Kainate-binding Proteins Are Rendered Functional Ion Channels upon Transplantation of Two Short Pore-flanking Domains from a Kainate Receptor*

Nathalie Strutz‡§, Carmen Villmann¶, Hans-Georg Breitinger¶, Markus Werner‡, Robert J. Wenthold, Pablo Kizelszteini**, Vivian I. Teichberg**, and Michael Hollmann‡

From the †Department of Biochemistry I: Receptor Biochemistry, Ruhr University Bochum, D-44780 Bochum, Germany, the ¶Institute for Biochemistry, University of Erlangen, D-91054 Erlangen, Germany, the ||Laboratory of Neurochemistry, NIDCD, National Institutes of Health, Bethesda, Maryland 20892-4162, and the **Department of Neurobiology, Weizmann Institute of Science, Rehovot 76100, Israel

Ionotropic glutamate receptors (GluRs) can be classified into three distinct pharmacological groups: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (GluR1–GluR4), kainate (KA) receptors (GluR5–GluR7, KA1 and KA2), and N-methyl-D-aspartate (NMDA) receptors (NMDAR1, NR2A–NR2D, and NR3A–B) (1–3). All of these subunits have been shown to form functional ion channels in heterologous expression systems, despite binding glutamate agonists with high affinity. To test the hypothesis that inefficient or interrupted signal transduction from the ligand-binding site via linker domains to the ion pore (gating) might be responsible for this apparent lack of function, we transplanted the short homologous linker sequences from the fully functional rat kainate receptor GluR6 into frog kainate-binding protein. We were able to generate chimeric receptors that are functional in the Xenopus oocyte expression system and in human embryonic kidney 293 cells. The linker domains A and B in particular appear to be crucial for gating, because a functional kainate-binding protein was observed when at least parts of both linkers were derived from GluR6. We speculate that to enable signal transduction from the ligand-binding site to the ion pore of the frog kainate-binding protein, the linker structure of the protein has to undergo an essential conformational alteration, possibly mediated by an as yet unknown subunit or modulatory protein.

Ionotropic glutamate receptors (GluRs) can be classified into three distinct pharmacological groups: α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors (GluR1–GluR4), kainate (KA) receptors (GluR5–GluR7, KA1 and KA2), and N-methyl-D-aspartate (NMDA) receptors (NMDAR1, NR2A–NR2D, and NR3A–B) (1–3). All of these subunits have been shown to form functional ion channels, either homomERICally, as part of heteromERIC subunit assemblies, or both. In addition to these functional subunits, several homologous subunits do not appear to form ion-conducting homomERIC receptors or heteromERIC complexes with other subunits. These include the kainate-binding proteins (KBPs) (4–8) and the orphan receptors δ1 and δ2 (2).

The KBPs have attracted much attention because they were shown to bind agonists such as glutamate, KA, and domoate with high affinity (5, 7, 9, 10), in contrast to the orphan receptors for which no ligand binding has yet been established (3). To date five different Kbp genes have been identified from five different species: one subunit each from the frog *Rana pipiens* (5), chicken (4), duck (12), *Xenopus laevis* (7), and two different subunits from the goldfish *Carassius auratus* (6). Chicken and duck KBP are 92.8% identical at the amino acid level, indicating that they represent the same gene. The other subunits share between 49.8 and 67.9% sequence identity and thus are derived from different genes. When compared with other GluRs the KBPs exhibit one compelling structural difference that is characteristic for all KBPs: a short N-terminal domain of only 128–148 amino acids, as opposed to ~520 amino acids for other GluR subunits.

The transmembrane topology of KBPs, however, was shown to be identical to that of other GluRs receptors (6). The pore domains of all five known KBPs have been investigated for intrinsic ion channel function by domain transplantation and were proven to be capable of conducting cations (11).

Because KBPs bind agonists with high affinity and possess functional ion channels, we speculated that inefficient “gating,” i.e. inefficient or interrupted signal transduction between the ligand-binding site and the ion pore, might be responsible for the observed lack of ion channel function of KBPs. The sequences linking the ligand-binding domains to the three transmembrane domains (linkers A–C; see Fig. 1A) were considered prime candidates for such gating domains. Because the kainate receptor GluR6 is fully functional and 41% identical at the amino acid level to frog Kbp (see Fig. 1B), we chose GluR6 as the donor for gating domains of proven functionality and transplanted putative GluR6 gating domains into frog Kbp. This approach for the first time succeeded in generating a mutant kainate-binding protein with demonstrable ligand-gated ion channel function.

**EXPERIMENTAL PROCEDURES**

Mutagenesis—Rat GluR6(Q) and frog Kbp wild type cDNAs were used for construction of chimeras between KBP and GluR6. In the present study, we define the connecting stretches of sequence between transmembrane domain (TMD) A and the S1 domain, between TMD B and the S2 domain, and between the S2 domain and TMD C as linkers A, B, and C, respectively (see Fig. 1A). The amino acid sequences of these three linker domains are GTNPVFSFLNPDLP (linker A, GluR6), AAESSYMGFLNFFSKE (linker A, Kbp), NLLAFLTVRMES (linker B, GluR6), SFSAYNSINTNQT (linker B, Kbp), EESKEASALG-

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*This work was supported by German-Israel Foundation Grant SFB 406 (to M. H. and V. I. T.) and by a grant from the Minerva Foundation (to V. I. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Notes:

1. The abbreviations used are: GluR, glutamate receptor; ConA, concanavalin A; HEK, human embryonic kidney; KA, kainate; KBP, kainate-binding protein; TMD, transmembrane domain; NMDA, N-methyl-D-aspartate; PCL, pore cassette large; PXL, pore cassette extra large; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid.

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VQN (linker C, GluR6), and GWVPQPHL (linker C, KBP) (see Fig. 1B).

The chimeras KBP-[linkerA]GluR6 (frog KBP with the linker A derived from GluR6; see Fig. 2), KBP-[linkerB]GluR6 (frog KBP with the linker B derived from GluR6; see Fig. 2), and KBP-[linkerC]GluR6 (frog KBP subunit with the linker C derived from GluR6; see Fig. 2) were generated by PCR. They do not contain any amino acid alterations except for the respective inserted native GluR6 linker sequences (see Fig. 1B). We furthermore engineered KBP constructs containing combinations of two or all three linker domains of GluR6, resulting in the constructs KBP-[linkerA]+B/GluR6, KBP-[linkerC]+C/GluR6, KBP-[linkerB]+C/GluR6, and KBP-[linkerA]+B+C/GluR6. For a graphic representation of the various linker transplacement constructs see Fig. 2. All of the mutated clones were sequenced across the PCR-amplified regions.

In preliminary experiments we had generated KBP-[linkerA]GluR6 and KBP-[linkerB]GluR6 constructs using a different approach that made use of intermediary constructs we had available from previous pore domain transplantation studies (11, 12). In that approach, we used four introduced restriction sites at homologous positions in KBP and GluR6 to allow easy construction of chimeras between these two receptors (see Fig. 1A): Kpn I and Nde I sites N-terminal of TMD A flanking linker A in KBP at amino acids 130–132 (AAE) and 146–148 (ELW), respectively, and in GluR6 at amino acids 516–518 (GTN) and 531–533 (DIW), respectively; furthermore, in KBP Nru I and EcoRV sites at C-terminal of TMD B flanking linker B in KBP at amino acids 241–243 (NQT), respectively, and in GluR6 at amino acids 627–629 (FLT) and 633–635 (MES), respectively. Numbering in each case starts with the first codon of the mature protein.

The mutant GluR6, which contains all four newly introduced restriction sites Kpn I, Nde I, Nru I, and EcoRV was named GluR6-PCL-PXL, and the equivalent KBP mutant was named KBP-PCL-PXL. These names are in keeping with our previously established nomenclature for mutants designed for ion pore exchange (13). They indicate that the constructs allow exchange of a large pore domain (a PCL, between sites Nde I and Nru I) as well as an extra large domain (a PXL, between sites Kpn I and EcoRV). These constructs, in addition to facilitating the exchange of ion pore inclusions, allow for easy manipulation of the pore domain, albeit at the expense of a few additional amino acid changes caused by the introduction of the four restriction sites (see Fig. 1B). Two additional KBP mutants were generated from KBP-PCL-PXL, which contain only the N-terminal linker A exchange sites Kpn I and Nde I (KBP-PCL-PXL/N) or the C-terminal linker B exchange sites Nde I and EcoRV (KBP-PCL-PXL/C).

To enable the exchange of PXL, Kpn I and EcoRV restriction sites (see above) were introduced into KBP and GluR6, resulting in the constructs KBP-PXL and GluR6-PXL. These constructs were then used as parent clones to create KBP-PXL/GluR6 (frog KBP with the extra large pore cassette derived from GluR6) and GluR6-PXL/KBP (rat GluR6 with the extra large pore cassette derived from frog KBP), respectively (see Fig. 1B).

cRNA Synthesis—cRNA synthesis was performed as described earlier (14). Briefly, template DNA was linearized with a suitable restriction enzyme. cRNA was synthesized from 1 µg of linearized DNA using an in vitro transcription kit (Stratagene) with a modified protocol that employs 800 µM GpppG (Pharmacia Corp.) for capping and an extended reaction time of 3 h with T7 polymerase. Trace labeling was performed with [35S]UTP to allow calculation of yields and evaluation of transcript quality by agarose gel electrophoresis.

Electrophysiological Measurements in Xenopus Oocytes—Oocytes of stages V and VI were surgically removed from the ovaries of X. laevis as described elsewhere (15). The oocytes were kept in Barth solution containing using a 10-µl Drummond (Broomall, PA) microdispenser. Two-electrode voltage clamp recordings were performed 4–8 days after cRNA injection with a TurboTec 10CD amplifier (npi, Tamm, Germany) by superfusion of the oocyte with glutamatergic agonists (300 µM) prepared in normal frog Ringer’s solution (115 mM NaCl, 1.5 mM CaCl2, 2.5 mM KCl, and 10 mM HEPES-NaOH, pH 7.2). Current electrodes were filled with 3 M CsCl and had resistances of ~0.5–1.5 MΩ. Voltage electrodes were filled with 3 M KCl and had resistances of ~4 MΩ. The oocytes were held at ~70 mV, and agonists (kainate and glutamate) were applied at 10 s at a flow rate of 10–14 ml/min. To minimize receptor desensitization, bath pretreatment of oocytes with concanavalin A (ConA, 10 µg/ml for 4 h) was carried out prior to agonist application. After 60 min, the oocytes were washed in 100 µl of Barth solution for 1 h, and the pellet was resuspended in 100 µl of 0.5 M NaOH. After a 1-h incubation, 75 µl of 12% acetic acid were added for neutralization. The samples were counted with scintillation fluid (Lumax-xylene). The specific binding of [3H]kainate was defined as the total binding protein complex. Briefly, intact oocytes were incubated in 10 µM bio-
Fig. 1. A, schematic representation of the structure of a typical ionotropic glutamate receptor. The N terminus is extracellular, the C terminus is intracellular, and there are three TMDs, A, B, and C. The pore-forming region consists of a hairpin loop plus two small intracellular loops (L1 and L2). The hairpin loop inserts into the membrane from the cytosolic side and is thought to line the ion permeation pathway (the pore), whereas loops L1 and L2 connect the pore to TMDs A and B, respectively. A large, extracellular domain resides between TMDs B and C. S1 and S2 are two extracellular domains homologous to bacterial amino acid-binding proteins that interact to form the ligand-binding site. Linkers A, B, and C are here defined as the connecting sequences between the S1 and S2 domains and TMDs A, B, and C, respectively (see also Fig. 1B). KpnI, NdeI, NruI, and EcoRV are introduced restriction sites used for constructing some of the chimeras between GluR6 and frog KBP. B, amino acid sequences of linkers A, B, and C of wild type rat GluR6 and frog KBP and linker transplantation mutants used in this study. The overall sequence identity between GluR6 and frog KBP subunits is 41.2%. Listed are the amino acid sequences of linkers A (16 amino acids in low affinity kainate receptors/17 amino acids in frog KBP), B (13/13 amino acids), and C (13/9 amino acids) of wild type GluR6, frog KBP, three linker exchange constructs, and the PCL-PXL and PXL mutants of GluR6 and frog KBP. The restriction sites, when present, are indicated by dashed lines below the respective sequence.

RESULTS

To determine the role of the linker regions (Fig. 1A) in KBP ion channel function, we transplanted linker sequences from the functional rat kainate receptor subunit GluR6(Q) into frog KBP (Fig. 2). We initially exchanged each linker region separately, generating the chimeras KBP-(linkerA)GluR6, KBP-(linkerB)GluR6, and KBP-(linkerC)GluR6, where the respective linker domains (in parentheses) were derived from GluR6 (Figs. 1B and 2). We also engineered KBP constructs containing combinations of two, or all three, linker domains of GluR6. Those constructs were named KBP-(linkerA+B)GluR6, KBP-(linkerA+C)GluR6, KBP-(linkerB+C)GluR6, and KBP-(linkerA+B+C)GluR6, respectively (Fig. 2).

All of the chimeras were expressed in both Xenopus oocytes and HEK 293 cells for electrophysiological analysis. Because it

minus the binding obtained in the presence of 1 mM kainate. All of the experiments were performed in triplicate. The binding data were analyzed using the PRISM program (GraphPad Inc., San Diego, CA).

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All of the chimeras were expressed in both Xenopus oocytes and HEK 293 cells for electrophysiological analysis. Because it
was not clear which agonist would evoke the largest current amplitude, we chose two agonists that were known to bind to KBPs (4, 8, 18, 22, 23), namely glutamate (300 μM) and kainate (300 μM). All three single linker transplantation constructs, KBP-(linkerA)GluR6, KBP-(linkerB)GluR6, and KBP-(linkerC)GluR6, were nonfunctional in Xenopus oocytes (n = 12, 16, and 10, respectively) as well as in HEK cells (n = 28, 22, and 25, respectively; Table I). We then tested constructs with the linker combination KBP-(linkerA+B)GluR6 (n = 12), KBP-(linkerA+C)GluR6 (n = 12), and KBP-(linkerB+C)GluR6 (n = 10). Of these, only KBP-(linkerA+B)GluR6 was functional in oocytes, showing current amplitudes of 8.4–8.7 nA (n = 12; Table I). Unfortunately, it was not possible to record dose-response curves of KBP-(linkerA+B)GluR6 in oocytes because of the small current amplitudes. In HEK 293 cells somewhat unexpectedly no significant currents could be detected in any of the three linker combination constructs KBP-(linkerA+B)-GluR6 (n = 30), KBP-(linkerA+C)GluR6 (n = 28), and KBP-(linkerB+C)GluR6 (n = 32). Interestingly, the substitution of linker C along with linker A and B from GluR6 (KBP-(linkerA+B+C)GluR6) resulted in loss of function compared with KBP-(linkerA+B)GluR6 (n = 11; Table I), suggesting that amino acids in linker C influence the structure of linkers A and B.

Surprisingly, the construct KBP-PCL-PXL, which carries four introduced restriction sites and which was used in preliminary experiments as an intermediary construct to enable the quick exchange of linkers A and B between KBP and GluR6, also gave small currents, of 2.8–5.2 nA (n = 18), in Xenopus oocytes after application of glutamate or kainate (Fig. 3 and Table I). This result was verified by expression of KBP-PCL-PXL in HEK 293 cells where the mutant also expressed functional ion channels. In these cells the construct showed current amplitudes of 107 ± 42 pA (n = 5 responding cells, of 39 tested). Steady state current amplitudes could be recorded without application of inhibitors of desensitization such as cyclothiazide or ConA. Treatment with ConA did not lead to any potentiation of current amplitudes, unlike what is observed for kainate receptors. The desensitization time constant for the construct KBP-PCL-PXL was 827 ± 332 ms (n = 8 traces averaged from three cells, S.D. given) and thus was significantly larger than in GluR6(Q) wild type receptors (19 ± 13 ms, n = 12 traces from four cells, S.D. given).

KBP-PCL-PXL contains a total of eight amino acid point mutations in linker domains A (three amino acids mutated) and B (five amino acids mutated), caused by the introduction of the four restriction sites in the cDNA to prepare this cDNA for domain transplantsations (see “Experimental Procedures”). The currents observed with KBP-PCL-PXL support the finding with KBP-(linkerA+B)GluR6 that a combination of amino acids in the linker domains A and B is indeed critical for gating. We found that the separation of the mutated amino acids in linker A of KBP-PCL-PXL from the mutated amino acids in linker B leads to loss of ion channel function; chimeras KBP-PCL-PXL/N and KBP-PCL-PXL/C, which contain only the three amino acid mutations in linker A and the five amino acid mutations in linker B, respectively, could not be activated by kainate or glutamate (data not shown). This parallels the finding that in KBP-(linkerA+B)GluR6 both linkers are required for function and supports our conclusion that linkers A and B interact directly or indirectly with each other. It also demonstrates that the linker domains are sensitive regions that indeed influence the gating mechanism.

The mutants KBP-(linkerA+B)GluR6 and KBP-PCL-PXL showed clear responses in only two of five batches of oocytes tested. However, nonfunctional chimeras never gave any measurable current. A similarly inconsistent expression of functional receptors was observed in HEK 293 cells, where only 25 of 55 cells for wild type GluR6 and 5 of 39 cells for KBP-PCL-PXL gave currents in response to stimulation by kainate or glutamate.

Western blot data demonstrated that all mutants and chimeras, functional as well as nonfunctional ones, were expressed and incorporated into the oocyte plasma membrane. Expression levels of chimeras were generally comparable with those of the wild type KBP receptor subunit (Fig. 4). To rule out the possibility that the limited reproducibility of ionic currents depended on varying expression levels of an endogenous glutamate receptor subunit, Xenopus KBP, also called XenU1, that is known to be expressed in Xenopus oocytes (7, 24), we coexpressed each of the functional chimeras KBP-(linkerA+B)-GluR6 and KBP-PCL-PXL with recombinant Xenopus KBP (11) in Xenopus oocytes. However, no currents could be measured under these conditions. This control experiment was repeated with the same result in a different batch of oocytes.

For KBP-(linkerA+B)GluR6 and KBP-PCL-PXL, the observed gain of function might potentially be explained by a simple change in agonist affinities. To test for this possibility, we performed ligand binding experiments with tritiated kainate to determine the K_d values for the chimeras analyzed here and compared those K_d values with K_d values of wild type KBP. The K_d value for kainate binding to wild type frog KBP was determined to be 20 ± 6 nM. Except for KBP-(linkerB)GluR6 (K_d = 178 ± 49 nM) and KBP-(linkerC)GluR6 (K_d = 104 ± 12 nM), the K_d values for kainate of all mutants were comparable.
Table I

| Construct | Oocytes | HEK 293 cells |
|-----------|---------|---------------|
|           | KA current | Glu current | n | KA current | n |
| KBR wild type | 0.0 ± 0.0 | 0.0 ± 0.0 | 20 | 0.0 ± 0.0 | 20 |
| KBR-PCL-PXL | 5.2 ± 2.0<sup>a</sup> | 2.8 ± 1.2<sup>a</sup> | 18 | 107.0 ± 42.0<sup>a</sup> | 38 |
| KBR-(linkerA)GluR6 | 0.0 ± 0.0 | 0.0 ± 0.0 | 12 | 0.0 ± 0.0 | 28 |
| KBR-(linkerB)GluR6 | 0.0 ± 0.0 | 0.0 ± 0.0 | 16 | 0.0 ± 0.0 | 22 |
| KBR-(linkerC)GluR6 | 0.0 ± 0.0 | 0.0 ± 0.0 | 10 | 0.0 ± 0.0 | 25 |
| KBR-(linkerA + B)GluR6 | 8.7 ± 1.1<sup>a</sup> | 8.4 ± 0.9<sup>a</sup> | 12 | 0.0 ± 0.0 | 30 |
| KBR-(linkerA + C)GluR6 | 0.0 ± 0.0 | 0.0 ± 0.0 | 12 | 0.0 ± 0.0 | 28 |
| KBR-(linkerB + C)GluR6 | 0.0 ± 0.0 | 0.0 ± 0.0 | 10 | 0.0 ± 0.0 | 32 |
| KBR-(linkerA + B + C)GluR6 | 0.0 ± 0.0 | 0.0 ± 0.0 | 11 | 0.0 ± 0.0 | 27 |

<sup>a</sup> Current amplitudes listed represent the mean current amplitudes of oocytes of batches where currents were measured for KBR-(linkerA + B) GluR6 or KBR-PCL-PXL; not included are oocytes of batches that did not show any currents for those receptors.

<sup>b</sup> Current amplitude listed represents mean current amplitude of five HEK cells that gave currents out of 39 tested cells. For an explanation of the constructs, see Figs. 1 and 2 and "Experimental Procedures."

DISCUSSION

The kainate-binding proteins are of interest because of their considerable sequence homology with mammalian glutamate receptor subunits and because high expression levels have been detected in nonmammalian vertebrate central nervous systems. Although researchers have put considerable emphasis on studying the molecular makeup of KBRs in the past, the physiological roles of these proteins are still unknown. Because these proteins are integral membrane proteins it appears unlikely that they serve as simple binding proteins for glutamatergic agonists, analogous to the recently discovered soluble glia-derived acetylcholine-binding protein that modulates synaptic transmission (26). Rather, it seems likely that they indeed serve some function in signal transduction across the plasma membrane.

Possible reasons for the lack of observable ion channel function of KBRs in heterologous expression systems in principle could be: 1) a lack of protein expression; 2) a lack of agonist binding; 3) an absence of a functional ion pore; or 4) a defect in translating the information of agonist binding into pore opening (gating). Protein expression of the recombinant KBR subunits has been demonstrated repeatedly (4, 8, 18, 22, 23). For the KBR chimeras in the present study, protein expression comparable with that of wild type was verified (Fig. 4). Similarly, high affinity agonist binding has been clearly shown for all KBRs (8, 9, 18, 22, 23). Furthermore, it has been demonstrated conclusively that domain swapping between different glutamate receptor subtypes is possible without loss of function (11, 13, 14, 27). With this domain swapping technique, the ion pores of all KBRs have been assayed for function and, without exception, were proven to be capable of conducting currents (11).

Obviously, homomerically expressed KBR subunits fail to translate ligand binding into channel opening. It is assumed that ligand binding causes a conformational change in the extracellular domain of the receptor (25, 28–30). This conformational change has been dubbed "venus fly trap" or "clamshell mechanism," alluding to the closure that, upon agonist binding, occurs between two extracellular domains. Although details of that mechanism remain controversial (25, 28), it presumably represents the gating step required to open the ion channel. In recent publications by Armstrong et al. (25, 30), data on the crystal structure of the ligand-binding domain of GluR2 complexed with kainate or other agonists strongly support the assumption that conformational changes take place after ago-
nist binding. For heterologously expressed KBP subunits, it appears that homomeric subunit assemblies either fail to generate the appropriate conformational change after binding of agonist or fail to communicate such change to the pore. In any case, this constitutes a disturbance in the gating mechanism.

Based on our previous studies showing that the pore domains of KBPs are inherently functional domains capable of fluxing ions (11), we used the frog KBP to investigate the function of the linker domains A, B, and C, which connect the TMDs and the ion pore domain to the ligand-binding domain. Our two functional KBP mutants, KBP-(linkerA+B)GluR6 and KBP-PCL-PXL, for the first time demonstrate directly that the linker domains are involved in the gating mechanism of KBPs. It is not clear whether exclusively the linker domains A and B are important for gating or whether linker C plays an additional role. The latter is suggested by the chimera KBP-(linkerA+B+C)GluR6, which is nonfunctional but shows a $K_d$ value for kainate binding comparable with that of wild type KBP despite the facts that KBP-(linkerC)GluR6 has decreased kainate affinity and that KBP-(linkerA+B)GluR6 agonist affinity is unchanged. It cannot be excluded off-hand that other regions of the receptor, previously undiscussed, play a role in gating. However, at least linkers A and B are clearly involved in and interact during the gating process, and, in wild type

**Fig. 3.** Sample current traces recorded with 300 μM kainate or 300 μM glutamate in *Xenopus* oocytes (A) or HEK 293 cells (B). Current traces shown are from different experiments. Note the different amplitude scales. The spikes at the beginning and end of the agonist application in *Xenopus* oocytes (A) represent switching artifacts of the perfusion system.
KBP, seem to be either disabled or extremely inefficient in triggering pore opening.

It is conceivable that, in the three-dimensional structure, the linker domains are in close proximity. The data on the linker chimeras KBP-(linkerA+B)GluR6, KBP-(linkerA)GluR6, and KBP-(linkerB)GluR6 as well as on KBP-PCL-PXL, KBP-PCL-PXLN, and KBP-PCL-PXL/C, indicate that amino acid sequences in the linker domains A and B influence each other. Thus, linkers A and B cannot be exchanged independently of each other and appear to be functionally coupled. Interestingly, if linker C of GluR6 is added to linkers A and B to produce the chimeric construct KBP-(linkerA+B+C)GluR6, the function gained by transplanting linkers A and B of GluR6 is lost again, suggesting that amino acids in linker C contribute to the gating domain structure. This latter conclusion is supported by additional data. In a recent publication, we presented evidence for the importance of the linker C domain of GluR7 for the gating mechanism (14). Single amino acid point mutations at three different positions within the linker domain C each converted the kainate receptor subunit GluR7, which, at least in oocytes, is nonfunctional under physiological conditions, into a clearly functional subunit. In a publication by Taverna et al. (31) the so-called Lurcher mutation (32) introduced into the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunit GluR1 is discussed as crucially influencing the binding/gating properties of GluR1. Because there are differences in the terminology and definition of the length of the transmembrane domains among researchers, the Lurcher mutation by Taverna et al. is considered to be located within transmembrane domain B (their definition) rather than in linker domain B (our definition). The results by Taverna et al. are further supported by a recent publication on NMDA receptors by Jones et al. (33), who proposed that the TMD B region (or M3) of NMDA receptors functions as a transduction element whose conformational change couples ligand binding to channel opening. The TMD B (or M3) region as defined in Jones et al. includes the first 5 amino acids of linker B, based on our definition of the linker regions. Whatever the exact definition of TMD B, the results by Taverna et al. and Jones et al. support our suggestion that the linker domains are crucial for gating in KBPs. However, in contrast to the data of Jones et al., who proposed the TMD B region (including part of the linker B sequences in our understanding) of NMDA receptors as the link between ligand binding and channel opening, Krupp et al. (34), suggested that linker A of NMDA receptors alone serves as a dynamic link between ligand binding and channel gating. Our data go beyond the data of Taverna et al., Krupp et al., and Jones et al. and in a sense may reconcile those apparently contradictory findings. We suggest that an appropriate linker B has to interact with an appropriate linker A to gate KBPs. It is certainly possible that KBPs and NMDA receptors differ in the mechanism of the linker structure required for successful gating. However, the contradictory data of Krupp et al. and Jones et al. together with our data may be taken to indicate that also for NMDA receptors there is not just one single determinant that mediates signal transduction between the ligand-binding site and the ion pore, as proposed by Krupp et al. (34) for linker A and by Jones et al. (33) for TMD B/linker B.

In another recent publication (35) transposon-mediated insertion of green fluorescent protein into GluR1 was used as a means to randomly fluorescence label the receptor protein. Interestingly, many of the insertion mutants were functional ion channels despite the large protein domain integrated into the sequence. However, green fluorescent protein insertion into linkers A and C were as incompatible with function as insertion into the pore domain or the ligand-binding domain, underscoring the importance of functional integrity of the linker domains.

Furthermore, Sun et al. (36) in their comparative analysis of the crystal structures of the ligand-binding domains of wild type GluR2 and a nondesensitizing mutant GluR2 recently suggested a crucial role for the linker regions in channel activation. They proposed that the ligand-binding cores are organized as dimeric units in a tetrameric assembly where interactions of the dimer interfaces determine the level of desensitization of the receptor. The mechanistic scheme the authors present entails an agonist-triggered increase in the separation of the linker regions resulting in the activation of the pore if a stable dimer interface is present. Our experimental data on KBP linker regions presented here, which suggest the participation of both linkers A and B in the gating process, are perfectly in line with the hypothesis of Sun et al. Our data as well as those of Sun et al. stress the need for structural models incorporating not only the ligand-binding domain of the receptor but also the linker domains and, ideally, the ion channel domain.

An unexpected observation in the present study was the unreliable current expression of KBP/GluR6 chimeras. Only in two of five experiments (two of five batches of *Xenopus* oocytes) did KBP-(linkerA+B)GluR6 and KBP-PCL-PXL show currents. In the two experiments in which these two constructs were functional, other chimeras were not. The question arises as to the reason for this phenomenon. Obvious explanations such as different concentrations of injected RNA or different protein expression levels can be excluded because these parameters were controlled for and monitored, respectively. We can also exclude a varying contribution by the endogenous *Xenopus* kainate-binding protein XenU1 (7, 24) being responsible for

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**Fig. 4.** Western blot demonstrating protein expression of the mutant frog KBP receptor subunits analyzed. Plasma membrane protein was labeled with biotinyl-ConA, solubilized, and then streptavidin-precipitated (11 oocytes/lane). Samples including controls from uninjected oocytes were separated on a SDS gel, Western blotted, and probed with a polyclonal antiserum against a C-terminal peptide of KBP (18). Arrows point to the position of the ~49-kDa band of wild type KBP and chimeras between KBP and GluR6. The asterisk denotes a known unspecifically cross-reacting protein band that is not found in control oocytes (11).
this effect. Coexpression of the functional chimeras KPB-(linkerA+B)(GluR6 and KPB-PCL-PXL with recombinant Xenopus KPB (7) did not increase the probability of current expression. Because the phenomenon of limited reproducibility has never been observed before for any wild type or mutant glutamate receptor subunit investigated in our laboratory, we conclude that lack of reproducibility might be a characteristic feature of our chimeric constructs or even of KPBs in general. Recently, Ayalon and Stern-Bach (37) have shown that the assembly of glutamate receptor subunits is determined by the linker domain (38–40). Interestingly, in contrast to the other vertebrate glutamate receptor subtypes, the kainate-binding pro-

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