Assembly and Ligand Binding Properties of the Water-Soluble Extracellular Domains of the Glutamate Receptor GluR1 Subunit

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SUMMARY

High-resolution structural studies of models of glutamate receptors (GluRs) have been limited to monomeric models of the ligand-binding site. To obtain oligomeric models of glutamate receptors that can reveal more complete structural information, we examined the assembly and ligand-binding properties of two truncated versions of the GluR1 subunit. The first version, GluR1-WS, consisted of only the N-terminal extracellular segment (Ala<sup>1</sup>-Glu<sup>520</sup>) bridged by a synthetic linker to the second extracellular domain (Asn<sup>615</sup>-Gly<sup>790</sup>). The second version, GluR1-M1, consisted of the first N-terminal extracellular domain (Ala<sup>1</sup>-Glu<sup>520</sup>) bridged by a synthetic linker to a second segment containing the second extracellular domain, the third transmembrane domain, and the intracellular C-terminal domain (Asn<sup>615</sup>-Leu<sup>889</sup>). When expressed in *Xenopus* oocytes, GluR-WS was secreted and water-soluble; GluR1-M1 was displayed on the surface of oocytes. GluR1-WS exhibited a velocity sedimentation profile that was consistent with assembly of homooligomers and bound the glutamate receptor agonist α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) with high-affinity. These findings show that the extracellular domains of GluR1 that are sufficient for ligand-binding, apparently are sufficient for subunit assembly, and might be a suitable target for structural studies of a water-soluble GluR1 oligomer.
INTRODUCTION

Ionotropic glutamate receptors (GluRs)\(^1\) mediate fast synaptic excitatory neurotransmission in the nervous system (1). Their biochemical, pharmacological, and physiological properties have been extensively studied (2-5) because they are important therapeutic targets for the treatment of many neurological disorders (6). The rational design of drugs that selectively target different subtypes of glutamate receptors critically depends on high-resolution structural studies. As membrane proteins, however, they belong to a general class of proteins for which there is a paucity of high-resolution X-ray crystallographic information. Structural studies of membrane proteins, in contrast to those of soluble proteins, have been hampered by numerous hurdles including those associated with obtaining large quantities of recombinant membrane proteins through expression in heterologous expression systems and those associated with the deleterious effects of detergents on the formation of crystals suitable for X-crystallography.

To circumvent some of these problems, water-soluble extracellular domains of many membrane proteins have been processed for crystallographic studies (7-15). Neurotransmitter-gated ion channels, however, represent a greater challenge for this strategy than monomeric single transmembrane domain membrane proteins because of the contribution of residues from multiple subunits to their pharmacological properties.

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\(^1\) The abbreviations used are: AChR, nicotinic acetylcholine receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; αBgt, α-bungarotoxin; Bmax, maximum binding sites; C, measured signal; C\(_o\), maximal signal; cpm, counts per minute; GluR, glutamate receptor; GPI, glycosylphosphatidylinositol; HEPES, N-[2-hydroxyethyl]piperazine-N'-2-ethane sulfonic acid; HRP, horseradish peroxidase; \(k_d\), equilibrium dissociation constant; L, ligand concentration; mAb, monoclonal antibody; n, Hill
Thus, establishing which domains of their subunits are required for assembly and assessing whether they are capable of forming water-soluble species with ligand-binding properties that mimic those of native receptors are essential intermediate goals towards establishing suitable models of neurotransmitter-gated ion channels for long-term structural studies.

In recent years it has become clear that for GluRs, two discontinuous extracellular domains of GluR subunits contribute to the formation of their ligand-binding site (16). In elegant work done since then, it has been shown that a fusion protein consisting of two short segments termed S1 and S2, one from each of these two discontinuous extracellular domains when bridged by an artificial linker, forms a monomer that is capable of binding GluR ligands (17,18). The crystal structure of a S1-S2 monomer derived from the GluR2 subunit has also been recently obtained (19). However, full-length glutamate receptors are thought to exist as either tetramers (20-22) or pentamers (23-25) in which neighboring subunits influence their ligand-binding properties (26,27). Thus a more complete understanding of the contributions of neighboring subunits and other regions of GluR subunits to their over all structure and pharmacological properties is desirable. Recently, the feasibility of obtaining extracellular domains of ligand-gated ion channels that assemble into an oligomer whose size and pharmacological properties are consistent with the formation of water-soluble receptors for the AChR α7 subunit was demonstrated (28).
Towards achieving a similar goal for GluR subunits, we have examined the assembly and ligand-binding properties of two truncated GluR1 subunits GluR1-WS and GluR-M1. Based on its velocity sedimentation profile on sucrose gradient, high-binding affinity for α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and competition for assembly with the full-length GluR1 subunit, we conclude that GluR1-WS, like the previously crystallized S1-S2 GluR4 monomeric protein, not only retains the ability to bind glutamatergic ligands but also possibly assembles into specific oligomers because of the inclusion of the N-terminal portion of the GluR1 subunit. Thus, our results suggest that N-terminal portions of the extracellular domains not included in the crystallized monomeric S1-S2 fusion protein, are important for assembly of GluR subunits and might be important for oligomeric structural models of GluRs.
EXPERIMENTAL PROCEDURES

Design of GluR1-142, GluR1-236, GluR1-M1, and GluR1-WS constructs. All site-directed mutagenesis was performed using the Altered Sites II in vitro Mutagenesis System (Promega, Madison, WI). The amino acid numbering system used refers to that of the mature GluR1 polypeptide (29). To tag the GluR1 subunit at its C-terminus, an Xba I restriction enzyme site was introduced into the GluR1 subunit cDNA by site-directed mutagenesis after the C-terminal residue 889 and then double-stranded synthetic DNA cassettes encoding epitope tags were ligated into the engineered Xba I sites. Two different epitopes and mAbs were used in this study and both correspond to well-characterized epitopes derived from the sequence of the Torpedo AChR α subunit (30). The sequence shown in bold within an epitope corresponds to the minimal epitope sequence necessary to bind the mAbs. The underlined sequences correspond to the respective epitopes. The amino acid sequence SSQVTGEVFQTPLIKNPSS corresponding to the insert containing the epitope (for mAb 142) was used to create the C-terminal tagged GluR1-142 subunit. The amino acid sequence SYSISPESDRPDLSTF VSISPESDRPDLSTFL corresponding to the insert containing two tandem epitopes (for mAb 236) was used to create the C-terminal tagged GluR1-236 subunit. The truncated epitope tagged GluR1 subunits were created using a similar strategy. GluR1-M1 was constructed by inserting a double-stranded synthetic DNA cassette corresponding to the amino acid sequence SSVSISPESDRPDLSTFSR between the Bgl II site immediately preceding the putative first TMD of the GluR1 subunit and the Bgl I site immediately after the putative last TMD of the GluR1-142 subunit. GluR1-WS was constructed by
inserting a double-stranded synthetic DNA cassette encoding the amino acid sequence LSLSNVAG* between the Hae II site preceding the last putative TMD of the GluR1-M1 subunit and the EcoRI cloning site, thus eliminating the putative last TMD. The asterisk represents the absence of an amino acid due to the introduced stop codon.

**GluR protein expression in Xenopus oocytes.** cRNA from linearized cDNA templates was 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 (31). 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 HEPES; pH 7.6, for 2-3 days prior to harvesting them.

**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 (100-200 µl) were incubated with approximately 10 µl of mAb-coupled Actigel ALD bead (0.5mg/ml)
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at 4°C using gentle agitation overnight. The beads were then washed five times with 1ml of solubilization buffer and eluted with sample buffer (lacking β-mercaptoethanol to avoid reduction of the disulphide linkage of the IgG chains) at 60°C for 30 min. and then β-mercaptoethanol was added to the eluted sample prior to analysis by SDS-PAGE. The proteins were electroblotted onto polyvinylidene difluoride membrane (IMMUN-BLOT; Bio-Rad Laboratories, Hercules, CA).

**Immunoblotting.** Where indicated, direct detection of the protein was performed by incubating the membranes with 20nM $^{125}$I-mAb 142 or $^{125}$I-mAb 236 in phosphate-buffered saline solution containing 0.1% Tween and 5% non-fat milk powder. In some cases, membranes were incubated with 20nM primary mAbs in phosphate-buffered saline solution containing 0.1% Tween and 5% non-fat milk powder and the binding of the primary mAbs was detected using goat anti-rat secondary Abs conjugated to horseradish peroxidase in conjunction with a chemiluminescence detection kit (SuperSignal, Pierce, Rockford, IL) after washing off the unbound primary mAb.

**Surface binding assays.** Oocytes (10 oocytes/200µl) were incubated with 20nM $^{125}$I-mAb 142 (Sp. Act. $\approx 10^{18}$ cpm/mol) in ND-96 containing 10% heat inactivated horse serum, washed five times with 1ml ND-96 and counted in a γ counter.

**Sucrose gradient sedimentation.** 200 µl of Triton X-100 solubilized membrane proteins from 10-20 oocytes expressing the various GluR1 proteins were layered onto 5-ml sucrose gradients (5-20% (w/v)) in 0.5% Triton solution containing (in mM): 100 NaCl,
10 sodium phosphate, 5 EGTA, 5 EDTA, and 1 NaNO₃, at pH 7.5. The gradients were centrifuged 50 min at 40,000 rpm at 4°C in a Beckman NVT90 rotor. Aliquots of 11 drops (approximately 130 µl) from the gradients were collected from the bottom of the tubes into Immulon 4 plastic microwells coated with the appropriate mAb. The entire gradient was collected in 40 fractions. After gentle agitation of the microwells for 24 h at 4°C, the microwells were washed and incubated with either 2 nM ¹²⁵I-mAb 142 or 2 nM ¹²⁵I-mAb 236 for 24 h, and following washing, the presence of immobilized protein detected by γ counting. For the GluR1-WS protein, the immunoisolated protein was fractionated by SDS-PAGE, and detected by immunoblotting in conjunction with a chemiluminescence kit (SuperSignal, Pierce, Rockford, IL). A non-saturating exposure of the chemiluminescent bands captured on X-ray film was scanned using the Gel Doc 1000 system and the digitized bands quantified using the Multi-Analyst software program (Bio-Rad, Hercules, California). To calibrate the sucrose gradient, approximately 200 µg of each of bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and apoferritin (443 kDa) were also sedimented on the sucrose gradients. The sedimentation of each of these proteins on the gradients was detected using a colorimetric assay in conjunction with the Dc protein assay kit (Bio-Rad, Hercules, Inc). The optical density of the colorimetric reaction was measured in microwells using the MR700 microtiter plate reader (Dyntect Laboratories, Chantilly, VA).

**Ligand-binding assays.** 2% Triton X-100 solubilized membrane proteins from oocytes expressing the GluR proteins were incubated (100 µl/well) overnight on Immulon 4 (Dyntect) wells coated with mAb 142 or mAb 236 as previously described (30). The
wells were washed three times with PBS containing 0.05% Triton and incubated with various concentrations of \[^{3}\text{H}]\text{AMPA}\ (49.3\ \text{Ci/mmol, Amersham})\) for 1 hr 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. Non-specific binding was measured with Triton X-100 membrane extracts of uninjected oocytes. The equilibrium dissociation constant \(K_d\) for the \[^{3}\text{H}]\text{AMPA}\ binding was determined by least-squares, non-linear fitting to a Hill-type equation \(C=C_0/(1+(K_d/L)^n)\), where \(C\) is the measured signal (cpm), \(C_0\) is the maximal signal, \(L\) is the concentration of \[^{3}\text{H}]\text{AMPA}\,\) and \(n\) is the Hill coefficient.
RESULTS

Expression of GluR1 proteins in Xenopus oocytes.

Schematic representations of the GluR1 subunit constructs used in this study are shown in Figure 1. GluR1-142 represents the full-length GluR1 subunit tagged with an epitope for mAb 142 and GluR1-236 represents the full-length GluR1 subunit tagged with an epitope for mAb 236. Both mAbs are against well-defined epitopes derived from the Torpedo AChR α subunit and have been previously described (32). They have also been shown to be topogenically neutral when introduced into the human AChR α1 subunit (30) and the GluR1 subunit (33). The GluR1-M1 subunit was derived from the GluR1-142 subunit by linking the entire first extracellular domain (Ala\(^1\) to Glu\(^{520}\)) of the GluR1 subunit to the second extracellular domain (Asn\(^{615}\) to Leu\(^{889}\)) via a 32 amino acid linker sequence containing epitopes for mAb 236, thus eliminating its first TMD, the reentrant loop and the second TMD but retaining the last TMD. The GluR1-WS is derived from GluR1-M1 and truncates at Gly\(^{790}\) thus eliminating the putative third TMD and the intracellular C-terminal domain.

These constructs were created to evaluate the necessity of different regions of the GluR1 subunit for subunit assembly and ligand binding. GluR1-WS would allow us to test whether the extracellular domains of the GluR1 subunit alone is sufficient for subunit assembly. The hyphenated "WS" of GluR1-WS refers to the expectation that this construct would be expected to be water-soluble. GluR1-M1 would allow us to evaluate
whether the presence of a single TMD was sufficient for oligomerization of GluR1 subunits. The expression levels of each of these subunits was checked by immunoblot analysis of Triton X-100 extracts from oocytes expressing the tagged subunit using either $^{125}$I mAb 142 or $^{125}$I mAb 236 as appropriate. The expression of the truncated subunits GluR1-WS and GluR1-M1 were expressed at approximately the same level (Figure 2) indicating that deletions of portions of the subunit did not grossly affect their stability in oocytes.

*GluR1-WS is a secreted water-soluble protein and GluR1-M1 is membrane-bound.*

The topology of GluR subunits has been extensively investigated (34-36). They are proposed to have an extracellular N-terminal domain, followed by a TMD, a membrane reentrant loop, a second TMD, a second extracellular domain, a third TMD and a C-terminal domain. Based on this topology, GluR1-WS was expected to be devoid of TMDs, and GluR1-M1 was expected to contain one TMD. Thus, we tested whether GluR1-WS would form a water-soluble secreted protein and whether GluR1-M1 as predicted would be expressed as a membrane-bound protein on the surface membrane of oocytes. The expression of GluR1-WS in the aqueous (secreted) and Triton X-100 solubilized fractions of oocyte proteins was monitored by immunoblot analysis. To eliminate the possibility that proteins in the aqueous media originated from cellular debris from unhealthy microinjected oocytes, care was taken to collect aqueous media only from oocytes that were deemed healthy by microscopic examination following an incubation period of 24 h. The surface expression of GluR1-M1 was monitored by the binding of
$^{125}$I-mAb 236 to whole oocytes expressing GluR1-M1. We found that GluR1-WS was secreted as a water-soluble protein in the aqueous media (Figure 3A) and that GluR1-M1 was expressed on the surface membranes of oocytes (Figure 3B).

The amount of secreted water-soluble protein was found to be at least 10-fold lower than the amount released after homogenization of the membranes and at least 50-fold lower than the amount solubilized by Triton X-100 as judged by the relative intensity of the immunoreactive bands. Two possible reasons could account for the relative difference in the yields of water-soluble GluR1-WS versus Triton-soluble GluR1-WS. The first possibility is that the detergent was required to release the GluR1-WS from membrane association that arose through hydrophobic patches of the protein that were left exposed by incomplete assembly of the truncated subunits. A second possibility is that the detergent was required to release the bulk of the water-soluble, non-membrane associated, GluR1-WS that was trapped inside membrane vesicles. Because the overall yield of protein expression in oocytes was low, we found it necessary to use Triton-solubilized proteins for all our further studies.

*Sedimentation profile of GluR1-WS suggests the formation of homooligomeric GluR species.*

To determine whether the truncated GluRs were capable of oligomerizing, we examined the velocity sedimentation profiles of Triton X-100 solubilized proteins from oocytes expressing GluR1-WS or GluR1-M1 on 5-20% (w/v) sucrose gradients. To
enable us to follow the sedimentation profiles of these protein species across the gradient, we used either $^{125}$I-mAbs or chemiluminescent assays to detect them. We followed the sedimentation profile of GluR1-WS across the gradient by first immobilizing protein species using mAb 236 followed by fractionation of the immobilized protein by SDS-PAGE and detection of the tagged GluR1-WS protein by immunoblotting using mAb 236. The sedimentation of four proteins of known molecular masses, bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), $\beta$-amylase (200 kDa), and apoferritin (433 kDa), was used to calibrate the gradient. The smaller fraction numbers correspond to the first fractions collected from the bottom of the gradient tube (i.e., faster sedimentation) and the larger fractions numbers correspond to later fractions from the top of the gradient tube (i.e., slower sedimentation).

GluR1-WS sedimted near the top of the gradient as at least two discernable protein species (Fig. 4A) defined by peaks on the sedimentation profile between fractions 26-31 and 32-37, respectively. The open arrows in Figure 4A indicate the fractions with the highest protein concentrations (30 and 34) that were used to estimate the apparent molecular masses of the two species. The major species around fraction 34 had an apparent molecular mass of 140 kDa, and the next most abundant species around fraction 30 had an apparent molecular mass of 255 kDa (Fig. 4B). The calculated molecular mass of a GluR1-WS monomer is 78.5 kDa. Thus, the molecular mass of the most abundant protein species (140 kDa) is close in value to the calculated molecular mass of a GluR1 dimer (157 kDa). Similarly, the molecular mass of the second most abundant protein species (255 kDa) is close in value to the calculated molecular mass of a GluR1-WS
trimer (235.5 kDa). These results suggest that the GluR1-WS formed dimers and trimers. A third, smaller peak around fractions 24-25 might represent a high order oligomer or mixture of oligomers. Notably, very little of GluR1-WS was retained at the very top of the gradient, where a monomeric form of the protein would be expected to run relatively close to the position of the bovine serum albumin of molecular mass of 66 kDa. Virtually no GluR1-WS was detected at the bottom of the gradient (i.e., fast sedimentation), where nonspecifically aggregated protein would be expected. In contrast, GluR1 and GluR1-M1 showed broad sedimentation profiles (data not shown). Anomalously fast sedimentation (i.e., faster than GluR1-142/236) was observed for GluR1-M1, possible due to nonspecific aggregation.

**GluR1-WS binds [³H]AMPA.**

To examine the maturation of the ligand-binding site in glutamate receptor species formed by GluR1-WS and GluR1-M1, we measured the ability of Triton X-100 solubilized species to bind [³H]AMPA. We solubilized oocytes with Triton X-100 to release all the intracellular GluR1-WS species because the secreted fraction of GluR1-WS protein was found to be too little to be used in ligand-binding assays. Specific high-affinity radioligand-binding was observed for both GluR1-WS and GluR1-M1 species in the solid-phase binding assays using 20nM [³H] AMPA (data not shown). To more precisely determine the affinity of GluR1-WS receptor species for [³H]AMPA, binding assays were carried out with varying concentrations of [³H]AMPA and yielded a Kₐ of ~32 nM (Figure 5). This value is within the nanomolar range of Kₐ values reported for
cloned AMPA receptors (2,5) as well as those reported for monomeric S1-S2 species derived from GluR2 (18) and GluR4 subunits (17). The ability of GluR1-WS to bind $[^{3}]$H]AMPA is consistent with the results of other investigators showing that only the S1-S2 portion of the extracellular domains is sufficient for binding glutamatergic ligands (17,18).

**GluR1-WS assembles with full-length GluR1 subunits.**

GluR1-WS is water-soluble and as such is of significant interest for further structural studies. Hence to obtain additional evidence for the ability of GluR1-WS to assemble into oligomers, we examined its ability to assemble with full-length GluR1 subunits. GluR1-WS was coexpressed with GluR1-142 in *Xenopus* oocytes and solubilized in Triton X-100. Solubilized GluR species were immunopurified using mAb 236-beads and fractionated by SDS-PAGE. The presence of coimmunopurified GluR1-142 subunits was examined by immunoblotting with mAb 142 to the engineered epitope in GluR1-142. As controls for nonspecific binding of proteins to the mAb-beads, we used beads coupled to rat IgG. The coimmunopurification of GluR1-142 with GluR1-WS (Figure 6) shows that GluR1-WS retained its ability to coassemble with GluR1-142 subunits. This result indicated that GluR1-WS appeared to have undergone sufficient conformational maturation to compete for assembly with the full-length GluR1 subunit.
DISCUSSION

In this study, we examined the assembly properties of two fusion proteins consisting of the two extracellular domains of the GluR1 subunit without TMDs (GluR1-WS) or with a TMD (GluR1-M1) with velocity sedimentation profiles on sucrose gradients, with the binding properties of [³H]AMPA, and with coimmunoprecipitation. We found that GluR1-WS appeared to be capable of assembling into homooligomeric GluR species and bound [³H]AMPA with high-affinity. The GluR-WS protein was water-soluble, and the GluR1-M1 protein was membrane bound at the cell surface. Collectively, these results demonstrate that GluR1-WS retains the ability to bind glutamatergic ligands and suggest that it also assembles into specific oligomers because of the inclusion of the N-terminal portion of the GluR1 subunit.

Both GluR1-WS and GluR1-M1 appeared to be stably expressed in oocytes. GluR1-WS was detected as a secreted water-soluble protein in keeping with the newer topology proposed for GluR subunits in which the two extracellular domain composing the GluR1-WS protein do not contain a TMD. Since GluR1-WS was secreted as a water-soluble protein, it provides a good starting point for exploring ways, in high-level expression systems, to circumvent problems associated with the use of detergents to solubilize and crystallize GluRs. Our results extend the work of others demonstrating that S1-S2 fusion proteins containing shorter portions of the extracellular domains of the GluR2 subunit (18) and the GluR4 (17) subunit are also processed as secreted water-soluble protein in heterologous expression systems.
The velocity sedimentation profile of the Triton X-100 solubilized GluR-WS on sucrose gradients was more compatible with that of a mixture of specific higher order oligomers than with that of monomers alone. We observed a close correlation between the apparent molecular masses (140 kDa and 255 kDa) of two peaks on the GluR1-WS sedimentation profile and the calculated molecular masses of GluR1-WS dimers (157 kDa) and trimers (235.5 kDa). Also, little GluR1-WS was detected at the bottom of the gradient where nonspecifically aggregated protein would be expected. These observations correlate well with a GluR1-WS dimer, and not a GluR1-WS monomer or nonspecifically aggregated protein, as the dominant species in the most abundant peak near the top of the gradient (fraction 34). The next most abundant peak with a faster sedimentation velocity (fraction 30) is consistent with the next higher order oligomer, i.e., a GluR1-WS trimer.

The ability of GluR1-WS to successfully compete for coassembly with the full-length GluR1-142 subunit also supported our conclusion that GluR-WS has the conformational maturation to form specific oligomers. This result also further demonstrated that the extracellular domains alone promote assembly of subunits. The ability of GluR1-WS to assemble into higher order homooligomers is a novel finding because it demonstrates that a fusion protein consisting of the complete extracellular portion of the GluR1 subunit polypeptide chain is not only water-soluble but also retains its ability to assemble. These results are also consistent with the earlier finding that the distal most N-terminal region of the GluR2 subunit promotes subunit assembly (37).
We showed that Triton X-100 solubilized GluR1-WS was capable of binding \(^3\)HAMPA on solid-phase radioimmunoassays, suggesting that this protein when synthesized in oocytes exhibited maturation of the ligand-binding site. Ligand binding in this case, however, does not distinguish between monomers and specific higher order oligomers of GluR1-WS. These results complement those of other investigators who have previously demonstrated that the S1-S2 portion of the extracellular domain of the GluR4 subunit alone is sufficient to bind several different glutamatergic ligands with high-affinity (17). Recently, while this work was in progress, a fusion protein similar in design to the GluR1-WS construct, but derived from the GluR4 subunit, was shown to assemble predominantly into dimers that exhibit high-affinity for glutamatergic ligands (38), when expressed at high-levels in insect cells. The authors speculated that the dimers might be an assembly intermediate in the formation of oligomers (tetramers or pentamers) of the GluR4 water-soluble fusion protein. Collectively, both these results suggest that the ability of the extracellular domains to oligomerize might be a general characteristic of all subunits within the GluR family.

The GluR1-M1 subunit was designed with the hope that it might more efficiently assemble than constructs lacking TMDs. This hope was not clearly borne out by the results. The anomalously fast sedimentation of GluR1-M1 as species that were apparently heavier than those of GluR1-142/236 might reflect a large fraction of GluR1-M1 that formed aggregates, perhaps in response to exposure of hydrophobic regions of its transmembrane domain that normally associate with the other transmembrane domains in
the full-length receptor. Nonetheless, a fraction of GluR1-M1 is transported to the surface membrane of oocytes. This transport raises the possibility that a fraction of GluR1-M1 subunits also formed either monomers or specific oligomers and not non-specific aggregates, since it is unlikely that such aggregates would be transported to the cell surface membrane. In addition, high-affinity $[^3H]$ AMPA binding was observed with Triton-solubilized GluR1-M1 species.

The results presented in this paper along with those of others obtained for water-soluble domains of GluRs (17-19,38) and AChRs (28) are encouraging starting points for producing a fully-assembled, water-soluble GluR. Our previous experience with the extracellular domain of the AChR $\alpha7$ subunit and the work described in this paper on the GluR1 subunit suggest that modifications in design are needed to successfully use this strategy for structural studies beyond a monomeric form of GluR. We suggest that water-soluble domains bridged by a protease-cleavable site to a TMD anchor derived from an integral membrane protein that contains only a single TMD, or membrane tethers such as a GPI moiety; and regulated expression of the extracellular domains in high expression systems might improve the efficiency of subunit assembly and thus yield larger amounts of higher order oligomers. These strategies are likely to yield intermediate milestones in the challenging task of obtaining high-resolution structural information for neurotransmitter-gated ion channels.
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FIGURE LEGENDS

Figure 1. Schematic representations of various GluR1 protein constructs.
GluR1-142 represents the GluR1 subunit tagged with an epitope for mAb 142 (142') after its C-terminal residue 889. Similarly, GluR1-236 represents GluR1 tagged with an epitope for mAb 236 (236') after residue 889. GluR1-M1 represents linkage of the residues Ala\(^1\)-Glu\(^{520}\) via a linker containing the epitope for mAb 236 to residues Asn\(^{615}\)-Leu\(^{889}\) and includes the C-terminal tag for mAb 142. GluR1-WS is derived from GluR1-M1 but truncates at residue Gly\(^{790}\).

Figure 2. Expression of the GluR1 protein constructs in oocytes.
2% Triton X-100 solubilized proteins from oocytes expressing the various GluR1 subunit constructs were immunopurified on mAb coated Immulon 4 wells and then eluted and fractionated by SDS-PAGE. The expression of GluR1-142, GluR1-236, and GluR1-M1 was detected by immunoblotting with the appropriate \(^{125}\)I-mAbs to the engineered epitopes tags, and mAb-236 binding to GluR-WS was detected using an HRP-conjugated secondary antibody in conjunction with a chemiluminescence detection method.

Figure 3. GluR1-WS is water-soluble and GluR1-M1 is membrane-bound.
A. The water-soluble nature of GluR1-WS was studied by immunopurifying (I.P.) proteins using mAb 236 beads from the aqueous oocyte incubation medium, the aqueous supernatant obtained after homogenization and centrifugation of oocyte membranes, and
from 2% Triton X-100 solubilized oocyte proteins expressing GluR1-WS, and then detecting them by immunoblotting (I.B.) with $^{125}$I-mAb 236. The controls correspond to proteins from non-injected oocytes treated similarly. The arrow indicates the GluR1-WS band. The amount of GluR1-WS detected on the immunoblot was isolated from 40 oocytes.

B. Surface expression of GluR1-M1 was detected using $^{125}$I-mAb 236 to individual oocytes. Non-specific binding was determined using noninjected oocytes. The error bars represent the standard error of binding to six oocytes. Approximately 1 fmol equivalent of $^{125}$I-mAb 236 binding sites to the GluR1-M1 protein is detected on the surface of each oocyte.

**Figure 4. Velocity sedimentation profile of GluR1-WS.**

A. The normalized sedimentation profile (top) of Triton X-100 solubilized GluR1-WS on 5-20% sucrose gradients containing 0.5% Triton X-100 was derived from quantitating the intensities of bands obtained for GluR1-WS by immunoblotting the fractions (bottom). The smaller fraction numbers corresponded to the first fractions collected from the bottom of the gradient tube (i.e., faster sedimentation) and the larger fractions numbers corresponded to later fractions from the top of the gradient tube (i.e., slower sedimentation). The open arrows at fractions 30, and 34 highlight two relatively abundant peaks in the sedimentation profile that correspond to apparent molecular masses of 140 kDa and 255 kDa. The apparent molecular masses corresponding to these two peaks suggest that the peaks could represent dimers and trimers, respectively, of GluR1-
WS. The solid arrows above the profile of GluR1-WS indicate the positions of the peaks of each of the four proteins used to calibrate the gradient.

B. The peak fraction number of each of the standard proteins, bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and apoferritin (443 kDa) is plotted against the log(Molecular Mass) of each of the standard proteins (filled circles). The line is a linear best fit of the points. The apparent molecular masses for the two peaks as calculated from this plot at fractions 30 and 34, are also shown (open circles).

Figure 5. Binding of [3H]AMPA to Triton X-100-solubilized GluR1-WS solubilized in Triton X-100.

Triton X-100 solubilized proteins from 10 oocytes expressing GluR1-WS tethered to mAb-coated microwells were incubated with various concentrations of [3H]AMPA in the presence of 100mM NaSCN for 2 h. Non-specific binding at each concentration was determined from wells containing Triton X-100 solubilized proteins from an equivalent number of uninjected oocytes. Following washing, the amount of bound radioactivity was determined by scintillation counting. The points shown correspond to the mean specific binding of duplicate determinations from one experiment after subtraction of the mean non-specific binding from each value at each of the concentrations. The error bars correspond to the standard error. The $K_d$ and $B_{max}$ were determined by least squares, nonlinear fitting of the binding data to a Hill-type equation.
Figure 6. Assembly of GluR1-WS with GluR1-142.

2% Triton X-100 solubilized proteins from oocytes coexpressing GluR1-142 and GluR1-WS were immunopurified (I.P.) on mAb 236-beads and rat IgG-beads (control), eluted, fractionated by SDS-PAGE, and sequentially immunoblotted (I.B.) with mAb 236 followed by mAb 142. Beads coupled to rat IgG were used as controls for non-specific binding. The upper band corresponds to GluR1-142 and the lower band corresponds to GluR1-WS. The GluR1-WS band weakly reappears in the right panel because the blot was not stripped prior to immunoblotting with mAb 142.
REFERENCEs


Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

\[ [\text{\textsuperscript{3}H}\text{AMP A Binding (pM)}] \]

\[ [\text{\textsuperscript{3}H}\text{AMP A Concentration (nM)}] \]

\( K_d = 32 \text{ nM} \)

\( B_{\text{max}} = 40 \text{ pmoles/L} \)
<table>
<thead>
<tr>
<th>I.B.</th>
<th>mAb 236</th>
<th>mAb 142</th>
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![Western Blot Image](image-url)

**Figure 6**