The extracellular part of ionotropic glutamate receptor (iGluR) subunits can be divided into a conserved two-lobed ligand-binding domain ("S1S2") and an N-terminal -400-residue segment of unknown function ("X domain") which shows high sequence variation among subunits. To investigate the structure and properties of the N-terminal domain, we have now produced affinity-tagged recombinant fragments which represent the X domain of the GluRD subunit of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-selective glutamate receptors either alone or covalently linked to the ligand-binding domain ("XS1S2"). These fragments were expressed in insect cells as secreted soluble proteins and were recognized by a conformation-specific anti-GluRD monoclonal antibody. A hydrodynamic analysis of the purified fragments revealed them to be dimers, in contrast to the S1S2 ligand-binding domain which is monomeric. The X domain did not bind radiolabeled AMPA or glutamate nor did its presence affect the ligand-binding properties of the S1S2 domain. Our findings demonstrate that the N-terminal domain of AMPA receptor can be expressed as a soluble polypeptide and suggest that subunit interactions in iGluR may involve the extracellular domains.

The ionotropic glutamate receptors (iGluRs) mediate the majority of fast synaptic transmission (1) and have been proposed to be involved in many pathological mechanisms. AMPA-, N-methyl-D-aspartate, and kainate-selective iGluR subclasses are all multisubunit membrane proteins composed of homologous subunits. Currently, the exact number of subunits is not clear, but the receptor is believed to be a tetramer (2-4) or a pentamer (5, 6).

The iGluR subunits (900–1500 residues) have a modular structure which consists of an N-terminal leucine/isoleucine/valine-binding protein (LIVBP)-like domain (7) and a bipartite ligand-binding domain (8) on the extracellular side, three transmembrane domains (M1, M3, and M4), an ion channel-forming re-entrant membrane-loop segment (M2), and a cytoplasmic C-terminal domain (9–11). The cytoplasmic tail has a role in synaptic clustering and in the formation of supramolecular signaling complexes via interactions with other postsynaptic proteins (reviewed in Ref. 12). The ligand-binding domain was first identified on the basis of sequence similarity between two discontinuous segments (S1 and S2) in iGluR subunits and bacterial periplasmic polar amino acid-binding proteins (7, 8, 13, 14). Subsequent expression of the native-like ligand-binding site of AMPA receptor as a soluble, secreted S1S2 fusion protein (15, 16), site-directed mutagenesis studies (8, 14, 17–19), and, most convincingly, the recently determined crystal structure of a GluRB S1S2-kainate complex (20), confirm the close structural and functional similarity between the ligand-binding domain of iGluRs and polar amino acid-binding proteins. In contrast to the other domains of iGluR subunits, very little is known about the structural and functional role of the N-terminal -400-residue segment (referred to here as "X domain"), which may be distantly related to bacterial LIVBP and homologous extracellular solute binding proteins (7, 21).

Exchange of the X domains between AMPA-selective GluRC (GluR3) and the glycine-binding NR1 subunit of the N-methyl-D-aspartate receptor had no effect on agonist affinities of the parent subunits (8), excluding direct participation in agonist binding. Recently, however, a role for the N-terminal segment in the modulation of glycine-independent desensitization of the N-methyl-D-aspartate receptor has been suggested (22, 23).

In the present study, we have characterized the properties of extracellular domains of the GluRD AMPA receptor, expressed in insect cells either as separate domains or as an XS1S2 ectodomain. We provide evidence that the presence of the X domain does not significantly affect the ligand-binding properties but it does contribute to dimerization of the ectodomain.

**EXPERIMENTAL PROCEDURES**

Recombinant DNA—DNA constructs for the expression of epitope-tagged rat GluRD and fragments of GluRD were generated using the polymerase chain reaction. Briefly, synthetic oligonucleotides incorporating appropriate restriction sites for further cloning served as primers to amplify GluRD fragments. A pBluescript plasmid encoding full-length GluRD cDNA was used as the template. The amplified fragments were cloned in a derivative of pFASTBAC1 (Life Technologies, Inc.) engineered to encode a signal peptide followed by an N-terminal FLAG epitope (15). The correctness of all polymerase chain reaction-derived sequences in the final construction was verified by DNA sequencing. For design of the expression constructs, see Fig. 1. The 13-residue peptide STEGEVNAEEEGF was used as a linker between the S1 and S2 segments as described previously (15). The recombinant baculoviruses were generated by using the Bac-to-Bac system (Life Technologies, Inc.), and Spodoptera frugiperda SF21 insect cells (In-
Expression in Insect Cells and Protein Purification—High-titer virus stocks were prepared from the recombinant viruses in SF21 insect cells growing in SF-900 II medium (Life Technologies, Inc.), and subsequently used to infect suspension cultures of High Five cells (Invitrogen) as described before (15, 18) or with L-[3H]glutamate. Protein concentration was determined by UV absorption at 280 nm or by monitoring the absorbance at 280 nm and band pass of 4 nm. All measurements were determined relative to a reference cuvette filled with water, adjusted to 1 M NaCl and 2.5 mM CaCl₂, and passed as 2-liter fractions through a NiCl₂-charged chelating Sepharose (Amersham Pharmacia Biotech) column (30 ml) at a flow rate of 1–2 ml/min. The column was washed with 20 mM Hepes, pH 7.4, 0.2 M NaCl, 0.25 mM phenylmethylsulfonyl fluoride and the bound proteins were eluted using a stepwise imidazole gradient prepared in the Hepes buffer. Eluates from 200 to 500 mM imidazole fractions were pooled, concentrated, and dialyzed against phosphate-buffered saline, pH 7.4, 10% glycerol. Protein concentration was determined by UV absorption at 280 nm or by using Pierce BCA (bicinchoninic acid) method according to manufacturer’s instructions.

Radioligand Binding Assays—Purified and dialyzed samples were assayed for AMPA and L-glutamate binding by incubating samples (up to 10 μg of protein) with [3H]AMPA (5 nM; 40.0 Ci/mmol, NEN Life Science Products Inc.) as described before (15, 18) or with L-[3H]glutamate (20 mM; 49.5 Ci/mmol, NEN Life Science Products Inc.) in 50 mM Tris-HCl, pH 7.4, for 1 h on ice, followed by rapid filtration through (0.3%) polyethyleneimine-treated Whatman GF/B filters. The filters were solubilized in 0.5% sodium dodecyl sulfate (SDS) high-salt, WALLAC and counted for 1 h at room temperature. Non-specific binding was determined in the presence of 1 mM t-glutamate.

For saturation binding, 3 μg of purified protein was incubated with 1–300 nM [3H]AMPA (diluted with unlabeled RS-AMPA to 8 μM/cuvette) in the presence or absence of 1 mM t-glutamate. The binding data were analyzed using nonlinear curve-fitting (GraphPad Prism) to yield the dissociation constant (Kd) and the specific binding activity (Bmax).

For ligand competition assays, the samples were incubated in the presence of 5 μM [3H]AMPA and increasing concentrations of unlabeled ligands. Kainate, L-glutamate, and 6,7-dinitroquinoxaline-2,3-dione (D-NTX) were obtained from RBI. The binding data were analyzed by nonlinear curve fitting using a model for one-site binding to yield IC50 values. All radioligand binding experiments were performed in triplicate and at least three times with essentially identical results.

Fluorescence Titration—All fluorescence titrations were measured with an SLM 8000 spectrofluorometer (SLM Instruments) at 5 °C with an excitation wavelength of 280 nm and band pass of 4 nm. All measurements were determined relative to a reference cuvette filled with rhodamine. The fluorescence change was followed at 336 nm. Aliquots of concentrated glutamate were added to a quartz cuvette containing 3 ml of protein (0.03–0.10 μM) in 10 mM NaPi, pH 7.3, under continuous mixing with a magnetic stirrer. For each ligand concentration 20 to 25 data points (each data point was a mean of 3 s) were averaged. The results were corrected for the dilution of the sample with ligand. The results were fitted by the equation,

\[
F = 1 + \frac{\Delta F_{max} \times (E_t + L + K_r)}{F_0 \times 2 \times E_t} \times \frac{(E_t + L + K_r)^2 - 4 \times (E_t \times L)}{\sqrt{(E_t + L + K_r)^2 - 4 \times (E_t \times L)}}
\]

where \(F_t\) is the fluorescence observed after the i-th addition of ligand and \(F_0\) the fluorescence of the free protein. \(E_t\) is the protein concentration, \(L\) the added ligand concentration, \(K_r\) the dissociation constant, and \(\Delta F_{max}\) is the maximum fluorescence change in going from unbound to completely bound protein.

Stopped-flow Kinetics—Rapid kinetic measurements were performed at 5 °C in 10 mM NaPi, pH 7.3, using an SF-61 stopped-flow fluorimeter (Hi-Tech Scientific) with an excitation wavelength of 280 nm. The fluorescence decrease was detected with a WG-320 filter (nominal cutoff 320 nm). The protein concentration was 50 nM and the glutamate concentration was at least 5-fold higher, so that pseudo-first order conditions were satisfied. To improve the signal-to-noise ratio, 5 to 7 individual traces were averaged for each ligand concentration.

The dissociation constant, \(K_d\), was calculated by fitting of the observed pseudo-first order rate constants as a function of ligand concentration; \(K_{ld}\) and \(K_{rd}\) are given by the slope and the intercept, respectively.

Immunoprecipitations—Five micrograms of purified protein was incubated with 5 μg of Fab212 for 1 h at 4 °C with end-over-end mixing. Thirty μl of Gammabind G-Sepharose (Amersham Pharmacia Biotech) were added and the incubation continued for another hour. The gel particles were separated by low speed centrifugation, washed three times in TBST (TBS, 0.05% Tween 20), and finally resuspended in Laemmli sample buffer.

Gel Filtration Chromatography—A Sephacryl S-300 HR 16/60 (120-ml bed volume) column (Amersham Pharmacia Biotech) was equilibrated in phosphate-buffered saline, pH 7.4, 10% glycerol. Standard proteins (50–500 μg of protein in 0.5 ml; HMW and LMW gel filtration kits, Amersham Pharmacia Biotech) and GluRD fragments (250–500 μg of protein in 0.5 ml) were run at 0.5 ml/min in a Pharmacia FPLC chromatography system operating at room temperature. Elution was monitored by UV absorbance (280 nm). Fractions (0.5–1 ml) were analyzed by immunoblotting and radioligand binding. At least three gel filtration experiments with essentially identical results were carried out for all fragments using independent purified preparations.

Intact FLAG/His-tagged GluRD was purified from SF21 insect cells as a Triton X-100 complex as described previously (24). For gel filtration experiments, a 1.5 × 100-cm column of Sephacryl S-300 HR, equilibrated in TBS (10 mM Tris, 150 mM NaCl), pH 7.4, 10% glycerol, 0.1% Triton X-100, was used. Purified receptor (100 μg in 1.0 ml of equilibration buffer) was injected by continuous flow and run using a flow-rate of 0.45 ml/min. As the high UV absorbance of Triton X-100 precluded UV detection, all fractions (0.9 ml) were subjected to dot immunoblotting and to radioligand binding assay. Standard proteins were run in the same buffer and detected by protein assay.

Sucrose Density Gradient Centrifugation—Sedimentation analysis of GluRD fragments was performed in linear 5–20% (w/v) sucrose gradients (phosphate-buffered saline, pH 7.4, 10% glycerol) run in Beckman SW 41 Ti rotor for 41 h at 180,000 × g (38,000 rpm) at 4 °C. Chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), aldolase (159 kDa), and catalase (232 kDa) from the HMW gel filtration kit (Amersham Pharmacia Biotech) were used as reference proteins. Fractions (0.5 ml) of the gradients were analyzed by SDS-PAGE. For cross-linked XS1S2, a 10–50% (w/v) sucrose gradient was used with the above mentioned parameters except for the run time which was 24 h.

Native molecular weights (M) were obtained from the Svedberg equation,

\[
M = \frac{rRT}{D(1 - vp)}
\]

where \(s\) is the sedimentation coefficient obtained from sucrose density gradient centrifugation experiments, \(R\) is the gas constant (8.314 J K⁻¹ mol⁻¹), \(T\) is temperature (293 K), \(D\) is the diffusion coefficient obtained from gel filtration experiments, \(v\) is the partial specific volume estimated from the amino acid sequence, and \(p\) is the density of solvent (for water \(\rho = 0.9982 \text{ g cm}^{-3}\)).
GluRD Extracellular Domains

Cross-linking Experiments—Three-hundred-microliter samples of GluRD fragments from the sucrose density gradient (100–200 μg/ml) were incubated with the covalent cross-linker glutaraldehyde (0–12 mM; EM Sciences) for 60 min at room temperature. Aliquots were removed from the reaction mixture at the time points indicated under “Results,” quenched with an excess of Tris, pH 8.0 (166 mM final concentration), and analyzed by SDS-PAGE (7%).

SDS-PAGE and Immunoblotting—Protein samples were denatured by heat treatment (95 °C, 5 min) in dithiothreitol-containing Laemmli sample buffer, and resolved by electrophoresis in a 7 or 10% gel. Gels were stained with silver nitrate or transferred to nitrocellulose, followed by immunostaining. Anti-FLAG M1 (10 μg/ml, Sigma), alkaline phosphatase-conjugated anti-mouse IgG (Bio-Rad), and 5-bromo-4-methylumbelliferylphosphate/nitro blue tetrazolium color substrates were used for the detection.

RESULTS

GluRD Extracellular Domains Expressed as Soluble Proteins—Recombinant GluRD fragments expressed in High Five insect cells were secreted as soluble proteins as demonstrated by an anti-FLAG immunoblot of the culture supernatants (Fig. 2A). The observed sizes of the immunoreactive bands correspond closely to the values expected from the sequence and from the likely presence of N-glycans (approximate contribution of 1.5 kDa per N-glycan); the XS1S2 fragment migrates as a 92-kDa band (lane 2); calculated size 82 kDa + maximally 6 N-glycans), the XS fragment migrates as a 67-kDa band (lane 3; 62 kDa + 6 N-glycans), the X fragment as a 50-kDa band (lane 4; 46 kDa + 4 N-glycans), and the XS1S2 fragment as a 42-kDa band (lane 5; 38 kDa + 2 N-glycans). For comparison, intact GluRD (subunit size 110 kDa), solubilized from insect cells is shown in lane 1.

The fragments were purified from culture supernatants using metal chelation chromatography (Fig. 2B). Purification yields were 100–400 μg of XS1S2, XS1, and X fragments, and 1–2 mg of S1S2 from 1 liter of culture supernatant. Initially, no signs of proteolytic degradation products were observed in the culture supernatants (see Fig. 2A), but proteolytic products started to appear, in particular with XS1 (60- and 42-kDa bands, lane 2, Fig. 2B) and XS1S2 (66-kDa band, lane 2, Fig. 4) after purification and storage for several weeks.

Radioligand Binding Characteristics of Purified GluRD Fragments—The purified proteins were examined for specific [3H]AMP (Fig. 2C) and 1-3H]glutamate binding (Fig. 2D).

The two fragments which contain the ligand-binding domain (XS1S2 and S1S2) bound both radioligands at levels exceeding the nonspecific background binding (in the presence of 1 mM unlabeled glutamate) by a factor of 10 (for γ-glutamate) to 50 (AMPA). The other fragments (XS1 and X) and control proteins (bovine serum albumin and an antibody fragment) did not show any specific binding with either radioligand. In a saturation binding assay, purified XS1S2 bound [3H]AMP (with a high affinity (Kd 42 nM) and capacity (Bmax 1 nmol/mg of protein) (Table I). In a ligand displacement assay, no significant differences in the relative affinities for γ-glutamate, kainate, and AMPA were observed between XS1S2, XS1, and the intact receptor (Table I).

Intrinsic Fluorescence-based Ligand Binding Measurements—Both XS1S2 and S1S2 show a decrease of intrinsic tryptophan fluorescence upon agonist binding.3 Since there are no tryptophan residues located within the immediate vicinity of the ligand-binding site (20), the fluorescence decrease presumably reflects conformational changes undergone following ligand binding and thus acts as a reporter for the ligand-binding mechanism of the S1S2 domain. Fluorescence titration measurements of glutamate binding to both XS1S2 and S1S2 (Table II, Kd values) confirm that their affinities for glutamate are comparable.

Finally, using stopped-flow rapid-mixing techniques, the time course of glutamate binding to both XS1S2 and XS1S2 was followed. Both constructs exhibited monophasic binding (Fig. 3A). Under pseudo-first order conditions, the observed rate constant exhibits a linear dependence on glutamate concentration, consistent with a one-step binding mechanism (Fig. 3B). The association and dissociation rate constants obtained for glutamate binding to XS1S2 and XS1S2 again show no significant difference (Table II). The Kd values for γ-glutamate binding calculated from the association and dissociation rate constants are in very good agreement with the values obtained from the fluorescence titration measurements, and only slightly higher (0.4–0.5 μM versus 0.3 μM) than the values obtained from radioligand displacement assays (Table I).

Immunoprecipitations with a Conformation-specific Fab Fragment—In the absence of any conventional assay to verify the structural integrity of the expressed and purified X domain, we used a recently described3 monoclonal antibody, designated as Fab21, which recognizes an epitope in the X domain of GluRD. Binding of Fab21 to GluRD is sensitive to acidic and alkaline treatments and to SDS, suggesting that the antibody is conformation-specific. All three soluble fragments which contain the X domain (XS1S2, XS1, and X) were immunoprecipitated by Fab21, whereas S1S2 was not (Fig. 4). Furthermore, X domain briefly heated to 65 °C was not immunoprecipitated, consistent with the conformation-specific nature of Fab21. Proteolytic degradation products of XS1S2, and especially of XS1

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| Table I Ligand binding characteristics of GluRD fragments |
|---|---|---|---|
| | L-glutamate | Kainate | DNQX* |
| | nm | μM | μM |
| XS1S2 | 41 ± 11 | 0.28 ± 0.05 | 2.1 ± 0.2 | 0.23 ± 0.07 |
| S1S2 | 33 ± 1 | 0.29 ± 0.02 | 2.3 ± 0.6 | 0.46 ± 0.17 |
| GluRD | 58 ± 15 | 0.45 ± 0.17 | 3.1 ± 1.1 | 0.58 ± 0.15 |

* DNQX, 6,7-dinitroquinoxaline-2,3-dione.
TABLE II
Rate constants and affinities of S1S2 and XS1S2 for glutamate

The dissociation rate constants $k_{off}$ and association rate constants $k_{on}$ were determined from the linear fits shown in Fig. 3. The equilibrium dissociation constants $K_d$ were determined by fluorescence titration measurements. The ratio of $k_{off}/k_{on}$ is shown for comparison with $K_d$.

|        | $k_{off}$ | $k_{on}$ | $k_{off}/k_{on}$ | $K_d$ |
|--------|-----------|----------|------------------|------|
| S1S2   | $16.0 \pm 0.9$ | $7.6 \pm 0.8$ | $0.47 \pm 0.06$ | $0.55 \pm 0.03$ |
| XS1S2  | $14.7 \pm 1.0$ | $5.9 \pm 0.9$ | $0.40 \pm 0.07$ | $0.49 \pm 0.02$ |

Hydrodynamic Properties of GluRD Fragments—In size exclusion chromatography on Sephacryl S-300, X and S1S2 fragments both eluted as single, relatively symmetric peaks corresponding to sizes of 87 and 59 kDa, respectively (assuming similar relation between $V_r$ and molecular size as for the globular protein standards) (Fig. 5, B and C, Table III). In contrast, the elution pattern of the XS1S2 fragment appeared less uniform, displaying a major peak corresponding to a size of 276 kDa and a minor shoulder at 137 kDa (Fig. 5A, Table III). Subsequent immunoblotting and radioligand binding assays showed that FLAG immunoreactivity and AMPA binding activity (XS1S2 and S1S2 fragments) were associated with the peak fractions of the UV absorbance and that both the main peak and the shoulder of XS1S2 samples consist of intact XS1S2 (results not shown). The XS1 fragment tended to aggregate during gel filtration as indicated by a remarkable loss of protein in the column and elution of the rest of the protein in the void volume (not shown), and therefore XS1 fragment was not analyzed further. We also studied whether the X and S1S2 domains would associate in vitro into a larger complex by mixing the two fragments in equivalent molar amounts (both at 0.5 mg/ml), and subjecting the mixture to gel filtration. No formation of bigger complexes was, however, observed (results not shown).

For comparison, the intact purified GluRD was analyzed by gel filtration in the presence of Triton X-100. Based on dot immunoblotting and radioligand-binding assays (Fig. 5E), GluRD eluted as a sharp ~500-kDa peak between ferritin (440 kDa) and thyroglobulin (669 kDa). Taking into account uncertainties in the amount of bound detergent and the contribution of molecular shape, this size estimate is consistent with 4–5 subunits per molecule.

The hydrodynamic properties of purified GluRD fragments were further analyzed by sucrose density gradient centrifugation (Fig. 6). S1S2 fragment sedimented with a rate close to that of ovalbumin ($s = 3.0$ S). The X fragment sedimented faster with a sedimentation coefficient of 5.3 S. In comparison to protein standards and to the S1S2 and X fragments, the XS1S2 fragment sedimented as less uniform population with a $s$ value of 6.7 S. From these values and the diffusion coefficients obtained from gel filtration calibration curve, the native molecular weights were calculated by using the Svedberg equation. The following molecular masses were obtained: 41 kDa for S1S2, 86 kDa for X, and 169 kDa for the XS1S2 fragment (Table III). These values are consistent with X and XS1S2 being dimers and S1S2 being a monomer.

Covalent Cross-linking Experiments—To further analyze the oligomerization of the XS1S2 fragment, we carried out covalent cross-linking experiments with the purified fragments. Treatment with dimethyl suberimidate, dimethyl-3,3-dithiobis(propionimide), or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide did not result in formation of any high molecular mass complexes under the conditions used, whereas glutaraldehyde treatment gradually converted the XS1S2 fragment into a 160–200-kDa species with concomitant disappearance of the 92-kDa species (Fig. 7A, upper panel). Under the same conditions, S1S2 did not form higher molecular weight complexes (Fig. 7A, lower panel). Somewhat unexpectedly X fragment produced only a very weak 100-kDa band upon treatment with glutaraldehyde (Fig. 7A, middle panel). Incubation with glutaraldehyde caused a diffuse appearance of the bands, most notably with XS1S2 and S1S2, presumably due to the effects of extensive (and nonuniform) intramolecular cross-links. Considered together with the hydrodynamic behavior of the fragments, these findings suggest that S1S2 exists predominantly in a monomeric state.
To resolve the cross-linking products of XS1S2 better, the cross-linked fragment was subjected to sucrose density gradient centrifugation followed by SDS-PAGE in a low percentage gel. Much like untreated XS1S2, the cross-linked XS1S2 sedimented as a heterogeneous broad population indicating that glutaraldehyde cross-linking does not induce an oligomerized or aggregated state which is not present in the original preparation (not shown). SDS-PAGE analysis of the fractions revealed that the slow sedimenting cross-linked species correspond largely to monomers whereas the peak fractions represent the dimer with an apparent size of 160–200 kDa (Fig. 7B). The cross-linked species present in the fast sedimenting fractions was not able to penetrate into the running gel (7% acrylamide concentration) representing larger aggregates. This experiment was also carried out in a reverse order, i.e., XS1S2 was first run in a sucrose gradient and fractions from the peak and before the peak were cross-linked. The XS1S2 dimer was produced from the peak fractions but not from the slowly sedimenting fractions of sucrose density gradient centrifugation (results not shown).

**DISCUSSION**

This study shows that the N-terminal LIVBP-like region, X domain, of iGluR subunits can be expressed in a soluble form allowing biochemical characterization of some of its properties. Separately expressed X domain did not show any measurable binding of L-glutamate or AMPA in a radioligand binding assay, whereas the XS1S2 fragment, which represents the entire extracellular region of GluRD subunit, bound both radioligands with properties indistinguishable from the soluble S1S2 ligand-binding domain and from the intact GluRD. Both S1S2 and XS1S2 showed similar fluorescence changes upon ligand binding and similar association and dissociation rates constants, consistent with a minimal or negligible contribution of the N-terminal domain to ligand binding. These findings are in agreement with earlier domain swap experiments, which showed that engrafting the N-terminal domain of NR1 on GluRC (GluR3) does not modify the agonist pharmacology of...
The ligand-binding S1S2 domain of GluRD behaves as a monomer in solution in agreement with the behavior of a similar fragment of GluRB (25). Also, small-angle x-ray scattering studies indicate that S1S2 of GluRD is a monomer even at protein concentrations as high as 20 mg/ml (26). Interestingly, insect cell-expressed S1S2 fragment of the NRII subunit of the N-methyl-D-aspartate receptor was reported to exist largely as multimers (27). The specific ligand binding activity of that preparation was, however, extremely low making it difficult to assess the significance of the difference.

Calculation of native molecular mass yielded a size of 86 and 169 kDa for the separately expressed X and XS1S2 domains, respectively, consistent with a dimeric structure. For some reason, however, glutaraldehyde was able to cross-link only a minority of X fragments. The purified XS1S2 preparations contained clearly at least two differently sized species. In gel filtration, the XS1S2 fragment eluted as a major 276-kDa and a minor 137-kDa peak. Based on cross-linking results, these are likely to correspond to dimers and monomers, respectively, of the ectodomain.

What is the relevance of our findings to subunit interactions in native AMPA receptors? Several facts support the view that the soluble recombinant XS1S2 and X fragments represent native-like protein domains. First, presence of a correctly folded ligand-binding domain in XS1S2 is indicated by the highly similar ligand-binding pharmacology and kinetics of XS1S2 and S1S2. In the absence of any functional signature for the X domain, we used binding to a monoclonal antibody which recognizes a conformational epitope present in the N terminus of native receptor as an alternative. The antibody was able to immunoprecipitate the X domain-containing fragments, demonstrating the presence of at least some structural characteristics of the native (but not denatured) receptor in the purified XS1S2 and X fragments. Furthermore, the secretion of the fragments as soluble proteins is consistent with a folded structure. Under similar conditions, several shorter versions of the X domain are not secreted at all, but accumulate in the cells as partly insoluble protein.

Despite the above discussed “native-like” properties of the soluble ectodomain, we found that the XS1S2 ectodomain is a dimer with no convincing evidence for the formation of specific higher oligomers, in particular, tetramers and pentamers. Some large molecular weight material was evident in the cross-linked XS1S2 preparations but this represented only a small fraction of the total and was not able to enter the 7% polyacrylamide separation gel at all. Our gel filtration experiments with intact GluRD suggest that the molecule forms tetramers or pentamers, consistent with observations of others (2–6). The absence of tetramers or pentamers from the GluRD ectodomain thus suggests that important determinants for oligomerization are either lacking (most notably transmembrane domains) or require a membrane environment to be fully manifested. Identification of the structures involved in XS1S2 dimerization, and analysis of their role in AMPA receptor assembly should help to resolve this issue. It is possible that the XS1S2 dimers may, however, represent a transient state present during the assembly of the receptor. For example, the assembly of the pentameric nicotinic acetylcholine receptor is guided by extracellular domain interactions and involves intermediates with a lower subunit number (28–31).

Interestingly, while the current manuscript was under revision, Leuschner and Hoch (32) reported that a membrane-anchored N-terminal fragment (X domain) of GluRB is able to associate with GluRA in transfected COS cells. This finding is consistent with our present results indicating that the X domain mediates dimerization of the ectodomain.

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