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A parallel panning scheme used for selection of a GluA4-specific Fab targeting the ligand-binding domain

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Abstract

A method for development of murine Fab fragments towards extracellular domains of a surface receptor is presented. The GluA4 ionotropic glutamate receptor is used as a model system. Recombinant GluA4 ectodomain comprising both the N-terminal domain (NTD) and the ligand-binding domain (LBD) in one molecule was used for immunization. A Fab-phage library was constructed and a parallel panning approach enabled selection of murine Fab fragments towards either intact ectodomain or the isolated LBD of the GluA4 receptor. One LBD-Fab (FabL9) showed exclusive selectivity for the GluA4 LBD, over a panel of LBDs from GluA2, GluK1, GluK2 and GluD2. Soluble FabL9 was produced in amounts suitable for characterization. Competitive ELISA and rat-brain immunoprecipitation experiments confirmed that the FabL9 epitope is conserved in the LBD and in the intact native receptor. By an alignment of GluA2 and GluA4, the likely binding epitope for FabL9 was predicted.

This study demonstrates a simple approach for development of antibody fragments towards specific sub-domains of a large ligand-gated ion channel, and this method could be utilized for all multi-domain surface receptors where antibody domain-selectivity may be desirable. Furthermore, we present for the first time a GluA4 subtype-specific murine Fab fragment targeting the LBD of the receptor.

Keywords

phage; Fab; GluA4

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1. Introduction

G-protein coupled receptors (GPCRs), ligand-gated ion-channels (LGICs), and voltage-gated ion-channels (VGICs) constitute ~ 40% of all currently known drug targets and thus constitute important classes of targets for small-molecule drug discovery and monoclonal-antibody (mAb) therapy [1]. Fragments of mAbs targeting intact cell-surface receptors are also excellent tools for the study of structure-function relationships. However, development of mAbs specific for cell-surface receptors is a challenge due to the difficulties associated with the expression and purification of suitable amounts of membrane-bound receptors of quality sufficient for immunization and panning schemes. Furthermore, membrane-embedded receptors are often unstable in detergent-solubilized form, which can lead to potential structural changes and epitope alterations [2]. Consequently, strategies for obtaining mAbs directed against surface receptors have primarily involved the use of intact cells as antigen. This approach can yield mAbs directed towards native epitopes in a random fashion, which can be useful for some applications [3, 4], but simultaneously yields mAbs against unwanted surface targets. An alternative approach is to use small peptides (conjugated to carrier proteins for immune response) comprising predicted loops from crystal structures of target receptors. However, such peptides often fail to recapitulate the three-dimensional structure of the native epitope.

Glutamate (Glu) is the major excitatory neurotransmitter in the central nervous system (CNS) [5–7]. Glu plays an important role in most of the normal brain functions due to its ubiquitous presence in the CNS, but it is also fundamentally involved in diseased states of the brain such as schizophrenia, epilepsy and Parkinson's disease [8, 9]. Glu mediates the communication between nerve cells by controlled release from presynaptic vesicles in a neuron, and upon release into the synaptic space the neurotransmitter is recognized by ligand-gated Glu-receptors expressed at the postsynaptic surface of a neighboring neuron.

Glu mediates its effect through both metabotropic and ionotropic receptors. The ionotropic effects are mediated by a heterogeneous family of Glu-gated ion channels assembled from a diverse collection of subunits. These receptor subunits are clustered into AMPA (GluA1-4), kainate (GluK1-5) and NMDA (GluN1, GluN2A-D, GluN3A-B) subfamilies based on sequence similarity and on their selective activation mediated by the agonists (*S*)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid (AMPA), kainic acid, and *N*-methyl-D-aspartic acid (NMDA), respectively. In addition, orphan Glu receptor-like delta subunits GluD1 and GluD2 exist, which form receptors that are not gated by Glu. GluD2 instead recognizes D-serine and glycine, a feature shared by the NMDA receptor subunits GluN1 and GluN3A/B [10, 11]. Functional Glu-receptors are tetrameric assemblies composed of homo- and heteromeric combinations of the cloned subunits within each class, generating considerable combinatorial diversity. The existence of subunit splice variants further increases the heterogeneity of the Glu-receptor population [11].

The AMPA receptors have been expressed and purified as full-length receptors, but the ectodomains (ED) and ligand-binding domains (LBD) have also been isolated as soluble truncated domain constructs (see cartoon in Figure 1A for AMPA receptor overview). It has been shown that the ectodomain forms a tetrameric dimer of dimers [12]. These soluble

domain constructs have contributed greatly to an understanding of receptor structure and the mechanisms of Glu-mediated receptor activation [13–16]. In particular the isolated GluA2 LBDs have been excellent tools for mechanistic and structural studies of small-molecule agonists, antagonists and modulators [15–17]. The GluA4 and GluA2 subunits exist in two different isoforms created by alternative splicing (termed flip and flop). The forms are equally abundant but display different distributions in the brain [18]. The two GluA4 isoforms differ in their time course of receptor desensitization and in their sensitivity towards the desensitization blocker cyclothiazide [19, 20].

At present only one study has described the development of murine Fab-fragments towards the GluA4 receptor. In the study, proteoliposomes with embedded full length GluA4 receptor were utilized for mouse immunization and subsequent Fab fragment selection. The Fab-fragments isolated in the study displayed a high degree of specificity, as Fab22 was GluA4-specific and Fab7 was GluA2/GluA4-specific. The binding epitopes for Fab7 and Fab22 were found to reside in the NTD and the epitopes disappear after receptor denaturation [21]. Fab22 has been used extensively in several studies as a probe to verify GluA4 conformational integrity [22, 23]. To the best of our knowledge, no antibodies exist that have the ability to bind the GluA4 LBD in a specific manner.

In this study we have devised a method for development of LGIC domain-specific antibody fragments using the GluA4 receptor as a model system. An immunized murine phage library was created and the library was used in a parallel panning approach, thereby enabling rapid selection of murine Fab fragments towards the intact ED and the LBD of the GluA4.

2. Materials & Methods

2.1 Expression of GluA4 ectodomain and ligand-binding domains

LBDs from rat GluA2_{flop} [24], GluA4_{flip} [13], GluK1 [20], GluK2 [25] and GluD2 [8] were expressed and purified as previously described. Rat GluA4 ectodomain was also expressed as described [12].

2.2 Mouse immunization

On day zero, two adult female BALB/c mice (Taconic) were injected intraperitoneally with a 200 µl solution of 50 µg GluA4 ectodomain in 50% (v/v) Freund's complete adjuvant (Statens Serum Institut, Copenhagen, Denmark). Further booster injections were performed on days 14, 34 and 45. Booster injections were performed with 50% Freund's incomplete adjuvant (Statens Serum Institut). Blood was drawn from the ocular vein, allowed to coagulate, and tested in indirect ELISA for binding to immobilized GluA4 ectodomain and LBD. The ELISA setup was as follows. GluA4 ectodomain or LBD was diluted to 1 µg/ml in PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and 100 µl was coated on maxisorp plates for 16 hours at 4 °C. The next day, wells were washed with PBS-T (PBS with 0.05% (w/v) Tween 20) and blocked with 2% (w/v) skimmed milk powder/PBS for 1 hour. Next, mouse serum, diluted to 1/64 with PBS-T, was added to the wells and incubation was performed for 1 hour, followed by washing four times with PBS-T. Finally the wells were incubated for 1 hour with rabbit anti-mouse Ig/HRP (P0260, Dako, Glostrup,

Denmark) diluted 1/1000 in PBS-T. Wells were washed four times and o-phenylenediamine (OPD, DAKO) was used as the substrate.

On day 48 the best-responding mouse was sacrificed, pinned down and cut open, and the spleen was removed. The spleen was cleaned for connective tissue and separated into four pieces of approximately 30 mg each. These were individually homogenized for 40 seconds with a mechanical homogenizer (T10 Basic, ICA®) in 600 µL Buffer RLT with guanidinium thiocyanate and β-mercaptoethanol on ice (all buffers and spin columns were from QIAGEN RNeasy Mini Kit™, Qiagen, Copenhagen, Denmark). The tissue was subsequently processed as described in the QIAGEN RNeasy Mini Kit™ manual. In the last step the mRNA was eluted with 50 µl RNase-free water (QIAGEN). The RNA preparations from the four spleen pieces were pooled and used as template for cDNA synthesis (iScript cDNA synthesis Kit, Biorad, Copenhagen, Denmark).

2.3 Fab library construction

The Fab library cloning cassette was constructed independent of immunoglobulin isotype by sequence-overlap PCR using libraries of primers in order to maximize the diversity of the final library [26]. 500 ng of the Fab-cassette was digested with *Sfi1/Not1* (New England Biolabs, Bionordika, Herlev, Denmark) and cloned into *Sfi1/Not1*-digested phagemid vector pFab74 [27] using T4 DNA Ligase (Invitrogen, Slangerup, Denmark). After ethanol precipitation, the DNA was re-dissolved in Milli-Q water and electro-transformed into ultra-competent TG1 *E. coli* cells prepared the same day in a total of 24 electroporations. The library was expanded in 500 ml of Luria–Bertani (LB) medium (Sigma–Aldrich, Brøndby, Denmark) containing 50 µg/ml ampicillin (Calbiochem, Merck Lifescience, Hellerup, Denmark) (LB-Amp) overnight at 37°C. From the overnight culture, glycerol stocks were made and stored at -80°C. The library size was 2.2×10^7 with an estimated 75 % of the clones having the correct insert.

2.4 Panning

From glycerol stocks, Fab-phage was produced by expanding the library in LB-Amp to a starting OD₆₀₀ of 0.05. At an OD₆₀₀ of 0.5, helper phage R408 (Stratagene, Agilent Technologies, Glostrup, Denmark) was added in 100× excess and allowed to infect for 20 min. Fab-phage expression was induced overnight at 22 °C with 1 mM isopropyl β-D-thiogalactoside (IPTG, VWR, Søborg, Denmark). Fab-phage was precipitated from the overnight culture supernatant with phage precipitation buffer (20% (w/v) polyethylene glycol 6000 and 2.5 M NaCl) on ice for 1 hour and re-dissolved in PBS. This Fab-phage stock was mixed 1:1 with 4% (w/v) skimmed milk powder (SM) in PBS, and 100 µl were transferred to a maxisorp plate that was coated overnight with 100 µl 10 µg/ml receptor fragment (GluA4 LBD and GluA4 ED, respectively) and blocked by adding 1:1 4% (w/v) SM. Fab-phage were allowed to bind for 2 hours followed by washing 10 times with PBS-T. Fab-phage were next eluted with 0.2 M glycine-HCl pH 2.0, which was neutralized with 1 µl 2M Tris-base. Finally, the eluted Fab-phage were transferred to TG1 cells in LB media and allowed to infect for 20 min. The infected cells were brought up in LB-Amp overnight, and after securing glycerol stocks, the next panning round was initiated using the same procedure. A total of four panning rounds were performed. Individual clones were amplified

from single colonies on LB-amp plates, and complementarity determining regions (CDR's) from positive clones (as judged by phage-ELISA, see below) were sequenced as described in [27].

2.5 Clone screening by phage ELISA

Single clones were isolated and superinfected for Fab-phage production overnight as described above. Overnight cultures were centrifuged at $10,000 \times g$ to remove cells, and the supernatants were diluted 1:1 in 4% (w/v) skimmed milk powder/PBS and applied directly to GluA4-coated and blocked maxisorp wells (as described above). The Fab-phage was allowed to bind for 2 hours at room temperature followed by washing 5 times in PBS-T. Fab-phage binding was detected by a monoclonal anti-M13 antibody-HRP conjugate (GE Healthcare, Brøndby, Denmark) diluted 1/1000 in PBS-T, which was allowed to incubate for 1 hour, followed by washing four times in PBS-T and ELISA development with OPD as the substrate.

2.6 FabL9 expression and purification

The pFab74 phagemid harboring the FabL9 DNA sequence fused to the DNA sequence encoding the protein III was isolated by maxiprep (Qiagen). The phagemid was digested with the restriction enzyme *EagI* to remove the protein III DNA sequence [27] and re-ligated with T4 DNA ligase. The ligation mixture was transformed into chemo-competent TG1 cells. Individual clones were isolated and sequenced to verify removal of protein III DNA. Delta protein III clones were isolated and used for FabL9 expression. The FabL9 clone was expanded in LB-media supplemented with 10 mM $MgCl_2$ and were induced at OD_{600} 0.6 with 1 mM IPTG for 16 hours at 30 °C. Cells were isolated by centrifugation, resuspended in PBS, and lysed by sonication of the cells. Lysates were clarified by centrifugation. The FabL9-containing supernatant was applied to a 1 ml HiTrap Chelating HP column charged with $NiSO_4$ (GE Healthcare). The column was washed extensively with 20 mM sodium phosphate, 500 mM NaCl, pH 7.4 (buffer A). The hexa-his tagged FabL9 fragments were eluted using a linear gradient of buffer B (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). FabL9 purity was confirmed by SDS-PAGE (Precise protein gels, 12%, Thermo Fisher Scientific).

2.7 Inhibition ELISA for estimation of fabL9 IC₅₀

Estimation of FabL9 IC₅₀ was performed essentially as described previously [28]. FabL9 was pre-incubated with various concentrations of GluA4 LBD in 2% SM for two hours and then transferred to maxisorp wells coated with GluA4 LBD and blocked with 2% SM as described above. Remaining free FabL9 was allowed to bind for two hours and wells were washed 5 times in PBS-T. FabL9 binding was detected by a polyclonal anti-murine IgG (Fab-specific)-HRP conjugate (diluted 1:5000, Sigma) and OPD (Dako) as the substrate. The absorbance values were measured at 490 nm after 30 min incubation at 22 °C in, each concentration in singlet. Absorbance values at each GluA4 LBD concentration (A) were divided by the absorbance measured in the presence of zero GluA4 LBD, which yielded normalized values (A/A_0). The normalized values were plotted against the GluA4 LBD concentration to construct the inhibition curve. GraphPad Prism 5.0 was used for nonlinear

regression to fit the data to the log(inhibitor) vs. response (variable slope) curve. The GluA4 concentration required for 50% inhibition was the IC₅₀ value as described [28].

2.8 GluA4 immunoprecipitation from rat brain by FabL9

Triton X-100 brain extract (BE) pooled from rat cortex hippocampus and striatum was prepared by standard methods and supplied to us by Dr. Niels Wellner and Dr. Harald S. Hansen (University of Copenhagen). 35 µg of purified FabL9 was incubated with 200 µl of BE for 1 hour at 4 °C. Separate positive (Fab22 [21]) and negative (MK16 [29]) controls were included. Simultaneously, Protein-G Sepharose (GE Healthcare) was coated with polyclonal Fab-specific goat-anti-mouse IgG (Sigma), and the Sepharose was washed extensively with PBS by repeated centrifugation and resuspension of the pellet. Next, 25 µl of ProteinG-Fab IgG Sepharose was added to the BE-FabL9 solution and BE-Fab controls and incubated for 1 hour at 4°C followed by washing 3 times with PBS. The captured proteins were eluted from Sepharose beads by incubation with SDS-sample buffer (62.5 mM Tris-HCl pH 6.8, 2.5% (w/v) SDS, 0.002% (w/v) Bromophenol Blue, 10% (w/v) glycerol), and boiling for 10 min. Proteins were resolved by SDS-PAGE as described above and blotted to PVDF membrane (GE Healthcare). Full brain extract was also included in the SDS-PAGE as a size control (i.e. with no immunoprecipitation). The blot was blocked overnight in 5% (w/v) skimmed milk/TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) at 4 °C followed by incubation with polyclonal rabbit-anti-GluR4 antibody (Chemicon, Merck Milipore) diluted 1:1000 in 5% (w/v) skimmed milk/TBS for 1 hour. The blot was washed with TBS-T (TBS with 0.05% (w/v) Tween 20) and incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Promega) diluted to 1:7000 for 1 hour. The blot was washed with TBS-T and developed using enhanced chemiluminescence (ECL) reagents (GE Healthcare).

3. Results and discussion

3.1 Fab-library construction and Fab selection

The GluA4 ectodomain can be expressed as an entity independent from the transmembrane part of the entire receptor. The ectodomain is a dimer in solution and fully capable of Glu binding [12]. By using the entire ectodomain for immunization, our goal was to raise antibodies towards the N-terminal domain (NTD) and LBD simultaneously. In our approach one mouse was immunized with GluA4 ectodomain and we could indeed demonstrate that antibodies were raised against both the ectodomain and LBD, as mouse serum could be used as the detection antibody in direct ELISA analyses with either domain (Figure 2). From the recovered mouse spleen, a Fab-library was constructed independent of immunoglobulin isotype [26] in the pFab74 phagemid system [27] (see Figure 1C for vector overview). The library size was 2.2×10^7 , and it was subsequently displayed on phage. The sizes of phage libraries depend on several factors, such as the number of B-cells used, extracted RNA quality, final Fab-cassette DNA quality, and electrotransformation efficiency. However, a reasonable immunized library typically contains 10^7 to 10^8 independent transformants [30] and Fab-phage have been successfully isolated from immune libraries with sizes of $\sim 10^7$ (see for example refs. [31, 32]). Thus our library was deemed large enough to proceed.

To select Fab from the phage-library, we performed a parallel panning scheme using immobilized GluA4 ectodomain and LBD, respectively (see Figure 1B). After four panning rounds the enrichment in the number of eluted phage between sample (Ectodomain/LBD) and control (panning on skimmed milk) was 752-fold and 36-fold for ectodomain and LBD, respectively. Ten single clones from the LBD panning (L1-10) and ten from the ectodomain panning (E1-10) were selected for further analysis. The twenty clones were analyzed by phage ELISA for GluA4 binding. Four L-clones (FabL5, FabL8, FabL9 and FabL10) bound the GluA4 LBD, whereas seven E-clones (FabE3, FabE4, FabE5, FabE6, FabE8, FabE9 and FabE10) were positive for binding to the GluA4 ectodomain (Figure 3).

3.2 Characterization of ectodomain binders

High amino-acid sequence similarities exist between the subunits forming AMPA (89% for GluA2_{flip} vs. GluA4_{flip}) and kainate (86% for GluK1 vs. GluK2) receptors. In contrast, the sequence similarity is much lower between the AMPA, kainate and orphan subgroups (e.g., 52% for GluA4 vs. GluK1, 52% for GluA4 vs. GluK2, 33% for GluA4 vs. GluD2). Direct ELISAs were performed to characterize the specificity of the selected Fab-phage towards the GluA4 LBD and ectodomain. All the ectodomain binders (E3, E4, E5, E6, E8, E9 and E10) displayed exclusive binding to the GluA4 ectodomain and not to the GluA4 LBD, thereby suggesting that the epitopes for these Fab-fragments require the GluA4 NTD (Figure 4). As GluA4-specific Fabs targeting the NTD already exist we did not focus further on the NTD-binding Fab fragments, but instead directed our attention to the new group of LBD binders.

3.3 Characterization of LBD binders

FabL5 bound exclusively to GluA4_{flip} LBD but with OD signals below 1, which was much lower than in the initial screening (Figure 4). FabL8 and FabL10 displayed insignificant binding after re-testing. The explanation for these results is likely instability of the FabL5/L8/L10 fragments. FabL7 targeted a common epitope present in the skimmed milk blocking solution. The most promising binder FabL9 bound GluA4_{flip} LBD specifically with high signals, but surprisingly did not bind GluA4_{flip} ectodomain (Figure 4). A likely explanation for this is presented below in section 3.4.

The complementarity-determining regions (CDRs) from Fab clones were sequenced to investigate the molecular basis for the GluA4 selectivity exhibited by FabL9 (Table 1). It is widely accepted that most antibody specificity resides in the heavy chain CDRs, primarily the CDRH3 [33]. The FabL5 heavy chain CDR1-3 sequences are highly similar or identical to FabL8 and FabL10 (CDRH1: GFKIKDTYIY, CDRH2: R*DPANGNTKYDPKF*G and CDRH3: YGNVYV, * represents a non-matching position). Hence, it is reasonable to conclude that these Fab-phage are essentially identical and a closer examination of the low FabL8/FabL10 signals in Figure 4 also suggest that FabL8 and FabL10 both bind more strongly to GluA4 LBD, as seen for FabL5.

FabL9 shares light-chain CDRs with the other Fabs (Table 1). It is highly likely that the PCR assembly has generated artificial Fd-L pairs and that a specific dominant light chain is widely used in this assembly, as this would explain why a specific light chain sequence is present in several of the Fabs. In addition, the specific FabL9 and the non-specific FabL7

share nearly identical CDRH1 sequences. Therefore, we conclude that the FabL9 GluA4 specificity is governed by the CDRH2 (EINPSNGRTNYNEKFKS) and CDRH3 (GLGQGTY). The FabL9 is to the best of our knowledge the first GluA4 LBD-specific Fab fragment that has been reported.

3.4 FabL9 expression, purification and characterization

FabL9 was engineered for soluble expression by performing a restriction-enzyme digest of the FabL9 phagemid DNA with *EagI*, followed by re-ligation, thus creating a FabL9 sequence devoid of protein III, but terminated by a hexa-histidine tag in the heavy chain [27]. PelB-directed periplasmic expression was induced, and the resulting Fab could be purified by immobilized metal affinity chromatography (IMAC; Figure 5A). A single contaminating band was present at ~ 25 kDa. This band most likely represents free heavy chain that co-elutes with Fabs.

The purified FabL9 was tested in competitive ELISA, and yielded an estimated IC₅₀ value of 260 nM (Figure 5B). The main purpose of the competitive ELISA experiment was to validate that the epitope on surface immobilized GluA4 also exists on GluA4 in solution, and competitive ELISAs is an efficient assay for this purpose. It is important to note that the reported IC₅₀ value is not a true affinity, but rather an indicator of relative binding. Recently, we used an antibody fragment to compare the K_D value obtained in competitive ELISA (20 nM) with that of detailed Biacore experiments (362 pM). These data underline that affinity measurements should be interpreted in the context of the method used [34]. The original *in vivo* affinity-matured VH-VL pair is likely to have a stronger binding affinity as an IC₅₀ of 260 nM is rather weak for a binder originating from an immune library.

This could suggest that the antigen-binding Fd binds promiscuously with an irrelevant (or less relevant) L chain as discussed in section 3.3 (Table 1). However, as demonstrated in the ELISA and in later experiments, the FabL9 affinity is sufficient for experimental use; in the future detailed surface-plasmon resonance experiments and light-chain replacements could shed light on the true affinity of FabL9.

As FabL9 did not bind microtiter plate-immobilized ectodomain (Figure 4), we were curious to test whether FabL9 was able to bind intact GluA4 in the context of rat-brain protein extract. By immunoprecipitation and immunoblotting, we did demonstrate that FabL9 could bind and pullout GluA4 from rat-brain extract, as seen by a strong band around 130 kDa that was also present in the positive control (Fab22), but absent in the negative control (MK16 Fab) (Figure 5C). The reason for the lack of GluA4 ectodomain binding is unclear, but as GluA4 LBD can be immobilized in microtiter plates without destroying the epitope, the most likely explanation is that the NTD shields the FabL9 epitope when the ectodomain is immobilized in microtiter plates, thereby rendering the epitope inaccessible for FabL9. Interestingly, our data suggest that this NTD-shielding does not occur in the intact receptor. It is possible that the presence of the transmembrane domain could exert strain on the NTD that permits display of the epitope in the LBD.

FabL9 was tested for modulation of GluA4 receptor by applying it in the presence of a saturating concentration of cyclothiazide to an HEK293 cell line stably expressing GluA4,

and measuring Ca^{2+} currents using a Fluo4-based fluorescence assay [35]. In this assay, FabL9 displayed neither agonist, antagonist, nor modulatory activity at the GluA4 receptor (data not shown). The observations in these experiments collectively strongly suggest that targeting the FabL9 epitope is unlikely to impair the functionality of the GluA4 receptor.

3.5 FabL9 epitope prediction

Since FabL9 binds GluA4_{flip} but not the highly homologous GluA2_{flop}, it is possible to estimate the binding epitope on GluA4. GluA4_{flip} and GluA2_{flop} have very similar linear sequences, but there are subtle differences (see Figure 6 for a linear sequence alignment). From this alignment the residues that differ between GluA4_{flip} LBD and GluA2_{flop} were identified.

GluA4_{flip} and GluA2_{flop} have homologous structures, and we could therefore use the structure of GluA4 LBD (Protein Data Bank ID: 3FAS) to investigate the positions of non-conserved residues. A surface representation of GluA4_{flip} LBD is seen in a front and top view in Figure 7. In the front view, it is evident that a clustering of non-conserved amino acids (yellow) is found above the Glu-binding site. The residues representing the potential epitope are M423, Y430, I464, A465 and P468 (GluA4_{flip} full-length receptor numbering, Uniprot ID P19493). The positions F437, A662, T705 and V755 also differ between GluA4_{flip} and GluA2_{flop} but these residues (blue) are either largely buried within the structure and are likely not accessible for FabL9, or are positioned far away from other residues as is the case for F437. A further residue clustering is found, also representing a potential epitope (red) comprising the residues S452, I458, I460, R765, T766, P767, S776, A778 and V780. From the structure it is evident that the I458 and I460 residues are brought into close proximity to R765, T766 and P767 in the folded GluA4_{flip} LBD, which could not be predicted from the linear sequence. Taken together, this epitope prediction represents a simple, yet powerful, way to narrow the list of potential target surfaces.

Using antigenic peptides comprising loops identified from crystal structures, polyclonal antibodies have been developed against AMPA receptors, including GluA4 [36–38]. Baude *et al.* and Wenthold *et al.* each used C-terminal sequences of GluA4 and developed rabbit polyclonal antisera that were specific for GluA4, but bound to the intracellular part of the receptor and could as such only be used for immunoprecipitation experiments with solubilized receptor [37, 38].

Based upon the data above, FabL9 joins the group of Fab-fragments with a GluA4 semi-specific/specific profile, namely Fab22 (GluA4-specific) and Fab7 (GluA2/GluA4-specific) [21]. The Fab7/22 were developed by a time-consuming process in which full length GluA4 receptors were produced in Sf21 insect cells, with a yield of ~100 µg/L [39], and incorporated into proteoliposomes. The proteoliposomes were subsequently used for immunization, and at the same time the proteoliposomes were biotinylated, thereby enabling capture on streptavidin during the panning procedure. The phage library was prepared as described in this manuscript. The isolated Fabs all targeted the NTD, which suggest one of two i) the LBD is shielded when the proteoliposomes are immobilized on the microtiter surface or ii) the LBD sequence is less immunogenic and no antibodies against the LBD are

produced in the mouse. The most likely explanation is the first, as we were able to generate LBD-binding Fabs in the present study using the same mouse strain (BALB/c).

The parallel panning approach described here enables for the first time selection of LBD-binders that were otherwise not selected by the proteoliposome approach. The conceptual idea of using proteoliposomes was that by immunizing with the intact receptor one would likely select antibodies with an affinity for the native protein. However, in this study we demonstrate that by using the simpler approach of immunizing with the GluA4 ectodomain, we were able to target native structures in the LBD. Due to the novel specificity of FabL9 for the GluA4 LBD, this molecule may supplement the existing Fabs in various biochemical applications.

Conclusion

The conclusions of the present study are two-fold. Firstly, we demonstrate that we can utilize a parallel panning approach to rapidly select murine Fab fragments towards individual domains of the GluA4 receptor. This approach of immunizing with a larger receptor fragment, creating one single phage library and subsequently screening for binders towards receptor sub-domains can be a viable approach for simplified antibody development towards other multi-domain surface receptors. Secondly, we have isolated a GluA4 LBD subtype-specific murine Fab fragment that targets the ligand-binding domain. Based upon the Fab specificity towards GluA4 LBD we propose a site for the binding epitope. This is the first report of an antibody with GluA4 LBD selectivity.

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Abbreviations

GPCR	G-protein coupled receptor
LGIC	ligand-gated ion-channels
VGIC	voltage-gated ion-channels
mAb	monoclonal-antibody
Fab	fragment antigen binding
ELISA	enzyme-linked immunosorbent assay
Glu	glutamate
AMPA receptor	(<i>S</i>)-2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propionic acid receptor

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Figure 1.

A) Cartoon overview of the iGluR receptor family. A dimer of dimer forms the active ion channel and both homomers and heteromers exist, dependent upon the receptor subtype assembly. The ectodomain comprises the amino-terminal domain (NTD) and the ligand-binding domain (LBD). Glutamate has high affinity for the LBD, and the binding leads to opening of an ion channel formed by the transmembrane domains (TMD). The carboxyl terminus is located in the cytoplasm, where it can interact with proteins of the postsynaptic density. **B) Overview of the immunization and parallel panning scheme.** A mouse was

immunized i.p. biweekly four times with rat GluA4 ectodomain (containing both the NTD and LBD in one molecule). Mouse-spleen RNA was subsequently isolated and the Fab cassette was constructed by jumping PCR from amplified heavy and light chains and ligated into the pFab74 phagemid. Electrotransformation into TG1 *E. coli* cells yielded a library size of 2.2×10^7 . The panning procedure was performed in parallel using intact ectodomain and LBD respectively. This enabled us to isolate Fab fragments towards GluA4 subdomains. C)

Detailed overview of the Pfab74 phagemid vector used in this study.

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Figure 2. Direct ELISA shows mouse immunoreactivity towards both intact ectodomain (ED) and ligand-binding domain (LBD)

Serum from 1st and 3rd bleed from the best responding mouse was used in the ELISA in plates coated with ED (panel A) or LBD (panel B). The signals are compared to empty wells coated with PBS. After the 3rd bleed, the mouse was put down and the spleen was used for Fab library construction.

Figure 3. Screening for GluA4 binding of twenty clones selected after four panning rounds Ten single clones from the LBD panning (L1-10) and ten from the ectodomain panning (E1-10) were selected for further analysis. The twenty clones were analyzed by phage-ELISA for GluA4 binding and four L-clones (L5, L7, L9 and L10) were positive for LBD binding (green). Furthermore, seven E-clones (E3, E4, E5, E6, E8, E9 and E10) were positive for binding to the GluA4 ectodomain (red). Buffer control (2% SM) values are shown in blue. Abbreviations: SM, skimmed milk powder; LBD, ligand binding domain; ED, ectodomain. Results are screening data from single measurements.

Figure 4. Specificity study of individual Fab fragments

LBD and ectodomain binders were tested in phage-ELISA for binding to GluA4 ED and LBD, as well as to LBD from GluA2, GluK1, GluK2 and GluD2. The cross-reactivity ELISA signals showed that L5 and L9 bound strongly and exclusively to GluA4 LBD, whereas L8 and L10 displayed a rather weak binding after re-testing. L7 seemed to target a common iGluR LBD epitope as it reacted strongly with all LBDs. All the ectodomain binders displayed binding exclusively to the GluA4 ectodomain and not to the GluA4 LBD, thereby suggesting that the epitopes all include the GluA4 NTD or a combination epitope including the NTD. ELISA signals shown are mean values and with SD error bars from triplicate measurements. Abbreviations: SM, skimmed milk; LBD, ligand binding domain; ED, ectodomain.

Figure 5. Purification and characterization of FabL9

A) FabL9 was expressed as a soluble fragment in the periplasm and purified by IMAC. The purified product is seen as a sharp band around 50 kDa by non-reducing SDS-PAGE eluting from the column in 60–96 mM imidazole. Lower levels of heavy and/or light chains are seen around 25 kDa. **B)** Inhibition ELISA demonstrates retained functional activity of purified FabL9 and estimated IC_{50} value. **C)** An immunoblot with rabbit α GluA4 confirms that FabL9 immunoprecipitates native intact GluA4 receptor from rat-brain extract, seen as a dominant band around 130 kDa. Parallel immunoprecipitations with Fab22 [21] and MK16 Fab [29], were used positive and negative controls. The lane with RB extract is the starting material here acting as a size control. As seen from the blot, some GluA4 degradation is present in the brain extract, as illustrated by smaller size protein bands on the blot. Abbreviations: Ctrl is short for control and RB is short for rat brain.

Figure 6. Alignment of LBD's from GluA2_{flop} and GluA4_{flip}

Shown is an alignment of the primary sequences of GluA2_{flop} and GluA4_{flip}. The alignment was prepared using T-Coffee [40] and Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Residues differing between GluA2_{flop} and GluA4_{flip} are displayed in light gray and white. Residues are labeled according to the GluA4_{flip} full-length receptor numbering (Uniprot ID P19493).

Figure 7. Structural placement of residues that comprise the FabL9 epitope

The structure of GluA4_{flip} LBD (PDB entry 3FAS) is shown as a van der Waals surface (grey). Residues highlighted in yellow and red represent the two potential epitopes highlighted by cluster residue differences between GluA4_{flip} and GluA2_{flop} (PDB entries 3FAS and 2CMO). Isolated sequence differences are shown in blue. PyMOL (Schrödinger, LLC, New York, NY) was used for the graphical illustrations.

Table 1
Sequence alignment of complementarity-determining regions for GluA4-specific LBD-binding Fab fragments

The heavy chain CDR1-3 and light CDR1-3 sequences are shown for fragments isolated by panning against the GluA4 LBD.

	Heavy chain			Light chain		
	CDR1	CDR2	CDR3	CDR1	CDR2	CDR3
Fab						
L5	GFKIKDTYIY	RVDPA NGNTKYDPKFQG	YGNVYV	KASHEIKSCLS	RVKRLVD	QNYLHPTAA
L7	GYTFTSYWMH	YINPSTGYTEYNQKFKD	SGGNCFDY	KASQDIKS YLS	YATSLAD	LRHGESPFF
L8	GFKIKDTYIY	RIDPANGNTKYDPKFQG	YGNVYV	KASQDINSYLS	RANRLVD	LQYDEFPR
L9	GYTFTSYWMY	EINPSNGRTNYNEKFKS	GLGQGTY	KASQDINSYLS	RANRLVD	LQYDEFPL
L10	GFKIKDTYIY	RIDPANGNTKYDPKFRG	YGNVYV	KASQDINSYLS	RANRLVD	LQYDEFPR