Two Regions in the N-terminal Domain of Ionotropic Glutamate Receptor 3 Form the Subunit Oligomerization Interfaces That Control Subtype-specific Receptor Assembly*

Received for publication, July 26, 2004, and in revised form, February 7, 2005
Published, JBC Papers in Press, February 8, 2005, DOI 10.1074/jbc.M408432000

Gai Ayalon‡‡, Eitan Segev‡, Sharona Elgavish¶, and Yael Stern-Bach§§

From the ‡Department of Anatomy and Cell Biology, Institute of Basic Dental Sciences, Hadassah Dental School and the ¶Structural Biology Bioinformatics Unit, Hadassah Medical School, The Hebrew University, Jerusalem 91120, Israel

The N-terminal domain (NTD) of α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA) and kainate glutamate receptors plays an important role in controlling subtype specific receptor assembly. To identify NTD subdomains involved in this process we generated AMPA glutamate receptor 3 (GluR3) mutants having intra-NTD substitutions with the corresponding regions of the kainate receptor GluR6 and tested their ability to form functional heteromers with wild-type subunits. The chimeric design was based on the homology of the NTD to the NTD of the metabotropic GluR1, shown to form two globular lobes and to assemble in dimers. Accordingly, the NTD was divided into four regions, termed here N1–N4, of which N1 and N3 correspond to the regions forming lobe-1 and N2 and N4 to those forming lobe-2. Substituting N1 or N3 impaired functional heteromerization but allowed protein-protein interactions. Conversely, exchanging N2 or N4 preserved functional heteromerization, although it significantly decreased homomeric activity, indicating a role in subunit folding. Moreover, a deletion in GluR3 corresponding to the hotspot mouse mutation of the glutamate receptor δ2, covering part of N2, N3, and N4, impaired both homomeric and heteromeric oligomerization, thus explaining the null-like mouse phenotype. Finally, computer modeling suggested that the dimer interface, largely formed by N1, is highly hydrophobic in GluR3, whereas in GluR6 it contains electrostatic interactions, hence offering an explanation for the subtype assembly specificity conferred by this region. N3, however, is positioned perpendicular to the dimer interface and therefore may be involved in secondary interactions between dimers in the assembled tetrameric receptor.

Glutamate, the major excitatory neurotransmitter, activates two receptor families: metabotropic glutamate receptors (mGLuRs),† which are coupled to G-proteins, and ionotropic glutamate receptors (iGluRs), which form cation selective ion channels. The iGluRs are further divided into three subtypes: AMPA, kainate, and NMDA receptors, originally named after their most selective agonist (1). Each subtype, in turn, consists of several subunits that assemble as tetramers in various combinations to form channels with distinct properties (2). Of the cloned subunits, GluR1–4 form the AMPA receptors, GluR5–7 and KA1–2 form the kainate receptors, and NR1, NR2A-D, and NR3A-B form the NMDA receptors. The δ1–2 subunits constitute an orphan group of iGluRs, and their relation to the three principal subtypes is unclear.

Overall, all iGluR subunits have a common domain organization (3). Each subunit contains four membrane regions (M1–M4), of which M1, M3, and M4 traverse the plasma membrane and M2 is a reentrant loop aligning the channel pore. The N terminus is extracellular and comprises an ~400-residue N-terminal domain (NTD; also termed ‘X domain’), which is homologous to bacterial periplasmic amino acid-binding proteins and to the N-terminal domain of mGluRs (4). Following the NTD is S1, an ~150-residue segment, which together with S2, the segment between M3 and M4, forms the glutamate-binding domain (5). The S1S2 domain, like the NTD, is related to bacterial periplasmic amino acid-binding proteins (4–6). The C terminus is intracellular and is involved in receptor trafficking, anchoring, and signal transduction (7).

Despite this common subunit organization and substantial sequence similarities, especially between AMPA and kainate receptor subunits, hetero-oligomers form exclusively within subtypes (2). In a previous study using AMPA/kainate chimeras, we showed that the NTD, the membrane sector and part of S2 determine the subunit compatibility in the assembly process (8). In addition, our data fit a model in which iGluRs assemble as dimer-of-dimers in a two-step basic process: 1) two monomers associate at their NTDs to form dimers; and 2) two dimers undergo a secondary dimerization at the glutamate-binding domains and at the membrane domains to form the tetramer, which is then stabilized by secondary NTD interactions. The notion that NTD compatibility is also required in the second step emerged from the observation that heteromerization was abolished between chimeras having incompatible NTDs, whereas the rest of their polypeptides were compatible (8). If the NTDs were playing a role only in the first step of monomer dimerization, the two different resulting homodimers could then co-assemble via their compatible C termini. These secondary NTD interactions likely take place after the interaction of the downstream regions, because when expressed as a soluble

* This work was supported by Grant 561/03 from the Israel Science Foundation and by the Bernard Katz Minerva Center for Cell Biophysics. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a Horowitz Foundation scholarship from The Hebrew University.

‡ To whom correspondence should be addressed: Inst. of Basic Dental Sciences, Hadassah Dental School, The Hebrew University, Jerusalem 91120, Isreal. Tel.: 972-2-6757444; Fax: 972-2-6758578; E-mail: yaelb@cc.huji.ac.il.

¶ The abbreviations used are: mGluR, metabotropic glutamate receptor; AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionate; iGluR, ionotropic glutamate receptor; NTD, N-terminal domain; LIVBP, leucine-isoleucine-valine-binding protein; HEK293, human embryonic kidney 293 cells; DTT, dithiothreitol; DN, dominant negative; NMDA, N-methyl-D-aspartic acid.
protein detached from the rest of the subunit, the NTD has been shown to form only dimers (9, 10). Therefore, the NTD seems to serve as a subtype compatibility gatekeeper throughout the assembly process. Interestingly, Pasterнак et al. (11) showed recently that AMPA receptor GluR4 subunits lacking their NTD are able to form functional homomeric receptors, and yet, the ability to form heteromers was not tested.

Given the important role that the NTD plays in iGluR restricted assembly, we further examined the contributions of NTD subdomains to this process. Employing electrophysiological experiments on *Xenopus laevis* oocytes expressing AMPA/kainate chimeras confined to the NTD, we found that compatibility in two regions is required for functional assembly. Computer modeling based on the solved crystal structure of the mGluR1-NTD (12) further suggests that one of these regions may comprise the initial dimerization interface, and the other may be attributed to a second interface formed between two dimers in the assembled tetramer.

**EXPERIMENTAL PROCEDURES**

Molecular Biology and in Vitro cRNA Transcription—All AMPA receptor constructs used here contained the flip isoform. Generation of R3(R6S1) and R6T1R3 (termed herein N3(3333) and N(6666), respectively) was described previously (5). Intra-NTD GluR3/GluR6 chimeras were constructed by a similar approach and subcloned in R3(R6S1) between Xmal and NruI sites. For each construct, the amino acids at the appropriate junction are indicated in Fig. 1 and are numbered relative to the first methionine in the open reading frame. GluR3 point mutations were constructed by the QuikChange method (Stratagene, La Jolla, CA). All mutations were confirmed by sequencing throughout the cassette insert. All constructs were inserted in pGEMHE, which was used for expression in *Xenopus* oocytes. Plasmids were linearized with Nhel, and capped cRNA was transcribed in *vitro* using T7 RNA polymerase (mMessage mMACHINE; Ambion, Austin, TX). GluR3(M599R) and GluR6(M589R) in pRK5 and pCDNA3 (termed here GluR2DN and GluR6DN, respectively) were a gift from James R. Howe (Yale University) (13). cRNAs were obtained by plasmid linearization with HindIII (GluR2DN) and XbaI (GluR6DN) followed by transcription using SP6 and T7 polymerase, respectively. The yield and quality of transcripts were assessed by agarose gel electrophoresis and ethidium bromide staining and recently also quantified by analytical UV spectrophotometry (ND-1000; NanoDrop Technologies, Inc. Rockland, DE).

Preparation of Oocytes and Electrophysiology—Stage V-VI *X. laevis* oocytes were prepared as described previously (5). Oocytes were injected up to 24 h after preparation, usually with 10–20 ng of cRNA in 50 nl/oocyte (unless stated), and assayed 3 days later. Two electrode voltage clamp recordings were carried out at room temperature using the two-electrode voltage clamp technique used (Protein Data Bank code 1EWK) gives root-mean-square deviation (ND-1000; NanoDrop Technologies, Inc. Rockland, DE).

Preparation of Oocytes and Electrophysiology—Stage V-VI *X. laevis* oocytes were prepared as described previously (5). Oocytes were injected up to 24 h after preparation, usually with 10–20 ng of cRNA in 50 nl/oocyte (unless stated), and assayed 3 days later. Two electrode voltage clamp recordings were carried out at room temperature using the two-electrode voltage clamp technique used (Protein Data Bank code 1EWK) gives root-mean-square deviation (ND-1000; NanoDrop Technologies, Inc. Rockland, DE).

**RESULTS**

**GluR3/GluR6 Intra-NTD Chimeric Design**—Applying the approach taken in our previous study (8) we further investigated the contribution of the NTD to subtype-specific assembly by generating chimeras containing AMPA/GluR3 and kainate- (GluR6) substitutions confined to the NTD and tested their ability to assemble with wild-type subunits.

The chimeric design was based on the weak but nonetheless significant homology of the NTD of the iGluRs to the NTD of the mGluRs, exemplified by mGluR1 (4), and to bacterial periplasmic amino acid-binding proteins, specifically the leucine-isoleucine-valine-binding protein (LIVBP) (4). Both the LIVBP and mGluR1-NTD fold into a structure containing two lobes, where each lobe is formed by two alternating regions of the S1S2 glutamate-binding domain (12, 23). The LIVBP and mGluR1-NTD structures available as potential templates for GluR3/GluR6 NTDs. Superimposition of the predicted GluR3-NTD structure with the structure of the GluR2 S12 domain, either in complex with glutamate or in its apo state (Protein Data Bank codes 1P73 and 1P7T, respectively), by exploring similar fragments using the Swiss-PdbViewer (22) showed only weak structural resemblance, mostly in lobe-2 (root-mean-square deviation > 5 Å).

**WNT-Related Domains Controlling Glutamate Receptor Assembly**

The WNT-Related Domains Controlling Glutamate Receptor Assembly are important for the assembly of GluR3 and GluR6 receptors. These domains play a crucial role in the assembly process by interacting with the NTD of the receptor. The WNT-Related Domains Controlling Glutamate Receptor Assembly are important for the assembly of GluR3 and GluR6 receptors. These domains play a crucial role in the assembly process by interacting with the NTD of the receptor.
S1S2 it involves both parts (25). However, all of the S1S2 structures currently available gave very poor fit compared with the mGluR1 structure (see “Experimental Procedures”), and therefore were excluded. It is also noteworthy that although the function of the NTD as a substance-binding domain in AMPA and kainate receptors is unclear, in NMDA receptors it has been shown to bind various allosteric modulators (26–28) in a region corresponding to the glutamate-binding cleft in mGluR1.

Taking all the above considerations into account, we divided the NTD into four parts, termed here N1–N4, according to its alignment with mGluR1-NTD (Fig. 1). N1 and N3 correspond to the regions forming the predicted lobe-1 and N2 and N4 to those forming lobe-2 (Figs. 1, 2A, and 5). N1, N2, and N3 each comprise approximately one-third of the NTD sequence, whereas N4 is only ~15 residues long and may function only as a connector between the NTD and S1. For practical reasons (see “Experimental Procedures”) these substitutions were made on the backbone of R3(R6S1), a GluR3 subunit having S1 from GluR6. This subunit is identical to GluR3 except for an increased affinity to some agonists (5) and a complete loss of receptor desensitization (29). The terminology used to describe the resulting intra-NTD chimeras is N(XXXX), where X stands for either “3” or “6,” representing a GluR3 or a GluR6 sequence in the corresponding N1–N4 regions. For example, a NTD chimera having N1 from GluR3 and N2, N3, and N4 from GluR6 is termed N(3666). The template, R3(R6S1), is therefore termed N(3333) throughout this article, and its NTD reciprocal chimera, having the entire NTD from GluR6 (originally named R6TM1R3 (5)), is termed N(6666).

**GluR3/GluR6 Substitutions Confined to the NTD Reduce Homomeric Activity—**

First, to evaluate the functionality of the different chimeras, X. laevis oocytes were injected with the respective cRNA (~10 ng/oocyte), and 3 days later whole-cell

### Fig. 1. Sequence and structure alignment of GluR3 and GluR6 NTDs based on mGluR1.

The alignment procedure is described under “Experimental Procedures.” Overlying bold lines represent the N1 and N3 regions comprising lobe-1, whereas overlying dotted lines correspond to the N2 and N4 regions comprising lobe-2. Underlying bars represent α-helices, and arrows represent β-strands. Gray background boxes account for residues participating in the dimer interface as suggested by homology modeling (see Fig. 5 and “Experimental Procedures”). Closed circles indicate cysteines in the dimer interface and open circles the two other iGluR conserved cysteines. The putative glycosylation sites are marked with triangles. A, B, and C mark the respective junctions of the different NTD-chimeras described in the text; A′ and A′ mark two alternative N1-N2 junctions that produced nonfunctional subunits; ho indicates the start of the hotfoot-like deletion made in GluR3. Amino acid numbering, shown at the left of each sequence, starts from the first methionine of the open reading frame.
would impair receptor function, may escape the oocyte quality control system, especially at high expression levels.

To further test this issue, we then injected oocytes with consecutive dilutions of cRNA of selected intra-NTD chimeras as well as N(3333) and N(6666), which served as controls. As shown in Fig. 2C, both the whole-cell current activity and the protein surface expression of N(3333) and N(6666) gradually decreased with decreasing cRNA concentrations. However, the activity as well as surface expression of N(3366), N(3633), and N(3633), but not total protein expression, was dramatically affected compared with the control subunits. Although at high concentrations (>5 ng/oocytes) surface expression was similar (or slightly lower) compared with N(3333), at low concentrations (<1.5 ng/oocytes) only a small fraction of surface protein was detected (Fig. 2C, middle panels). Finally, we also measured glutamate EC50 values, which were all found to be similar (N(3333) = 46 ± 5 μM; N(3366) = 62 ± 4 μM; N(6333) = 42 ± 5 μM; N(6363) = 80 ± 4 μM; n = 5), thus excluding problems in agonist recognition as the cause of reduction in homomeric activity. Altogether, based on these results we may conclude that the observed lower homomeric activity is probably due to problems in subunit assembly/folding or trafficking rather than a direct effect of receptor-channel properties.

Functional Heteromerization of NTD Chimeras With GluR2R Requires Compatibility in the N1 and N3 Regions—Functional heteromerization was determined by the current rectification assay as was done in our previous work (8). Briefly, in AMPA receptors, current rectification and single channel conductance are inversely controlled by a single amino acid located in M2, which as a result of RNA editing can be either a glutamine (Q) or an arginine (R) (30). The unedited Q-form exhibits high conductance and strong inward current rectification, which is due to an intracellular block by polyamines. In contrast, the edited R-form (in nature only GluR2) exhibits very low conductance and lack of sensitivity to polyamines, thus generating a linear current/voltage (IV) relationship. Heteromers made of R- and Q-form subunits are highly conducting and exhibit a clear linear IV relationship. Because of its low single channel conductance, the activity of homomeric GluR2R R-form (GluR2R) is hardly detectable (<10 nA), and thus a linear IV relationship obtained for a Q-form chimera co-expressed with GluR2R is an indication of functional heteromerization.

As shown previously (8) when N(3333) was expressed with GluR2R at 1:1 ratio (~20 ng of total cRNA/oocyte) we observed large macroscopic currents with a linear IV relationship, reflecting the predominant heteromeric formation between these two subunits (Fig. 2, B, right, and D). At similar expression conditions, Q-form chimeras having either N1 or N3 from GluR6 exhibited strong inward current rectification in the presence of GluR2R, indicating that compatibility in each one of these regions is required for subtype-specific functional assembly. Conversely, chimeras N(3633) and N(3363), in which N2 or N4 where exchanged, did undergo linearization, indicating their ability to functionally heteromerize with GluR2R. In all cases the presence of GluR2R and its ratio to the chimeric subunit were verified by Western blot analysis (Fig. 2D, inserts). Substitution of Either N1 or N3 Results in Partial Protein-Protein Interactions—The apparent lack of functional heteromerization when either N1 or N3 was substituted could result from a complete lack of interaction between the chimera and GluR2R, or from partial interactions that were not sufficient to generate functional channels. To test this issue, we first co-expressed the intra-NTD chimeras with dominant-negative (DN) mutants of GluR2 and GluR6 (carrying a mutation of a conserved methionine in the M2 channel pore to arginine), which were shown to knock down AMPA and kainate receptor
activity in a subtype-specific manner (13). As expected, 1:1 co-expression of GluR2DN with N(3333) reduced the peak current ~10-fold compared with N(3333) expressed alone (Fig. 3B), whereas co-expression with GluR6DN had no effect. Also consistent with our previous study (8), the activity of the reciprocal chimera N(6666) was not attenuated by GluR2DN but was reduced to 50% by GluR6DN (note that lack of full inhibition of N(6666) by GluR6DN is due to lack of compatibility in the region C-terminal to M1).

In contrast to N(3333) and N(6666), 1:1 co-expression of N(3666) and N(6666) with either GluR2DN or GluR6DN resulted in a 40–50% reduction in peak current (Fig. 3B), indicating an intermediate degree of protein-protein interaction between the chimeras and each one of the dominant-negative subunits. For chimera N(3633) we recorded relatively low currents, which prevented a reliable quantification of the activity attenuation by the dominant-negative subunits, and therefore a 1.5:1 ratio in favor of N(3633) was tested. Despite the favorable conditions GluR2DN reduced the activity of N(3633) by 66%, significantly more than the attenuation observed for the other chimeras, thus indicating a strong interaction with GluR2DN. On the other hand, co-expression with GluR6DN had no significant effect on N(3633) activity.

To further examine protein-protein interactions, we performed co-immunoprecipitation experiments on oocytes co-injected with the AMPA receptor subunit GluR1 and the chimeras (1:1 ratio; Fig. 3C). As expected, N(3333) for the most part co-precipitated with GluR1, whereas N(6666) did so only marginally (8). Under the same conditions, however, chimeras N(3666) and N(6633) significantly co-precipitated with GluR1, as compared with N(6666), but not as efficiently as N(3333). Chimera N(3633), on the other hand, exhibited a degree of precipitation similar to that of N(3333). Overall these results support the observations drawn from co-expressions with GluR2DN in the dominant-negative functional assay.

A Deletion in GluR3 Corresponding to the Hotfoot Mouse Mutation of the Glutamate Receptor δ2 Disrupts Functional Oligomerization—The hotfoot mouse mutations are deletions within the NTD of the orphan δ2 glutamate receptor subunit (31). According to their amino acid sequence, δ2 subunits are part of the iGluR family. However, they do not form functional glutamate-gated ion channels, nor do they bind any other known agonist (32, 33). From a different mouse mutation, the Lurcher mutation, it is known that δ2 subunits can function as ion channels because this mutation results in constitutively active receptors in the absence of ligand (34). δ2 is expressed in Purkinje cells in the cerebellum and is crucial for normal cerebellar activity because mutations in δ2 result in ataxia (35).

The ataxic hotfoot-4J mice have been shown to carry a 170-amino acid deletion in the C-terminal half of the δ2-NTD (31), which covers part of N2, N3, and N4 in our GluR3/GluR6 NTD-chimeras (Figs. 1, 4, and 5). Functionally this mutant is similar to our chimera N(3666), which has N2, N3, and N4 from GluR6 (Figs. 2 and 3). Using biochemical assays, Matsuda and Yuzaki (36) suggested that the hotfoot-4J δ2 mutant (δ2ho) may be impaired in its ability to form oligomers. First, they found that in HEK293 cells δ2ho is retained in the endoplasmic reticulum, whereas the full-length δ2 (δ2wt) reaches the plasma membrane, even when co-expressed. Second, in co-immunoprecipitation experiments δ2ho precipitated only about half the amount of δ2wt, whereas full interaction between the two subunits was expected to result in full precipitation. This result is similar to the ~50% reduction in activity we obtained for chimera N(3666) when co-expressed with GluR2DN as compared with the almost complete knock-down of N(3333) activity (Fig. 3). Third, co-precipitation of differentially tagged δ2ho subunits was very weak, indicating problems with homomeric interaction. This result is in line with the very low homomeric activity we found for chimera N(3666) (Fig. 2).

Because the activity of δ2 receptors cannot be measured in vitro, to further test the possible functional similarity between δ2ho and chimera N(3666) we introduced the corresponding deletion into wild-type GluR3 (Fig. 4A). Homeric specific activity of the resulting mutant, R3ho, was extremely low, ~5% compared with wild-type GluR3 (Fig. 4B, R3wt). Surface labeling of intact oocytes with biotin showed that the R3ho protein reaches the plasma membrane (unlike the δ2ho in HEK293 cells (36)). However, it constitutes only ~5% of its whole-cell fraction compared with ~12% observed for GluR3 (Fig. 4C), thus indicating some problems in exiting the endoplasmic reticulum. We then co-expressed R3 wt with R3ho to assess their ability to interact with each other. When expressed in a 1:1 ratio, R3wt still retains ~75% of its homomeric activity (Fig. 4B, left). At this subunit ratio a full interaction would be expected to retain only ~6% of the R3wt activity. Even at a 3:1 ratio in favor of R3wt, R3wt activity was reduced to only 30% (Fig. 4B, right). These results confirm at the functional level that the deletion mutant has very weak oligomerization capabilities as inferred from the biochemical assays (36).

Computer Modeling of GluR3-NTD and GluR6-NTD Sug-gests an Explanation for the Subtype-specific Assembly Con-formation—Based on the results shown in Figs. 2 and 3, subunit compatibility in regions N1 and N3 was found to be crucial for subtype-specific heteromerization. As mentioned previously, these two regions together correspond to the regions forming lobe-1 of the mGluR1-NTD, the lobe that creates the dimer interface in both glutamate-bound and unbound states (12). Therefore, to better understand the role of N1 and N3 in iGluR assembly, we generated homology models.
of GluR3-NTD and GluR6-NTD homodimers based on the homodimeric structure of mGluR1-NTD in complex with glutamate (Fig. 5). This particular template of mGluR1 was chosen because it represents the maximum intersubunit contacts at this domain (12, 37).

As indicated in Fig. 1 by gray boxes, the predicted dimer interface, which is formed mainly by N1 (Fig. 5), is more hydrophobic in GluR3, whereas in GluR6 it is more polar and contains charged amino acids. Sequence alignment shows that the N1 interface is highly conserved within each subtype. The similarity and identity values obtained for the AMPA receptor subunits GluR1–4 are 83 and 78%, respectively. For the kainate receptor subunits GluR6–7, they are 93 and 86%. GluR6–7 and KA1–2 share only ~35% similarity; however, the KA1–2 N1 interface, as in GluR6–7, contains charged residues. Between GluR3 and GluR6 the similarity and identity values drop to 26 and 20%, respectively. Overall, the different characteristics of the N1 interface between AMPA and kainate receptor subunits may be one of the factors responsible for their apparently restricted assembly.

The other region, N3, according to the models, does not seem to participate in the dimer interface (Fig. 5) but rather is positioned perpendicularly to it. Therefore, N3 may be involved in interactions between two dimers in the assembled tetrameric receptor. Within each subtype the sequence similarity of N3 is high, 70–85%, but drops to less than 30% between subtypes.

Finally, it has been also shown that the mGluR1-NTD homodimer is stabilized by a disulfide bond formed by cysteine 140 from each monomer. In NMDA receptors, it was recently proposed that a similar bond may be formed by Cys79 between two NR1 subunits (38). In AMPA and kainate receptors there are four conserved cysteines, which in GluR3 correspond to Cys85 and Cys111 (closed circles in Fig. 1) are depicted in yellow ball-and-stick form and Cys215 and Cys334 (open circles in Fig. 1) are cyan. The consensus N-linked glycosylation sites are shown only in A. For clarity, the Ca of the respective asparagine residues (triangles in Fig. 1) are shown in black Corey-Pauling-Koltun (CPK) presentation.

**FIG. 4.** Insertion of the hotfoot δ2 mouse deletion into GluR3 impairs receptor assembly. A, the hotfoot δ2 mouse deletion was inserted into the GluR3 NTD between Val227 and Ser387. B, upper panels show representative Western blots of whole-cell homogenates of oocytes injected with wild-type GluR3 (R3wt) and GluR3 carrying the deletion (R3ho) alone or together at 1:1 (left) and 1:3 (right) ratios. Lower panels show the relative activity measured for each respective injection (in the presence of cyclothiazide to block receptor desensitization; average of 10 oocytes). 100% represents R3wt homomeric activity. C, surface protein expression of R3wt and R3ho when expressed alone or together at 1:3 ratio, as determined by labeling intact oocytes with biotin. The lower panel shows the calculated surface fraction of each subunit from its total whole-cell protein expression shown in B.

**FIG. 5.** Computer model of GluR3-NTD homodimer. Three orientations of the homodimer are shown: A, front view; B, top view (generated by rotating view A 90° along the x axis); C, side view (generated by rotating figure A 90° along the y axis). The model is presented as solid ribbons. N1–N4 regions in one monomer (as shown on Fig. 1) are colored blue (N1), red (N2), green (N3), and magenta (N4). N1 and N3 form the corresponding lobe-1, and N2 and N4 form lobe-2. The second monomer is colored gray and orange, where the orange region labels residues Val227–Ser387, which correspond to the hotfoot δ2 mouse mutation. In both monomers residues Cys85 and Cys111 (closed circles in Fig. 1) are depicted in yellow ball-and-stick form and Cys215 and Cys334 (open circles in Fig. 1) are cyan. The consensus N-linked glycosylation sites are shown only in A. For clarity, the Ca of the respective asparagine residues (triangles in Fig. 1) are shown in black Corey-Pauling-Koltun (CPK) presentation.
circles) are too far apart to form such a bond. Cys$^{215}$ is positioned in N2 at the bottom of the structure, and Cys$^{354}$ is located in N3 at the top (Fig. 5, ball-and-stick residues (cyan)). To test a possible role of Cys$^{215}$ and/or Cys$^{354}$ in forming a disulfide bond, we generated GluR3 mutants carrying a point mutation to alanine in each one of these positions alone and the double mutant R3(C85A,C111A) (4). Western blot analysis on whole-cell homogenates obtained from the respective oocytes described in A (15-ng injection) and separated on 7% SDS-PAGE under nonreducing (−DTT) and reducing (+DTT) conditions. Blots were reacted with anti-GluR2/3 antibodies. The arrows indicate the difference in mobility of complexes, presumably dimers, obtained for the different mutants compared with wild-type GluR3. The bands migrating near the 117-kDa marker are monomers. In the presence of DTT degraded fragments are also seen, which may represent the complexes migrating near the 171-kDa marker in the absence of DTT. An identical pattern (but with weaker intensity) was obtained for oocytes injected with 1.5 ng cRNA/oocytes (not shown).

DISCUSSION

Previously, we and others have shown that the NTD plays an important role in controlling iGluR subtype-specific receptor assembly (8, 39). We also proposed that iGluRs assemble as dimer-of-dimers and that NTD compatibility is required both at the initial step of subunit dimerization and at the second step of dimerization of dimers (8). The NTD is a large portion of the subunit protein consisting of ~400 residues. Therefore, in this study we further analyzed the contribution of specific NTD subdomains to the assembly process by generating intra-NTD GluR3/GluR6 chimeras.

Our results demonstrate that any attempt to make changes in the NTD result in significant reduction in homomeric activity as compared with the parental subunit or with a chimera having the entire NTD from GluR6 (N(3333) and N(6666), respectively; see Fig. 2). Initial surface biotinylation experiments showed that all chimeras expressed at the oocytes surface similar to the control subunits. This result would argue against severe problems in assembly or trafficking, suggesting rather that intrinsic receptor-channel properties have been altered. In support of this idea, functional allosteric interactions between the NTD and the glutamate-binding core have been found in the NMDA receptor (40). However, these surface protein measurements were done on oocytes injected with high amounts of cRNA (~10 ng), and therefore, at these expression levels it could be that misfolded proteins may have escaped the oocyte quality control system. Indeed, at lower expression levels, both activity and surface protein expression were largely inhibited, whereas total protein expression was not different from the control subunits. Although these findings cannot distinguish between problems in assembly or trafficking, the observation that some of these chimeras fail to fully co-purify with GluR1 (see below) would favor incorrect assembly. The importance of NTD integrity was also observed for intra-NTD truncations made in the AMPA receptor GluR4, which resulted in loss of receptor homomeric activity (11).

Although homomeric activity of the intra-NTD chimeras produced in this study was in general lower than the parental activity, at high expression levels (>5 ng cRNA/oocytes), the currents recorded were large enough (>500 nA) to allow us to test for functional heteromerization as we had done previously (8). We found that functional heteromeric assembly requires compatibility in two regions, which approximately comprise the N- and C-terminal thirds of the NTD sequence (assigned here as N1 and N3) (Fig. 2). Compatibility in only one of these regions lead to weak protein-protein interactions as determined by eletrophysiological and biochemical assays (Figs. 3). Together with the observed reduction in homomeric assembly, these results demonstrate the importance of N1 and N3 in both intra- and intersubunit contacts.

Based on sequence similarity (Fig. 1) N1 and N3 were designed to correspond to the regions forming lobe-1 of the mGluR1-NTD structure, the lobe that forms the dimer interface of this domain (12). Computer modeling based on the mGluR1 crystal structure suggested that the dimer interface, which is composed mainly of N1 (Fig. 5), is markedly different between GluR3 and GluR6. In GluR3 the interface is more hydrophobic, and in GluR6 it is more polar and contains electrostatic interactions (Fig. 1, gray boxes). That this interface is highly conserved within subtypes and differs considerably between subtypes may be one of the factors responsible for the lack of compatibility between AMPA and kainate receptor subunits. The models also predict that at the dimer interface two pairs of cysteines may be close enough to form disulfide bonds (Fig. 1, closed circles; Fig. 5, yellow ball-and-stick), as shown for Cys$^{140}$ in mGluR1 and as recently proposed for Cys$^{79}$ in NMDA receptor subunit NR1 (38). Indeed, mutating either or both of these cysteines to alanine in GluR3 (C85A/C111A) resulted in an almost complete block of activity. SDS-PAGE analysis under nonreducing conditions was not, however, consistent with a formation of inter-NTD disulfide bond(s). Nevertheless, the mutants did exhibit altered migration profiles, which may reflect stronger association with cellular chaperones or other interacting proteins, consistent with problems in subunit folding. Improper positioning of these cysteines (singularly or to-
biochemical assays, as the deletion into GluR3, which can be assayed functionally. This our chimera N(3666) for which we also found impaired assem-

tionally (42). In principle, the hotfoot deletion corresponds to regu-

lation.  

erning subunit composition within iGluR subtypes and their 

ibility. Since both subunit assembly and AMPA/kainate assembly incompat-

have identified two subdomains that are critical for functional 

NTD in the subtype-specific assembly of AMPA receptors. We 

and S. F. Heinemann for wild-type AMPA and kainate receptors. 

170-residue deletion in the NTD of the null-like phenotype of the hotfoot mice. These mice carry a closed-closed state by allosteric regulators. 

function (26–28), the NTD may normally adopt a relaxed 

bound, respectively (12, 41) (37). In iGluRs, the NTD does not 

each monomer: open-open, open-closed, and closed-closed, 

nera, respectively (1, 26–28) (27). In iGluRs, the NTD does not 

10. Wells, G. B., Lin, L., Jeanclos, E. M., and Anand, R. (2001) 

15060