Crystal structure and association behaviour of the GluR2 amino-terminal domain

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Fast excitatory neurotransmission is mediated largely by ionotropic glutamate receptors (iGluRs), tetrameric, ligand-gated ion channel proteins comprised of three subfamilies, AMPA, kainate and NMDA receptors, with each subfamily sharing a common, modular-domain architecture. For all receptor subfamilies, active channels are exclusively formed by assemblages of subunits within the same subfamily, a molecular process principally encoded by the amino-terminal domain (ATD). However, the molecular basis by which the ATD guides subfamily-specific receptor assembly is not known. Here we show that AMPA receptor GluR1- and GluR2-ATDs form tightly associated dimers and, by the analysis of crystal structures of the GluR2-ATD, propose mechanisms by which the ATD guides subfamily-specific receptor assembly.

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Introduction

Chemical synapses are the primary sites of communication between nerve cells and are specialized junctions where a presynaptic cell releases vesicles loaded with neurotransmitter opposite clusters of receptors localized in the postsynaptic cell density (Lisman *et al*, 2007; Chen *et al*, 2008). At canonical synapses in the mammalian nervous system, the areas of the active zones of transmitter release and receptor localization are of the order of $0.07\ \mu\text{m}^2$ (Harris and Stevens, 1989), and the separation of the presynaptic and postsynaptic membranes is $\sim 0.024\ \mu\text{m}$ (Zuber *et al*, 2005). Within this $\sim 0.0017\ \mu\text{m}^3$ volume of the synaptic cleft reside the

extracellular domains of pre and postsynaptic receptors participating in an extensive yet largely undefined array of intermolecular contacts (Chen *et al*, 2005, 2008), interactions that help define the architectural and dynamical properties of chemical synapses (Newpher and Ehlers, 2008).

Ionotropic glutamate receptors (iGluRs) are a family of ligand-gated ion channels that are primarily localized to chemical synapses and that mediate fast excitatory neurotransmission in the mammalian nervous system (Dingledine *et al*, 1999). Composed of three subfamilies— α -amino-3-hydroxyl-5-methyl-4-isoxazole (AMPA; GluR1–4), kainate (GluR5–7, KA1, 2) and *N*-methyl-D-aspartate (NMDA; NR1, NR2A–D and NR3A, B)—tetrameric iGluRs share a common, modular receptor architecture in which each subunit of these receptors possesses an ~ 45 -kDa amino-terminal domain (ATD) that resides within the synaptic cleft (Wo and Oswald, 1995; Paas, 1998; Mayer, 2006). Within this cleft, the ATD interacts with synaptic proteins including N-cadherin (Saglietti *et al*, 2007), as well as with neuronal pentraxins NARP (O'Brien *et al*, 1999) and NP1 (Sia *et al*, 2007) in the case of AMPA receptors and the ephrin receptor in the case of NMDA receptors (Dalva *et al*, 2000). The GluR2-ATD specifically induces the formation of dendritic spines (Passafaro *et al*, 2003), whereas the ATDs generally play a fundamental role in determining subtype-specific receptor assembly, defining that only subunits within a subfamily assemble with one another (Ayalon and Stern-Bach, 2001; Ayalon *et al*, 2005) although the ATD itself is not essential for receptor assembly and function (Pasternack *et al*, 2002; Horning and Mayer, 2004; Armstrong *et al*, 2006). In GluR1 and GluR2 AMPA receptors, the ATD also encodes an anterograde trafficking signal, a tripeptide IQI motif near the amino terminus that participates in receptor trafficking from the endoplasmic reticulum to plasma membrane (Xia *et al*, 2002). For NMDA receptors, the ATD modulates receptor function, providing binding sites for allosteric modulators that include protons, zinc ions, polyamines and small organic molecules such as ifenprodil (Paoletti and Neyton, 2007).

To define the molecular architecture of the iGluR ATD, we exploited the modular nature of the receptor and genetically excised selected ATDs from the AMPA receptor subfamily, ending each ATD construct at or near the beginning of the S1 segment of the agonist-binding domains (Stern-Bach *et al*, 1994). The recombinant ATD constructs were secreted from insect cells and examined for favourable biochemical behaviour. The ATD domains of the rat GluR1 and GluR2 receptors expressed well, were stable and monodisperse, and were amenable to biophysical characterization and crystallization. To accurately define the subunit stoichiometry of these AMPA receptor ATDs, we first carried out sedimentation equilibrium studies, showing that the ATD forms a well-defined dimer and accurately determining the dimer dissociation constant. These studies were complemented by high-resolution crystallographic experiments on the GluR2-ATD in which we not

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only elaborated the subunit structure and the dimer architecture but also suggested why receptor assembly is subfamily specific.

Results and discussion

Isolated ATDs form tightly associated dimers in solution

To explore the association behaviour of the isolated GluR1-ATD (Asn1–Asp375) and GluR2-ATD (Val1–Ser383) in solution, we first carried out analytical size-exclusion chromatography at a loading concentration of 1 mg/ml. Both ATDs migrated with an estimated molecular weight about 1.6–1.7 times greater than that of the subunit molecular weight as determined by mass spectrometry, presumably because of dimer formation (data not shown). We next carried out sedimentation equilibrium studies in an analytical ultracentrifuge using multiple protein concentrations and rotor speeds to determine subunit association behaviour (Howlett *et al*, 2006). Initial experiments, carried out at relatively high protein concentrations (~ 1 mg/ml), show that both the GluR1- and GluR2-ATDs behaved as dimeric assemblies, in agreement with previous studies on rat GluR4 (GluRD) (Kuusinen *et al*, 1999) and GluR1 (Wells *et al*, 2001). At these relatively high protein concentrations, there was no

evidence for either monomeric species or higher-ordered oligomers.

As the ATD dimers did not measurably dissociate under the conditions of the initial experiments, we carried out sedimentation studies at lower protein concentrations (0.01–0.05 mg/ml), exploiting the robust absorbance of the peptide bond at 229 nm to determine protein concentrations. On the basis of equilibrium experiments carried out at three rotor speeds and three protein concentrations, for both GluR1- and GluR2-ATDs, the combined data were best fit to a reversible monomer–dimer equilibrium model. The estimated dimer dissociation constants for both of the ATD domains were similar, with K_d values of 270 nM for GluR1- and 152 nM for GluR2-ATD (Figure 1). Fitting the equilibrium sedimentation data to a fixed single monomeric or dimeric species resulted in poorer square root of variance statistics and residuals (Supplementary Table S1).

iGluRs are tetrameric receptors (Rosenmund *et al*, 1998) and experimental data suggest that the ATD plays a crucial role in receptor oligomerization (Kuusinen *et al*, 1999; Leuschner and Hoch, 1999; Ayalon and Stern-Bach, 2001). Tetramerization seems to occur in two discrete steps, with the first step involving interactions between the ATDs of adjacent subunits to form dimers (Ayalon and Stern-Bach, 2001) and

Figure 1 The isolated GluR1 and GluR2 ATDs are dimers. (A) Sedimentation equilibrium studies of the GluR1 ATD. Lower panel: absorbance data (red dots) fit to a monomer–dimer model (black line), showing calculated populations of monomer (green) and dimer (blue). The loading concentration was 0.01 mg/ml and rotor speed was 12 500 r.p.m. Upper panel: Residual from the fit. The estimated GluR1 dimer K_d is ~ 270 nM. (B) Similar experimental data and analysis for the GluR2 ATD. Lower panel: absorbance data (red dots) fit to a monomer–dimer model (black line), showing calculated populations of monomer (green) and dimer (blue). The loading protein concentration was 0.01 mg/ml and the rotor speed was 13 000 r.p.m. Upper panel: Residual from the fit. The estimated GluR2 ATD dimer K_d is ~ 152 nM. (C) Residuals in absorbance units at 229 nm versus GluR1-ATD concentration (also in absorbance units) from a global fit of three concentrations (0.01, 0.02, 0.05 mg/ml) and three rotor speeds (10 000, 12 500, 17 000 r.p.m.). (D) Residuals in absorbance units at 229 nm versus GluR2-ATD concentration (in absorbance units) from a global fit of three concentrations (0.01, 0.02, 0.04 mg/ml) and three rotor speeds (10 000, 13 000, 18 000 r.p.m.).

the second step comprising the association of two dimers to form the functional, tetrameric receptor. Our analytical ultracentrifugation results showing that the GluR1- and GluR2-ATDs form tightly associated dimers buttress the contention that ATD plays a crucial role in receptor assembly.

Structure determination

To provide an atomic structure of an iGluR ATD we subjected GluR1- and GluR2-ATDs to crystallization trials. Although both formed crystals, the GluR1-ATD crystals diffracted to ~ 6 Å resolution, whereas the GluR2-ATD crystallized in two different space groups, one of which ($P2_12_12$) diffracted X-rays to high resolution (Table I). The GluR2-ATD structure was solved by single-wavelength anomalous diffraction using a selenomethionine (SeMet) derivative (Hendrickson *et al*, 1990; Hendrickson, 1991). A partial model was built from maps in which we combined the SeMet phases and amplitudes from the Native 1 data set. Using this partial model and the higher-resolution, more redundant Native 2 data set (Table I), we constructed a nearly complete atomic model through iterative cycles of manual model building and crystallographic refinement. There are three protomers in one asymmetric unit of the $P2_12_12$ crystal form, two forming a non crystallographic symmetry (NCS) dimer (subunits A and B) and the third located on the crystallographic two-fold axis (subunit C). As the A and B subunits are better ordered than subunit C, subunit A and the AB dimer are used for figures and analysis. We then employed the subunit A of the $P2_12_12$ crystal form to solve the structure of the $P2_12_12$ form by molecular replacement, which has two protomers in one asymmetric unit. The conformation of the five independent protomers and the dimeric assemblies are similar between the two crystal forms. The $P2_12_12$ structure was refined to good crystallographic and stereochemical statistics (Table II).

Clamshell-like structure of the GluR2-ATD protomer

The GluR2-ATD protomer has a clamshell-like shape in which the amino-terminal portion of the sequence defines most of one lobe (L1) and the carboxyl-terminal region composes most of the second lobe (L2; Figure 2A and B). The L1 and L2 domains, each with α/β topology, are connected by three short loops. The first ordered residue of the ATD N-terminus (Ser 3; numbering according to the mature polypeptide sequence; Keinänen *et al*, 1990) is located in the L1 domain

and includes the trafficking sequence IQI (Xia *et al*, 2002) in the first β -strand, whereas the last ordered residue at the carboxyl terminus is Leu 378, located at the end of $\beta 15$ in the L2 domain, 15 residues from the beginning of the S1S2 agonist-binding domain (Lys 393) (Kuusinen *et al*, 1995; Armstrong *et al*, 1998; Armstrong and Gouaux, 2000). The ATD is post-translationally modified by an intraprotomer disulphide bond between Cys57 and Cys309 and by predicted N-linked glycosylation at residues Asn 235 and Asn 349. Clear electron density is present for the disulphide link and for two N-acetyl glucosamine moieties at Asn 235, but there is no clear density for the remainder of the carbohydrate at Asn 235 or for the carbohydrate at Asn 349. As the $P2_12_12$ crystals diffracted to ~ 2.3 Å resolution, we were able to visualize the electron density for ~ 368 water molecules. Careful inspection of difference maps, especially within the clamshell-like cleft or subunit interfaces, did not show electron density for other bound molecules or ions.

Over 15 years ago O'Hara and colleagues predicted that the extracellular, ligand-binding domains (LBDs) of metabotropic glutamate receptors (mGluRs) and the ATDs of iGluRs had a fold similar to that of the bacterial leucine-isoleucine-valine

Table II Refinement statistics

Resolution (Å)	20.0–2.33
Number of reflections	62 450
$R_{\text{work}}/R_{\text{free}}$ (%) ^a	18.6/23.5
<i>RMS deviations from ideal geometry</i>	
Bond length	0.008 Å
Angle	1.089
<i>Number of atoms</i>	
Protein	9392
NAG	8968
Water	56
Mean main chain B value (Å ²)	368
Mean side chain B value (Å ²)	82.2
Ramachandran plot statistics	87.6
Residues in most favored regions	(1060) 94.7%
Residues in allowed regions	(59) 5.3%
Residues in disallowed regions	0

The formula for R_{free} is the same as that for R_{work} . R_{free} was calculated using 7.5% of the reflection data chosen randomly and omitted from refinement at the start of the refinement.

$$^a R_{\text{work}} = \sum_{hkl} |F_{\text{obs}}(hkl) - |F_{\text{calc}}(hkl)|| / \sum_{hkl} F_{\text{obs}}(hkl).$$

Table I Data collection statistics

Data set	Native 1	Native 2	Native 3	SeM15	SeM30
Wavelength (Å)	1.0000	1.0000	1.0000	0.9787	0.9789
Space group ^a	$P2_12_12$	$P2_12_12$	$P2_12_12$	$P2_12_12$	$P2_12_12$
<i>Cell dimensions</i>					
a, b, c (Å)	64.5, 363.5, 61.7	64.0, 363.0, 61.4	108.2, 85.4, 95.2	62.8, 360.7, 61.1	63.5, 361.8, 61.3
α, β, γ (deg)	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution (Å) ^b	50.00–2.40 (2.44–2.40)	50.00–2.33 (2.41–2.33)	50.00–2.68 (2.74–2.68)	50.00–2.85 (2.95–2.85)	50.00–3.05 (3.16–3.05)
R_{merge} (%) ^c	4.9 (51.8)	6.1 (60.5)	4.5 (60.0)	5.4 (45.5)	10.5 (62.5)
$I/\sigma I$	26.4 (2.0)	35.8 (2.2)	20.9 (2.9)	16.2 (2.0)	21.7 (2.1)
Completeness (%) ^c	99.8 (100.0)	99.9 (100.0)	99.8 (100.0)	98.6 (87.8)	99.2 (93.2)
Redundancy ^c	3.9 (4.0)	7.3 (7.4)	6.0 (6.2)	4.1 (2.6)	10.5 (5.3)

$$R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl).$$

^aAsymmetric unit contents: three protomers in the $P2_12_12$ cell and two protomers in the $P2_12_12$ cell.

^bValues in parentheses define the highest resolution shell.

^cValues in parentheses are for the highest resolution shell.

Figure 2 Structure of the GluR2-ATD protomer. (A) Ribbon representation of the GluR2 ATD protomer showing the clamshell-like organization of the ATD, domains L1 and L2, α -helices (blue), β -strands (gold) and non α , non β 'loops' in green. The clamshell-like cleft between domains L1 and L2 is defined by a red star, the amino terminus (N) is at the 'top', the carboxyl terminus (C), which couples to the S1S2 portion of the receptor, is near the ATD 'bottom', and two N-acetylglucosamine groups at the well-ordered carbohydrate site (Asn 235) are shown in stick representation. Shown is chain A from the Native 2 crystal structure. (B) View of the protomer following a rotation of $\sim 90^\circ$ around a vertical axis. (C) Superposition of the GluR2-ATD protomer (A chain; colours as in panel (A)) and an mGluR1-LBD protomer in an 'open' conformation (grey; PDB code 1EWK chain B). (D) Similar superposition as in panel (C), except with the closed, glutamate-bound form of an mGluR1-LBD protomer (grey; PDB code 1EWK; chain A).

binding protein (LIVBP) (O'Hara *et al*, 1993). Accordingly, a structural similarity search using the DALI server (Holm *et al*, 2008) showed that the structure of the GluR2-ATD is similar to that of the ligand-binding domain of mGluRs (Kunishima *et al*, 2000; Muto *et al*, 2007), the extracellular domain of the natriuretic peptide receptor (He *et al*, 2001) and *Escherichia coli* LIVBP (Trakhanov *et al*, 2005), even though the amino-acid sequence identity between the GluR2-ATD and these proteins is $<15\%$. The closest structural homologue to the GluR2-ATD is a protomer from the mGluR7-LBD (Muto *et al*, 2007), with a r.m.s. deviation of 2.9 Å over 333 aligned residues; the mGluR1-LBD is also closely related, with a r.m.s. deviation of 3.1 Å over 338 residues.

There are, however, significant structural differences between the GluR2-ATD and the mGluR-LBDs that suggest the domains have different functions in metabotropic and iono-

tropic receptors. First, from studies on LIVBP, other bacterial orthologues and the mGluRs, it is well known that the L1 and L2 lobes can adopt open, typically apo or ligand-free conformations and closed, generally ligand-bound states. For LIVBP and the mGluRs, the rotation angle that describes this open-cleft to closed-cleft conformational change is large and ranges from 25° to 50° (Kunishima *et al*, 2000; Tsuchiya *et al*, 2002; Trakhanov *et al*, 2005; Muto *et al*, 2007). In the five crystallographically independent GluR2-ATD protomers reported here, however, we see only one conformation. Superposition of the L1 and L2 domains of GluR2-ATD onto the corresponding domains of either LIVBP or mGluR1 (Kunishima *et al*, 2000) clearly shows that the GluR2-ATD adopts a conformation that is intermediate between the canonical open-cleft and closed-cleft states of LIVBP and mGluR1 (Figure 2C and D). Here we used the mGluR1-LBD

for comparisons instead of mGluR7-LBD because multiple conformations are only available for mGluR1.

There are also multiple differences between the GluR2-ATD and the mGluR1-LBD, the first being that in mGluR1 there is a long disordered segment (Asp125–Lys153) between helix-B and strand-d that includes a crucial Cys residue (Cys140) proposed to form an interprotomer disulphide bridge. The corresponding segment on the GluR2-ATD is between $\alpha 2$ and $\beta 3$, is much shorter, and is not disordered. A second departure is that the GluR2-ATD has a well-structured loop (Gln296–Trp317), or ‘flap’ that lies between $\alpha 9$ and $\alpha 10$. This flap adopts an extended conformation and ‘sits’ on the top of the GluR2-ATD and is linked to the $\alpha 2$ helix by an intraprotomer disulphide-bond bridge formed between Cys57 and Cys309. The corresponding region on mGluR1-LBD (residues Cys432–Asp446, between helix M and O) is much shorter and forms an internal disulphide bond between Cys432 and Cys439. A third distinction is that there are only three residues between $\beta 1$ and $\alpha 1$ on the GluR2-ATD. In contrast, the equivalent region in the mGluR1-LBD (Ser52–Gly75) contains 24 residues. Finally, the region between helix-K and helix-M on mGluR1-LBD (55 residues between Leu355 and Lys409, including a disulphide bond between Cys378 and Cys394) is much longer than the equivalent structure on GluR2-ATD (17 residues between $\alpha 8$ and $\alpha 9$). Thus, even though the core structural fold of the GluR2-ATD is similar to that of mGluRs, there are substantial differences in the lengths and structures of protruding loops—differences that are likely to affect the conformations that the GluR2-ATD can adopt and the manner in which the ATD participates in interactions within a fully-assembled receptor and between an assembled receptor and other synaptic proteins.

Inspection of the ATD protomer structure in combination with the aligned amino-acid sequences of AMPA, kainate and NMDA receptors allows several striking observations to be made, perhaps the most prominent being the predicted complete absence in NMDA receptors of helix 8 (Figure 2A and B; Supplementary Figure S1), one of the three structural elements that couples domains L1 and L2 in GluR2-ATD. An additional prominent difference is localized between $\beta 4$ and $\beta 5$, near the juncture between the L1 and L2 domains, where, in NMDA receptors, there are as many as six additional residues (Figure 2A and B; Supplementary Figure S1). In

NMDA receptors, this region is near the putative clamshell ‘hinge’ and is likely to undergo conformational changes on binding of allosteric modulators.

Architecture of the GluR2-ATD dimer

In agreement with the GluR1- and GluR2-ATD solution behaviour, the GluR2-ATD crystallizes as a dimer in both crystal forms (Figure 3). For the $P2_12_12$ crystals, there are three subunits in the asymmetric unit: subunits A and B form a NCS-related dimer (AB dimer) whereas molecule C, positioned on the crystallographic two-fold axis, forms a dimer with its symmetry mate (CC dimer). For the $P2_12_12_1$ crystals, there is one NCS dimer (A'B') in the asymmetric unit. All three crystallographically independent dimers have a similar conformation, and pairwise superposition of C α atoms for residues 6–378 yields r.m.s. deviations of 0.9, 1.5 and 1.5 Å between the AB and CC, the AB and A'B', and the CC and A'B' dimers, respectively. The equivalence in the conformations among the three independent dimers thus suggests that under the conditions of crystallization neither the dimer nor the individual subunits readily adopt multiple, unliganded conformational states.

There are extensive protein–protein interactions between the two protomers of the GluR2-ATD dimer that involve multiple L1–L1 and L2–L2 domain contacts (Figure 3). On dimer formation, each subunit buries about 1408 Å² of solvent-accessible surface area at the dimer interface, consistent with the low dimer dissociation constant observed in sedimentation studies. The dimer interface between the two-fold-related L1 domains can be divided into a region at the very ‘top’ of the dimer that is mediated by the tips of the ‘flap’ (Figure 4) and the region immediately below the loop that makes contacts involving extensive interactions between the $\alpha 2$ and $\alpha 3$ helices (Figure 5A and B).

The flap is an element of extended polypeptide between helices $\alpha 9$ and $\alpha 10$, includes residues Gln296 to Trp317 (Figure 4; Supplementary Figure 1), and is more well-conserved within receptor subtypes than between receptor subtypes. The flap covers the ‘top’ of the L1 domain and at the turn of the loop, forms hydrophobic (Leu 310) contacts with the two-fold-related Leu310 at the dimer interface. Most importantly, the flap harbours Cys309, which forms an intraprotomer disulphide bridge with Cys57, a covalent cross-

Figure 3 Architecture of the GluR2-ATD dimer. (A) View of dimer perpendicular to the molecular two-fold axis. Boxed regions are the crucial L1–L1 and L2–L2 interprotomer interfaces. Helices and strands in a darker shade are either involved in domain–domain interactions (L1–L1: $\alpha 2/\alpha 3$; ‘flaps’; L2–L2: $\alpha 5/\beta 7$) or bridge L1 and L2 domains ($\alpha 8$). (B) View of dimer parallel to the molecular two-fold axis looking onto the ‘top’ of the L1 domains and into the L1–L1 interface, showing the ‘flaps’ and the essential disulphide bonds between the $\alpha 2$ helices and the ‘flaps’.

Figure 4 The L1 'Flap'. (A) The L1 'flap' folds over the 'top' of the L1 domain, spans residues Ile298–Trp317, and includes residue Cys309, which makes a disulphide bond to Cys57, as well as Leu310, which forms a hydrophobic contact in the L1–L1 interface. (B) Multiple amino-acid-sequence alignment of the 'flaps' from AMPA, kainate and NMDA receptors shows that even though the loop varies substantially in length and residue composition from subtype to subtype, the cysteine involved in disulphide bond formation is conserved.

link within a subunit that is conserved across receptor subtypes. We suggest that the disulphide bridge constrains the conformation of the flap and thus the disulphide bridge indirectly plays an important role in subunit assembly. Furthermore, the well-defined intraprotomer disulphide bridge in the GluR2-ATD, in combination with amino-acid sequence alignments (Supplementary Figure S1), suggests that in NMDA receptors the corresponding cysteine residues are involved in a disulphide bond within rather than between subunits (Papadakis *et al*, 2004).

At the dimer interface and immediately below this flap are multiple subunit–subunit contacts mediated by residues on helices $\alpha 2$ and $\alpha 3$ and involving both nonpolar and polar contacts (Figures 5A, B, and 6A, B). The most prominent nonpolar contacts are aromatic edge-to-face contacts between Phe50 ($\alpha 2$) and Phe82 ($\alpha 3$). This specific interaction is unique to AMPA receptors; in kainate receptors, the amino-acid equivalent of Phe82 is an Ile and in NMDA receptors the equivalent amino acids are Ile, Lys, or Thr. There is a substantial amount of solvent in the L1–L1 dimer interface and this is illustrated by the coordination of a well-ordered water molecule by four Thr residues, one from each of $\alpha 2$ and $\alpha 3$ (Thr54 and Thr78). Additional polar interactions that knit the dimer interface together are formed from Asn54 (N $\delta 2$) to

Leu310 (O) and Ser81 (O γ) to Asn48 (O $\delta 1$)/Phe50 (N). The crystallographically defined interface also allows us to correct amino-sequence alignments and definitions of residues in the L1 interface based on homology models (Ayalon *et al*, 2005). We now see that the sequence alignment of Ayalon *et al* (2005) is not correct, and that the L1 interfaces of AMPA and kainate receptors are similar in terms of the balance of hydrophobic and hydrophilic contacts.

Extensive interactions are observed between the GluR2 L2 domains, a conclusion that is also in opposition to homology models of the AMPA-receptor ATD (Ayalon *et al*, 2005). Most of the interactions are hydrophobic contacts involving residues on $\alpha 5$ and $\beta 7$ (Figure 5C and D). Specifically, four residues on the $\alpha 5$ helix facing the dimer interface, Leu137, Leu140, Leu144, and Ala148, as well as residues Ala156 and Ile157 on $\beta 7$, form a large hydrophobic patch, which is buried after dimerization of the GluR2-ATD. These nonpolar residues on $\alpha 5$ are conserved or conservatively substituted between AMPA and kainate receptors, whereas in NMDA receptors, the extent of sequence conservation is lower, with a glycine residue in the NR1 subunit at the location equivalent to Leu 137 (GluR2), for example. The departure in amino-acid conservation at the L1 and L2 interfaces between non-NMDA and NMDA receptors is consistent with the idea that the binding of modulators to NMDA receptors results in conformational changes not only within subunits but also between subunits, probably at the L2–L2 interface.

Comparison between the dimer interfaces of GluR2-ATD and mGluR1-LBD suggests a molecular mechanism by which conformational changes in NMDA ATDs might arise. In contrast with the extensive interactions at the L2–L2 interface in the GluR2-ATD, most of the dimer interactions on mGluR1-LBD are mediated by residues on domain L1. In fact, the charged residues on helix F and strand H of mGluR1-LBD (equivalent to $\alpha 5$ and $\beta 7$ on the GluR2-ATD), especially the cluster of four acidic residues (Asp 191, Glu 233, Glu 238 and Asp 242), make mGluR1 L2–L2 interactions unfavourable (Kunishima *et al*, 2000). Significant interactions between L2 domains of mGluR1-LBD are only observed in the glutamate/ Gd^{3+} complex, where Gd^{3+} binds at the centre of the acidic patch and alleviates the electrostatic repulsion between the two subunits (Tsuchiya *et al*, 2002). In harmony with the mechanisms of NMDA receptor modulation proposed earlier (Gielen *et al*, 2008), we suggest that the 'weak' L2–L2 interface in the NMDA receptor is akin to that of mGluRs, thereby allowing for the movement on closure of the L1–L2 clamshell and allosteric modulation of the ion channel by way of the intervening ligand-binding domains (Figure 8).

Intra- and intersubunit interactions stabilize a partially closed conformation

What interactions stabilize the unliganded, 'empty' GluR2-ATD in a partially closed conformation? We suggest that there are two basic classes of interactions that stabilize the GluR2-ATD in a partially closed conformation, the first being interactions within a subunit and the second involving interactions between subunits. Within a subunit, there are clusters of residues located at a position equivalent to the hinge of the mGluR-LBD (Kunishima *et al*, 2000). The first cluster is in the concave groove of the clamshell and involves a direct hydrogen bond between the carboxylate of Asp223 (L2) and the NH group of Tyr274 (L1) together with a water-mediated

Figure 5 Nature of interprotomer contacts. (A) L1–L1 interface illustrating the proximity of the Cys57–Cys309 disulphide bond and the roles of aromatic–aromatic (Phe50–Phe82) and polar contacts (Thr53, Thr78, Asn54 and Leu310 and the water molecule, HOH63). (B) Multiple amino-acid-sequence alignment of the residues in helices $\alpha 2$ and $\alpha 3$, the two crucial helices comprising the four-helix bundle, L1–L1 interdomain interface. (C) Contacts at the L2–L2 interprotomer interface showing non-polar interactions between the side chains of Leu137, L140 and L144 of helix $\alpha 5$, together with a hydrogen bond between Tyr131 and Gln141, two residues conserved between AMPA and kainate receptors. Here, the molecular two-fold axis is oriented approximately perpendicular to the plane of the page from the vantage point of the ‘top’ of the interface, proximal to Leu137. Protomer A is gold, protomer B is blue. The van der Waals surfaces of the leucine side chains are shown as dots. (D) Multiple amino-acid-sequence alignment of residues in helix $\alpha 5$.

hydrogen bond network connecting residues Ile272 and Tyr274 on the L1 domain and residues Leu219, Asp223, Gln240, Ile241 and Val242 on the L2 domain (Figure 7A). A second cluster of interdomain interactions involves Leu111 and Ile352 (L2 domain) and Pro109 and Tyr274 (L1 domain). A third cluster involves Phe95 (L1) acting like a ‘pebble in the clamshell cleft’, packing against the main-chain atoms of residues 135–136 (L2), propping the cleft partially open. On the convex side of the clamshell cleft is a fourth set of interactions, formed by the highly conserved Asp281 and main-chain NH groups of Leu335 and Ser336, contacts that probably further stabilize the clamshell cleft. The L1–L2 domain interactions in the GluR2-ATD are not nearly as extensive as those seen in the glutamate-bound mGluR-LBD in which there are nine pairs of interdomain hydrogen bonds distributed widely over the interfacial surfaces of the L1 and L2 domains, surrounding the bound glutamate.

The second class of interactions that stabilize the GluR2-ATD are intersubunit contacts mediated by the L2 domains. To illustrate the likely role that these contacts play in defining the GluR2-ATD conformation, and to answer the question of whether the GluR2-ATD is likely to undergo conformational changes on interactions with other synaptic proteins, it is helpful to compare the GluR2-ATD to the mGluR1-LBD. The mGluR1-LBD adopts two distinct conformations: the ‘A’ conformation represents the activated state and the ‘R’ conformation corresponds to the resting state (Kunishima *et al*, 2000). These two conformations are defined by distinct intersubunit orientations of the L1 domain. The GluR2-ATD adopts a conformation similar to the ‘A’ form of the mGluR1-LBD. Unlike the mGluR1-LBD, however, the GluR2-ATD

dimer is held together by extensive interactions involving both the L1 and L2 domains. In the mGluR-LBD, the L2 domain is not constrained by intersubunit interactions, and is thus relatively free to undertake ligand-dependent conformational changes, movements that are likely crucial for transducing the signal of ligand binding to the transmembrane domain (Jingami *et al*, 2003). For the GluR2-ATD, the L2–L2 interaction buries a large hydrophobic surface composed of residues that are mostly conserved among the AMPA subfamily. When we model the dimeric GluR2-ATD into the ‘R’ form of mGluR1-LBD (data not shown), a large hydrophobic patch on the L2 domain is exposed, suggesting that the GluR2-ATD is constrained to the ‘A’-like dimer configuration.

iGluR-ATD does not contain a canonical amino-acid binding site

If the GluR2-ATD has a protein fold that is closely related to the mGluR-LBD and to the bacterial LIVBP, might it also possess a binding site for amino acids? We can clearly answer this question by inspection of the superimposed structures and the aligned amino-acid sequences. In the mGluR1-LBD there are six residues, invariant among mGluRs1–8, that coordinate the α -amino and α -carboxyl groups of the bound glutamate molecule: Ser 165 (main chain N and OG), Ser 186 (main chain O), Thr 188 (OG1), Asp 208 (OD2), Tyr 236 (cation- π interaction) and Asp 318 (OD2) (Kunishima *et al*, 2000; Bräuner-Osborne *et al*, 2007). Interestingly, all six residues are highly conserved in the distantly related amino-acid-binding protein LIVBP, where the corresponding residues are Ser 79, Ala 100, Thr 102, Asp 121, Tyr 150 and

Figure 6 Conservation of amino-acid residues and hydrophobic character at the L1-L1 and L2-L2 interfaces. **(A)** Conservation of amino-acid residues of rat AMPA receptors GluR1-4 mapped onto the protein surface. Red is most conserved and blue is least conserved. **(B)** Conservation of amino-acid residues of rat AMPA receptors GluR1-4 and rat kainate receptors GluR5-7 mapped onto the solvent-accessible protein surface. Residue colouring is the same as in panel (A). **(C)** Mapping of residue polarity onto the GluR2-ATD protein surface, coloured accordingly: white, hydrophobic (Ala, Gly, Ile, Leu, Met, Pro and Val); yellow, semipolar (Cys); cyan, polar (His, Asn, Gln, Ser and Thr); red, acidic (Asp and Glu); blue, basic (Lys and Arg); wheat, aromatic (Phe, Tyr and Trp).

Glu 226, respectively (Trakhanov *et al*, 2005). In LIVBP, these six equivalent residues form the same hydrogen network with the α -amino-acid moiety of ligands as the corresponding residues in the mGluR1-LBD form with glutamate. In spite of the structural similarity among GluR2-ATD, mGluR-LBD and LIVBP, however, the six highly conserved residues that interact with the α -amino and α -carboxyl groups of a bound ligand are not conserved in the GluR2-ATD sequence and are, instead, Lys 73, Ser 94, Pro 96, Leu 111, Arg 135 and Asn 218. Furthermore, the crucial residues required for amino-acid binding are not conserved in any AMPA, kainate and NMDA receptor ATD. Therefore, the ATDs of iGluRs from animals are devoid of canonical mGluR/LIVBP-like amino-acid binding sites and are thus incapable of binding amino-acid ligands through a similar mechanism, in agreement with recent and extensive amino-acid sequence analysis (Acher

and Bertrand, 2005). The ATD of predicted plant iGluRs, however, contains elements of an eight-residue signature sequence associated with known amino-acid binding, and thus might, in fact, bind amino acids in their LBD as well as in the classical S1S2 ligand-binding domain (Acher and Bertrand, 2005).

Conclusion

The GluR2-ATD structure provides an atomic model of the last remaining extracellular domain of iGluRs and suggests possible roles that residues, located at the dimer interface, might play in defining receptor subtype-specific assembly (Figure 8). Together with the association analysis by analytical ultracentrifugation, the crystal structures show that extracellular domains of tetrameric iGluRs are composed

Figure 7 Clamshell cleft and hinge. (A) Residues that probably stabilise the partially closed conformation and that are located within the clamshell cleft and near the juncture between the L1 and L2 lobes. (B) Conserved Ala 277, Asp281 and Phe 341 define crucial interactions between helix $\alpha 9$ and the 'loop' that includes $\beta 11$ and $\beta 12$, interactions that also bridge lobes L1 and L2 and modulate possible closed/open cleft conformations.

Figure 8 Role of ATD in iGluR assembly and function. Illustration of two subunits of an iGluR tetramer. Crucial interfaces between ATD subunits are at the L1-L1 and L2-L2 interfaces, emphasised in rectangles. In AMPA and kainate receptors, we suggest that neither of these interfaces nor the 'clamshell' itself undergoes substantial conformational changes. In NMDA receptors, however, modulatory ligands might induce conformational changes in the clamshell and at the L2-L2 interface (rectangle with hatched lines). These changes, in turn, might be transmitted to the agonist-binding and the ion-channel domains, perturbing the balance between closed and open states.

of tightly associated dimeric assemblies. Tetramer formation in iGluRs, therefore, likely derives from combined interactions between the transmembrane domains together with interactions between the ATD and S1S2 domains.

Materials and methods

Expression, purification and crystallization

The amino-terminal domain (ATD; Val1-Ser383) of the rat AMPA (α -amino-5-methyl-3-hydroxy-4-isoxazole propionic acid)-sensitive GluR2 receptor was secreted from *Spodoptera frugiperda* insect cells (Sf9) as a carboxyl-terminal His₈-tag fusion and purified by metal

ion affinity chromatography and size-exclusion chromatography (SEC). GluR2-ATD protein used to obtain crystals for data sets Native 2 and Native 3 (Table 1) was treated with thrombin to remove the His₈-tag.

GluR1-ATD (Asn1-Asp375), fused with carboxyl-terminal 1D4 peptide (MacKenzie *et al*, 1984) and a His₈-tag, was expressed and purified in a similar manner, except that the SEC step was substituted with a cationic ion-exchange chromatography step. GluR1-ATD was bound to a SP Sepharose column (GE Healthcare) in 50 mM MES (pH 6.0), 25 mM NaCl, 1 mM EDTA, eluted with 90–120 mM NaCl and dialysed overnight in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 1 mM EDTA.

L-selenomethionine (SeMet)-substituted GluR2-ATD was produced by expression using Sf9 cells adapted to methionine-free media (Expression Systems), infection with recombinant baculovirus, and depletion of residual methionine for 18 h before the addition of SeMet to a final concentration of 100 mg/l at 24, 48 and 72 h post-infection. To minimize oxidation of the SeMet residues, purification was carried out in the presence of 1-mM β -mercaptoethanol and 5-mM L-methionine. SeMet incorporation was evaluated by amino-acid analysis and was typically greater than 90%.

GluR2-ATD crystals used for native data sets 1 and 2 (P₂₁₂₁₂ crystal form) were grown by hanging-drop vapour diffusion at 4°C by mixing protein (3–6 mg/ml) with a reservoir solution containing 14–16% PEG 3350, 100 mM Tris-HCl (pH 8.0–8.5), 10 mM MgCl₂ in 1:1, 1:2 or 2:1 ratios (v/v). SeMet-GluR2-ATD crystals were grown under similar conditions. Crystals used for native data set 3 (P₂₁₂₁₂ crystal form) were prepared similarly but grown from a reservoir solution containing 20% PEG 3000, 100 mM tri-sodium citrate (pH 5.5). All crystals were cryoprotected with the respective reservoir solution supplemented with glycerol, added gradually over 10 min in 3% increments, to a final concentration of 24%.

Data collection, structure determination and refinement

The structure of the GluR2-ATD in the P₂₁₂₁₂ crystal form was solved by a single anomalous diffraction (SAD) experiment on SeMet-labeled crystals (Hendrickson, 1991). The first SeMet data set (SeM30) was collected at the Se peak using an inverse beam data-collection strategy and the diffraction data were processed using HKL2000 (Otwinowski and Minor, 1997). Selenium sites and initial phases (figure-of-merit (FOM) of 0.28) were determined with SOLVE (Terwilliger and Berendzen, 1999) and phases were improved by density modification that included non-crystallographic symmetry (NCS) averaging among the three NCS-related protomers, solvent flattening and histogram matching using DM (Cowtan and Zhang, 1999). The SeM30-derived phases were then applied to the Native 1 data set and extended to the resolution limit of the data, by density modification. This map was used to build a partial model using O (Jones *et al*, 1991) and COOT (Emsley and Cowtan, 2004), which was subsequently employed with the Native

2 data set to yield a model that was ~70% complete. However, this partial model yielded Rfree value around 50% and could not be improved by further manual model building and refinement.

To provide additional experimental phases, diffraction data on another SeMet-labelled crystal, which diffracted to slightly higher resolution (SeM15), were collected. SAD phasing was carried out with SOLVE (Terwilliger and Berendzen, 1999), and 14 out of 15 selenium sites were identified. The electron-density map clearly showed that two molecules (Mol-A and B) formed an NCS dimer. Weak electron density was observed for the third molecule (Mol-C) that formed a dimer with its symmetry mate. The electron density was improved by density modification and NCS averaging in DM (CCP4 Project, 1994). A partial model was built automatically by RESOLVE (Terwilliger, 2003), including 50% of the residues in Mol-A and -B but only a few residues for Mol-C. However, using the partial model obtained from the SeM30 dataset as a reference, we were able to manually build an ~70%-complete poly-Ala model of Mol-A using COOT.

This partial model was next employed in molecular replacement (MR) using Phaser (McCoy, 2007) and the Native 2 data set (Table I). Although only two out of three molecules were found (Mol-A/B), the quality of the electron-density map for Mol-A/B was good and most of the side chains were visible. The structure was improved by iterative manual model-building with COOT and refinement with Crystallography and NMR System (CNS) (Brunger *et al*, 1998) until the Rfree reached 0.41. At this point, the structures of Mol-A and B were over 90% complete and the electron density for Mol-C was well defined. One copy of Mol-A was then manually moved and fit into the electron density of Mol-C using COOT. Refinements with all three molecules were begun with rigid body minimization followed by a slow-cooling simulated annealing protocol at 5000K in CNS (Brunger *et al*, 1998) to reduce model bias. Iterative rounds of positional and B-factor refinement were carried out in conjunction with manual model building until Rfree converged. Further refinements were carried out in Phenix (Adams *et al*, 2002) with three TLS groups for Mol-A and B, and four TLS groups for Mol-C, as identified by TLSMD (Painter and Merritt, 2006). During the final cycles of refinement, NCS restraints were released and each protomer was refined independently, ultimately yielding a structure with excellent stereochemistry and good crystallographic statistics (Table II).

The structure of the GluR2 ATD in the P2₁2₁2₁ crystal form was solved by molecular replacement with Phaser (McCoy, 2007) using Mol-A from the P2₁2₁2 crystal structure as the search probe. The structure was subjected to one round of crystallographic refinement using data to 2.7 Å resolution. This refinement resulted in *R*_{work} and *R*_{free} values of 0.22 and 0.29 (no solvent), respectively, and root mean square deviations on bond lengths and angles of 0.008 Å and 1.10°, respectively. The conformation of the protomers in the P2₁2₁2₁ cell is similar to that of the protomers in the higher-resolution, well-refined P2₁2₁2 crystal form, and thus further refinement of the P2₁2₁2₁ form was not pursued.

Analytical ultracentrifugation

Sedimentation equilibrium experiments were carried out in a Beckman Coulter Optima XL-I analytical ultracentrifuge at 4°C in an An50Ti rotor with absorbance optics (229 and 280 nm) and quartz windows. For these experiments, GluR1- and GluR2-ATD were further purified by SEC, concentrated and dialysed overnight against 20 mM sodium phosphate (pH 7.5), 150 mM NaCl, 1 mM EDTA. Samples were loaded into six-sector, 12-mm charcoal-filled Epon centerpieces at 0.01, 0.02, 0.05, 0.25, 0.5 and 0.75 mg/ml for GluR1-ATD and run at speeds of 10 000, 12 500 and 17 000 r.p.m.; for GluR2-ATD, concentrations included 0.01, 0.02, 0.04, 0.07, 0.1 and 0.2 mg/ml run at speeds of 10 000, 13 000 and 18 000 r.p.m.

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The instrument was programmed to scan the cell at 2-h intervals with 0.005-cm spacings and 10 replicates per point.

Equilibrium runs using high concentrations (1, 2.5 and 5 mg/ml) of GluR2-ATD, interference optics and speeds of 8000, 12 000, and 18 000 r.p.m. were also carried out in an effort to find evidence of higher-order assembly, that is, tetramer formation, but no oligomer greater than a dimer was observed.

The programme WinMATCH was employed to determine when the balance between protein sedimentation and diffusion had reached equilibrium. WinREED was used to remove menisci from the scans before data were analysed through nonlinear regression in WinNONLIN (Johnson *et al*, 1981). Solvent density, solvent viscosity and protein partial specific volume (*V*_{protein}) were calculated with Sednterp (Cohn and Edsall, 1943). *V*_{protein} was then adjusted for glycosylation by comparing the monomer molecular weights determined from amino-acid sequence with those obtained from MALDI-TOF mass spectrometry and then adding the contribution of an estimated carbohydrate partial specific volume (*V*_{carbohydrate}) of 0.63 ml/g, according to Lewis and Junghans (2000). This resulted in new partial specific volumes for the glycosylated proteins (*V*_{glycos-prot}) of 0.725 ml/g for GluR1-ATD and 0.729 for GluR2-ATD, respectively.

Association constants for any monomer ↔ dimer equilibrium obtained from the analysis were converted from absorbance (*K*_{2,abs}) to molar units (*K*_{2,M}) with the equation *K*_{2,M} = *K*_{2,abs}(*εl*)/2, where *l* is the path length of the cell (1.2 cm) and *ε* is the molar extinction coefficient at 229 nm (359 210 and 318 045 M⁻¹ cm⁻¹, respectively, for GluR1- and GluR2-ATD). The *ε*₂₂₉ value was obtained by first calculating the *ε*₂₈₀ value with Sednterp and then extrapolating to 229 nm after comparing absorbance scans in the XL-I at 229 and 280 nm.

Mass spectrometry

To assess the relative homogeneity and glycosylation state of the ATDs, mass spectrometric analysis (MALDI-TOF) was carried out. These experiments showed that the GluR1-ATD has a subunit mass of 48 394 Da and GluR2-ATD has a mass of 47 988 Da.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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