An investigation of the membrane topology of the ionotropic glutamate receptor subunit GluR1 in a cell-free system

Andrew J. SEAL,*† Graham L. COLLINGRIDGE† and Jeremy M. HENLEY†

Department of Pharmacology, The Medical School, Edgbaston, Birmingham B15 2TT, U.K.

We have utilized cell-free translation in rabbit-reticulocyte lysate supplemented with canine pancreatic microsomal membranes to study the processing and membrane topology of the rat ionotropic glutamate receptor subunit GluR1. *In vitro*-synthesized RNA encoding GluR1 was translated to yield a primary translation product with an apparent molecular mass of 99 kDa. In the presence of microsomal membranes this protein was processed to give a band of 107 kDa. Treatment with peptide-N-glycosidase F showed that this increase in molecular mass was due to N-linked glycosylation. Incubation of the processed receptor with proteinase K revealed the presence of a 68 kDa protease-resistant band which decreased to 56 kDa following deglycosylation. A deletion mutant (GluR1M1) lacking the

predicted transmembrane domains was fully translocated across the microsomal membrane and protected from the action of the protease. The mutant and wild-type receptor could be immunoprecipitated by anti-peptide antibodies directed against the C-terminus. Following translocation of the wild-type and mutant receptor across the microsomal membrane and treatment with proteinase K the antibody binding to GluR1 was abolished, but was retained for GluR1M1. These data allow identification of the orientation of the N- and C-termini of GluR1 within the microsome; results which are consistent with an extracellular N-terminal and intracellular C-terminal localization following incorporation into the plasma membrane.

INTRODUCTION

Glutamate ionotropic receptors are the principal mediators of fast excitatory synaptic transmission in the vertebrate central nervous system [1]. These receptors are conventionally classified as being of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, or *N*-methyl-D-aspartate (NMDA) type. The first member of the AMPA family of receptors to be cloned was designated GluR-K1 and later as GluR-A, although it is now most commonly referred to as GluR1. Functional AMPA receptors are composed of heteromeric assemblies of different subunits, of which four have been identified (GluR1–4) [2,3]. While much has been learnt about the electrophysiology and pharmacology of these receptors, rather less is known about their post-translational modification, cellular trafficking and membrane topology.

The two original models of topology for AMPA receptor subunits, proposed by homology to other ligand-gated ionotropic receptors, both included four transmembrane domains (TMDs) with both N- and C-termini exposed on the extracellular surface [2,3]. However, they differed in the placement of TMDs, with hydrophobicity profiles identifying five regions as potential TMDs [4]. More recently experimental evidence has led to a variety of topological models being proposed, the most current of which includes three TMDs with the N-terminus placed extracellularly and the C-terminus intracellularly [5]. Based on this model and sequence homology, a modular design has been proposed for ionotropic glutamate receptor subunits in which the functional domains are related to prokaryotic precursor proteins [6].

Several approaches to the biochemical mapping of transmembrane topology have been employed in the study of native or recombinant glutamate receptors. These include: immunocytochemical detection of regions accessible to antibodies [7–9];

protection of segments of the peptide from degradation or modification by added reagents [10–13]; the analysis of the post-translational modification of specific sites on the protein [14–18]; and functional analysis of mutagenized receptor [19,20]. One system in which these assays may be used to study the processing of receptors in the early stages of the secretory pathway is an *in vitro* coupled translation–translocation system [21]. With respect to glutamate receptors, this technique has been used to study goldfish kainate-binding proteins [10], rat metabotropic glutamate receptor 1 α (mGluR1 α) [11] and rat GluR3 [12]. Here, we have utilized cell-free translation in rabbit-reticulocyte lysate supplemented with canine pancreatic microsomal membranes to study the processing and membrane topology of rat GluR1.

EXPERIMENTAL

In vitro transcription

A plasmid containing the coding sequence for rat GluR1 (flop) was supplied as a gift by Dr. Boulter (Salk Institute, San Diego, CA, U.S.A.) and subcloned into the vector pcDNA1/Neo (Invitrogen). For transcription the construct was linearized with *Xho*I and cRNA was synthesized using 30 units of T7 RNA polymerase in a 50 μ l reaction mixture containing 10 mM Tris, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 1 mM CTP, 1 mM UTP, 1 mM ATP, 0.5 mM GTP, 0.5 mM m⁷(5')ppp(5')G (sodium salt), 10 mM dithiothreitol, 20 units of RNAGuard (Pharmacia) and 1 μ g of plasmid DNA. The resulting cRNAs were used directly in the *in vitro* translation reaction.

Mutagenesis

To delete the region of the protein encoding the TMDs the restriction enzymes *Sma*I and *Bal*I were used to cleave the

Abbreviations used: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluR1, AMPA receptor subunit; GluR1M1, deletion mutant of GluR1; PNGF, peptide-N-glycosidase F; TMD, transmembrane domain; ER, endoplasmic reticulum.

* To whom correspondence should be addressed.

† Present address: Department of Anatomy, The Medical School, University of Bristol, Bristol BS8 1TD, U.K.

cDNA. The *SmaI*–*BalI* fragment between nucleotides 1345 and 2404 [2] was then removed by gel purification and the construct religated. The resultant mutant form of GluR1 (GluR1M1) lacked the cDNA encoding the native amino acids 449 to 802 but acquired a new codon encoding proline at position 449 (see Figure 2).

In vitro translation

Commercial micrococcal nuclease-treated rabbit-reticulocyte lysate and canine pancreatic microsomes (Promega) were used. Translation reactions were carried out according to the manufacturers' recommendations in a total reaction volume of 25 μ l. Reactions supplemented by the inclusion of canine microsomal membranes contained 2 equivalents of activity [21]. Translation products were labelled by the inclusion of [35 S]methionine in the reaction mixture at 0.8 mCi/ml.

Protease protection

Protease protection assays were based on the method of Morimoto et al. [22]. Briefly, following the standard translation reaction, aliquots of lysate were chilled on ice and CaCl_2 was added to 10 mM to help stabilize vesicle structure [21]. Proteinase K (Sigma), preincubated for 15 min at 37 °C in 10 mM Tris/HCl (pH 7.5) containing 10 mM CaCl_2 , was then added to the translation products to give a concentration of 1 mg/ml. Proteolysis was allowed to proceed for 60 min on ice before the addition of 5 mM PMSF. Following a further 10 min incubation on ice samples were solubilized by the addition of SDS/PAGE loading buffer, heated to 95–100 °C for 2–3 min and analysed by SDS/PAGE.

Peptide-N-glycosidase F (PNGF) treatment

Incubation of membrane fractions with PNGF (Oxford GlycoSystems) was performed according to the manufacturer's instructions. Samples of translation products were centrifuged for 5 min in a Microcentaur, the pellet was washed with PBS and resuspended in 10 μ l of 0.5% SDS containing 3 mM leupeptin. Incubations were then performed in 25 μ l of the buffer recommended by the manufacturer containing 0.6% octylglucoside and 40 units/ml PNGF at 37 °C for periods up to 5 h. PMSF (2 mM) was included in the incubation of protease-treated samples.

Immunoprecipitation

Rabbit anti-GluR1 antibody, raised against synthetic peptides corresponding to amino acids 877–889 of the predicted sequence of GluR1 [2], was purchased from Chemicon. Aliquots (5 μ l) of the *in vitro* translation/translocation reaction were diluted to 200 μ l in PBS, and incubated with 1 μ g of normal rabbit immunoglobulin fraction or 1 μ g of anti-GluR1 polyclonal antibody, with and without detergent (0.3% Triton X-100 and 0.3% Tween 20). For immunoprecipitation of protease-treated samples 2 mM PMSF was included in the incubation mixture. Following overnight incubation at 4 °C, 10 μ l of insoluble Protein A cell suspension (Sigma) was added and incubation was continued for a further 2–3 h. The immune complex was then pelleted by spinning for 0.5 min in a microcentrifuge and washed twice in PBS. SDS/PAGE sample buffer containing 5% 2-mercaptoethanol was then added and the samples heated to 95–100 °C for 5 min. Following centrifugation, aliquots equivalent to 1 μ l of the original translation reaction were loaded on to the gel.

SDS/PAGE analysis

The products of the translation and protease protection experiments were analysed by SDS/PAGE using a Bio-Rad mini-gel apparatus. The gels were fixed in acetic acid, prepared for fluorography with 2,5-diphenyloxazole (PPO), and exposed to X-ray film.

Replicates

Each experiment was performed on between 2 and 6 separate occasions with similar results.

RESULTS

Translation and deglycosylation of GluR1

In vitro-synthesized RNA encoding GluR1(flop) was translated to yield a dominant primary translation product with an apparent molecular mass of 99 kDa (Figure 1, lane 3). In the presence of microsomal membranes this protein was processed to give an additional band with an apparent molecular mass of 107 kDa (lane 4). These apparent molecular masses correspond well to those predicted from the sequence and found in other studies on the rat GluR1 [7]. The lower-molecular-mass proteins seen correspond to partial translation products which may arise from premature termination of translation or initiation from internal ATG codons. Processing was shown to involve N-linked glycosylation at multiple asparagine residues. As shown in Figure 1 (lane 6), deglycosylation of the processed form by treatment with PNGF resulted in an increase in mobility so that the deglycosylated form ran with the same apparent molecular mass as the

Figure 1 *In vitro* translation and glycosylation of GluR1

In vitro-transcribed RNA (cRNA) was used as template in a rabbit-reticulocyte translation/translocation system containing 0.8 mCi/ml [35 S]methionine. Translation products were analysed on a 7.5% SDS/PAGE gel and processed for fluorography. Lane 1, 14 C-labelled molecular-mass markers; lane 2, control reaction which contained water instead of cRNA; lanes 3–6, cRNA encoding GluR1 was included in the reactions which were supplemented in lanes 4–6 with 2 equivalents of microsomal membranes, in lanes 5 and 6 translation products were pretreated and incubated with a buffer blank (lane 5) or PNGF at 40 units/ml (lane 6). In all Figures equivalent amounts of material from the original translation reaction were loaded in all lanes. However, the deglycosylation procedure involved washing of the microsomal fraction prior to incubation and therefore some loss of material. Therefore in this Figure lanes 5 and 6, taken from the same gel, were exposed for a longer period than the others to allow clear visualization of the bands. Arrowheads indicate the full-length primary translation product and processed protein.

Figure 2 Schematic illustration of the predicted topogenic domains in GluR1 and GluR1M1

The positions of consensus sites for N-linked glycosylation, which all occur in the region preceding the first predicted TMD, are indicated by asterisks. Predicted TMDs are shown stippled and the putative signal peptide is shown filled in. The five putative TMDs shown are those that were predicted by Keinänen et al. and Hollmann et al. [2,3] and are numbered according to Gasic and Hollmann [4]. The cross-hatched region on GluR1, which includes all the predicted TMDs, corresponds to the portion of the protein which was removed to create GluR1M1. To create this mutant the cDNA between the *Sma*I site at nucleotide 1345 and the *Bal*I site at nucleotide 2404 was excised and the construct religated. This resulted in the deletion of codons for 354 native amino acids and the creation of a proline codon at position 449. The protein encoded by the GluR1M1 cDNA would therefore contain 536 amino acids, following signal peptide cleavage, and have a predicted molecular mass of approx. 60 kDa. It should be noted that GluR1M1 retains the last 6 amino acids of TMD IV but that such a small stretch of hydrophobic residues would not be expected to act as a TMD.

primary translation product. The samples in lane 5 were treated identically except that PNGF was replaced by a buffer blank during incubation. PNGF is a glycosidase which cleaves specifically between asparagine and the innermost *N*-acetylglucosamine residue of *N*-linked glycans [23]. Given that glycosylation only occurs within the lumen of the endoplasmic reticulum (ER) [24], it can be deduced that in the presence of microsomes GluR1 is, at least partially, translocated across the microsomal membrane.

Protease resistance of GluR1 and GluR1M1

According to the predicted model (Figure 2) a large N-terminal region of the receptor will be translocated across the ER membrane as it transits through the secretory pathway. If this is the case, in an *in vitro* translation/translocation system the N-terminus should be protected from the action of added proteases as it will lie within the sealed lumen of the microsome. To investigate this prediction the translation products were treated with the non-specific proteinase K. Protease treatment of the processed receptor revealed the presence of a 68 kDa protease-resistant band (Figure 3a, lane 6). This protease resistance was not found in the absence of microsomal membranes and was abolished by the inclusion of 1% Triton X-100 in the proteolysis reaction (Figure 3, lanes 5 and 7). The size of this protected fragment was consistent with translocation of the predicted N-terminal domain into the lumen of the microsome.

In a polytopic transmembrane protein, insertion of the polypeptide into the membrane is generally initiated by the signal peptide and translocation proceeds up until the insertion of the first TMD. The normal topology of the protein is obtained by the function of regions of hydrophobic amino acids acting alternatively as start and stop signals for the translocation process [25]. The process of translocation can therefore be manipulated by the insertion or deletion of hydrophobic regions into a recombinant protein. We predicted that deletion of the portion of GluR1 cDNA encoding the transmembrane domains would result in the subsequent translation of a protein that would be fully translocated across the membrane of the microsomal vesicle. A mutant of GluR1, designated GluR1M1, was constructed in which all the TMDs were removed (see Figure 2). As seen in Figure 3(b) this mutant translated to give a primary translation product with an apparent molecular mass of 60 kDa which was

Figure 3 *In vitro* translation and protease resistance of GluR1 and GluR1M1

In vitro-transcribed RNA (cRNA) encoding (a) GluR1 or (b) GluR1M1 was used as template in a rabbit-reticulocyte translation/translocation system containing 0.8 mCi/ml [35 S]methionine. Translation products of GluR1 and GluR1M1 were analysed on 7.5% SDS/PAGE gels and processed for fluorography. (a) Lane 1, 14 C-labelled molecular-mass markers; lane 2, control reaction containing water instead of cRNA. Lanes 3–7 contained cRNA and in lanes 4, 6 and 7 the reactions were supplemented with 2 equivalents of canine pancreatic microsomal membranes. Samples were incubated with proteinase K (1 mg/ml) in lanes 5–7 with the addition of 1% Triton X-100 to the sample run in lane 7. (b) The treatment of the samples was identical for GluR1M1 but a water blank was not loaded on the gel. Arrowheads indicate the protease-resistant fragments.

processed in the presence of microsomal membranes to give a product of 68 kDa. The extent to which this protein was translocated was assessed by protease treatment. When incubated with protease a predominant protease-resistant band was observed corresponding to the molecular mass of the full-length processed mutant (Figure 3b, lane 5). This indicated that, as predicted, the protein was fully translocated across the microsomal membrane. The lower-molecular-mass bands seen in lane 5 may be due to the presence of partial-length translation products which were translocated into the microsome. This result indicates that the initial translocation of GluR1 is mediated by the N-terminal signal peptide independently of the predicted TMDs.

To investigate the identity of the wild-type protease-resistant fragment further it was subjected to deglycosylation by PNGF

Figure 4 Deglycosylation of the protease-resistant fragment of GluR1

In vitro-transcribed RNA (cRNA) was used as template in a rabbit-reticulocyte translation/translocation system containing 0.8 mCi/ml [35 S]methionine. Translation products were analysed on a 7.5% SDS/PAGE gel and processed for fluorography. Lanes 1–3, cRNA encoding GluR1 was included in the reaction and supplemented with 2 equivalents of microsomal membranes. Lane 2, after translation samples were incubated with proteinase K (1 mg/ml); lane 3, protease-treated translation products were then pretreated and incubated with PNGF at 40 units/ml. Arrowheads indicate the protease-resistant fragments before and after deglycosylation.

(Figure 4). Complete deglycosylation of all peptides was not achieved even after extended incubation, the doublet seen in lane 3 always being observed. The pattern of bands obtained from deglycosylation of this protease-resistant fragment indicated the presence of multiple N-linked glycosylation sites. The lowest band corresponded to an apparent molecular mass of 56 kDa, and suggested the presence of at least five N-linked glycones. Analysis of the published sequence of GluR1 indicated the existence of six consensus sites for N-linked glycosylation, these sites all lying within the region of the protein preceding the first predicted TMD (see Figure 7). These data therefore demonstrated that the protease-resistant fragment corresponds to the N-terminus and, taken together with the size of the deglycosylated protected fragment, suggest that the N-terminus is translocated across the microsomal membrane up until at least the end of the hydrophobic region originally predicted by Hollmann et al. to form TMD1 [2].

Immunoprecipitation of GluR1 and GluR1M1

Immunoprecipitation of GluR1, translated in the presence of microsomes, was accomplished by using a commercial polyclonal antibody raised against a peptide corresponding to amino acids 877–889 [7]. Precipitation of the receptor was achieved by incubation with a Protein A cell suspension under washing conditions in which microsomes were not pelleted unless bound by immune complex. In the absence of detergent both the 99 kDa and the 107 kDa bands were precipitated by the C-terminal-directed antibody, but with low efficiency (Figure 5, lane 4). In the presence of detergent the 107 kDa band, corresponding to the glycosylated form of GluR1, was precipitated with increased

Figure 5 Immunoprecipitation of the translocated form of GluR1 and GluR1M1

In vitro-transcribed RNA (cRNA) encoding GluR1 or GluR1M1 was used as template in a rabbit-reticulocyte translation/translocation system containing 0.8 mCi/ml [35 S]methionine and supplemented with 2 equivalents of microsomal membranes. Lane 1, 14 C-labelled molecular-mass markers; lanes 2 and 6, total translation products; lanes 3 and 7, with control rabbit immunoglobulin; lanes 4 and 8, translation products were immunoprecipitated with polyclonal antibodies directed against the C-terminus of GluR1. 0.3% Triton X-100 and 0.3% Tween 20 were included in the reactions run in lanes 5 and 9. Arrowheads indicate the primary translation product and processed form of the wild-type and mutant proteins.

Figure 6 Immunoprecipitation of the protease-resistant forms of GluR1 and GluR1M1

In vitro-transcribed RNA (cRNA) encoding GluR1 or GluR1M1 was used as template in a rabbit-reticulocyte translation/translocation system containing 0.8 mCi/ml [35 S]methionine and supplemented with 2 equivalents of microsomal membranes. Samples were analysed on a 5% SDS/PAGE gel and processed for fluorography. Lane 1, 14 C-labelled molecular-mass markers; lanes 2 and 5, total translation products. Following digestion with proteinase K translation products were immunoprecipitated with control rabbit immunoglobulin (lanes 3 and 6) and with polyclonal antibodies directed against the C-terminus of GluR1 (lanes 4 and 7). The arrowhead indicates the protease-resistant fragment of the mutant protein.

efficiency, while the 99 kDa band showed little change (Figure 5, lane 5). As precipitation of the translocated protein is greatly enhanced in the presence of detergent it suggested that at least some of the peptide epitopes were within the lumen, or masked by the membrane, of the microsome. However, the effect could also be due to an enhancement of antibody binding independent of any effect on the membrane. GluR1M1 also showed the same characteristics as GluR1 under these conditions in that antibody binding to the glycosylated protein occurred in the absence of detergent, but was increased in the presence of detergent (Figure 5, lanes 8 and 9). However, antibody binding to GluR1M1 in the absence of detergent was unexpected as we had already demonstrated that the mutant was fully translocated into the microsome. An explanation for this was that the lumen of the microsome, under conditions used for the immunoprecipitation, was partially accessible to antibodies in the absence of detergent. The less than complete protection of translocated protein from the action of protease (see Figure 3) suggested that there were two populations of microsomes, one of which was permeable to exogenous proteins.

From the above data it was evident that immunoprecipitation alone could not provide a clear-cut answer. To address more clearly the question of whether the C-terminus of the wild-type protein was located on the exterior or interior of the microsome, immunoprecipitation following protease treatment was attempted. If the C-terminus was located on the interior of the microsome then we expected to be able to immunoprecipitate the fragment of the C-terminus extending as far as the N-terminal

side of TMD IV (approx. 12 kDa). If, on the other hand, the C-terminus is exposed on the exterior of the microsome we would not expect to detect antibody binding as the antigenic site would be destroyed. The inclusion of GluR1M1 served as a positive control for the immunoprecipitation as we knew that the antigenic site on this protein would be protected within the microsome. As seen in Figure 6 no band could be precipitated following protease digestion of the translocated wild-type protein, whereas efficient precipitation of protease-treated GluR1M1 was seen. The autoradiograph was exposed for an extended period to ensure detection of any low-molecular-mass peptides. The absence of any immunoprecipitated material from wild-type GluR1 demonstrated the exterior location of the C-terminus antigenic sites. The immunoprecipitation of protease-resistant GluR1M1 also confirmed the result that in the absence of the TMDs the receptor is fully translocated across the membrane of the microsome.

DISCUSSION

Since the cloning of GluRs, a number of different techniques have been used to investigate the predicted models of topology. Our findings from the current study demonstrate that, in a cell-free system, the N-terminus of GluR1(flop) is located within the microsome and the C-terminus is extramicrosomal. Recently, similar results have been obtained utilizing the same cell-free system combined with protease protection, deglycosylation and mutagenesis to analyse the membrane topology of other iono-

Figure 7 Schematic representation of structures proposed for GluR1 and GluR1M1

The membrane topology of the receptor is shown as it is predicted to occur in the plasma membrane (**a** and **c**) and in the microsomal vesicle (**b** and **d**). (**a**) and (**b**) represent the four-TMD model proposed in the original cloning papers [2,3], while (**c**) and (**d**) represent the current model based on the latest experimental evidence (see Discussion for details). The findings of this paper are consistent with the structure shown in (**c**) and (**d**). The predicted topology of the GluR1 mutant GluR1M1 is shown in (**e**) and (**f**). Note that the microsomal form of GluR1M1 is completely translocated into the microsome and therefore should be inaccessible to antibody binding and protected from the action of the protease. When transported to the cell surface we predict that the protein would be secreted. Consensus sites for N-linked glycosylation are marked with an asterisk. As can be seen they all occur within the protease-protected N-terminal domain which lies within the lumen of the microsome.

tropic glutamate receptor subunits. Wo and Oswald [10] investigated the topology of goldfish kainate-binding proteins, while Bennett and Dingledine [12] investigated the topology of the rat GluR3 subtype of the AMPA receptor. The later group also used immunological techniques but engineered reporter epitopes into the receptor rather than utilizing native epitopes.

The orientation of the glutamate receptor subunits within the microsome corresponds to an expected extracellular location for the N-terminal and intracellular location for the C-terminal regions following incorporation into the plasma membrane. Recent studies using anti-peptides antibodies [8,9] and the engineering of synthetic N-glycosylation sites followed by expression in oocytes [18] have produced findings consistent with this prediction for GluR1.

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