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Topology Profile for a Glutamate Receptor: Three Transmembrane Domains and a Channel-Lining Reentrant Membrane Loop

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Summary

We investigated the transmembrane topology of the GluR3 subunit that was translated in rabbit reticulocytes supplemented with microsomal membranes. A prolactin reporter epitope was fused to GluR3 at six locations, bracketing each of the proposed transmembrane domains. The sidedness of the epitope in the microsomal membrane was then assessed by proteinase K sensitivity. The N terminus and the entire region between M3 and M4 was extracellular, and the C terminus was intracellular by this method. Four native N-linked glycosylation sites in the amino terminus and one introduced site between M3 and M4 were utilized, confirming the extracellular location of these regions. Epitopes inserted upstream and downstream of M2 were protease sensitive and thus intracellular. Our results support a topological model for glutamate receptor subunits that consists of three transmembrane domains, M1, M3, and M4, and another domain, the proposed channel-lining M2, which forms a reentrant membrane segment with both ends facing the cytoplasm.

Introduction

Glutamate receptors are formed by the union of individual subunits, each containing multiple membrane-spanning segments; similar to other ligand-gated ion channels, both the ligand recognition site and the ion channel are found in each receptor molecule. Understanding the mechanism of activation of these receptors by agonists requires a detailed knowledge of the structure of each subunit. The assignment of intracellular, extracellular, and membrane domains is a necessary step towards identifying the ligand recognition sites and is essential for elucidating the coupling mechanism between agonist binding and channel opening. Furthermore, elucidation of structural information about these receptor subunits may assist in the targeting of specific drugs to these receptors and lead to novel therapeutic treatments for stroke, epilepsy, and memory deficits (Dingledine et al., 1990; Beal, 1992; Gill, 1994).

Site-directed mutagenesis has identified a sequence upstream of the proposed first transmembrane domain M1a (Figure 1A) that appears to be important for agonist binding in N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropimate (AMPA), and kainate receptors (Uchino et al., 1992; Kuryatov et al., 1994; Stern-Bach et al., 1994). Homology mapping of the glutamate receptors onto the crystal structure of bacterial amino acid–binding proteins and chimeric receptor expression suggest that agonist recognition in GluR3 and GluR6 receptors requires portions of both the large N terminus and the region between M3 and M4 (O'Hara et al., 1993; Stern-Bach et al., 1994). These findings are consistent with an extracellular orientation of the N terminus and the region between M3 and M4; however, it is clear from the β-adrenergic receptors that part of the ligand-binding site can be buried within a membrane-spanning domain (Strader et al., 1987).

Two topological maps were originally proposed for the glutamate receptors based on hydrophobicity analysis and by analogy to the well defined topology of the nicotinic receptor (Figure 1B). Each contained four transmembrane segments, positioned the N terminus and the C terminus on the extracellular surface, and contained a large intracellular loop between the third and fourth transmembrane domains, but differed in the assignment of the first and second putative transmembrane domains, labeled 1a and 1b in Figures 1A–1C (Hollmann et al., 1989; Keinänen et al., 1990; Gasic and Hollmann, 1992). The four transmembrane model was challenged recently by results from the analysis of endogenous glycosylation and phosphorylation sites in the kainate receptor subunit GluR6 and the NMDA receptor subunit NR1, shown schematically in Figure 1A (Raymond et al., 1993; Tingley et al., 1993; Wang et al., 1993; Roche et al., 1994; Taverna et al., 1994). These results suggested an additional transmembrane domain, M3′, between the original third and fourth transmembrane segments, which positions the carboxyl terminus on the intracellular surface (Figure 1C; Tingley et al., 1993; Roche et al., 1994; Taverna et al., 1994).

Studies of the voltage-dependent block of these receptor channels provide indirect topological information. A single amino acid site, occupied by glutamine (Q) or arginine (R) located in the M2 domain of the AMPA and kainate receptors, controls not only divalent ion permeation (Hume et al., 1991; Burnashev et al., 1992a) but also the degree of voltage- and use-dependent block of AMPA receptor channels by polyamine spider toxins (Blaschke et al., 1993; Herlitze et al., 1993; Washburn and R. D., unpublished data). Similarly, the homologous site in the NMDA receptors contains an asparagine (N) residue that was shown by site-directed mutagenesis to control the degree of voltage-dependent Mg^{2+} block of these receptors (Burnashev et al., 1992b; Mori et al., 1992; Kawajiri and Dingledine, 1993; Sakurada et al., 1993). The high degree of voltage dependence for Mg^{2+} block of native or recombinant NMDA receptors predicted that the Mg^{2+} blocking site was located near the intracellular surface of the channel (Ascher and Nowak, 1988; Jahr and Stevens, 1990; Kawajiri and Dingledine, 1993; Zarei and Dani, 1994). Based on these functional results, the so-called Q/R/N site (asterisks in Figures 1A–1C) of glutamate receptors was proposed

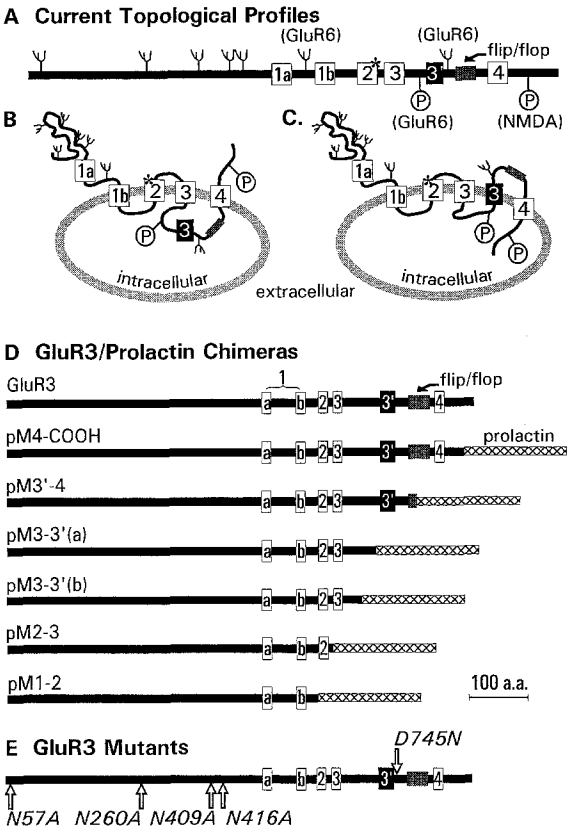

Figure 1. Current GluR3 Topology Profiles and the GluR3-Prolactin Chimeras and GluR3 Mutants Used to Test Them

(A) Linear representation of the position of topologically sensitive native sites throughout the glutamate receptor family. In this and subsequent portions of the figure, glycosylation sites are indicated by "trees" and phosphorylation sites by P; the asterisk denotes the position of the Q/R/N site, the small, darkly hatched box denotes the flip/flop locus, and the larger white or black boxes represent putative membrane domains. The box labeled 1a denotes the M1 domain proposed by Hollmann et al. (1989), and the box labeled 1b denotes the M1 domain proposed by Keinenän et al. (1990). The box labeled 3' represents the proposed additional transmembrane domain (Roche et al., 1994; Taverna et al., 1994).

(B) Schematic representation of the four-transmembrane topological model (Keinenän et al., 1990) and the orientation of the topologically sensitive sites.

(C) Schematic representation of the five transmembrane topological model (Roche et al., 1994; Taverna et al., 1994) and the orientation of topologically sensitive sites.

(D) GluR3-prolactin chimeras used in the epitope protection assay to test the current glutamate receptor topological models. A 195 amino acid prolactin reporter epitope was fused in frame to GluR3 at the positions indicated.

(E) Schematic of GluR3 glycosylation mutants. Upward arrows indicate N-linked glycosylation sites that were removed by mutation. Downward arrow indicates the insertion of a new N-linked glycosylation site in GluR3.

to reside near the cytoplasmic mouth of the channel (Dingledine et al., 1992), contrary to the original topological models.

All of the above studies indicate that in the glutamate receptors, hydropathy analysis may be misleading regarding transmembrane topology just as it is for voltage-dependent K^+ channels (MacKinnon and Yellen, 1990; Yellen et al., 1991) and the p-glycoprotein transporter (Skach et al., 1993; Skach and Lingappa, 1994). In the present study, we investigated glutamate receptor topology by two independent biochemical approaches. The most direct method, which has been used successfully for topological mapping of a variety of polytopic membrane proteins (Chavez and Hall, 1992; Skach and Lingappa, 1993, 1994; Skach et al., 1993, 1994; De Fea et al., 1994), analyzes the protease sensitivity of a topologically neutral reporter epitope strategically placed to bracket the proposed transmembrane domains. This approach was complemented by determination of the utilization of endogenous N-linked glycosylation sites and an additional glycosylation site engineered at a position analogous to a native glycosylation site in the kainate receptor subunit GluR6. Our results with the AMPA receptor subunit GluR3 demonstrate the intracellular orientation of the C-terminal region and the extracellular location of the N-terminal region, and they show that the entire region between the third and fourth transmembrane domains is extracellular. Most importantly, our results directly demonstrate that M2 does not transverse the membrane but may instead form a reentrant domain. These results suggest a novel topological profile for GluR3 that helps to explain some of the functional properties of the glutamate receptors.

Results

Large N-Terminal Domain Is Extracellular

Our goals were to identify which of the previously proposed transmembrane regions actually span the membrane and to determine the cellular orientation of membrane flanking regions. We determined the amino-terminal orientation of the AMPA receptor subunit GluR3 by analyzing the glycosylation of native N-linked glycosylation sites scattered throughout this region (tree-like structures in Figure 1A). Lane 1 of Figure 2A shows that the predominant translation product of GluR3 mRNA in reticulocyte lysate had a molecular weight of 99.5 kDa (closed arrowhead). When translated in the presence of microsomal membranes, protein size increased to 109.5 kDa (Figure 2A, open arrow in lane 2), which is similar to the molecular weight of the GluR3 protein identified in brain (Hampson et al., 1992). When GluR3 protein translated with microsomes was treated with endoglycosidase H to remove N-linked sugars, its molecular weight was reduced to that in the absence of microsomes (Figure 2A, lanes 3 and 4). This demonstrates that the shift up in molecular weight was entirely due to N-linked glycosylation. The absence of an appreciable smear or multiple bands between the unglycosylated and the glycosylated bands indicates that the protein was either fully glycosylated or fully unglycosylated. To determine which, if any, of the amino-terminal consensus sites for N-linked glycosylation were utilized, four of the five sites were individually mutated to disrupt the consensus sequence (see Figure 1E). Each mutation resulted in a protein product that, when translated in the presence of microsomes, migrated at a lower molecular weight than wild-type GluR3 (Figure 2B); the unglycosylated proteins,

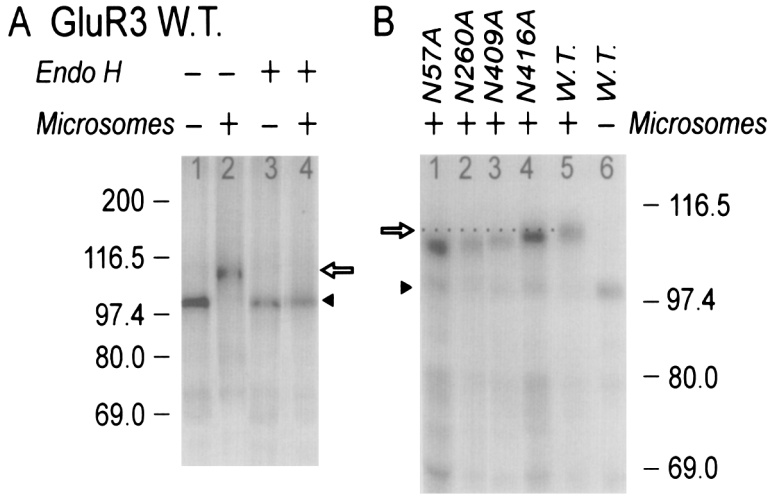

Figure 2. Glycosylation of In Vitro–Translated Native and Mutant GluR3 Subunits

(A) GluR3 was translated in the presence or absence of microsomal membranes, and proteins were separated by SDS-PAGE on an 8% acrylamide gel run at 250 V for 2 hr at room temperature. An increase in molecular weight of 10 kDa was observed when mRNA was translated with microsomes (lanes 1 and 2). The closed arrowhead indicates the position of the unglycosylated protein, whereas the open arrow points to the glycosylated species. If the translation product was treated with endoglycosidase H, its molecular weight was shifted downward to the level of the unglycosylated protein (lanes 3 and 1). Endoglycosidase H did not reduce the size of GluR3 in the absence of microsomes (lanes 4 and 1).

(B) Four of five N-linked glycosylation sites located in the N terminus of GluR3 were individually destroyed by changing asparagine to alanine. Each mutant was translated in the presence of microsomes and proteins were separated by SDS-PAGE on an 8% acrylamide gel run at 70 mA for 4.5 hr at 4°C. The molecular weight of the glycosylated form decreased in each mutant as compared with wild-type GluR3. No individual mutation resulted in a decrease to the level of the unglycosylated form. GluR3(N57A) decreased by approximately 3.0 kDa, N260A by 2.5 kDa, N409A by 1.5 kDa, and N416A by 0.5 kDa, which together account for 7.5 kDa of the 10 kDa total weight produced by glycosylation.

indicated by a closed arrowhead, showed no difference in molecular weight. Therefore, the amino terminus through amino acid 416 is extracellular.

C Terminus Is Intracellular

The principal method we employed for the topological mapping of GluR3 used a reporter of translocation derived from the secretory protein prolactin, in which the N-terminal third of the prolactin was removed to eliminate endogenous topogenic elements (Rothman et al., 1988). This reporter was fused in frame to the GluR3 subunit at positions bracketing the proposed transmembrane regions (see Figure 1D), and the chimeras were translated in vitro with microsomes. Translation in the presence of microsomal membranes results in a glycosylated protein product threaded through the microsomal vesicles in a topological orientation inverted from that in the plasma membrane. Subsequent treatment with proteinase K digests only protein segments located extramicrosomally (Perara and Lingappa, 1985). The reporter epitope protection assay allows one to determine which protein segments actually transverse the membrane and the cellular orientation of the regions between the membrane segments.

Our major concern with this method was that the large prolactin epitope, 195 amino acids, might interfere with the normal threading of the nascent polypeptide chain through the membrane. We addressed this issue in part by comparing the functional properties of the C-terminal GluR3/prolactin chimera pM4-COOH with those of wild-type GluR3 (Figure 3). When expressed in Xenopus oocytes, the construct pM4-COOH (see Figure 1D) responded to the agonist kainate (Figure 3A, inset). The kainate dose-response relationship was the same for GluR3 and pM4-COOH (Figure 3A). Similarly, the pM4-COOH chimeric receptors demonstrated the same inward rectification as wild-type GluR3 receptors (Figure 3B). However, the magnitude of the response to 300–1000 μM kainate for the prolactin-containing construct was 10%–30% of the wild-type response when approximately equal molar amounts of the two mRNAs were injected (Figure 3B, inset). The difference in maximal responses may be due to prolactin partially blocking ion flow through the channel or to decreased receptor assembly. Nevertheless, our results show that the insertion of the prolactin epitope into the carboxyl terminus of GluR3 did not alter the agonist recogition or ion permeation properties and therefore did not interfere with the translocation of upstream protein regions through the membrane. Chimera pM4-COOH was the only construct studied functionally because it was the only one in which a putative transmembrane segment had not been deleted.

The prolactin epitope in the construct pM4-COOH was positioned within the region of GluR3 homologous to the C-terminal region of the NMDA receptor subunit NR1 that contains several protein kinase C phosphorylation sites (Tingley et al., 1993). This epitope was highly sensitive to proteinase K following translation in the rabbit reticulocyte system supplemented with canine pancreatic microsomes (Figure 4A, lanes 2 and 4). This finding indicates that the C terminus of GluR3 is extramicrosomal, which corresponds to an intracellular location in the plasma membrane. To ensure that degradation of the epitope was not due to access of proteinase K to intramicrosomal protein segments, an antibody that recognizes the amino terminus of GluR3 was used to immunoprecipitate the proteolysis product. The amino terminus of pM4-COOH was protected from proteolysis under nondetergent conditions (Figure 4B, lanes 2 and 4), but was digested in the presence of detergent (Figure 4B, lane 6), which validates the extramicrosomal location of the C terminus. These results also confirm the extracellular location of the amino terminus of GluR3.

The sizes of the primary immunoprecipitation products

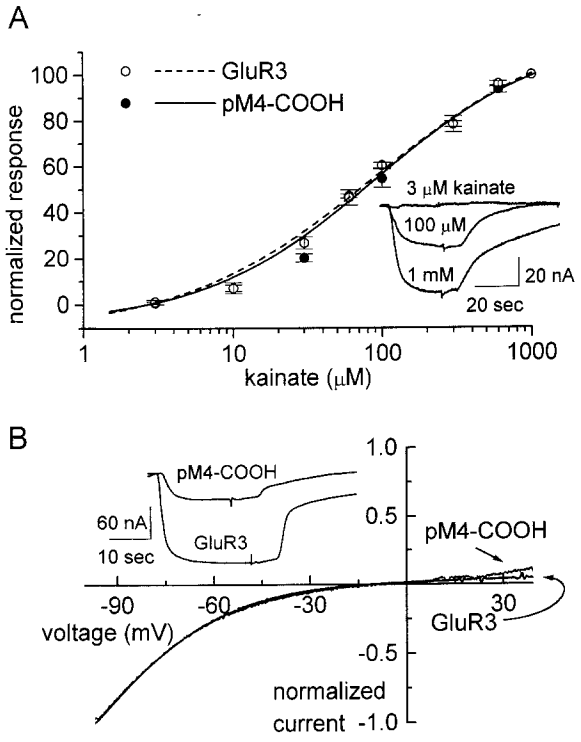

Figure 3. Kainate Dose–Response and I–V Curves for GluR3 and the C-terminal Chimera pM4-COOH

Xenopus oocytes were injected with equal molar amounts of either wild-type GluR3 or pM4-COOH mRNA.

(A) Dose response curves to kainate for GluR3 and for pM4-COOH were obtained at −100 mV. Responses were normalized to the 1 mM kainate response. Each point represents the mean and standard error of measurements from three oocytes. The insert shows responses of pM4-COOH to three kainate applications.

(B) Averaged current–voltage (I–V) relationships for GluR3 (n = 4) and the C-terminal chimera pM4-COOH (n = 3). I–V currents were normalized to the kainate current of each cell at −100 mV. The insert shows a typical response to 300 μM kainate for GluR3 and pM4-COOH.

isolated by the amino-terminal antibody following proteolysis were approximately 101.5 kDa and 73.5 kDa (Figure 4B, asterisks in lane 4). The lower band migrates as expected for a glycosylated protein segment consisting of the amino-terminal region through M1. The upper band corresponds to the predicted size of a glycosylated protein extending from the amino terminus through M4, lacking only the protein region from M4 through the prolactin. This finding suggests that the short peptide loops between the M1 and M3 domains may not be readily accessible to proteinase K. Our findings demonstrate that the C terminus of the AMPA receptor subunit GluR3 is intracellular, which contradicts the original topological profiles but agrees with the proposed intracellular orientation of the C terminus of the NMDA receptor (Tingley et al., 1993).

M2 Is a Reentrant Loop Facing the Cytoplasm

The orientation of the second membrane domain is of particular interest because site-directed mutagenesis combined with functional expression suggests that at least one amino acid in this region, the Q/R/N site, faces the open channel. Constructs pM1-2 and pM2-3 (see Figure 1D) were designed to assess the orientation of M2. The prolactin epitope was positioned in each construct at the furthest downstream site before the next transmembrane region to ensure that the minimum number of residues within the loops between membrane domains were deleted (Figure 5C). This was of concern, since the number of positively charged amino acid residues within a region might influence the cellular orientation of that region (Parks and Lamb, 1993; Gafvelin and von Heijne, 1994). The prolactin epitope in constructs pM1-2 and pM2-3 was susceptible to proteinase K in the absence of detergent following translation in the presence of microsomes (Figure 5A, lanes 2 and 5). The N-terminal region remained intact under the same conditions (Figure 5B, lanes 2 and 5), indicating that the protease had not degraded protein segments located within the microsome. The 22 kDa downward shift in the molecular weight of the protein product immunoprecipitated by the N-terminal antibody is consistent with the loss of the extramicrosomal prolactin epitope due to proteolysis (Figure 5B, lanes 1, 2, 4, and 5). The protease sensitivity of the prolactin epitope in both pM1-2 and pM2-3 indicates that the protein regions preceding and following M2 are both oriented towards the intracellular surface; these results are consistent with M2 forming a reentrant membrane domain (Figure 5D).

Extracellular Orientation of the Flip/Flop Region

Constructs pM3-3′(b), pM3-3′(a), and pM3′-4 (see Figure 1D) were designed to bracket the additional transmembrane domain, M3′, (see Figure 1A) proposed by Roche et al. (1994) and Taverna et al. (1994). The prolactin reporter epitope in construct pM3′-4 was positioned in the middle of the flip/flop region to test the orientation of this region known to be involved in AMPA receptor desensitization (Sommer et al., 1990) and its modulation by cyclothiazide (Partin et al., 1993). It is unclear precisely where M3′ might be located, as hydrophobicity analysis predicts no potential membrane-spanning regions. Since this transmembrane domain was initially proposed to lie between the protein kinase A (PKA) phosphorylation site and the glycosylation site in GluR6, the reporter in construct pM3-3′(a) was placed at the homologous position in GluR3 to the PKA phosphorylation site in GluR6 (Raymond et al., 1993; Wang et al., 1993). A second construct, pM3-3′(b), was made in which the epitope was placed 28 amino acids upstream to ensure that it did not fall in the middle of the actual transmembrane region. The prolactin epitope in all three chimeras was protected from proteolysis following translation in the presence of microsomes (Figure 6A, lanes 2, 5, and 8). The epitope was destroyed when proteolysis was carried out in the presence of detergent (Figure 6A, lanes 3, 6, and 9), indicating that protection was not due to an inherent protease resistance of these constructs. The unglycosylated proteins, indicated by the closed arrowheads (Figure 6A, lanes 2, 5, and 8), were rapidly degraded by proteinase K, demonstrating that extramicrosomal proteins were fully digested.

It is clear from Figure 6A that the protease protected fragments from constructs pM3′-4, pM3-3′(a), and pM3-

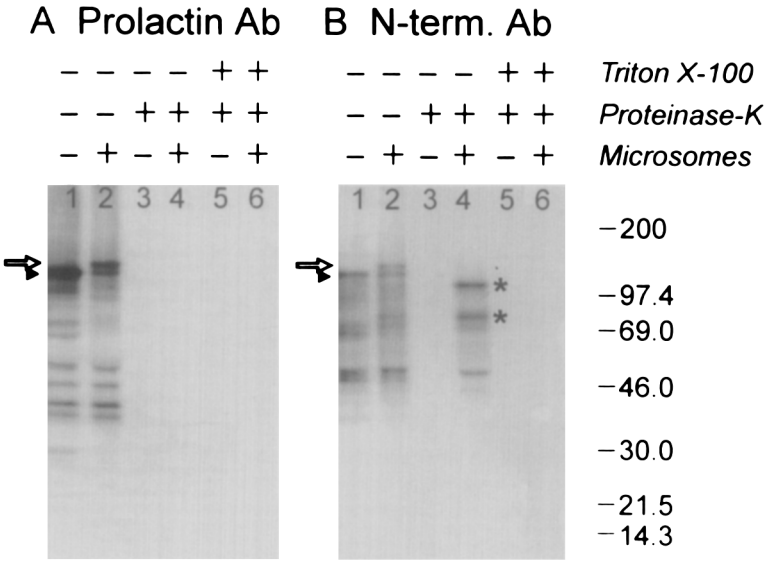

Figure 4. Proteolysis and Immunoprecipitation of the C-Terminal GluR3-Prolactin Chimera pM4-COOH

The chimera was translated in the presence or absence of microsomes, then either sham treated, proteinase K treated, or treated with proteinase K plus Triton X-100, as indicated above the lanes. Digestion was arrested after 60 min, and the product immunoprecipitated with either a prolactin antibody or a glutamate receptor N-terminal antibody and analyzed by SDS-PAGE on a 5%–15% acrylamide gel at 250 V for 2 hr.

(A) The prolactin antibody immunoprecipitated a protein product of correct molecular weight. The closed arrowhead indicates the position of the unglycosylated protein, whereas the open arrow points to the glycosylated species. The prolactin epitope of pM4-COOH was highly sensitive to proteinase K under nondetergent conditions following translation in the presence of microsomes (lane 4).

(B) The N-terminal antibody immunoprecipitated the same glycosylated and unglycosylated proteins as the prolactin antibody. Following proteinase K, the N-terminal antibody immunoprecipitated a 101.5 kDa and 73.5 kDa protein (asterisks in lane 4) when translated in the presence of microsomes. No protein was immunoprecipitated following proteinase K plus Triton X-100 (lane 6) or following proteinase K treatment after translation in the absence of microsomes (lanes 3 and 6).

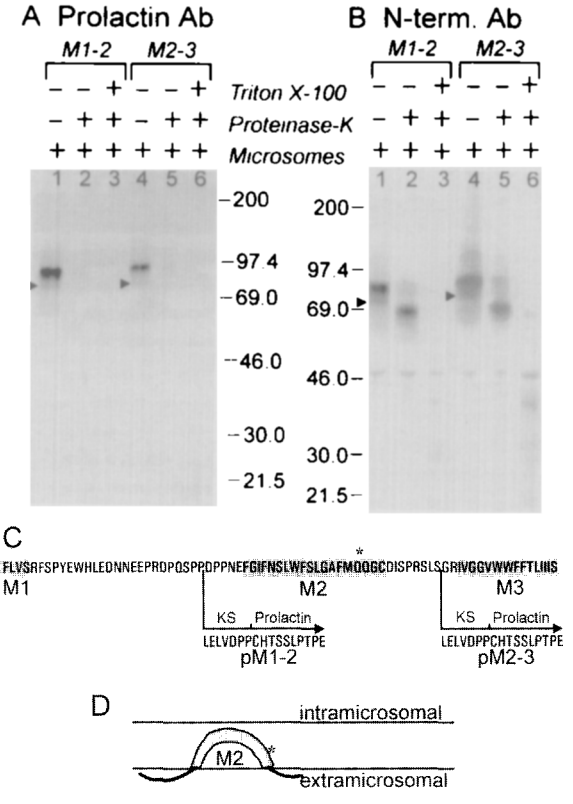

Figure 5. Proteolysis and Immunoprecipitation of pM1-2 and pM2-3 Bracketing the Proposed M2 Domain

Protein chimeras were translated in the presence of microsomes then either sham treated, proteinase K treated, or treated with proteinase K plus Triton X-100. Digestion was arrested after 60 min. The product was immunoprecipitated with either the prolactin antibody (A) or glutamate receptor N-terminal antibody (B) and analyzed by SDS-PAGE on a 5%–15% acrylamide gel run at 250 V for 2 hr. The closed arrowhead indicates the unglycosylated protein.

(A) The prolactin epitope in both chimeras was susceptible to protease under nondetergent conditions (lanes 2 and 5).

(B) The N-terminal region was reduced in size under the same conditions (lanes 2 and 5) but was destroyed following proteinase K plus Triton X-100 (lanes 3 and 6).

(C) Amino acid sequence for GluR3 showing the prolactin sequence and insertion positions for chimeras pM1-2 and pM2-3. Shaded boxes denote proposed membrane domains, and the asterisk denotes the Q/R site.

(D) Schematic representation of the microsomal orientation of M2 as determined by epitope protection. The asterisk denotes the Q/R site.

3′(b) are the same size as the full-length proteins. If the M2 domain and flanking regions had been accessible to proteinase K, these bands would have been approximately 38 kDa, 27 kDa, and 23 kDa, respectively, which correspond in size to the prolactin reporter up to M2. Immunoprecipitation of the amino terminus following proteinase K treatment also resulted in protein products of identical molecular weight to the full-length products (data not shown). The N-terminal antibody would have precipitated a fragment of approximately 73 kDa had the region between M1 and M2 been accessible to proteinase K. These results reinforce the conclusion from the pM4-COOH construct that much of the M2 region and its flanking sequences must reside within the membrane or be closely associated with it. The combined results from pM3′-4 and pM4-COOH indicate that the proposed M4 is indeed a transmembrane region, crossing from the extracellular to the intracellular surface. The protease resistance of pM3-3′(a), pM3-3′(b), and pM3′-4 demonstrate that the entire region of GluR3 between M3 and M4 is intramicrosomal, corresponding to an extracellular location in the plasma membrane. These results are consistent with an extracel-

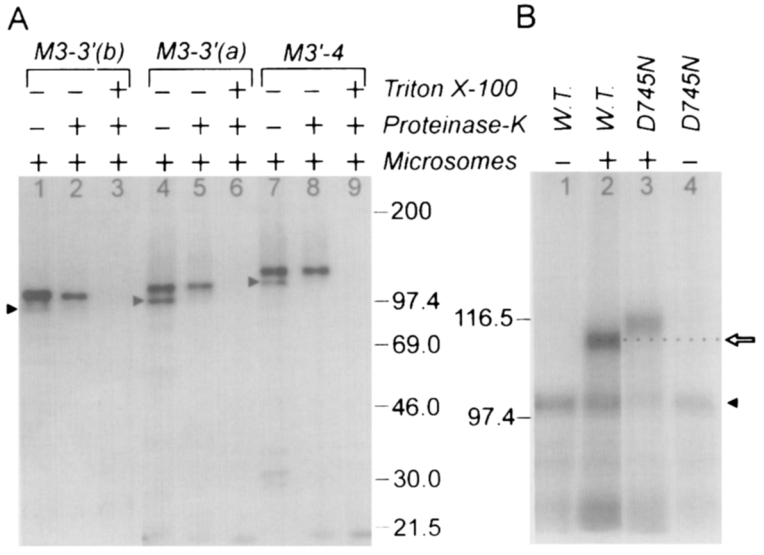

Figure 6. Proteolysis and Immunoprecipitation of Prolactin Chimeras between M3 and M4

(A) Chimeras pM3-3′(a), pM3-3′(b), and pM3′-4 were translated in the presence of microsomes, then either sham treated, proteinase K treated, or treated with proteinase K plus Triton X-100. Digestion was arrested after 60 min, and the product immunoprecipitated with a prolactin antibody and analyzed by SDS-PAGE on a 5%–15% acrylamide gel run at 250 V for 2 hr. The closed arrowhead indicates the position of the unglycosylated protein in each lane. The prolactin epitope in all three constructs was protected from proteolysis but was destroyed when proteolysis was carried out in the presence of Triton X-100 (lanes 3, 6, and 9).

(B) An N-linked glycosylation site was engineered within this same region resulting in GluR3 mutant D745N. When translated in the presence of microsomes, there was a shift up in molecular weight as compared with wild type (lanes 2 and 3), but there was no difference when translated in the absence of microsomes (lanes 1 and 4).

lular location for the flip/flop region, the cysteine pair involved in redox regulation of the glutamate receptors (Sullivan et al., 1994), and part of the ligand-binding domain of GluR3 and GluR6 (Stern-Bach et al., 1994) but are inconsistent with the existence of an additional transmembrane domain in GluR3 as proposed for GluR6 (Roche et al., 1994; Taverna et al., 1994). The protease sensitivity of the prolactin epitope in pM2-3 (see Figure 5A), but not in pM3-3′(a) or pM3-3′(b) (Figure 6A), indicates that M3 is a transmembrane region that crosses from the intracellular to the extracellular surface.

To provide an independent test of the orientation of the flip/flop region, we engineered an N-linked glycosylation site at amino acid position 745 (see Figure 1E), corresponding to a utilized glycosylation site in GluR6 (Roche et al., 1994; Taverna et al., 1994). This engineered site was indeed glycosylated as demonstrated by the increase in molecular weight as compared with wild-type GluR3 when translated in the presence of microsomes (Figure 6B, lanes 2 and 3). This supports the previous conclusion based on our epitope protection assay that this region is extracellular in GluR3.

Discussion

Approaches to Transmembrane Topology Mapping

Our results support a topological model for GluR3 that includes three transmembrane segments (M1, M3, and M4) plus a reentrant membrane domain (M2; Figure 7). This conclusion is at variance with most previous topology maps of glutamate receptors (Hollmann et al., 1989; Keinänen et al., 1990; Dingledine et al., 1992; Gasic and Hollmann, 1992; Molnar et al., 1993, 1994; Tingley et al., 1993; Roche et al., 1994; Taverna et al., 1994), but agrees with predictions of two recent studies (Wo and Oswald, 1994; Hollmann et al., 1994). Methodological limitations of the different approaches used may explain the differences. In the absence of more direct information provided by X-ray crystallography (Deisenhofer et al., 1985; Weiss et al., 1990; Kreusch et al., 1991) or averaging of two-dimensional high resolution electron micrographs (Henderson and Unwin, 1975; Henderson et al., 1990), the transmembrane topology of polytopic proteins must be inferred from the sidedness of identified protein regions. Three biochemical strategies have been employed for studying ligand-gated ion channels; each has its own advantages and limitations that must be appreciated to evaluate the resulting topological maps.

A commonly used method is immunocytochemical localization of peptide epitopes at the light or electron microscopic level (Criado et al., 1985a, 1985b; Young et al., 1985; Ratnam et al., 1986a, 1986b; Pederson et al., 1990; Molnar et al., 1993, 1994). This method allows topological mapping to be carried out without modifying the amino acid sequence of the target protein, but two concerns limit its usefulness. First, extracellular epitopes that are buried cannot be distinguished unambiguously from intracellular epitopes because detergents used to permeabilize the membrane may unmask buried epitopes. Second, it has proven difficult in practice to verify antibody selectivity under the conditions needed for immunocytochemistry. This could be done, in principle, by identifying the recognition sites of the antibodies and then genetically modifying the epitope sequences to demonstrate antibody selectivity. In practice, however, Western blots of denatured proteins are used instead. Antibodies have often been used for immunocytochemistry at concentrations much higher than those determined for antigenic specificity using Western blots. Early studies of nicotinic receptor topology epitomize these problems. The initial four-transmembrane model was challenged by immunocytochmical studies, which proposed a five- (Young et al., 1985) or a seven-transmembrane model (Criado et al., 1985a, 1985b; Ratnam et al., 1986a, 1986b; Pederson et al., 1990). These models were later found by more direct biochemical meth-

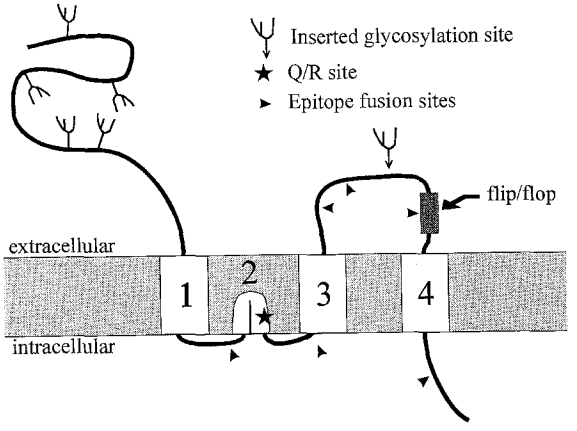

Figure 7. Proposed GluR3 Topology in the Plasma Membrane

Tree-like structures denote N-linked glycosylation sites, the tree atop an arrow denotes the glycosylation insertion site at position 745, the arrowheads denotes the approximate position of prolactin epitope fusion sites, the star denotes the Q/R site, and the hatched box denotes the flip/flop locus.

ods to be in error (McCrea et al., 1987; DiPaola et al., 1989; Chavez and Hall, 1991, 1992).

A more secure approach is to identify posttranslationally modified amino acid residues, usually N-linked glycosylation and phosphorylation sites, that have an expected membrane sidedness. This approach has been used for the glutamate receptors (McGlade-McCulloh et al., 1993; Moss et al., 1993; Raymond et al., 1993; Wang et al., 1993; Roche et al., 1994; Tan et al., 1994; Taverna et al., 1994; Hollmann et al., 1994). This method is most conservatively used to assess topology of endogenous sites in their native cellular environment. Glycosylation or phosphorylation sites engineered throughout the protein to obtain a more complete picture of the topological profile have the potential to alter topology. Moreover, although N-linked glycosylation typically occurs on extracellularly targeted domains, and kinases act on cytoplasmic sites, there may be exceptions. N-linked glycosylation of a cytoplasmic domain on the α-subunit of Na/K ATPase has been reported (Pedemonte et al., 1990), and PKA secreted by activated platelets is reported to phosphorylate an extracellular site on vitronectin (Korc-Grodzicki et al., 1988, 1994; Shaltiel et al., 1993). These examples limit the reliability of approaches relying solely on glycosylation or phosphorylation.

We have depended primarily on an epitope protection assay (Chavez and Hall, 1992; Skach and Lingappa, 1993, 1994; Skach et al., 1993, 1994; De Fea et al., 1994). In our assay, a well defined prolactin epitope was fused to GluR3, and the protease sensitivity of the epitope was determined when the chimera was expressed in microsomal vesicles. Intramicrosomal epitopes (topologically extracellular) are protected from protease, whereas extramicrosomal epitopes (topologically cytoplasmic) are rapidly digested. In this method, the detection antibody is used under exactly the same conditions used to determine its antigenic specificity; buried epitopes are of no concern because antibody–antigen recognition is carried out on fully denatured proteins. The major assumptions with our use of this approach are, first, that topogenesis in isolated microsomes is identical to that in endoplasmic reticulum in situ and, second, that transmembrane topology of the GluR3 protein is not altered by truncation and fusion of the large prolactin epitope. In response to the first issue, the membrane orientation of a variety of proteins translated in microsomes was indistinguishable from that of the proteins synthesized in cells (Blobel and Dobberstein, 1975; Katz et al., 1977; Anderson and Blobel, 1981; Goldman and Blobel, 1981; Braell and Lodish, 1982). Moreover, the epitope protection assay of p-glycoprotein–prolactin chimeras expressed either in isolated canine pancreatic microsomes or the endoplasmic reticulum of intact Xenopus oocytes produced identical results (Skach and Lingappa, 1993; Skach et al., 1993, 1994; De Fea et al., 1994), as expected from our understanding of topogenesis of membrane proteins (Blobel, 1980; Walter and Lingappa, 1984). Regarding the second issue, gene fusion systems have been used extensively for mapping the transmembrane topology of Escherichia coli proteins with considerable success. A similar approach to ours is used in which a reporter molecule, such as alkaline phosphatase, β-lactamase, β-galactosidase, or an antigenic tag, each lacking its signal sequence, is fused to carboxy-terminally truncated versions of the membrane protein (Hennessey and Broome-Smith, 1993). Since the factors involved in signal-sequence recognition, membrane targeting, and translocation are very similar in E. coli and eukaryotes (Dobberstein, 1994), the eukaryotic system should handle the gene-fusion products equally well as E. coli. Furthermore, our truncated prolactin epitope has been found to be topologically neutral when fused to various topogenic sequences (Rothman et al., 1988). The most cogent example is that of the α and δ nicotinic receptor subunits, in which the pattern of proteolysis following translation in microsomes was not altered by fusion of a prolactin epitope to the receptor subunit (compare Anderson et al., 1983 with Chavez and Hall, 1992). Additionally, the prolactin epitope fused in frame to the region downstream of M4 in GluR3 resulted in a functional ion channel (see Figure 3). This demonstrates that the large prolactin sequence did not alter the transmembrane topology of upstream protein segments in this chimera. In all other constructs, however, predicted membrane domains had been deleted, and the topologic neutrality of the prolactin epitope could not be tested. Given the limitations of each of the methods described here, the most compelling conclusions would be those supported by convergent results of independent methods. Our results with native and engineered N-linked glycosylation sites in all cases confirmed the topological assignments from the epitope protection assay.

GluR3 Topology: Relation to Other Glutamate Receptor Models

Much of the available topology data suggests that the entire glutamate receptor family may share a similar profile, as was originally predicted based on hydropathy plots.

Indeed, the intracellular C terminus of GluR3 (see Figure 4) and GluR1 (Molnar et al., 1993, 1994) accords with predictions for the NMDA receptor subunit NR1 based on phosphorylation studies (Tingley et al., 1993). At least part of the M3–M4 loop is extracellular in the kainate receptor subunit GluR6 (Roche et al., 1994; Taverna et al., 1994), as is the whole region in GluR3 (see Figure 6).

The protease sensitivity of constructs pM1-2 and pM2-3 (see Figure 5) indicates that M2 is not a transmembrane domain and that both ends of M2 are intracellular. Because the entire region between M1 and M3 is resistant to proteolysis (see Figure 5B and Figure 6A), this sequence must be closely associated with the membrane. Functional evaluation of M2 mutations suggests that at least the Q/R/N site of M2 (asterisk in Figure 7) may be located within a channel-lining membrane segment. First, the size of the amino acid side chain at this position influences ionic selectivity of the GluR3 channel (see Figure 5 in Dingledine et al., 1992), as expected if this site contributes to the narrow part of the open channel. Second, the voltage- and use-dependent block of the GluR3 ion channel by polyamine spider toxins can be abolished by mutations at this site (Blaschke et al., 1993; Herlitze et al., 1993; Washburn and R. D., unpublished data), as can the voltage-dependent Mg^{2+} block of NMDA receptors (Burnashev et al., 1992b; Mori et al., 1992; Kawajiri and Dingledine, 1993; Sakurada et al., 1993). Our model positions the Q/R/N site near the cytoplasmic surface of the channel in agreement with the Woodhull prediction for the Mg^{2+} blocking site of NMDA receptors (Ascher and Nowak, 1988; Jahr and Stevens, 1990).

It is worth noting that the amino acid sequence of the M2 region shows limited homology to the channel-lining P domain of the voltage-gated K^+ channels (A. VanDongen, personal communication). The P domain is thought to be a reentrant membrane segment that spans the entire pore (MacKinnon and Yellen, 1990; Yellen et al., 1991). However, as our results clearly demonstrate, homology mapping, mutagenesis, and functional evaluation provide only indirect evidence regarding membrane topology. It is clear that more direct approaches are needed to determine how deep within the membrane M2 actually penetrates and which residues contribute to the channel wall.

The entire region between M3 and M4 of GluR3 is extracellular, based on epitope protection in three prolactin chimeras blanketing this region and the utilization of an N-linked glycosylation site inserted here. The extracellular orientation of the flip/flop region is consistent with its involvement in the block of desensitization by the polar compound cyclothiazide (Partin et al., 1993). The redox-sensitive cysteine pair, identified in AMPA and NMDA receptors (Sullivan et al., 1994), would also be extracellular in this model. An extracellular loop between M3 and M4 accords with the involvement of this region in agonist recognition (Stern-Bach et al., 1994) and is in complete agreement with glycosylation patterns of GluR1 mutants (Hollmann et al., 1994).

A different conclusion has been reached by Raymond et al. (1993) and Wang et al. (1993), who reported an intracellular PKA phosphorylation site between M3 and M4 in the kainate receptor subunit GluR6, although the amino acid residues phosphorylated were not positively identified. This apparent discrepency between the AMPA and kainate receptor topologies has not been resolved. It might be explained if the phosphorylation site in GluR6 is extracellular (cf. Korc-Grodzicki et al., 1988, 1994; Shaltiel et al., 1993) or if glutamate receptor topology is dynamic, like that of colicin, a bacterial membrane channel protein that upon activation translocates a 31 amino acid segment from the extracellular to the intracellular surface (Slatin et al., 1994). Alternatively, although there is sequence homology across the entire receptor family in addition to nearly identical hydropathy profiles, the topology of the three glutamate receptor classes may not be the same. A more thorough investigation of the topological profiles of the kainate and NMDA receptors is needed to address this possibility.

Ligand-Gated Ion Channel Superfamily

Our study allows for the direct comparison of the transmembrane topologies of the glutamate receptor subunit GluR3 and the α and δ subunits of the nicotinic receptor (Chavez and Hall, 1992) since identical methods were employed. The nicotinic receptor contains four transmembrane domains, extracellular N and C termini, and a large intracellular loop between M3 and M4. Despite similarities in hydropathy plots of glutamate and nicotinic receptors, there are distinct differences in their transmembrane organization. This finding questions the concept of a single ligand-gated ion channel superfamily in which all members have the same transmembrane topology. However, there appear to be some structural similarities between the two classes of receptors beyond the level of topological organization. Both the nicotinic receptors (McCrea et al., 1987; DiPaola et al., 1989; Chavez and Hall, 1992; Anand et al., 1993) and the AMPA receptors likely have four membrane domains. A single amino acid residue located in M2 near the cytoplasmic surface of the nicotinic receptor α subunit was proposed to play a prominent role in forming the ion selectivity filter, based largely on the effect of side chain volume on ion permeation (Villarroel et al., 1991). Similar experiments on GluR3 indicate a comparable role for the Q/R site (Dingledine et al., 1992). Thus, the overall architecture of both groups of ligand-gated ion channels may be similar.

Experimental Procedures

DNA-modifying enzymes and endoglycosidase H were obtained from New England Biolabs, [^{35}S]methionine from Amersham, Taq DNA polymerase, RNasin, rabbit reticulocyte lysate, and canine pancreatic microsomes from Promega, and proteinase K from Fisher Scientific. Rabbit polyclonal antibody to bovine prolactin was purchased from USB, rabbit polyclonal antisera to the glutamate receptor N-terminal region (numbers 290 and 295) were generously provided by S. Rogers (University of Utah), and protein A sepharose was purchased from Promega. Prestained protein molecular weight markers were purchased from Amersham and BioRad, and the fluorographic reagent Amplify and Hyperfilm from Amersham. All other chemicals were purchased from Aldrich and Sigma.

Construction of Chimeric Proteins

The glutamate receptor subunit pSK-GluR3 (obtained from J. Boulter)

was digested with EcoRI and SmaI. The supF gene in pcDNAI (Invitrogen) was replaced by an ampicillan resistance gene, which resulted in pcDNA-Amp. The glutamate receptor fragment was cloned into the EcoRI and EcoRV sites of pcDNA-Amp, resulting in pcDNA-AmpR3. A section of the prolactin gene from the plasmid pSP-BPI (Rothman et al., 1988) between the PvuII and PstI sites, lacking the N-terminal third of the prolactin molecule that contains the topogenic signal sequence, was subcloned into pBluescript KS$^-$ (Stratagene) between the SmaI and PstI sites, resulting in pKS-BplTR. The 195 amino acid prolactin fragment generated an immunoprecipitable protein product large enough to detect by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

The chimera pM4-COOH was constructed by inserting a NotI site in the GluR3 sequence at position 2780, which replaced GCTACATA with GCGGCCGC. The NotI site was introduced by polymerase chain reaction (PCR) mutagenesis (Higuchi, 1990) with Taq DNA polymerase. The prolactin epitope of pKS-BplTR was cloned in frame into the sequence between the NotI site and XhoI sites of pcDNA-AmpR3(Not2780). The constructs pM1-2, pM2-3, pM3-3′(b), pM3-3′(a), and pM3′-4 were made by first removing the XbaI site in the polylinker region of pcDNA-AmpR3 with Bal 31 nuclease and then inserting a XbaI site by PCR mutagenesis in the GluR3 sequence at positions 1939, 2035, 2191, 2275, and 2512. Thus, the sequences TGATCC, TGGGCG, CCTGGA, AGAGCC, and AGGCCT were each replaced by TCTAGA. These positions were selected to bracket the proposed transmembrane domains. The prolactin epitope was then cloned in frame into the sequences between the XbaI and XhoI sites. A schematic of the constructs is shown in Figure 1D. Restriction enzyme analysis and sequencing were employed to verify the constructs.

Deletion and Insertion of Glycosylation Sites

PCR mutagenesis was used to either delete or insert N-linked glycosylation sites. Four of the five endogenous glycosylation sites in the N-terminal region of GluR3 were individually removed by replacing the asparagines at positions 57, 260, 409, and 416 (numbered from the translational start site) with alanines. A unique glycosylation site was introduced at position 745, just upstream of the flip/flop region, by inserting an asparagine-leucine-threonine sequence in place of the naturally occurring aspartate-cysteine-asparagine. A schematic of the constructs is shown in Figure 1E. All mutations were verified by sequencing.

RNA Transcription

Plasmids were linearized with XhoI. Methyl-diguanosine triphosphate-capped RNA was synthesized in vitro with an RNA transcription kit from Stratagene using T7 or T3 polymerase (Dingledine et al., 1992). A trace amount of [^{32}P]CTP was incorporated in the transcription reaction. RNA quality was checked by gel electrophoresis and autoradiography, and the amount of RNA was quantified by determining the fractional incorporation of [^{32}P]CTP.

Xenopus Oocyte Expression

Xenopus oocytes were harvested and injected with 50 ng of in vitro transcribed GluR3 or an equimolar amount, 55 ng, of pM4-COOH as described previously (Kawajiri and Dingledine, 1993). Oocytes were voltage clamped at −100 mV, and kainate was bath applied in an external solution containing 90 mM NaCl, 1 mM KCl, 1.8 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 15 mM HEPES (pH 7.6). Current–voltage (I–V) relationships were obtained by applying a 2 s voltage ramp from −100 to +40 mV before and during kainate application. The I–V relationship of kainate-evoked current was then obtained by subtracting the control I–V relationship from that in the presence of kainate. Currents for the I–V relationship were normalized to the kainate current of each cell at −100 mV.

Cell-Free Translation

In vitro transcribed RNA (250–500 ng) was translated using a micrococcal nuclease treated rabbit reticulocyte lysate system in the presence or absence of canine pancreatic microsomes according to the instructions of the manufacturer. The translation reaction was optimized for the GluR3 transcript and contained 20 μM of each amino acid except methionine, 0.8 μM [^{35}S]methionine, 1.7 mM magnesium acetate, 2 mM dithiothreitol, 50 mM KCl, and 13 U of RNasin ribonuclease inhibitor. The volume of microsomes added was optimized at 5 μl for a 25 μl reaction. Microsomal membranes were not allowed to undergo more than a single freeze-thaw before use to preserve the integrity of the microsomes. The translation reaction was incubated at 30°C for 30–60 min.

Proteinase K Digestion

Immediately following the translation incubation, the reaction was placed on ice and the $CaCl_2$ concentration adjusted to 12 mM. To a 10 μl aliquot of the translation product either 1.1 μl of H_2O, 1.1 μl of 100 μg/ml of proteinase K, or 1.1 μl of 100 μg/ml of proteinase K plus 1.2% Triton X-100 was added, and the reaction was incubated on ice for 60 min. Proteolysis was arrested by adding phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 2 mM. Microsomes were permeabilized before immunoprecipitation by the addition of 100 μl of 1% SDS, and adjusted to a final concentration of 1 M Tris-HCl (pH 8.0), followed by an incubation for 10 min at room temperature. Proteinase K was stored at −20°C in aliquots sufficient for a single experiment.

To optimize the proteolysis incubation time, a submaximal amount of microsomes was added to an in vitro translation reaction of full-length prolactin containing the native signal sequence. This yielded approximately half of the protein product on the extramicrosomal surface and half on the intramicrosomal surface. Proteinase K was added as above, and aliquots were taken at 10 time points between 0 min and 150 min. The half-life of extramicrosomal protein was <5 min, whereas it was ~150 min for intramicrosomal protein. These experiments also indicated that at the 60 min time point, >80% of protein located on the interior of the microsome remained intact, whereas >95% of protein outside the microsome was proteolytically digested.

Immunoprecipitation and Analysis

The proteolysis product was mixed with 1.2 ml of immunoprecipitation buffer consisting of 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.0), 10 mM EDTA, 1% Triton X-100, and 1 mM PMSF. Either rabbit polyclonal antisera raised against bovine prolactin at 1:1000 dilution or rabbit polyclonal antisera to the N-terminal domain of the AMPA receptors at 1:375 dilution was added, and the reaction mixture was incubated for 10 min on ice; 12 μl of a 1:1 slurry of protein A–sepharose was then added, and the incubation continued at 4°C for 14–18 hr with gentle agitation. The immunoprecipitate was washed three times with immunoprecipitation buffer and twice with 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.0). Samples were resuspended in 10 μl of loading buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 1% SDS, and 0.004% bromophenol blue, heated to 100°C for 4 min, then immediately loaded onto and analyzed by SDS-PAGE. Stacking gels contained 0.04% polyacrylamide, 0.5 M Tris-OH, and 0.4% SDS, and the separating gels contained a 5%–15% polyacrylamide gradient, 0.4% SDS, 1.5 M Tris-OH (pH 8.8). Gels were run at 250 V for 2 hr at room temperature, fixed for at least 6 hr in isopropanol:water:acetic acid (25:65:10), soaked 30 min in Amplify, dried, and exposed to film. Experiments for each construct were replicated four to nine times, giving similar results.

Enzymatic Deglycosylation

The translation reaction (7 μl) was denatured in 0.5% SDS and 1% β-mercaptoethanol for 10 min at room temperature, then incubated in 50 mM sodium citrate (pH 5.5) with 500 U endoglycosidase H at 37°C for 4 hr. The product was analyzed by SDS-PAGE and fluorography.

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