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Crystal structures of the glutamate receptor ion channel GluK3 and GluK5 amino terminal domains

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Abstract

Ionotropic glutamate receptors (iGluRs) mediate the majority of fast excitatory synaptic neurotransmission in the central nervous system. The selective assembly of iGluRs into the AMPA, kainate and NMDA receptor subtypes is regulated by their extracellular amino terminal domains (ATD). Kainate receptors are further classified into low-affinity (GluK1-3) and high-affinity (GluK4-5) receptor families based on their affinity for the neurotoxin kainic acid. These two families share 42% sequence identity for the intact receptor but only 28% sequence identity at the level of ATD. We have determined for the first time high-resolution crystal structures for the GluK3 and GluK5 ATDs, both of which crystallize as dimers, but with a strikingly different dimer assembly at the R1 interface. By contrast, for both GluK3 and GluK5 the R2 domain dimer assembly is similar to that reported previously for other non-NMDA iGluRs. This observation is consistent with the reports that GluK4-5 cannot form functional homomeric ion channels and require obligate coassembly with GluK1-3. Our analysis also reveals that the relative orientation of domains R1 and R2 in individual non-NMDA receptor ATDs varies by up to 10°, in contrast to the 50° difference reported for the NMDA receptor GluN2B subunit. This restricted domain movement in non-NMDA receptor ATDs seems to result from both extensive intramolecular contacts between domains R1 and R2, and from their assembly as dimers which interact at both the R1 and R2 domains. Our results provide the first insights into the structure and function for GluK4-5, the least understood family of iGluRs.

Keywords

kainate receptor; glycosylation; structural biology; cell surface receptor; synaptic plasticity

Introduction

Excitatory synaptic transmission in the brain of vertebrates is mediated by a family of 18 glutamate receptor ion channel genes (iGluRs), the products of which coassemble to form three major functional classes named AMPA, kainate and NMDA receptors.^{1,2} Within each major subtype four subunits coassemble to form a large and diverse family of native iGluRs, the molecular identity of which has yet to be fully established. Within an iGluR tetramer the

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Accession codes. Coordinates and structure factors have been deposited with the Protein Data Bank with accession numbers of 3OLZ for GluK3; 3OM0 for the GluK5 orthorhombic form; and 3OM1 for the GluK5 monoclinic form.

individual subunits share a common modular structure, which differs from that found in all other ligand gated ion channels.^{3,4} After signal peptide cleavage, the first 380 residues encode an extracellular amino terminal domain (ATD), which is coupled, via a short linker, to a 280 residue ligand binding domain (LBD). The LBD is in turn attached via three short linkers to a membrane embedded ion channel, which is followed by a cytoplasmic domain of variable length.⁵ This modular assembly of semi-autonomous extracellular domains has permitted genetic excision and large scale expression of individual ATDs and LBDs for biochemical and structural studies on representative AMPA, kainate and NMDA receptor subunits. The validity of this approach was recently confirmed by the structure of a full length tetrameric AMPA receptor at 3.6 Å resolution, which established that the isolated ATDs and LBDs are accurate models of the structure of the extracellular domains in an intact receptor.⁵ Because structural studies on full length iGluRs are exceedingly difficult, and at present have yielded only a single low resolution structure of the resting state of an AMPA receptor, experiments using the isolated ATDs and LBDs still have the potential to provide new insights into iGluR structure and function.

Using recombinant proteins overexpressed in *Escherichia coli*, crystal structures have been solved for the LBDs of 10 different iGluR subunits, including more than 80 ligand complexes with the AMPA receptor GluA2 subunit.^{4,6} In combination, these structures have established in depth the mechanism of binding of the neurotransmitters glutamate, glycine and D-serine; the basis of subtype selectivity; and how agonist binding triggers activation and desensitization.^{3,4,7–11} Although the role of the LBD is well understood, both at a functional and structural level, much less is known about the molecular properties of the ATD. A growing body of evidence indicates that the ATD mediates multiple functions, including control of receptor assembly, protein trafficking, trans-synaptic interactions with other proteins, and in NMDA receptors ion channel open probability.¹² In part, our limited knowledge of the molecular properties of the ATD is a consequence of limited success in preparing protein for structural and biochemical analysis. Numerous prior attempts at expression in *E. coli* have failed to yield soluble monodisperse protein, and only recently has crystallization succeeded following large scale expression in insect and mammalian cells. Currently, only three ATD structures have been solved. These are for the GluA2 (GluR2) AMPA,^{13,14} GluK2 (GluR6) kainate,^{15,16} and GluN2B (NR2B) NMDA receptor subunits.¹⁷ In combination, these studies have established directly that the ATD of homomeric AMPA and kainate receptors encodes an assembly apparatus responsible for tetramer formation by a dimer of dimers assembly. By contrast, the NMDA receptor GluN2B subunit is monomeric in solution and likely requires the GluN1 (NR1) subunit for ATD dimer assembly, although this has not been directly established. These crystal structures of representative AMPA, kainate and NMDA receptor ATDs reveal differences in subunit interactions, domain closure, and the conformation of loops proposed to play a role in oligomer assembly; the origin and biological significance of this diversity is unknown.

To extend our knowledge of the structural biology of iGluRs we have solved crystal structures for additional kainate receptor ATDs that are encoded by genes from two different families, named GluK1–3 (previously called GluR5–7) and GluK4–5 (previously called KA1 and KA2). The GluK1–3 and GluK4–5 families form receptors that have low and high affinity respectively for the prototypical agonist kainic acid.^{18–21} The GluK4–5 subunits are the least understood members of the iGluR genome, and the only family for which no crystal structures have been solved for either the ATD or LBD. Here we report two crystal structures of the GluK5 ATD at resolutions of 1.40 Å and 1.68 Å, together with a structure of the GluK3 ATD at a resolution of 2.75 Å.

Results and Discussion

Purification, crystallization and structure determination

HEK 293 GnT1⁻ cells grown in adherent culture were transfected with plasmids encoding cDNAs with native signal peptides for the GluK3 (Met1–Arg423) or GluK5 (Met1–Ile406) ATDs, together with a carboxy-terminal thrombin site and His₈-tag. The secreted glycoproteins were purified by affinity and ion exchange chromatography and digested with thrombin followed by endoglycosidase H (Endo H). Final yields were 0.5 and 3.5 mg of purified protein per 450 ml of conditioned medium for GluK3 and GluK5, respectively. N-terminal sequencing and comparison with cDNA translations established that the GluK3 signal peptide is cleaved between Gly31 and Met32 while for GluK5 the 19 residue signal peptide is cleaved between Cys19 and Val20. Following the convention used in our prior structural studies on iGluRs, amino acid numbering in the following sections is given for the mature protein, with the 1st residue after signal peptide cleavage assigned the identity one. ESI mass spectrometry was used to verify that after cleavage of N-linked glycans the proteins had the correct mass predicted from their amino acid sequence. GluK3 and GluK5 structures were solved by molecular replacement using a GluK2 ATD monomer (PDB 3H6G) as a search probe. Refinement of data with Bragg spacings of 2.75 Å for GluK3, $R_{\text{work}}/R_{\text{free}} = 19.5/25.2\%$; 1.40 Å for the orthorhombic GluK5 crystal form, $R_{\text{work}}/R_{\text{free}} = 17.5/19.6\%$; and 1.68 Å for the monoclinic GluK5 crystal form, $R_{\text{work}}/R_{\text{free}} = 16.8/19.6\%$, resulted in models with good stereochemistry and geometry (Table 1). Electron density maps for the final refined models are shown in Supplementary Fig. S1.

Kainate receptor ATDs show diversity within a conserved core structure

Sequence alignments using ClustalW22 reveal that the GluK1–3 and GluK4–5 ATDs share 68–75% and 65% within family amino acid sequence identity respectively, but only 25–27% identity between families (Supplementary Fig. S2), comparable to the 21–24% between family sequence identity for AMPA and kainate receptor ATDs. Despite this low homology, our results reveal that the overall structures of the GluK3 and GluK5 ATDs are closely related, and formed by a two domain clam shell like structure, in which three short loops connect domains R1 and R2, both of which have $\alpha\beta$ folds with a central β -sheet core flanked by α -helices (Fig. 1a). This motif is also found in type 1 periplasmic binding proteins and the ligand binding domains of type 3 G-protein coupled receptors (GPCRs).^{23–25} However, the GluK3 and GluK5 ATDs adopt a partially closed conformation, similar to that observed previously for the GluK2 and GluA2 ATDs,^{13–15} and distinct from the canonical open and closed cleft conformations found for the apo and ligand bound states of periplasmic binding proteins.^{23,26}

For the GluK3 ATD there are two molecules in the asymmetric unit; for both protomers the 1st two residues in the N-terminus; Leu273–Leu284 in loop 2; and Thr386–Arg392 in the carboxy terminus were disordered; the two GluK3 subunits form a dimer with 2-fold molecular symmetry. Following least squares superposition, the rmsd of 0.38 Å for 322 Ca atoms indicates that two subunits in the GluK3 dimer assembly have essentially identical conformations. The GluK3 dimer is similar to that observed previously for the GluK2 ATD, which crystallizes in the same hexagonal space group, but with a 43 Å elongation of the *c* axis.¹⁵ The GluK5 monoclinic crystal form also contained two molecules in the asymmetric unit, arranged as a dimer, with quasi 2-fold symmetry. For both protomers, the 1st residue in the N-terminus; residues Asp176–Arg178 in the loop connecting β strand 7 with α -helix G; and residues Thr377–Ile387 in the carboxy terminus were disordered; in the B subunit Ile35–Lys41 in the loop connecting α -helix A with β strand 2 were also disordered. For the two GluK5 protomers the rmsd of 0.39 Å and 0.38 Å for individual superposition of domains 1 and 2 increased to 1.1 Å for a global superposition, and a rotation of 9.7° was required to

superpose domain R1 after prior superposition of domain R2, indicating a change in lobe orientation for the two GluK5 subunits in the dimer assembly. The GluK5 orthorhombic crystal form contained one molecule in the asymmetric unit, but with a dimer created by crystal symmetry operations. The 1st residue in the N-terminus; Pro107-Leu112 in loop 1; Asp 175-Leu177 in the loop connecting β strand 7 with α -helix G; and Arg376-Ile387 in the carboxy terminus were disordered. The rmsds of 0.36 and 0.52 Å (domain R1) and 0.32 and 0.31 Å (domain R2) for individual superposition on each of the two protomers in the GluK5 monoclinic form, increased to 0.50 and 0.83 Å for a global fit. Rotations of 4° and 7° rotations, in opposite directions, were required to superimpose domain R1 after prior superposition of domain R2, indicating that all three crystallographically independent GluK5 protomers have different conformations.

A close inspection of the structures reveals that, as expected from 68–75% sequence identity within the GluK1–3 gene family, the structure of the GluK3 ATD is almost identical to that of GluK2, with the exception of a 3 amino acid insert from Gly16-Asn18, which forms a 3_{10} helix in the loop connecting β -strand 1 with α -helix A in GluK3 (Fig. 1). Least squares superpositions, using equivalent C α positions for the GluK3 and GluK2 ATDs (PDB 3H6H), gave rmsd values of 0.74 Å (171 C α) for domain 1, and 0.50 Å (151 C α) for domain 2. When the calculation was repeated using C α positions in both domains R1 and R2, the rmsd value was 0.65 Å, indicating that the two lobes have similar orientations in GluK3 and GluK2 (Fig. 1a); the disordered segments of loop 2 in GluK3 likely have the same conformation as found for GluK2.

Similar calculations revealed larger differences in the conserved core structure of GluK5 versus GluK3. Least squares superpositions on a GluK3 protomer, using equivalent domain 1 C α positions for the three crystallographically independent GluK5 protomers, gave rmsd values of 1.2–1.3 Å (132 C α) for domain 1 and 1.1–1.3 Å (122 C α) for domain 2; when the calculation was repeated using C α positions in both domains R1 and R2, the rmsd values increased to 1.7–1.8 Å. Analysis for the two GluK5 ATD protomers of the monoclinic crystal form reveals that the increase results from 8.6° and 6.6° differences in the twist angle between domains 1 and 2 compared to GluK3 (Fig. 1a). This twist does not result in a change in domain closure, like that found in periplasmic binding proteins and the iGluR LBDs, and is instead produced by a lateral movement of domain R2 in the GluK5 structure, such that aligned C α positions in α -helices G and H move by 5–6 Å (Fig. 1a). A similar 8.3° rotation of domain R2 was found for the GluK5 orthorhombic crystal form.

Although individual superposition of domains R1 and R2 revealed conserved structural cores for GluK2, GluK3 and GluK5, with changes in twist angle between the two lobes of the ATD, two differences stand out. First, in GluK5 there is a 22° change in tilt of α -helix I compared to the orientation found in GluK3 and GluK2 (Fig. 1a). This likely results from the presence of a disulfide bond that links the C-termini of α -helices A and I, and which is absent in GluK3 and GluK2, also from the four amino acid deletion in loop 2 of GluK5. Second, the nine amino acid loop which connects α -helix A with β -strand 2 has a different conformation in GluK3 and GluK2 compared to that in GluK5, creating a binding site for a cation in GluK3 and GluK2 but not GluK5. The ion binding site is created by the main chain carbonyl oxygen atoms of Ile36, Asn39, Leu42 and Leu43 in GluK3. The identity of the bound ion is uncertain, but Mg²⁺ is a likely candidate, since the crystallization solution contained 100 mM MgCl₂. Consistent with this, a Mg²⁺ ion positioned by real space refinement in Fo-Fc maps lies 2.1 Å from the carbonyl oxygen atoms of Ile36 and Leu42 (Fig. 1b), and it is likely that water molecules not resolved at 2.75 Å fill in the remaining 2 coordination positions. It is noteworthy that a similar ion binding site was observed in the GluK2 structure,¹⁵ and that in GluK1-GluK3 the loop connecting α -helix A with β -strand 2 also contains two N-linked glycosylation sites. By contrast, in GluK5 the loop moves

upwards by 8 Å, disrupting the carbonyl cage which forms the ion binding site, and changing the packing of the loop against α -helix J. Notably, in the GluK3 structure, the dipped conformation of the ion binding site loop is stabilized by packing of the Leu43 side chain against α -helix J. In the GluK5 structure the side chain guanidinium group of Arg300 in α -helix J projects upwards, to within 4 Å of the bound ion in the GluK3 structure, creating an unfavorable electrostatic profile for cation binding; at the equivalent position in GluK2 and GluK3 there is a Gln in α -helix J. What role might the ion binding site play in the GluK2 and GluK3 ATDs? One possibility would be that it stabilizes a loop conformation required for interaction of the ATD with the extracellular domains of other membrane proteins. If the site can bind Ca^{2+} it is plausible that the strength of such interactions could be modulated by changes in Ca^{2+} activity resulting from activation of calcium channels and pumps. Further work will be required to explore this.

The ATDs of the two kainate receptor gene families also show differences in post translational modification. The GluK3 and GluK2 ATDs both contain a single disulfide bond linking α -helix B with loop3; sequence analysis indicates conservation in GluK1 (Supplementary Fig. S2). By contrast, in addition to the disulfide bond linking α -helix B with loop3, GluK4 and GluK5 contain two additional disulfide bonds, one between Cys17 and Cys273, which links the N-terminus of α -helix A with loop 2, and one between Cys146 and Cys151, which forms a short loop in domain 2 linking β -strand 6 with α -helix E (Fig. 1a). The disulfide bond formed between Cys17 and Cys273 likely plays a role in the change in orientation of α -helix I in the GluK5 structure. The unique disulfide bonding pattern observed in GluK4 and GluK5 distinguishes this family from other kainate receptors formed by the GluK1–3 gene family, and also from the AMPA and NMDA iGluR subtypes which contain only a single disulfide bond.

Unique glycosylation sites in kainate receptor sub families

Sequence analysis indicates that the ATDs of the GluK1-GluK3 gene family contain five potential N-linked glycosylation sites. For GluK3, modification of all five sites was established by mass spectrometry, which gave a peak of 47551, compared to the value of 46563 predicted from the amino acid sequence; the difference of 1015 daltons is consistent with addition of five N-linked N-acetylglucosamine (NAG) molecules, each of mass 203 daltons. For GluK3, electron density for single NAG molecules was visible at Asn 39, 247 and 350 but not Asn 45 and 384. All five sites are remote from the cleft that separates lobes 1 and 2 of the iGluR ATDs (Fig. 1a). By contrast, GluK5 contains seven potential N-linked glycosylation sites, each of which is located at a different position from those found in GluK1–3, including one which occurs in a four amino acid insertion at the start of loop 3 (Supplementary Fig. S2). Of note, at two of the glycosylation sites found in GluK1–3, those adjacent to β -strands 10 and 12, GluK5 also contains an asparagine residue, but the third amino acid in the NXS/T consensus sequence for N-linked glycosylation is either leucine, or lysine (Supplementary Fig. S2); a similar exchange occurs at the Asn247 glycosylation site in GluK5, where the third amino acid in GluK1–3 is either aspartate or glutamate.

Mass spectrometry for the GluK5 ATD construct digested with endoglycosidase H (Endo H) yielded an unexpectedly complex pattern, with four peaks of mass 46340, 46502, 46664 and 46827 (Fig. 2a). The mass difference of 2591 between peak 1 and the predicted value of 43749 for the GluK5 ATD after signal peptide cleavage is consistent with a single NAG molecule at six of the seven of N-linked glycosylation sites, combined with an intact $\text{MAN}_6\text{GlcNAc}_2$ glycan resistant to Endo H digestion at the seventh site. The mass difference between peaks 1 & 2, peaks 2 & 3, and peaks 3 & 4 is 162–163 daltons, which corresponds to the sequential addition of mannose residues. Based on inspection of electron density maps at 1.4 Å resolution, for which two NAG molecules and a partially disordered mannose were visible at Asn200 in the orthorhombic GluK5 crystal form, with single NAGs

at Asn 252, 266, 303, and 353, the mass of peaks 1–4 can be explained by a disordered $\text{MAN}_{6-9}\text{GlcNAc}_2$ glycan at Asn200, with two single additional NAGs at Asn 375 and 381 that were also disordered in electron density maps. Prior to experimental digestion with Endo H, N-linked glycans produced in HEK cells deficient in N-acetylglucosaminyltransferase I would be expected to have the composition $\text{MAN}_5\text{GlcNAc}_2$, but in overexpression systems using strong viral promoters it is probable that the cellular capacity of golgi α -mannosidase activity becomes limiting, resulting in incomplete digestion of the $\text{MAN}_9\text{GlcNAc}_2$ precursor at Asn200. Consistent with this, for a second GluK5 preparation we observed only three species in the mass spectrum, corresponding to peaks 1, 2 and 3, indicating more complete processing by α -mannosidase. To confirm our interpretation of the mass spectrometry results for GluK5, we prepared the N200D mutant, expressed this in HEK 293 GnTI⁻ cells, and performed mass spectrometry after affinity tag cleavage & Endo H digestion. In contrast to the complex spectrum observed for the wild type protein, the GluK5 N200D mutant gave a single peak of mass 44961 corresponding to the addition of single N-linked NAG molecules at the remaining six glycosylation sites (Fig. 2b).

The presence of a glycosylation site resistant to Endo H digestion was unexpected, and inspection of the GluK5 crystal structure suggests that access of the enzyme to the glycan at Asn200, which is located in the interface between domains R1 and R2, is prevented by steric hindrance (Fig. 2c). The glycan at Asn200 is partially buried, and butts up against Gln14 and Thr15 in the loop connecting β -strand 1 with α -helix A, and likely plays a role in limiting domain closure in GluK5. Insensitivity to digestion by Endo H further suggests that in the isolated GluK5 ATD domains R1 and R2 can not move far apart enough to allow access by enzyme, suggesting that the ATD is a relatively static structure in solution, even when liberated from conformational restraints present in an intact receptor assembly. Of interest, there is a glycan residue in the interdomain cleft in the structurally related human natriuretic peptide receptor NPR-C.27 However, NPR-C is a dynamic structure in which the closed-cleft apo conformation is stabilized by intramolecular contacts made by the glycan, which are disrupted upon ligand binding. In NPR receptors, ligand binding occurs in the intermolecular interface of the domain R2 surface of the dimer assembly, producing a 13.5° change in lobe orientation in each subunit. In the NPR-C peptide complex each of the subunits in the dimer opens, due to a conformational change in which the equivalent of domain R2 clamps down on and traps the peptide ligand. By contrast, in the GluA2, GluK2, GluK3 and GluK5 ATD dimer assemblies, the domain R2 surface where the natriuretic peptide binds in the NPR-C dimer is already closed. While it is tempting to speculate that NMDA receptor ATD dimers may undergo movements like those which occur in NPR-C, further structural work is required to establish this.

Trans-domain interactions within a partially closed ATD subunit

It is intriguing that the ATDs of GluK3 and GluK5 adopt partially closed conformations similar to those found for GluK2 and GluA2. What elements in the ATD structures underlie this and what insight does this give into whether the ATDs of kainate and AMPA receptor iGluRs can adopt different conformations? At the center of the interface between domains R1 and R2, a bowl shaped cavity about 12 Å in diameter is formed by residues mediating interdomain contacts in GluK3 (Fig. 3a). The base of the bowl is formed by the side chains of Tyr122 and Thr290 from domain 1, and by the main chain and side chains of Thr22, Thr230 and Leu231 in domain 2; contributing to the sides of the bowl are Arg105, Trp106 and Asp291 in domain 1, and Arg156, His204, Asp232 and Ala235 in domain 2 (Fig. 3c). On one side of the bowl, Trp106 in domain 1 stacks against the side chain of Arg156 in domain 2; on the opposite side, the side chain of Asp232 in domain 2 forms a hydrogen bond with the main chain NH group of Thr290 in domain 1; located above this pair, the side

chains of His204 and Ala235 in domain 2 are stacked against Met289 in domain 1. Distal from the center, the side chain of Tyr234 in helix η 2 makes a hydrogen bond contact with the main chain amide of Met288 in domain 1; moving further outwards, the side chains of Asp257 in domain 1 and Lys373 in domain 2 form a salt bridge. In combination, these interactions likely stabilize the partially closed cleft conformation, and also prevent further domain closure by steric hindrance. The resolution of the GluK3 structure was not sufficient to resolve many solvent molecules, but it is likely that interdomain interactions across the sides of the bowl include solvent mediated contacts, as found in the higher resolution GluK5 structures.

In the GluK5 ATD there is a deeper but smaller bowl, which is connected to a groove running across the interdomain interface (Fig. 3b). Three well ordered water molecules fill the bowl, and these are linked to a complex hydrogen bonded solvent network formed by more than 20 well ordered water molecules which fill the groove (Fig. 3d). In combination, these solvent molecules mediate interdomain interactions via contacts with Ser79, Tyr120, Pro121 and Ser284 in domain 1, and with Asn123, Glu150, Arg154, Asp198, Thr 226, Thr227 and Asp229 in domain 2. Of note, loop 1 contains a one amino acid deletion and is missing the conserved tryptophan residue found in GluK1–3 at position 106, which if present would stack against the arginine residue found at position 154 in GluK5. However, the glycan at Asn200, which acts as a wedge between domains R1 and R2 (Fig. 2c), likely prevents further domain closure necessary to close the groove adjacent to Pro81 in domain 1 and Glu150/Arg154 in domain 2. Remote from the center of the GluK5 inter domain interface, the side chain amide of Asn123, at the N-terminus of α -helix D in domain 2, makes a hydrogen bond with the side chain hydroxyl group of Thr106 in domain 1; adjacent to this, the main chain amide of Asn123 makes a hydrogen bond with side chain carboxylate of Glu104 in domain 1 (Fig. 3d). On the other side, 24 Å away, the side chain of His234 in domain 2 forms a hydrogen bond with the main chain carbonyl of Pro279 in domain 1.

In none of the GluK3 and GluK5 structures was there any electron density corresponding to an unidentified ligand in the cleft between domains 1 and 2; in addition, in the high resolution GluK5 structures no ions were identified in the interdomain cleft, although in both the 1.4 Å and 1.7 Å resolution structures Na^+ and acetate ions were found elsewhere in the structures. Surface potential analysis revealed that the bowl and surrounding groove for GluK3 has a negative charge, generated by aspartate residues at positions 149, 150, 201, 232 and 291. By contrast, in the related region of AMPA receptors,¹⁴ the interdomain groove has a strong positive charge and binds a SO_4^{2-} ion (PDB 3HSY). The binding site for the SO_4^{2-} ion in AMPA receptors is created by two conserved Arg residues which are replaced in GluK1–5 by a Tyr residue at position 122 in GluK7 (His in GluK4), and by either a Thr in GluK1–3, or a Glu in GluK4–5, at positions 152 and 150 in GluK3 and GluK5, respectively. Related to this, although structurally homologous to the ligand binding domain of GPCRs and leucine/isoleucine and valine binding proteins, the ATDs of iGluRs do not bind glutamate,²⁸ and lack a conserved 8 amino acid sequence motif present in the active site of amino acid binding proteins.²⁹

Overall, our results indicate that the ATDs of the GluK1–3 and GluK4–5 kainate receptors can undergo moderate domain twisting movements on the order of up to 10°, and are thus not locked into a single conformation; an analysis of six GluA2 protomers in the PDB (codes 3H5V, 3HSY and 2WJW) reveals a smaller, 3–6° range of movement. However, large conformational movements, on the order of the 50° twists associated with the binding and unbinding of ligands in structurally related periplasmic binding proteins and type 3 GPCRs, are likely prevented in kainate, and AMPA receptor ATDs by two mechanisms. The first, as discussed above, are contacts at the interface of domains 1 and 2 that prevent full domain closure, and which likely help to stabilize the partially close conformation. Large

scale movements required for opening are in addition hindered by the assembly of the ATDs as dimers linked by domain R2, as discussed below.

Dimer assemblies of the GluK3 and GluK5 ATDs

Dimer assemblies were found in each of the three crystal forms reported here, but the relative orientation of the protomers in the two GluK5 ATD dimers differed from that for GluK3. Most striking was a change in orientation of the R1 domains, such that after superposition on the two R2 domains in the GluK3 dimer assembly, rotations of 16.1° and 13.4° were required to superimpose the R1 domains of the A and B subunits in the GluK5 monoclinic form dimer assembly; for the GluK5 orthorhombic form dimer assembly generated by crystal symmetry operations, the rotation required was 15°. By contrast, when similar calculations were performed for the GluK2 dimer assembly (PDB 3H6H) the rotation required to superimpose domain R1 was only 0.5°. These differences are illustrated in Fig. 4, which shows nearly perfect superpositions of domains R1 and R2 in the GluK3 and GluK2 dimer assemblies, but large movements for the R1 domains of the GluK5 monoclinic crystal form dimer. For example, the distance between Tyr57/Phe61 in α -helix B and Ile90/Ile93 in α -helix C increases from 5.6 Å in the GluK3 dimer, to 10.7 and 11.4 Å for the pair of subunits in the GluK5 monoclinic crystal form dimer, and to 10.7 Å for the orthorhombic form (Supplementary Fig. S3).

As a result of these movements, there is a large difference in the buried surface area of the R1 domain in the three dimer assemblies. Solvent accessible surface analysis reveals that dimer formation buries 1560 and 1690 Å² for the two subunits in the GluK3 dimer interface, contributed almost equally by the R1 and R2 domains (Fig. 4c). However, for the GluK5 ATD structures the buried surface area decreases by 275 Å² per subunit for the monoclinic crystal form, and 600 Å² per subunit for the orthorhombic crystal form. This loss in contact surface for the GluK5 ATD results from different dimer packing at the R1 interface, as evident from solvent accessible surface analysis for the individual domains. Thus, the GluK5 dimer assembly is held together primarily by the R2 domains, which have a buried surface of 720 Å² and 690 Å² for the monoclinic and orthorhombic crystal forms respectively, similar to the value of 750 Å² for GluK3, while for the R1 domains the values are only 490 Å² and 260 Å² respectively (Fig. 4c).

Interactions at the R1 dimer interface

In GluK3 the R1-R1 dimer interface is formed primarily by α -helices B and C and is capped by loop 3 that projects into the dimer interface. The loop is attached to α -helix B by a disulfide linkage between Cys319 and Cys68; Cys315 and Cys64 form the corresponding disulfide bond in GluK5. In the GluK3 ATD the aromatic ring of Phe61 at the base of α -helix B is inserted into a hydrophobic pocket formed by residues Ile93 and Ala96 from α -helix C and by the Cys68-Cys319 disulfide bond (Fig. 5a). These nonpolar interactions are stabilized by polar interactions both upstream and downstream of Phe61. The side chain of Lys65 in α -helix B forms a hydrogen bond with the main chain carbonyl of Cys319 on the loop 3, while the main chain amide of Phe61, and the side chain carboxyl group of Asp59, are connected by hydrogen bonds to the hydroxyl group of Ser92 on α -helix C from the interacting protomer. The interactions in the R1-R1 dimer interface of GluK3 are two fold symmetric, and stacking of Phe61 with Ile93 seems to be important for optimal packing at this interface. This packing is similar to that observed in the GluK2 ATD where the corresponding residues are Phe58 from one subunit, which stacks with Ile90 from interacting protomer.¹⁵ Similarly, in the GluA2 ATD Phe50 stacks with Phe82 at positions that correspond to Phe61 and Ile90 in GluK3, and to Phe58 and Ile90 in GluK2, respectively.^{13,14} However, in the GluK5 dimer structures these interactions are disrupted

due to the 6–9° twist in the R1 domain away from the dimer interface (Fig. 5), resulting in unique interactions present only in the GluK5 ATD structures.

In the GluK5 monoclinic crystal form, polar inter subunit interactions are formed between residues Arg53 and Ser55 at the base of α -helix B in chain B, with His89 and Glu93 on α -helix C, and with Tyr111 from loop1 of the interacting protomer (Fig. 5b). These contacts stabilize a dimer conformation which prevents optimal stacking of Tyr57 in chain B with Ile90 in chain A, as occurs in the GluK3 and GluK2 dimer assemblies, although the aromatic ring adopts the same rotamer in both structures. The dimer interface in the GluK5 monoclinic crystal form is not 2-fold symmetric and contacts made by Tyr57 in GluK3 differ in the two subunits. In chain A, Tyr57 adopts a different rotamer and is aligned nearly perpendicular to α -helices B and C of the partner subunit, where it is trapped between the side chain methyl groups of Thr60 and Thr316 in the interacting protomer (Fig. 5c). In combination, this series of unique GluK5 interactions likely prevents the close positioning of α -helices B and C, which in turn prevents optimal phenyl ring stacking interactions with Ile90, as observed in the GluK3 and GluK2 ATD dimer structures. These differences are even more extreme in the GluK5 ATD orthorhombic crystal form. In the GluK5 orthorhombic crystal form most of the R1 domain is accessible to solvent and the intersubunit space is filled with numerous water molecules (Supplementary Fig. S1). By contrast to the monoclinic crystal form, loop 1 and the Tyr57 side chain are disordered in the GluK5 orthorhombic crystal form and make no intermolecular contacts (Supplementary Fig. S1). This further disruption of R1 dimer interface is the result of additional twists of the R1 domain away from the axis of dimer symmetry. Consistent with this, after least squares superposition of 244 core C α atoms in domain R2 of the dimer assemblies (rmsd 0.72 Å), rotations of 5.2° and 8.6° were required for individual superposition of the R1 domains of the orthorhombic crystal form dimer assembly on the monoclinic crystal form dimer assembly (Supplementary Fig. S3).

The non-optimal assembly of the R1 dimer interface observed in the GluK5 structures is of interest, since the R1 domain contains structural elements and ‘specificity loops’ that have been proposed to underlie subtype-specific assembly.^{13–15,30} GluK1–3 each form functional homomeric ion channels,^{31–33} while GluK4–5 require obligate coassembly with GluK1–3 for surface expression of functional receptors.^{20,34–36} Our results suggest that non optimal dimer assembly observed for GluK5 might play a role in this.

Interactions at the R2 dimer interface are 2-fold symmetric

In both the GluK3 and GluK5 ATD dimers interactions at the R2 dimer interface are mediated by residues from α -helices E and F and β -strands 6 and 7 of both protomers. The interactions at this interface are primarily hydrophobic contacts, and in GluK3 involve Leu154, Leu155, Ile161, Met162, Ile173, which form a large hydrophobic patch that is buried in the dimer assembly (Fig. 6a). Similarly, in GluK5 the hydrophobic interaction surface is formed by residues Leu152, Leu153, Val159 and Leu163 on α -helices E and F and residues Leu169 and Val171 on β 7 (Fig. 6b). Interestingly, these hydrophobic residues are conserved in all 11 non-NMDA receptor subunits, including the δ subunits. These central hydrophobic contacts are further stabilized by peripheral, mainly polar interactions. In GluK3, the hydroxyl group of Tyr148 from β -strand 6 makes a hydrogen bond with side chain of Gln154 in α -helix F of the dimer partner, and the side chain of Met162 stacks against Gln175 in the dimer partner. In GluK5 the main chain carbonyl of Val171 makes a hydrogen bond with the side chain of Arg160, while the main chain carbonyl and amide groups of Leu169 make direct and solvent mediated hydrogen bond contacts with the peptide bonds of Leu163 and Arg160. There might be similar solvent mediated interactions in GluK3, which are not observed at 2.75 Å resolution.

It is striking that, despite the different conformations of protomers in the GluK3 and GluK5 dimer assemblies, contacts mediated by the hydrophobic patch in domain R2 are preserved at the expense of contacts mediated by domain R1. By contrast, in NMDA receptor ATDs, several hydrophobic residues at the R2 interface are replaced by polar or charged amino acids. In the only ATD structure of an NMDA subunit solved to date, NR2B crystallizes as a monomer and the R2 domains are twisted by a striking rotation of 50° with respect to the GluK2, GluK3 and GluA2 ATDs.¹⁷ Further, it has also been shown recently, by functional cysteine-crosslinking experiments, that this twist observed in the isolated GluN2B ATD structure is also present in the ATD domain of the GluN2A subunit in an intact NMDA receptor.³⁷ These observations are consistent with the fact that while there are no known physiological ligands that bind to non-NMDA ATDs, large number of small molecules and ions including polyamines, phenylethanolamines, and Zn²⁺ bind to the ATD of NMDA receptors and allosterically modulate ion channel activity.^{12,38–40} It has been suggested that binding of these allosteric inhibitors to the ATD promotes a closed-cleft conformation, pulling the R2 domains apart thereby decreasing the ion channel open probability.^{41,42} For this to occur, the ATD dimer interface in NMDA receptors would need to be mediated primarily by domain R1 contacts. In this context, the structurally homologous ligand binding domains of GPCRs are established examples where the interactions at domain 2 are disrupted, and the dimer surface is formed primarily by domain 1, allowing ligand induced conformational changes by movement of domain 2.^{25,43}

GluK3 Tetramer assemblies

A GluK3 tetramer with a global 2-fold axis of molecular symmetry is created by crystal symmetry operations (Fig. 7a). A similar tetramer assembly occurs for GluK2¹⁶, and least squares superpositions using 624 structurally conserved C α positions in core α -helices and β -strands gave an rmsd of 2.5 Å² for the two structures. The buried surface area in the B and D subunits, which mediate the GluK3 dimer of dimers assembly, is 590 Å² per subunit, which is unusually small compared to that in most oligomeric assemblies. It is thus remarkable that the isolated ATDs of three different iGluR subunits that form functional homomeric ion channels, i.e. GluK3, GluK2 and GluA2, crystallize as tetrameric assemblies with similar structure and symmetry. The structures of the isolated ATD tetramers replicate that found in full length GluA2,⁵ suggesting that the ATD plays a key role in tetramer assembly *in vivo*. In the GluK3 structure reported here, the tetramer assembly is mediated by 2-fold symmetric contacts between α -helices G and H, including a salt bridge between Lys191 and Glu222; a hydrogen bond between Lys191 and the main chain carbonyl of Ser244; and a potential cation π bond between Arg184 and Tyr243. van der Waals contacts are formed among the side chains of Lys191, Ala216, Met215, Glu222, Thr221, and Tyr243 (Fig. 7b). By contrast, for both GluK5 crystal forms, tetramers like those found for GluK3 and GluK2, were not generated by symmetry expansion of the unit cell contents. Consistent with this, examination of the lateral surface of GluK5 protomers reveals key amino acid substitutions which cause a loss of contacts observed in the GluK3 dimer of dimers assembly.

Conclusions

This study reports the first crystal structures for ATDs of the GluK3 and GluK5 subtypes of iGluRs. Our observation that the three independent GluK5 ATD subunit structures, in two different crystal forms, have different degrees of twist, larger than that observed for GluK3, GluK2 or GluA2, was unexpected. This conformational heterogeneity probably results from changes in contacts at the R1 dimer interface, including the novel interaction of loop1, especially Tyr111, with residues from α -helices B and C of the dimer partner. These contacts likely prevent formation of a tight assembly at the R1 dimer interface as observed

in the GluK3, GluK2 and GluA2 structures.^{13–15} This observation is consistent with the obligate heteromeric assembly of GluK4–5 subunits which form functional heteromeric ion channels only when coexpressed with GluK1–3.^{34, 365} A heterodimer structure formed between these two families could possibly provide further insights into the importance of specificity determining elements in kainate receptor assembly. Overall, our analysis across all the ATD structures for which structures have been solved suggests that non-NMDA ATDs can undergo only moderate 4–10° twists in contrast to GluN2B ATD, where a twist of 50° is observed and the R2 domain seems to be free to oscillate between open and closed conformations.^{17,37} The restricted domain movement in non-NMDA ATDs seems to result in part from extensive intramolecular contacts at the lips of the clamshell. Non-accessibility of the N-linked glycan at Asn200 in the GluK5 ATD to Endo H digestion, further demonstrates the rigidity of the R1 and R2 domains to large movements. In GluK3 and GluK2, extensive protein-protein interactions at both the R1-R1 and R2-R2 dimer interface also seem likely to further restrict large amplitude movements. The observation of a tetrameric dimer of dimers assembly in the GluK3 crystal lattice, similar to that present in GluK2^{15,16} and GluA2¹⁴, suggests a similar packing and domain organization in full length kainate and AMPA receptors.

Materials and Methods

Protein expression and purification

Construct design of the GluK3 and GluK5 ATDs was based on that previously reported for GluK2.¹⁵ Both constructs were cloned into the pRK5-IRES-EGFP expression vector with a C-terminal LELVPRGS-His₈ affinity tag and thrombin cleavage site.⁴⁴ HEK293 cells lacking N-acetylglucosaminyltransferase I (GnT1[−])⁴⁵ activity were cultured as adherent monolayers in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine. For large scale purification cells were grown to ~90% confluency in 5 Nunc triple layer tissue culture flasks (500 cm²/flask) with 90 ml medium per flask and were transiently transfected with the “PEI-MAX” form of polyethyleneimine (Polysciences, Inc Warrington, PA). Plasmid DNA (240 µg/flask) and PEI (720 µg/flask) were added to 25 ml aliquots of pre-warmed serum-free medium and incubated separately for 10 minutes. DNA and PEI containing medium were mixed together, vortexed briefly and incubated for 15 min at room temperature to allow DNA-PEI complex formation. During complex formation, medium from the plates to be transfected was replaced with 90 ml/flask of fresh medium containing fetal bovine serum at a concentration of 2% (v/v). The DNA-PEI mixture was added directly to the flask and was briefly rotated to allow mixing. The conditioned media was harvested 5–8 days after transfection for purification of secreted glycoproteins. Tris-HCl (pH 8.0) and NaCl were added from 1 and 5M stock solutions to final concentrations of 50 and 200 mM, respectively; the volume was reduced by ultrafiltration (Millipore LabScale TFF system, Pellicon Ultracel 10 kDa), and the concentrate clarified by centrifugation at 40,000 rpm for 20 minutes at 4 °C before loading onto a Ni²⁺ charged 1 ml HiTrap chelating HP column (Amersham). ATD proteins were eluted using a linear gradient of imidazole and the pooled fractions were dialyzed extensively against 20 mM HEPES pH 7.4, 0.2 M NaCl and 1mM EDTA. To remove the affinity tag, CaCl₂ was added to a final concentration of 10 mM and the protein was digested with thrombin at a 1:400 w:w ratio (Enzyme:Protein) at 25 °C for 90 minutes. The thrombin-digested protein was dialyzed overnight against a buffer containing 20 mM sodium acetate, 200 mM NaCl & 1 mM EDTA, pH 5.0, and then digested with Endo H at a 1:10 w:w ratio (Enzyme:Protein) for 120 minutes at 25 °C to trim N-linked glycans. The protein was then further purified by cation exchange chromatography on an SP Sepharose column and analyzed for homogeneity using 20% SDS-PAGE. Purified protein was concentrated by shock elution from an SP Sepharose ion-exchange column, dialyzed against

crystallization buffer containing 20 mM sodium acetate, pH 5.0, 200 mM NaCl, 1 mM EDTA, flash frozen in liquid nitrogen at 2 mg/ml and stored at -80°C . The final yields were 0.5 and 3.5 mg of purified protein per preparation for GluK3 and GluK5, respectively.

Crystallization and data collection

Purified GluK3 and GluK5 ATDs at 2.0 mg/ml were screened against high throughput sparse-matrix screens using a nanolitre pipetting robot (Mosquito, TTP, LabTech) and MRC 3 well crystallization plates (Swissci). Sitting drops of 0.1 μl protein were mixed at a 1:1 volume ratio with reservoir solution and equilibrated against 45 μl of reservoir solution at 20°C . Potential hits were then optimized in 24 well plates (Hampton Research) by the hanging drop vapor diffusion method at 20°C , with 1 μl protein solution mixed with 1 μl reservoir solution, and equilibrated against 500 μl of reservoir solution. GluK3 crystals grown in 0.1 M MgCl_2 , 0.1 M MES pH 6.8, 10% isopropanol and 5% PEG 4K were cryo-stabilized by gradual transfer to mother liquor supplemented with increasing concentrations of glycerol to a final value of 15% v/v. GluK5 crystals were obtained in two different conditions; 0.1 M CHES pH 9.5, 20% PEG 6K (orthorhombic form) and 0.1 M BICINE pH 8.8, 20% PEG 8K (monoclinic form) and were cryo-protected by gradual transfer to mother liquor containing 15% and 20% glycerol respectively.

X-ray diffraction data were collected from single crystals at 100 $^{\circ}\text{K}$ at APS 22-ID and 23-B-ID beamlines (Table 1). Data sets were indexed, scaled and merged using DENZO and SCALEPACK from the HKL2000 suite.⁴⁶ GluK3 crystals belonged to space group P6_1 with cell parameters $a = 171.2 \text{ \AA}$, $b = 171.2 \text{ \AA}$, $c = 68.2 \text{ \AA}$ and $\alpha = \beta = 90^{\circ}$, $\gamma = 120^{\circ}$. GluK5 crystals grown in CHES/PEG 6K belonged to space group C222_1 with cell parameters $a = 66.6 \text{ \AA}$, $b = 102.1 \text{ \AA}$, $c = 116.0 \text{ \AA}$ and $\alpha = \beta = \gamma = 90^{\circ}$. A second GluK5 crystal form grown in BICINE/PEG 8K belonged to space group C2 with cell parameters $a = 109.3 \text{ \AA}$, $b = 65.6 \text{ \AA}$, $c = 113.8 \text{ \AA}$ and $\alpha = \gamma = 90^{\circ}$, $\beta = 95.8^{\circ}$. None of the datasets showed twinning as analyzed by Phenix xtriage.⁴⁷

Structure solution and refinement

The GluK3 ATD structure was solved by molecular replacement using PHASER⁴⁸ and a chainsaw (CCP4)⁴⁹ generated search probe from a GluK2 ATD monomer.¹⁵ The solution contained 2 monomers in the asymmetric unit, corresponding to a solvent content of 60% (Matthews coefficient 3.0), with rotation (RFZ) and translation function (TFZ) scores of 12.3, 23.1 and 11.6, 35.4 respectively for the first and second protomer. The GluK5 ATD orthorhombic form at 1.4 $\text{\AA}$ resolution was also phased by molecular replacement using PHASER⁴⁸ and a GluK2 ATD poly Ala model of domain R1 (residues 4–104, 114–120 and 288–354) and R2 (residues 122–254) as separate search probes; the solution contained 1 molecule in the asymmetric unit (Matthews coefficient 2.13, solvent content 42%). Although a unique solution was found, the Z scores were low (RFZ = 4.2 and TFZ = 6.3) for the R1 domain but significantly better for the R2 domain (RFZ = 5.7 and TFZ = 12.4). ARP/wARP⁵⁰ run remotely on the EMBL Hamburg cluster built 82% of the model. This partially built model was used as a search probe for molecular replacement of the GluK5 monoclinic crystal form at 1.68 $\text{\AA}$ resolution. The solution contained 2 molecules in the asymmetric unit, with a solvent content of 44% (Matthews coefficient 2.2). All the structures were initially refined using simulated annealing to remove model bias, and then subjected to several rounds of manual building into σA weighted Fo-Fc and 2Fo-Fc maps in COOT⁵¹ iterated with cycles of crystallographic refinement with PHENIX.⁴⁷ TLS groups were identified by motion determination analysis⁵² and were used along with riding hydrogens in refinement. The final refined models have $R_{\text{work}}/R_{\text{free}}$ values of 19.5/25.2 for GluK3 structure, 17.5/19.6 for the GluK5 orthorhombic crystal form and 16.8/19.6% for the GluK5 monoclinic crystal form (Table 1). Calculations with MOLPROBITY⁵³ revealed that

97.3%, 98.3% and 98.6% of residues were in the preferred regions of the Ramachandran plot⁵⁴ for the GluK3 and two GluK5 structures. Solvent accessible surface area was calculated using the CCP4 program areaimol; additional crystallographic calculations were performed using CCP4⁴⁹ and the USF suite.⁵⁵ Surface potential analysis was performed with APBS⁵⁶ and PDB2PQR.⁵⁷ Figures were prepared using PyMol.⁵⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Conservation of core structure for kainate receptor ATDs

(a) Stereoview of ribbon diagrams for GluK3 (left) and GluK5 (right) protomers, with domains R1, R2 and loop 3 colored wheat, yellow and green for GluK3, and red, violet and green for GluK5, respectively. N-linked glycans are drawn as sticks for residues with electron density; for other glycosylation sites only the Asn side chains are shown. A GluK2 ATD protomer, superimposed by least squares using domain R1 C α coordinates, is drawn using transparent green shading, and illustrates the nearly identical conformations of the GluK2 and GluK3 ATDs, and the change in inter-lobe twist angle for the GluK5 ATD. (b) Stereoview of the divalent ion binding site in domain R1 of the GluK3 ATD; secondary structure elements are drawn with wheat shading; a GluK5 ATD protomer, superimposed by least squares using domain R1 C α coordinates, is drawn with red shading, and illustrates the different conformation of the loop connecting α -helix A with β -strand 2 in GluK3 and GluK5. Residues forming the ion binding site are drawn as sticks. A cation positioned by real space refinement in the Fo-Fc map contoured at 4 σ is shown as a pink sphere, connected by dashed lines to protein ligand atoms.

Fig. 2. The GluK5 ATD contains a glycan wedge in the cleft between domains R1 and R2
 (a) ESI mass spectrum for Endo H digested GluK5 expressed in HEK 293 GnTI⁻ cells reveals 4 peaks of mass 46340, 46502, 46664 and 46827. (b) ESI mass spectrum for the GluK5 N200D mutant expressed in HEK 293 GnTI⁻ cells and digested with Endo H reveals a single peak of mass 44961. (c) Stereoview of the cleft formed at the interface between domains R1 and R2 of a GluK5 protomer with the molecular surface drawn using transparent shading; secondary structure elements are drawn using ribbon representation. The N-linked glycan at position 200 is drawn using sticks and transparent VDW spheres, and shows the 2 NAG and 1 MAN residues visible in electron density maps.

Fig. 3. Interdomain interactions in the GluK3 and GluK5 ATDs

(a) Molecular surface for a GluK3 protomer reveals a bowl shaped depression in the interdomain boundary, the location of which is marked by a black box; domains R1 and R2 are colored wheat and orange, respectively. (b) Molecular surface for a GluK5 protomer; domains R1 and R2 are colored red and violet, respectively; the location of the interdomain bowl shown below is indicated by a yellow box. (c) Stereoview of the bowl region for a GluK3 protomer, with the molecular surface drawn as a transparent object; the ribbon diagram for domains R1 and R2 is colored wheat and orange, respectively; amino acids lining the interdomain interface are drawn in stick representation; the hydrogen bond linking Asp232 with the main chain amide of Thr290 is drawn as a dashed line. (d) Stereoview of

the GluK5 bowl region with a ribbon diagram colored as in (b); water molecules lining the cleft are drawn as red spheres; a subset of the hydrogen bonds linking the interdomain solvent network is drawn as dashed lines; for clarity the glycan at Asn200 is not shown.

Fig. 4. Dimer assemblies of the GluK3 and GluK5 ATDs

(a) The GluK3 ATD is shown with α -helices drawn as chocolate and wheat colored cylinders for the A and B subunits in a dimer assembly, superimposed by least squares on the GluK2 dimer assembly (PDB 3H6H) colored in green. (b) The GluK5 monoclinic crystal form ATD is drawn with raspberry and red colors for the A and B subunits in a dimer assembly, superimposed by least squares on the GluK3 dimer assembly shown in (a). (c) Molecular surface of subunits in the GluK3 and GluK5 dimer assemblies with solvent inaccessible surfaces colored green; the buried surface for the GluK5 orthorhombic crystal form (C222₁) is reduced compared to that for the monoclinic (C2) crystal form.

Fig. 5. Domain R1 intersubunit contacts in the GluK3 and GluK5 dimer assemblies

(a) The GluK3 dimer interface drawn as a ribbon diagram with subunits A and B colored chocolate and wheat respectively; residues involved in intermolecular contacts and disulfide bonds are drawn in stick configuration, with transparent CPK spheres for non polar residues; residues in subunit B are labeled with a prime; hydrogen bonds are shown as dashed lines; loop3 is colored green. (b and c) Dimer interface for the GluK5 monoclinic crystal form, with the A and B subunits colored red and raspberry respectively; due to the lack of perfect 2-fold rotational symmetry contacts made by residues in α -helix B with residues in α -helices B, C and loop 3 in the dimer partner differ in the two protomers; the view in panel c is rotated by 180° to that in panel b.

Fig. 6. Domain R2 intersubunit contacts in the GluK3 and GluK5 dimer assemblies
 (a) GluK3 dimer interface drawn as a ribbon diagram with subunits A and B colored chocolate and wheat respectively. (b) Dimer interface for the GluK5 monoclinic crystal form, with the A and B subunits colored red and raspberry respectively. In both panels, residues involved in intermolecular contacts are drawn in stick configuration, with transparent CPK spheres showing residues forming non polar contacts; solvent molecules are shown as red spheres; residues in subunit B are labeled with a prime; and hydrogen bonds are shown as dashed lines.

Fig. 7. Tetrameric assembly of the GluK3 ATD

(a) The GluK3 ATD tetramer is formed by a dimer of dimers assembly mediated by contacts between the B (wheat) and D (cyan) subunits in the AB and CD dimer pairs. The GluK2 ATD structure colored in green and superimposed by least squares using core C α atoms is shown to illustrate the conserved tetramer assembly. (b) Stereoview of the GluK3 ATD tetramer interface colored as in (a), illustrating intermolecular contacts made by residues in α -helices G, H and the loop connecting β -strand 9 with α -helix H with their symmetry mates.

Table 1

Data collection and refinement statistics

Data Set	GluK3 (2.75 Å)	GluK5 (1.40 Å)	GluK5 (1.68 Å)
DATA COLLECTION			
Space group	P6 ₁	C222 ₁	C2
Unit cell <i>a</i> , <i>b</i> , <i>c</i> (Å)	171.2, 171.2, 68.2	66.3, 102.0, 116.2	109.3, 65.6, 113.8
α , β , γ (°)	90, 90, 120	90, 90, 90	90, 95.8, 90
Number per a.u.	2	1	2
Wavelength (Å)	1.03320	1.0000	1.0000
Resolution (Å) ^a	45.00 – 2.75 (2.85)	50.00 – 1.40 (1.45)	30.00 – 1.68 (1.74)
Unique observations	29, 967	77, 538	91, 506
Mean redundancy ^b	4.5 (4.4)	5.7 (4.9)	3.9 (3.5)
Completeness (%) ^b	98.0 (97.3)	98.6 (96.4)	96.6 (77.3)
R _{merge} ^{bc}	0.091 (0.640)	0.043 (0.645)	0.065 (0.401)
I/ σ (I) ^b	16.0 (2.3)	29.2 (2.5)	18.1 (2.4)
Beamline	APS-23-B-ID	APS-22-ID	APS-22-ID
REFINEMENT			
Resolution (Å)	43.31 – 2.75	31.89 – 1.40	29.89 – 1.68
<i>No. atoms</i>			
Protein	5975	3029	5834
NAG/BMA	70/-	83/11	168/-
Acetate / Na ⁺ / Cl ⁻	- / - / 2	4 / - / -	- / 2 / -
Glycerol	-	18	12
Water atoms	14	437	609
R _{work} / R _{free} (%) ^b	19.5 (24.6) / 25.2 (34.6)	17.5 (20.7) / 19.6 (28.3)	16.8 (24.7) / 19.6 (29.6)
<i>rms deviations</i>			
Bond lengths (Å)	0.007	0.014	0.006
Bond angles (°)	0.956	1.590	1.077
<i>Mean B-Values (Å²)</i>			
Protein overall	64.9	30.0	37.9
MC / SC ^d	60.4 / 69.4	25.4 / 34.4	33.2 / 42.8
NAG/BMA	114.6/-	43.6/80.7	94.2/-
Acetate / Na ⁺ / Cl ⁻	- / - / 65.6	33.9 / - / -	- / 34.7 / -
Glycerol	-	41.8	53.7
Water	45.2	38.8	41.5
Ramachandran % ^e	97.3 / 0.1	98.3 / 0.0	98.6 / 0.2

^aValues in parenthesis indicate the low resolution limit for the highest-resolution shell of data.

^bValues in parenthesis indicate statistics for the highest-resolution shell of data.

^cR_{merge} = $(\sum |I_i - \langle I \rangle|) / \sum I_i$, where $\langle I \rangle$ is the mean I over symmetry-equivalent reflections.

^d Main chain/Side chain

^e Preferred/Disallowed conformations