# Characterization of the kainate-binding domain of the glutamate receptor GluR-6 subunit

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Recombinant fragments of the kainate-selective glutamate receptor subunit GluR-6 were expressed in insect cells and analysed for [$^3$H]kainate binding activity in order to characterize the structural determinants responsible for ligand recognition. Deletion of the N-terminal ~ 400 amino-acid-residue segment and the C-terminal ~ 90 residues resulted in a membrane-bound core fragment which displayed pharmacologically native-like [$^3$H]kainate binding properties. Further replacement of the membrane-embedded segments M1–M3 by a hydrophilic linker peptide gave rise to a soluble polypeptide which was accumulated in the culture medium. When bound to chelating Sepharose beads via a C-terminal histidine tag, the soluble fragment showed low-affinity binding of [$^3$H]kainate, which was displaced in a concentration-dependent manner by unlabelled domoic acid, L-glutamate and 6-cyano-7-nitroquinoxaline-2,3-dione. Our results indicate that the kainate-binding site is formed exclusively by the two discontinuous extracellular segments (S1 and S2) which are homologous to bacterial amino-acid-binding proteins. Ligand binding characteristics of soluble S1–S2 chimaeras between the GluR-6 and GluR-D subunits showed that, whereas both S1 and S2 segments contribute to agonist-selectivity, the N-terminal one-third of the GluR-D S2 segment is sufficient to confer α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate-binding capacity to the chimaeric ligand-binding domain.

## INTRODUCTION

Ionotropic glutamate receptors (iGluRs) are multimeric ligand-gated cation channels composed of homologous subunits (for reviews see [1] and [2]). Homo- and hetero-meric co-assembly of specific subunit sets gives rise to three pharmacological glutamate receptor (GluR) subclasses, named according to their agonist selectivities as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), *N*-methyl-D-aspartate (NMDA) and kainate receptors. According to the current model of the transmembrane topology of iGluR subunits, each of the ~ 900–1300-residue subunits harbours three transmembrane segments (M1, M3 and M4), and one membrane-embedded channel-forming loop (M2) [3–5].

The N-terminal ~ 550 amino acid residues preceding M1, and the hydrophilic segment between M3 and M4, are predicted to be extracellular and should contain the binding site(s) for the natural synaptic transmitter, L-glutamate. The segment comprising ~ 400 N-terminal residues bears distant sequence-similarity to the extracellular domain of metabotropic GluRs, and to bacterial Leu-Ile-Val-binding proteins [6]. This segment is not, however, present in the low-molecular mass kainate-binding proteins of chick [7], frog [8] and goldfish [4], which are otherwise similar in sequence to iGluR subunits. This fact, together with the finding that the N-terminal segment of the NR1 subunit of the NMDA receptor can replace the corresponding segment in GluR-6 without major effects on ligand pharmacology [9], speaks against participation of the N-terminal segment in ligand recognition. In contrast, the ~ 150 residues immediately preceding the M1 membrane-spanning domain and the hydrophilic segment between the M3 and M4 membrane-spanning domains, show clear sequence similarity to bacterial proteins, which bind polar amino acids [6,9,10] and contain structural determinants involved in ligand pharmacology in NMDA [11–13], AMPA [9,14–16] and kainate receptors [9]. Indeed, in AMPA receptors, these segments, designated S1 and S2 [9], can be separated from the receptor and linked together covalently to form a functional, water-soluble ligand-binding domain [17,18].

In the present study, we have addressed the structural basis of ligand recognition in the kainate-selective GluR-6 receptor [19,20] by analysing the ligand-binding properties of recombinantly expressed GluR-6 fragments and soluble binding-site chimaeras. Our results indicate that the S1 and S2 segments exclusively form the binding site for kainate agonists and for quinoxalinedione antagonists in the GluR-6 subunit.

## EXPERIMENTAL

### Expression plasmids

The DNA constructions for the expression of epitope-tagged rat GluR-6 and GluR-6 fragments were generated by using PCR. Briefly, synthetic oligonucleotides incorporating appropriate restriction sites for further cloning served as primers to amplify GluR-6 sequences. A pBluescript plasmid encoding full-length GluR-6 cDNA [20] was used as the template. The amplified fragments were cloned in a derivative of pFASTBAC1 (Gibco-BRL) engineered to encode a signal peptide followed by an N-terminal FLAG-epitope [21,22]. Whenever possible, restriction fragments from the original GluR-6 cDNA clone were used to substitute for the corresponding PCR-generated frag-

Abbreviations used: AcNPV, *Autographa californica* nuclear polyhedrosis virus; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; GluR, glutamate receptor; iGluR, ionotropic GluR; NMDA, *N*-methyl-D-aspartate.

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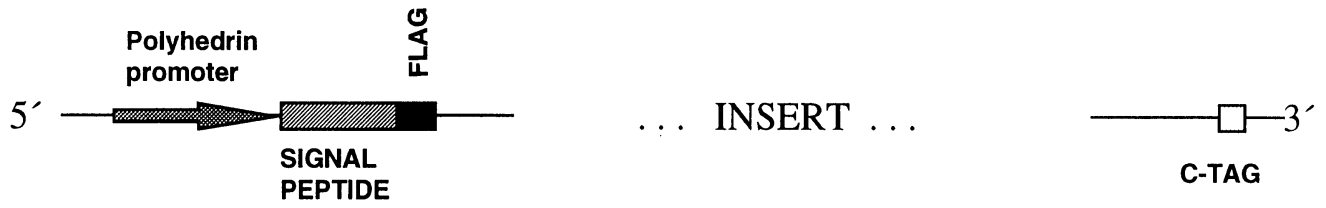



*GluR-6 fragments:*

| | | | | | |
|---|---|---|---|---|---|
| GluR-6 | EGT | TTHV... | | ...ETMA | HIS |
| GluR-6/S1[M1-M3]S2 | EGT | PASG... | | ...GVQN | HIS |
| GluR-6/S1S2-M4 | GluR | PASG... | ...PLSPestERME... | ...ETMA | HIS |
| GluR-6/S1S2 | GluR | PASG... | ...PLSPestegevnaeeegfERME... | ...GVQN | HIS |
| *Chimeras:* | | | | | |
| D/S1:6/S2 | EGT | PTLG... | ...PLAYEstegevnaeeegfERME... | ...GVQN | HIS |
| 6S1:D/S2 | GluR | PASG... | ...PLSPestegevnaeeegfERMV... | ...SLSN | MYC |
| D/S1:D6/S2(EcoRI) | GluR | PTLG... | ...GSTKEF/FKKSKI... | ...GVQN | HIS |
| D/S1:D6/S2(PvuII) | GluR | PTLG... | ...TRTTA/EGIQR... | ...GVQN | HIS |
| 6/S1:D6/S2(EcoRI) | GluR | PASG... | ...GSTKEF/FKKSKI... | ...GVQN | HIS |
| 6/S1:D6/S2(PvuII) | GluR | PASG... | ...TRTTA/EGIQR... | ...GVQN | HIS |

**Figure 1 Schematic structure of the expression cassette**

EGT, signal peptide of AcNPV ecdysone-*S*-glycotransferase; GluR, signal peptide of GluR-D. The S1 and S2 segments of GluR-6, as defined here, comprise the amino-acid residues 407–561 and 662–819 respectively. Of the inserts, the first and last four amino acid residues and relevant internal sequences are indicated. Small letters represent the artificially engineered sequence in the region linking the S1 and S2 segments. HIS, C-terminal 6 × His-tag; MYC, C-terminal Myc tag.

ments. The correctness of all PCR-derived sequences in the final constructions was verified by DNA sequencing. Chimaeric soluble binding sites between the S1 and S2 segments of GluR-6 and GluR-D [17,23] were prepared by fragment exchange, taking advantage of the unique *Sal*I site engineered in the sequence encoding the junction between the S1 segment and the linker peptide. To generate internal S2 chimaeras, the C-terminal part of the GluR-D S2 segment was replaced by the corresponding PCR-derived fragment of GluR-6 cDNA by using the unique *Eco*RI (at GluR-D residue 680) or *Pvu*II sites (at residue 709), and a unique 3′ *Hin*dIII site in the vector. For detailed information on the structure of the expression constructs, see Figure 1 and Figure 6.

### Recombinant baculoviruses and expression in insect cells

The recombinant baculoviruses were generated by using the Bac-to-Bac system (Gibco-BRL) which involves site-specific transposition of the expression cassette into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome maintained in *Escherichia coli* DH10Bac [24], followed by introduction of the resulting recombinant bacmid into Sf21 cells by lipofection (Insectin, Invitrogen). The recombinant viruses were used to infect Sf21 and HighFive insect cells growing in SF-900 II medium (Gibco-BRL) as described previously [17]. The cells were collected 3–4 days after the infection by centrifugation, and were analysed by immunoblotting and prepared for radioligand binding as described below.

### Radioligand binding assays

Insect cells were homogenized in 20 mM Hepes, pH 7.4/5 mM EDTA. The particulate fraction was pelleted (30000 ***g***, for 30 min) and washed several times by repeated homogenization and centrifugation in 20 mM Hepes, pH 7.4/0.5 mM EDTA/0.2 M NaCl, and finally suspended in 50 mM Tris/citrate, pH 7.0, for the kainate binding assay, which was performed essentially as described previously [20]. For qualitative measurement of the binding activity and for ligand competition experiments, 10 nM [$^3$H]kainate (DuPont-NEN, 58 Ci/mmol) was used, whereas for saturation analysis (1–300 nM [$^3$H]kainate), the radioligand was diluted with unlabelled kainate to a specific activity of 5.8 Ci/mmol. Non-specific binding was determined in the presence of 1 mM L-glutamate. With soluble samples, a modified assay was used in which the fragment was bound to $Ni^{2+}$-charged chelating Sepharose (Pharmacia) or to Anti-FLAG (M1) affinity gel (Kodak), and was washed briefly with Tris/citrate, whereafter the agarose beads were divided into equal sized aliquots and incubated with [$^3$H]kainate as described above. [$^3$H]AMPA (DuPont-NEN, 52 Ci/mmol) binding was measured as described previously [17]. The Whatman GF/B filters were pretreated with 0.3 % poly(ethyleneimine) [17,25]. The ligand-binding data were fitted to a one-site model by using non-linear curve-fitting (GraphPad Prism 2.0) to derive the $K_d$ and $IC_{50}$ values. The Cheng–Prusoff equation [26] was used to calculate the $K_i$ values. SDS/PAGE and immunoblotting were performed as described previously [17].

## RESULTS

### Expression of GluR-6 in insect cells

Membranes prepared from Sf21 cells expressing epitope-tagged (FLAG) GluR-6 bound [$^3$H]kainate with a high affinity ($K_d$ 18 ± 3 nM, $n = 4$, Figure 2a), whereas no specific binding was observed with uninfected control cells. In a ligand competition assay, domoate, L-glutamate and the competitive antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) inhibited the binding with $K_i$ values of 7 ± 1 nM, 0.4 ± 0.03 μM and 2.2 ± 0.2 μM,

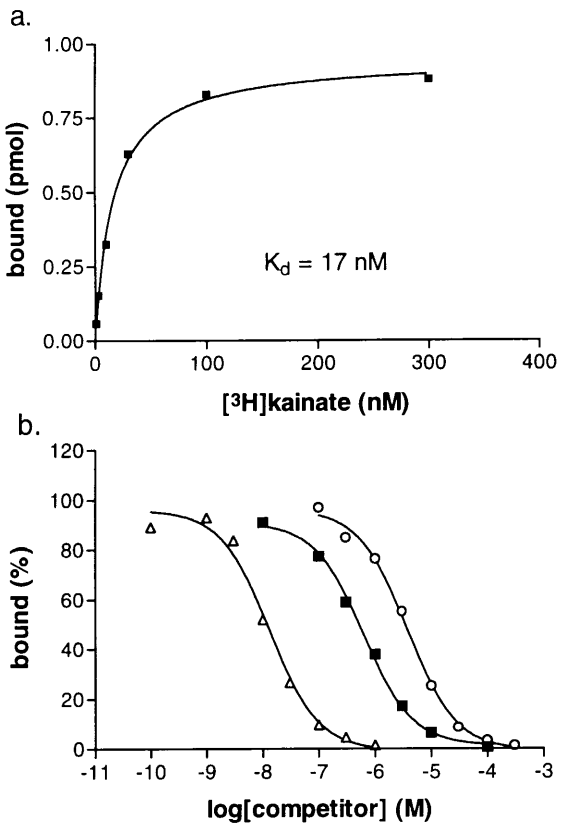



**Figure 2 Binding of [$^3$H]kainate to membranes prepared from GluR-6-expressing insect cells**

(**a**) Saturation binding isotherm. (**b**) Ligand displacement profile. Inhibition of binding of 10 nM [$^3$H]kainate by domoate (△), L-glutamate (■) and CNQX (○) is shown. The $IC_{50}$ values were: 13 nM (domoate), 0.6 $\mu$M (L-glutamate) and 3.8 $\mu$M (CNQX).

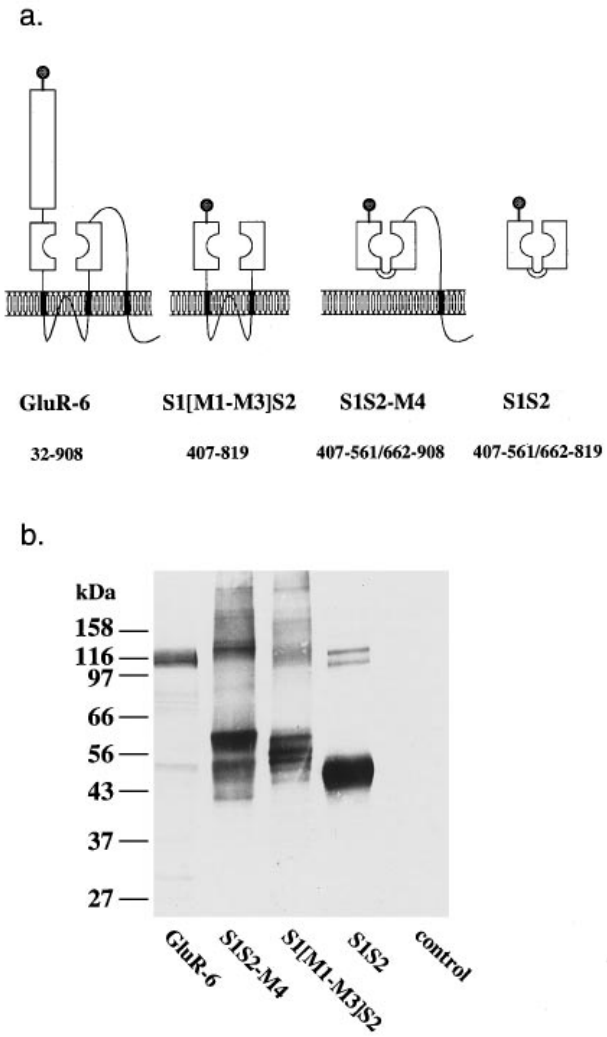



**Figure 3 Expression of GluR-6 fragments in insect cells**

(**a**) Molecular design of the fragments in relation to the current model for membrane topology of the GluR-6 subunit. (**b**) Anti-FLAG immunoblot analysis of the cells expressing GluR-6 and the indicated recombinant fragments. As control, uninfected Sf21 cells were used. Molecular-mass markers (in kDa) are indicated at the left.

respectively ($n = 3$; Figure 2b). In contrast, AMPA and NMDA did not inhibit the binding at concentrations of 100 $\mu$M. These binding properties are in reasonable agreement with those reported previously for GluR-6 expressed in mammalian cells [19,20] or in insect cells [27]. Solubilization of GluR-6 in Triton X-100 resulted in a slight increase in affinity ($K_d$ $11 \pm 1$ nM; $n = 3$) but did not produce any significant changes in the ligand-binding profile (results not shown). In SDS/PAGE, GluR-6 migrated as a broad 100–112 kDa species, detected by anti-FLAG immunoblotting (Figure 3b, lane 1).

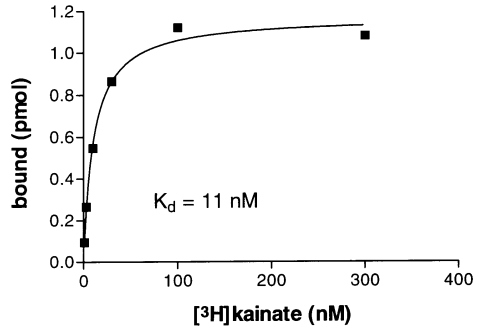
## Expression of GluR-6 fragments

In order to analyse the structural determinants responsible for ligand binding, in particular the role of the S1 and S2 segments, recombinant fragments of GluR-6 were designed and engineered for expression in insect cells (see Figure 3a for an outline). The effect of deleting the N-terminal ~ 400-residue segment and the C-terminal tail, including the M4 transmembrane segment, was studied with GluR-6–S1[M1–M3]S2, which encompassed the amino-acid residues 407–819 including the S1 (407–561) and S2 (662–819) segments and the cluster of three membrane-embedded segments (M1–M3) between them. To delineate the ligand-binding domain in more detail, two further constructs were prepared. In one of these (GluR-6–S1S2-M4) the membrane-associated segment from M1 to M3 was deleted and the protein was anchored to the membrane by the M4 segment and the C-terminal tail of GluR-6. In the other construct, GluR-6–S1S2, the M1–M3 part was replaced by a linker peptide with the sequence STEGEVNAEEEGF [17], to produce a soluble protein (Figure 3a). In the anti-FLAG-immunoblot (Figure 3b), the insect-cell-expressed recombinant fragments often appeared as multiple bands with a size range of 48–60 kDa (GluR-6–S1[M1–M3]S2), 54–60 kDa (GluR-6–S1S2–M4), and 43–54 kDa (GluR-6–S1S2), possibly reflecting glycosylation differences and/or proteolytic degradation. In addition, slowly migrating immunoreactive bands, probably indicative of dimerization of the respective recombinant proteins, were observed (Figure 3b). In agreement with the design of the proteins, GluR-6–S1[M1–M3]S2 and GluR-6–S1S2–M4 were membrane-bound, whereas GluR-6–S1S2 accumulated in the culture medium as a soluble protein (results not shown).

a.

| Construct | [$^3$H]kainate binding activity |
|---|---|
| GluR-6 | + |
| S1S2-M4 | + |
| S1[M1-M3]S2 | + |
| S1S2 | - |
| GluR-D | - |

b.




c.

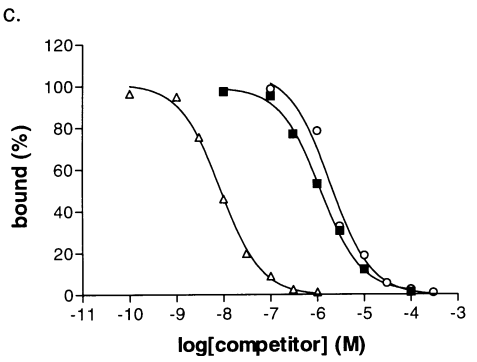



**Figure 4 Ligand-binding properties of the membrane-bound GluR-6 fragments**

(**a**) Binding of [$^3$H]kainate (10 nM) to membranes from cells expressing the indicated receptor fragments. The plus sign indicates consistent, specific binding and the minus sign indicates no binding. (**b**) Saturation binding isotherm of [$^3$H]kainate binding to GluR-6–S1[M1–M3]S2. (**c**) Inhibition of [$^3$H]kainate (10 nM) binding to GluR-6–S1[M1–M3]S2 by unlabelled domoate (△), L-glutamate (■) and CNQX (○). The $IC_{50}$ values were: 8.7 nM (domoate), 1.2 $\mu$M (L-glutamate) and 1.9 $\mu$M (CNQX).

## Kainate-binding activity of the membrane-bound receptor fragments

The ligand-binding activity of the receptor fragments was studied by using washed membrane preparations in a [$^3$H]kainate (10 nM)-binding assay. Membranes prepared from cells expressing GluR-6–S1[M1–M3]S2 or GluR-6–S1S2–M4 consistently bound [$^3$H]kainate (Figure 4a). This binding was displaced by the presence of 1 mM L-glutamate, and no specific binding was observed with membranes from cells expressing GluR-6–S1S2 or from uninfected control cells. The ligand-binding properties of GluR-6–S1[M1–M3]S2 were further characterized by saturation-binding and ligand-displacement analyses. [$^3$H]Kainate bound with high affinity ($K_d$ 15 $\pm$ 9 nM, $n = 3$; Figure 4b) and the binding was inhibited by domoic acid, L-glutamate and CNQX, with $K_i$ values of 6 $\pm$ 1.8 nM, 0.8 $\pm$ 0.06 $\mu$M and 1.2 $\pm$ 0.3 $\mu$M respectively ($n = 3$, Figure 4c). AMPA and NMDA did not inhibit the binding at concentrations of 100 $\mu$M (results not shown).

## Analysis of the ligand-binding activity of GluR-6–S1S2

To determine whether the secreted GluR-6–S1S2 fragment is capable of ligand binding, the culture supernatant was dialysed against Tris/citrate buffer to remove any endogenous L-glutamate and was assayed with [$^3$H]kainate. However, no binding activity was observed, even at 100 nM radioligand concentration (results not shown). The ligand-binding activity of GluR-6–S1[M1–M3]S2 and GluR-6–S1S2–M4 suggested that membrane-anchorage may be necessary for ligand-binding activity. This possibility prompted us to use a modified binding assay in which the soluble GluR-6–S1S2 fragment was first bound to Chelating Sepharose beads (as a substitute for membrane anchorage) via the C-terminal His-tag, followed by incubation with [$^3$H]kainate and separation of the bound from the free radioligand by filtration (Figure 5a). With this modification, specific binding of [$^3$H]kainate (but not of [$^3$H]AMPA) to GluR-6–S1S2 was consistently observed. Free (untreated) Chelating Sepharose beads or beads conjugated to detergent-solubilized His-tagged GluR-D did not show any specific [$^3$H]kainate binding but beads conjugated to detergent-solubilized His-tagged GluR-6 bound [$^3$H]kainate specifically (results not shown). Similar results were obtained when anti-FLAG immunoaffinity gel was used instead of the Chelating Sepharose for the immobilization of the soluble binding site (results not shown). Binding of [$^3$H]kainate to Sepharose-bound GluR-6–S1S2 was of low affinity with a $K_d$ of 300–400 nM, although obtaining accurate $K_d$ values proved difficult as the specific binding did not reach saturation at the range of radioligand concentrations used (up to 300 nM; Figure 5b). In a competition assay (Figure 5c), domoate, L-glutamate and CNQX inhibited [$^3$H]kainate binding in a concentration-dependent manner, whereas AMPA (300 $\mu$M) did not inhibit. Domoate was the most potent, exhibiting sub-micromolar $IC_{50}$ values, whereas L-glutamate had an $IC_{50}$ value of 15 $\mu$M. Interestingly, the apparent affinity of CNQX ($IC_{50}$ in the low $\mu$M range) was much less affected than that of the agonists domoate and glutamate (Figure 5c).

## Chimaeric GluR-6–GluR-D ligand binding domains

Chimaeric soluble fusion proteins containing S1 and S2 segments from GluR-6- and AMPA-selective GluR-D were generated in order to examine the relative roles of the two segments in ligand selectivity. In addition to chimaeras harbouring intact segments, further constructions carrying a chimaeric S2 segment with the N-terminal 61 amino acid residues from GluR-D were prepared. Upon infection of insect cells with the respective recombinant baculoviruses, all S1S2 fusion proteins accumulated in the culture medium as soluble FLAG-immunoreactive 42–49 kDa species (Figure 6). The ability of the fusion proteins to bind [$^3$H]AMPA was determined after extensive dialysis of the samples, whereas [$^3$H]kainate binding activity was determined after binding the fragment to agarose beads. The parental S1S2 proteins showed specific binding of their respective radioligands exclusively, whereas the chimaeric polypeptide harbouring the S1 segment of GluR-6 and the S2 segment of the AMPA-selective GluR-D (6/S1:D/S2) bound both [$^3$H]AMPA and [$^3$H]kainate. In contrast, no specific binding of either radioligand was obtained with

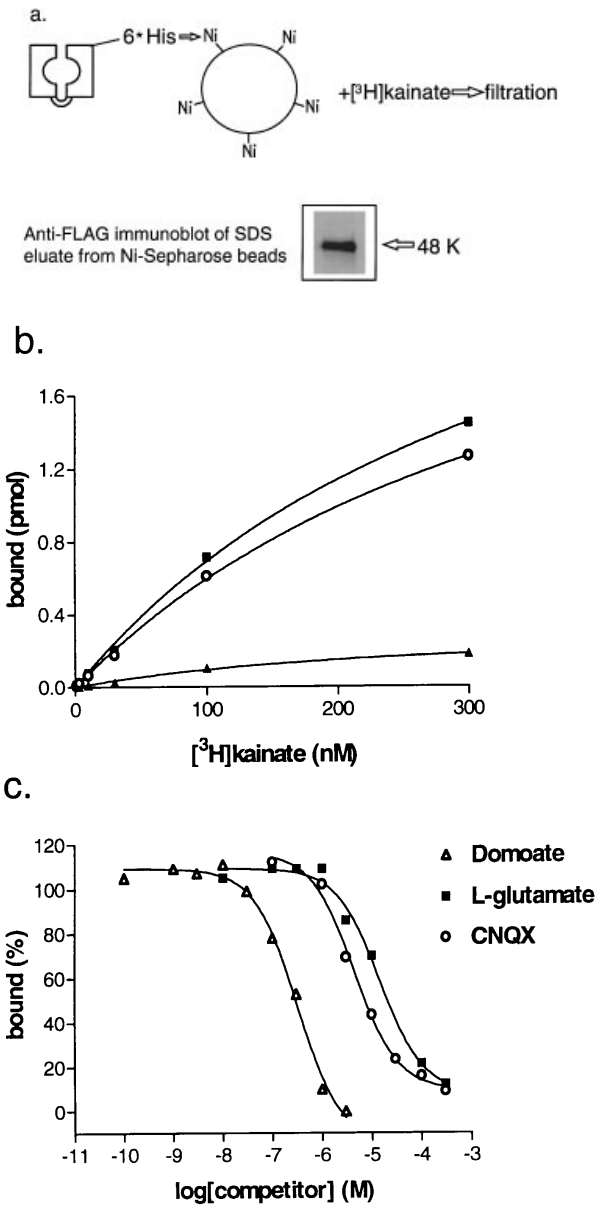



**Figure 5 [$^3$H]kainate binding to soluble GluR-6–S1S2**

(**a**) A scheme outlining the principle of the binding assay. The insect shows an immunoblot of a representative preparation used in the binding assays. (**b**) [$^3$H]Kainate binding isotherm. Total binding (■), binding in the presence of 1 mM L-glutamate (▲) and the specific binding (○) as the difference between the two former, are shown. (**c**) Displacement of [$^3$H]kainate binding by unlabelled domoate (△), L-glutamate (■) and CNQX (○). The $IC_{50}$ values were: 0.33 $\mu$M for domoate, 4.5 $\mu$M for CNQX and 15 $\mu$M for L-glutamate.

the opposite chimaeric construction, D/S1:6/S2, suggesting a structural incompatibility between the S1 segment of GluR-D and the S2 segment of GluR-6 in forming a functional ligand-binding site (Figure 6). Replacement of the 61 N-terminal amino-acid residues of GluR-6 S2 with the corresponding segment from GluR-D produced a chimaera (D/S1:D6/S2) which was able to bind AMPA but not kainate. Subsequent exchange of the S1 segment gave rise to a chimaera (6/S1:D6/S2) in which the only part originating from GluR-D was the 61-residue segment in the N-terminus of S2. This construct was able to bind [$^3$H]kainate and, surprisingly, also [$^3$H]AMPA (Figure 6). The AMPA-binding properties of this chimaera were studied further by using saturation-binding and ligand-competition assays. The affinity for AMPA was too low to be measured accurately ($K_d > 200$ nM), but inhibition of [$^3$H]AMPA (5 nM) binding by unlabelled L-glutamate, kainate and CNQX yielded $IC_{50}$ values of $0.07 \pm 0.03$ $\mu$M, $0.37 \pm 0.19$ $\mu$M and $1.3 \pm 0.7$ $\mu$M respectively (mean $\pm$ S.D. of three measurements).

## DISCUSSION

The major findings of the present study are: (1) direct demonstration that the S1 and S2 segments alone dictate the ligand binding pharmacology of the GluR-6 kainate receptor; (2) production of the ligand-binding domain of GluR-6 in a soluble, secreted form; and (3) use of soluble chimaeric binding sites to localize an important AMPA-binding determinant in the N-terminal one-third of the S2 segment. Consistent with earlier studies [9,17,18,28], our results indicate that the $\sim$400 N-terminal amino-acid residue segment (X domain; [17]), the membrane-embedded region (M1–M3), and the 90 C-terminal residues including the transmembrane segment M4, are not necessary for the folding or maintenance of the kainate-binding site.

Successful expression of the AMPA-binding domain of GluR-D [17] initially prompted us to see if the kainate-binding site of GluR-6 could be produced in a similar manner as a soluble S1S2 fusion protein. In agreement with the predicted transmembrane topology of iGluR subunits [3–5], but inconsistent with suggestions of a membrane-spanning segment within the S2 of GluR-6 [29,30], the resulting fusion protein was soluble and was secreted into the culture medium. In the standard filtration assay, however, the soluble fragment showed no specific kainate-binding activity. Several possible mechanisms responsible for this failure were considered. First, direct contribution to ligand binding of structures within the M1–M3 segment or within M4 and the C-terminal tail is extremely unlikely, as membrane-bound recombinant fragments lacking either one of these domains were able to bind kainate. Secondly, a slight structural change might decrease the binding affinity below a detectable level. We were unable to detect any specific binding even at higher radioligand concentrations but, due to sensitivity of the filtration assay to fast dissociation rate, a substantial decrease in the binding affinity cannot be ruled out. The fact that the two kainate-binding fragments S1[M1–M3]S2 and S1S2–M4 were both membrane-bound invoked the possibility that membrane anchorage may be needed for the maintenance of a high-affinity ligand-binding site. We reasoned that similar physical anchorage may be provided by binding the S1S2 fragment to Chelating Sepharose (via a His-tag) or to M1 immunoaffinity gel (via the FLAG epitope), and, indeed, observed consistent and specific binding of [$^3$H]kainate to the gel-bound S1S2 fragment. The reason why adsorption of the S1S2 fragment to a solid support improved the binding is unclear but decreased flexibility of the immobilized binding site may be an important factor. In this respect, it might be also possible to increase the ligand-binding affinity of the S1S2 construct by slightly modifying the linker area.

The soluble ligand-binding domain bound kainate with an affinity in the $K_d$ range of 300–400 nM, although a precise value was not obtained. In ligand-displacement analysis, the relative affinities of domoate and L-glutamate were also decreased by a factor of 20–30 (when compared with the affinities to the intact GluR-6), but the apparent affinity of CNQX was only little decreased (about two-fold). From these data it can be concluded

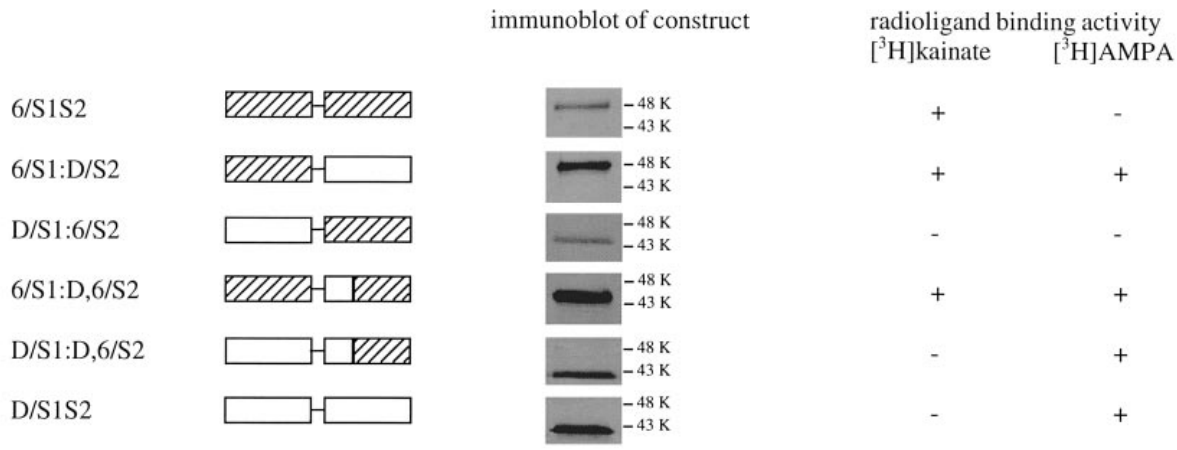



**Figure 6 Binding site chimaeras between GluR-6 and GluR-D**

Schematic structure of the chimaeric constructs, an anti-FLAG immunoblot analysis of the respective culture supernatants and binding of [$^3$H]labelled kainate and AMPA are shown. Thje plus sign indicates consistent, specific binding and the minus sign indicates no binding.

that the binding site for kainate agonists and for the quinoxalinedione antagonist CNQX are present in the S1S2 fusion protein but the conformation is slightly altered. Furthermore, the structures involved in antagonist binding are different and less affected by the removal of the transmembrane domains than the agonist binding structures. It will be interesting to see whether other kainate receptor antagonists also behave in a manner similar to CNQX.

The ligand-binding properties of soluble chimaeric GluR-6–GluR-D S1S2 binding sites are in agreement with the results obtained for the chimaeric GluR-6–GluR-C receptors [9]: a binding site formed by the GluR-6 S1 segment and GluR-D S2 segment bound both AMPA and kainate, whereas the opposite chimaera did not bind either of the radioligands. The structural incompatibility of the GluR-D S1 and the GluR-6 S2 segments in forming a functional ligand-binding pocket was relieved by replacing the 61 N-terminal residues in the S2 segment by the corresponding GluR-D residues from GluR-D. The participation of this area in ligand binding was further highlighted by the ability of the chimaera (6/S1:D6/S2) in which this 61-residue segment alone was from GluR-D but all other sequences were from GluR-6, to bind not only [$^3$H]kainate but also [$^3$H]AMPA. This result agrees with the report that introduction of a 107-amino-acid-residue segment including 81 N-terminal residues of S2 from GluR-C conferred AMPA-responsiveness to a chimaeric GluR-6–GluR-C receptor [9]. The short segment which imparts AMPA-binding capacity to the soluble binding site includes 29 differences between GluR-D and GluR-6 and contains an area, in particular the GluR-D residues 671–678, which corresponds to a region involved in extensive hydrogen bonding with the ligand in the bacterial amino-acid-binding proteins [31–33]. Evidence for the actual involvement in ligand binding of residues (or their homologous counterparts) within this 61-amino-acid segment have been presented for the NR1 subunit [11,13], and for the chick kainate-binding protein [34]. We attempted to decrease the contribution of GluR-D from 61 to 32 residues (residues 649–680) by use of another restriction site, but the resulting chimaeras did not have any ligand-binding activity.

In conclusion, our results indicated that the kainate-binding site is formed exclusively by the S1 and S2 segments, consistent with the presumed close structural relationship between the ligand binding domains of iGluRs and the bacterial polar amino acid-binding proteins. The secreted S1S2 fusion protein should provide a simplified model system for the structure–function analysis, in which the effects of mutations on binding of agonists and antagonists can be studied in relative isolation from (mutational) effects on folding, assembly and transport of the iGluR complex to the cell surface. Furthermore, the small size, water-solubility and relatively high expression level (in the order of 1–10 mg/l) of the ligand-binding domain provides further benefits for the biochemical and pharmacological analysis of ligand recognition in kainate receptors.

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