligand-binding.

In conclusion, our main findings regarding the topology of the GluR subunits are consistent with the new model proposed for GluR subunits. We have provided further experimental evidence for some features of this model and our results provide further support the use of the other methods discussed earlier as being suitable for determining the topology of other membrane proteins.

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