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Asn-265 of frog kainate binding protein is a functional glycosylation site: implications for the transmembrane topology of glutamate receptors

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Abstract Kainate binding proteins (KBPs) from frog and goldfish brain are glycosylated, integral membrane proteins. These KBPs are homologous (35–40%) to the C-terminal half of AMPA and kainate receptors which have been shown to form glutamate-gated ion channels. We report here that the frog KBP has three functional *N*-glycosylation sites. Of particular interest, Asn-265, a residue located between two putative membrane spanning regions of the frog KBP, is a functional *N*-glycosylation site. A mutation of Ser-267 to Gly renders this site non-functional as shown using an in vitro translation system and by transient expression in human embryonic kidney (HEK 293) cells. The mutant receptor protein (S267G), when expressed in HEK cells, binds kainate with high affinity (K_d = 16 nM). These results further support a topology with three transmembrane segments for KBPs and, by sequence homology, for glutamate-gated ion channels.

Key words: Glutamate receptor; N-linked glycosylation; Transmembrane topology; Ligand binding domain

1. Introduction

Glutamate receptors are the major excitatory neurotransmitter receptors in the vertebrate central nervous system. The ionotropic glutamate receptors (iGluRs, ligand-gated ion channels) can be divided into AMPA (α-amino-3-hydroxy-5-methylisoxazole-propionate), kainate and NMDA (*N*-methyl-D-aspartate) subtypes based on pharmacological data [1]. Molecular cloning and functional analysis have yielded a wealth of knowledge about iGluRs [2–4]. Functional ion channels are formed by subunits of 100 kDa either alone or in various combinations. AMPA receptors are formed by GluR1–GluR4 subunits, kainate receptors are formed by GluR5–GluR7 and KA-1 and KA-2 subunits. NMDA receptors are formed by NR1 and NR2A-2D. In addition to these functional iGluRs from various species, high affinity kainate binding proteins have been purified and the cDNA cloned from several nonmammalian vertebrate species (e.g. fish, frog, bird, [5–7]), these proteins (40–50 kDa) are homologous (35–40%) to the C-terminal half of subunits (100 kDa) of AMPA and kainate receptors.

iGluRs have long been thought to be structurally similar to other ligand-gated ion channels having four transmembrane segments with the ligand binding domain in the N-terminal extracellular region (e.g. nicotinic acetylcholine receptors, GABA receptors, glycine receptors). This long-held, pervasive assumption has recently been questioned [8], largely due to the study of the transmembrane topology of various subtypes of iGluRs. Recent evidence has indicated that previously proposed topological models with four or five transmembrane segments are incorrect. Based on our topological analysis of goldfish kainate receptors, including the study of native and engineered *N*-glycosylation sites and proteolytic patterns of GFKARα and GFKARβ, we have proposed a topological model with three transmembrane segments for kainate receptors [5,9]. An identical model was subsequently proposed based on the study of engineered *N*-glycosylation sites in GluR1 [10] and constructs of epitope-tagged GluR3 [11]. This 3-TM model has provided important insight into the modular structure of iGluRs and will serve as a framework for future detailed structure-function analyses.

In the present study, we analyze the *N*-glycosylation of the frog KBP in both an in vitro translation/membrane translocation system and an HEK cell expression system. We provide further evidence in support of the 3-TM model by identifying a native and functional *N*-glycosylation site (Asn-265) of frog KBP. This is the first demonstration of a *native* glycosylation site in the region of iGluRs proposed to be cytoplasmic in models with four and five transmembrane segments.

2. Material and methods

2.1. Materials

[^{3}H]Kainic acid (60 Ci/mmol) was purchased from Dupont NEN. L-[^{35}S]Methionine (1100 Ci/mmol) was purchased from Amersham Corp. Autoradiography enhancer INTENSIFY was purchased from DuPont NEN. Endo-β-*N*-acetylglucosaminidase H (Endo H) and *N*-glycosidase F were obtained from Boehringer-Mannheim. The monoclonal antibody KAR-B1 was a gift from Dr. David R. Hampson (Univ. Toronto).

2.2. Site-directed mutagenesis and truncation mutant

(A) The S267G mutation was prepared using PCR. The T7 primer and an antisense oligonucleotide (5′-CCGAATTCTTAAAGAAATTTAAAGTGGCAGAG-3′) were paired to amplify a fragment of 960 bp from frog KBP cDNA in pBluescript. The PCR fragment was digested with *Xba*I and *Eco*RI and was used to replace the corresponding fragment of wild-type frog KBP cDNA. The mutation was confirmed by DNA sequencing. (B) cDNA encoding the N-terminal 118 amino acid residues of frog KBP was subcloned into pcDNAII vector (Invitrogen). An antisense oligonucleotide (27-mer, 5′-GCGCTCGAG(*Xho*I site)TTA(stop codon)CATTGTTAAAGGTGC-3′) was paired with a

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionate; Endo H, endo-β-*N*-acetylglucosaminidase H; iGluR, ionotropic glutamate receptor; KBP, kainate binding protein; FKBP, frog kainate binding protein; GFKARα, 45 kDa kainate receptor subunit from goldfish brain; GFKARβ, 41 kDa kainate receptor subunit from goldfish brain; HEK, human embryonic kidney; NMDA, *N*-methyl-D-aspartate; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TM, transmembrane domain; TMI, TMII, TMIII, TMIV, first, second, third and fourth putative transmembrane domain as originally defined by hydropathy plots.

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T7 (pBluescript) primer to amplify a 480 bp cDNA fragment from the frog KBP cDNA clone (in pBluescript) by PCR. This PCR fragment was digested with SpeI and XhoI, then subcloned into pcDNAII vector (Invitrogen). This construct was termed $N118_{(FKBP)}$.

2.3. In vitro translation and translocation

Promega's TNT coupled rabbit reticulocyte lysate system was used as described previously [5]. T7 polymerase was used to transcribe frog KBP and KBP(S267G) mutant cDNA in pBluescript vector. SP6 polymerase was used to transcribe GFKARα cDNA and the construct $N118_{(FKBP)}$ in pcDNAII vector. For *N*-glycosylation, canine pancreatic microsomal membranes (Promega) were added to the translation system as indicated previously [5]. The translational efficiency of frog KBP and FKBP(S267G) cDNAs is about 5-fold lower than GFKARα and GFKARβ cDNAs. To load a similar amount of [^{35}S]Met-labeled protein, samples of frog KBP and FKBP(S267G), translated in the presence of microsomes, were centrifuged, and the pellets (MM fractions) were then resuspended in a 5-fold smaller volume. Samples were analyzed on SDS-polyacrylamide gels (8% or 14%). Gels were treated with the enhancer and [^{35}S]methionine-labeled bands were visualized with fluorography on Kodak XAR film.

2.4. Expression in cultured HEK 293 cells

Both frog KBP and KBP(S267G) mutant cDNA inserts (1.6 kb, with *Xba*I and *Xho*I ends) were subcloned from pBluescript into an expression vector, pRBG4 [12]. HEK 293 cells (a transformed human embryonic kidney cell line) grown to 6×10^5 cells/10-cm dish were transfected by these expression constructs (20 μg plasmid DNA/dish) using the calcium phosphate precipitation method [12]. HEK 293 cell membranes were prepared 48 h after transfection as described previously [9]. Membrane pellets from each dish were resuspended in 0.5 ml of 1 mM PMSF and stored at −20°C.

2.5. Western blotting and gel electrophoresis

As described previously [9] samples of the transfected HEK 293 cell membrane preparations were solubilized in SDS loading buffer (8% β-mercaptoethanol) and separated by 10% SDS-polyacryamide gel electrophoresis. Proteins were electrophoretically transferred from the gel to Immobilon-P membrane (Millipore Corp.). The membrane was blotted sequentially by the monoclonal antibody FKAR-B1 and a second antibody (horseradish peroxidase-conjugated goat anti-mouse IgG). Proteins recognized by the mAb FKAR-B1 were visualized using Renaissance Chemiluminescence reagent (Dupont NEN).

2.6. [^{3}H]Kainate binding assay

[^{3}H]Kainate binding to transfected HEK 293 cell membrane preparations was measured as described previously for membranes [13,14]. The additional presence of 100 μM kainate in the assays defined nonspecific binding.

3. Results

Native frog KBP is a glycoprotein with an apparent molecular mass of 48 kDa on SDS-PAGE [15–17]. The deduced sequence of frog KBP cDNA indicates that there are two putative

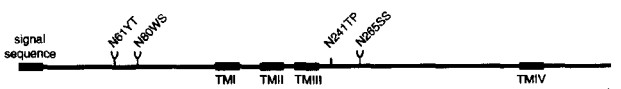

Fig. 1. Schematic depiction of frog KBP. The signal sequence and three proposed transmembrane segments (TMI, TMIII and TMIV) are shown as black boxes. The originally designated TMII (likely not a true transmembrane segment) is shown as a gray box. Three consensus *N*-glycosylation sites (Asn-X-Ser/Thr) are shown with a ⑂; a fourth site which has a proline following the Asn-X-Ser/Thr motif is indicated with a |. Two regions of frog KBP homologous to the bacterial periplasmic amino acid binding proteins are indicated as LAOBP/QBP (lysine-, arginine- and ornithine-binding protein and glutamine-binding protein) regions. The region comprising TMI to TMIII is homologous to the channel pore of K^+ channels [8].

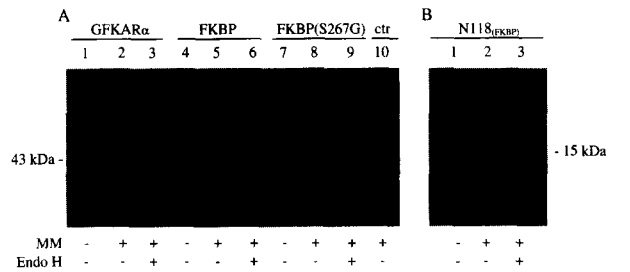

Fig. 2. (A) In vitro translation and glycosylation of goldfish and frog kainate binding proteins and the frog KBP(S267G) mutant. Samples were separated on 8% SDS-PAGE. Note that an endogenous 52 kDa band is visible (lane 10) in a 5-fold concentrated control sample (e.g. no cDNA template). (B) In vitro translation and glycosylation of the N-terminal 118 amino acids of frog KBP. Samples were separated on 14% SDS-PAGE. Microsomal membranes are noted with MM. The glycosylated species are indicated by an asterisk.

N-glycosylation sites (N61YT and N81WS) in the N-terminal extracellular region preceding TMI ([6]; Fig. 1). Our proposed 3-TM model [5,8] would suggest that in addition to these two sites, N265SS may also be a functional *N*-glycosylation site. This site is 35 residues downstream from the C-terminal end of the proposed TMII. A coupled in vitro translation/membrane translocation system was used to determine the number of the core oligosaccharide chains transferred to the newly synthesized frog KBP. We have previously used this system to characterize the *N*-glycosylation sites in goldfish kainate receptors (GFKARα and GFKARβ; [5,9]), which are 55% homologous to frog KBP. GFKARα has three functional *N*-glycosylation sites. Each uniform core oligosaccharide chain transferred from a lipid donor to acceptor Asn residues has a apparent mass of 3 kDa [18]. Frog KBP translated in vitro in the absence of microsomes was about 3 kDa larger than non-glycosylated GFKARα (44 kDa vs. 41 kDa; Fig. 2, lane 4 vs. lane 1), consistent with the fact that frog KBP is 30 residues longer than GFKARα [5,6]. In the presence of microsomes, GFKARα translated in vitro exhibited two glycosylated bands, corresponding to species with two and three core oligosaccharide chains [9]. Whereas, in the presence of microsomes, frog KBP has two glycosylated species, the fully glycosylated form is approximately 9 kDa larger than the non-glycosylated form (Fig. 2, lanes 4 and 5), indicating the presence of three functional sites for *N*-glycosylation. In contrast, the KBP(S267G) mutant, in which the N265SS site has been changed into a non-consensus sequence, has only two attached oligosaccharide chains when translated in the presence of microsomes (Fig. 2, lane 8). Therefore, Asn-265 is a functional *N*-glycosylation site in this in vitro translation/translocation experiment, indicating that sequence surrounding Asn265 is translocated into the lumen of microsomes (equivalent to the rough endoplasmic reticulum).

With this in vitro translation system, we found that the translational efficiency of frog KBP and KBP(S267G) cDNA is about 5-fold lower than GFKARα and GFKARβ cDNA. This may be due to a number of factors; for example, the translational initiation could be affected by structural features of the mRNA [19,20]. Apparently associated with this relatively low efficiency of the translation initiation site of frog KBP cDNA, a second band was consistently observed (approximately 3 kDa

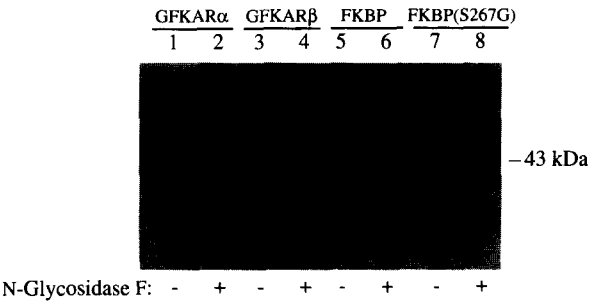

Fig. 3. Immunoblotting of kainate binding proteins expressed in HEK 293 cells. Samples (10 μl) of a resuspended transfected cell membrane pellet (the pellet of each 10 cm dish is resuspended in 0.5 ml of 1 mM PMSF), with or without *N*-glycosidase F treatment, were separated on 10% SDS-PAGE (under reducing conditions). Receptor proteins were recognized by the specific monoclonal antibody (mAb KAR-B1), and visualized by chemiluminescence.

smaller than the major 43 kDa band, see Fig. 2A, lanes 4 and 7). This second band may be the product of translational initiation at a second site, which may be an internal AUG or a non-AUG codon [21].

The most likely candidates for the additional two functional glycosylation sites left in frog KBP(S267G) were N61YT and N81WS. To test this, a cDNA fragment encoding the N-terminal 118 amino acid residues of frog KBP was subcloned into the pcDNAII vector with a stop codon introduced at the end of this DNA fragment ($N118_{(FKBP)}$). This region comprises the signal sequence (17 aa residues) and 101 amino acids of the N-terminal portion of the mature protein. In vitro translation of $N118_{(FKBP)}$ in the absence of microsomes yielded a major species of 10 kDa (Fig. 2B, lane 1). In the presence of microsomes, a major species of 15 kDa was produced (Fig. 2B, lane 2), indicating a modification by glycosylation. This 15 kDa species was converted into 9 kDa species when treated with Endo H (Fig. 2B, lane 3), confirming that it was *N*-glycosylated. The 6 kDa difference in mobility is consistent with the attachment of two core oligosaccharide chains. The Endo H deglycosylated from (9 kDa) is slightly smaller than the product (10 kDa) in the absence of microsomes (Fig. 2B, compare lane 3 vs. lane 1), indicating the N-terminal signal sequence (17 aa) was cleaved upon translocation across the membrane of microsomes. Therefore, N61YT and N81WS in the N-terminal extracellular region are functional glycosylation sites and are likely to be glycosylated in the native protein.

Kainate receptors (GFKARα, GFKARβ, frog KBP and the mutant FKBP(S267G)) were transiently expressed in HEK 293 cells. Cell membrane preparations were analyzed by immunoblotting using the monoclonal antibody KAR-B1, which was raised against frog KBP [16] and recognizes goldfish kainate receptors (GFKARα, 45 kDa, and GFKARβ, 41 kDa) as well [22]. Enzymatically deglycosylated samples (using *N*-glycosidase F) were also analyzed. The fully glycosylated form of GFKARα and GFKARβ are 45 and 41 kDa, respectively (Fig. 3, lane 1 and 3), consistent with previously determined apparent molecular mass of native kainate receptor proteins in goldfish brain [22]. Fully glycosylated frog KBP is 48 kDa (Fig. 3, lane 5), consistent with the molecular mass of the native protein in frog brain [16]. The glycosylated KBP(S267G) mutant is smaller than frog KBP, indicating that Ser-267 to Gly mutation rendered N265 non-functional and that N265 is extracellular. An increase in mobility of all four preparations was observed after treatment with *N*-glycosidase F. Deglycosylated GFKARα and GFKARβ have the same molecular mass (about 40 kDa), and deglycosylated FKBP and FKBP(S267G) mutant have the same molecular mass (about 43 kDa).

The binding of [^{3}H]kainate to the membrane preparations of HEK 293 cells transfected with frog KBP or FKBP(S267G) mutant was measured (Fig. 4). The Scatchard plots were linear for both, with a K_d of 6.6 nM for FKBP and 16 nM for FKBP(S267G) mutant. These results indicated that the S267G mutation, while eliminating the carbohydrate chain on Asn-265, did not significantly affect the proper folding and the assembly of the receptor. The slight decrease (approximately 2.5-fold) in K_d is probably due to the Ser-267 to Gly mutation *rather than* the loss of one carbohydrate chain, since in case of GluR3 and GluR6, this region is directly involved in forming the ligand binding pocket [23]. The Ser-267 to Gly mutation may have a subtle effect on the structure of the ligand binding domain. [^{3}H]Kainate binding is comformationally dependent, since denaturation (boiling 1 min) of the receptor preparation completely eliminated the [^{3}H]kainate binding activity (data not shown). Judging from both immunoblotting and the [^{3}H]kainate binding, GFKARα, GFKARβ, FKBP and the FKBP(S267G) mutant were all effectively expressed in the transfected HEK 293 cells. In each case, the maximum binding (B_{max}) is in the range of 2–5 × 10^5 sites per cell. The Ser-267 to Gly mutation did not appear affect the expression of the mutant FKBP.

4. Discussion

Asn-265 of frog KBP is a functional native *N*-glycosylation site. Therefore, the sequence surrounding Asn-265 is located extracellularly in the mature receptor protein (Fig. 5). This result is consistent with recent topological studies on several

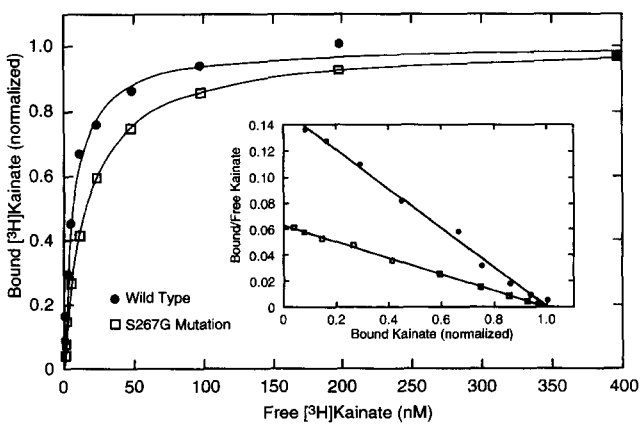

Fig. 4. Saturation binding analysis of transfected HEK 293 cell membrane preparations. Untransfected HEK 293 cells have no binding activity for [^{3}H]kainate. The curves are the average of triplicates (S.E.M. are less than 10%). The Scatchard plots derived from the saturation curves are shown in the insert. The K_d for [^{3}H]kainate binding at equilibrium is 6.6 nM for frog KBP and 16 nM for FKBP(S267G) mutant. Binding parameters were determined using a non-linear least squares fit to the data. The maximum binding (B_{max}) corresponded to approximately 2 to 5 × 10^5 sites per transfected cell for both wild and mutant frog KBPs.

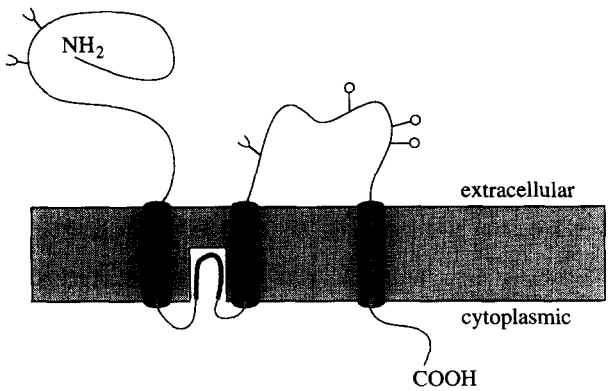

Fig. 5. Transmembrane topology (3-TM model) of kainate binding proteins. Native functional *N*-glycosylation sites in frog KBP and GFKARα are shown as Υ and Υ, respectively. The previously designated TMII region is proposed to dip into the center of the oligomeric receptor due to subunit folding and assembly in vivo.

members of the glutamate receptor family (e.g. GFKARα, GFKARβ [5,9], GluR1 [10] and GluR3 [11]). While the 4-TM and 5-TM models [2,24–26] all place this site on the cytoplasmic side, only a topology with three TMs [5,9–11] place this site on the extracellular side of the plasma membrane. The proposed 3-TM topology model raises questions concerning the reported identification of regulatory phosphorylation sites in iGluR subunits, in particular the PKA sites (Ser-648 and Ser-666, [27,28]) and Ca^{2+}/calmodulin-dependent kinase site (Ser-627, [29]) of GluR6.

The sequence of Asn-X-Ser/Thr-Y is a necessary, but not sufficient condition for the attachment of oligosaccharide chains. In order to produce a functional site, the following conditions should also be satisfied: (a) the site has to be translocated across the membrane of the rough endoplasmic reticulum (ER) at the early step of receptor biosynthesis, because oligosaccharidyl transferase is an integral membrane protein located in the lumen of ER [30,31]; (b) the consensus site has to be at least 12–14 residues away from the lumenal side of ER membrane [32]; (c) residues X and Y cannot be proline [33]. Therefore, N241QTP of frog KBP is not a functional *N*-glycosylation site because both the short distance to the proposed TMIII (only 10 residues away) and the presence of the adjacent Pro-243 on the C-terminal side of N241QT would render it nonfunctional. Generally, when the above three requirements are satisfied, a consensus *N*-glycosylation site (either native or engineered by mutagenesis) will be used in the in vitro coupled translation/membrane translocation system in the presence of microsomes. This makes the in vitro translation/translocation system a very useful tool for deducing the transmembrane topology of membrane proteins, which is a crucial step for understanding the structure-function in the absence of direct three-dimensional structure-determination.

In addition to the in vitro translation/translocation system, the *N*-glycosylation of the proteins expressed in a mammalian cell line also lends valuable information to the identification of the glycosylation site. Since N-linked oligosaccharide chains can affect protein folding [34], an engineered site (introduced without proper consideration of 3D structure of the protein) upon glycosylation could disrupt the correct folding of nascent polypeptide chain in vivo, resulting in the failure of the expression of the mutated protein or the expression of a non-functional protein in heterologous expression system (e.g. oocytes and transfected cell lines). In previous studies, native and functional glycosylation sites have been identified in the C-terminal half of the large loop between TMIII and TMIV [5,24,25], and mutations adding additional glycosylation sites to the N-terminal half of this loop have been used to indicate that it too is extracellular [5,9,10]. One caveat is that these mutations have not been demonstrated to result in a correctly folded protein. Thus, the identification of the native site, in particular Asn-265 of frog KBP, is of particular importance in testing the 3-TM model. We demonstrate here that the frog KBP has a native and functional consensus *N*-glycosylation site (Asn-265) only 35 residues from TMIII in the N-terminal portion of this loop and that, in both the glycosylated and the less glycosylated form of the protein, the binding site remains intact. The identification of native *N*-glycosylation sites of GluR6 [24,25] and frog KBP in this study also showed that members of the family of iGluRs have functional *N*-glycosylation sites in both the extracellular N-terminal and the large loop between TMIII and TMIV, which indicates that Asn-linked oligosaccharides are not necessarily restricted to a single extracellular segment as suggested by Landolt-Marticorena and Reithmeier [35].

Defining the transmembrane topology is critical for understanding the structure and its relationship to function of iGluRs. The 3-TM model has led to a drastically revised view of the evolutionary origin and the modular structural design of iGluRs [8] in two major respects. (1) The model predicts two large extracellular regions (the N-terminal region preceding TMI and that between TMIII and TMIV). Interestingly, these two regions were found to be homologous to bacterial periplasmic amino acid binding proteins [36,37]. Study of the glycine binding site of the NMDA receptor and recent domain-swapping experiments between GluR1 and GluR6 showed that these two regions together form the ligand binding pocket [23,38]. (2) We found that TMII, the region likely involved in forming the channel pore of iGluRs, is homologous to the pore-forming P-segment of various K^+ channels [8]. Therefore, iGluRs are likely to have evolved by splicing together several primordial structural modules with distinct functions. While the structural relationship between these lower molecular weight kainate binding proteins (e.g. GFKARα, GFKARβ, frog KBP and chick KBP) and subunits of AMPA and Kainate receptors is evident; thus far, ion channel activity has not been demonstrated when these KBPs were expressed in heterologous expression systems (e.g. oocytes and transfected mammalian cells). Therefore, the physiological role of this class of receptor proteins remains to be determined. With regard to this important and unsolved issue, it is of interest to note that, although GluRδ shows no ligand binding activity for various glutamatergic ligands and no ion channel activity when expressed in heterologous expression system, GluRδ has significant physiological importance as indicated by the study of transgenic mice with mutations in GluRδ [39].

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