α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptor Channels Lacking the N-terminal Domain*

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Ionotropic glutamate receptor (iGluR) subunits contain a ~400-residue extracellular N-terminal domain (“X domain”), which is sequence-related to bacterial amino acid-binding proteins and to class C G-protein-coupled receptors. The X domain has been implicated in the assembly, transport to the cell surface, allosteric ligand binding, and desensitization in various members of the iGluR family, but its actual role in these events is poorly characterized. We have studied the properties of homomeric α-amino-3-hydroxy-5-methylisoxazolepropionate (AMPA)-selective GluR-D glutamate receptors carrying N-terminal deletions. Our analysis indicates that, surprisingly, transport to the cell surface, ligand binding properties, agonist-triggered channel activation, rapid desensitization, and allosteric potentiation by cytochrome c oxidase can occur normally in the complete absence of the X domain (residues 22–402). The relatively intact ligand-gated function of a homomeric AMPA receptor in the absence of the X domain indirectly suggests more subtle roles for this domain in AMPA receptors, e.g. in the assembly of heteromeric receptors and in synaptic protein interactions.

Ionotropic glutamate receptors (iGluR) mediate the majority of fast excitatory neurotransmission in the mammalian central nervous system. These ligand-gated channels are believed to be tetramers consisting of subclass-specific sets of homologous subunits, each ~900–1300 amino acid residues in length (1, 2). Previous studies have identified three segments in iGluR subunits that show sequence similarity to bacterial proteins involved in the transport of extracellular solutes (3). Two segments, S1 and S2, separated by the membrane-associated channel domain, form a two-lobed agonist-binding domain, which is homologous to bacterial polar amino acid-binding proteins specific for leucine (LBP) and leucine/isoleucine/valine (LIVBP) and to the N-terminal extracellular N-terminal segments of Class C G-protein-coupled receptors, which include the metabotropic glutamate receptors (3). The three-dimensional structure of this domain has not been determined, but homology models predict an LIVBP-like structure consisting of two lobes surrounding a central cleft (8, 9).

The N-terminal domains of human and rat AMPA receptor subunits are ~95% identical. This high degree of sequence conservation is certainly indicative of an essential physiological function. In contrast to the agonist-binding domain and the transmembrane channel region, however, the functional role of this domain, which in AMPA and kainate receptors may represent up to 45% of the mature polypeptide, is poorly understood, hence it is referred to as the “X domain.” In principle, functional activities assigned to or suggested for the N-terminal domain fall into three different classes. First, this domain is implicated as a determinant in the assembly of oligomeric channels (10–12). Second, the X domain may mediate the allosteric transitions involved in the channel activation, desensitization, or modulation by ions and drugs as has been observed for NMDA receptors (8, 13–17). Third, the X domain may provide docking sites for extracellular proteins, which serve to cluster the receptors or stabilize their localization. In support of the latter possibility is a recent report of an extracellular interaction of AMPA receptors with a synaptic protein Narp, although the detailed site of the interaction was not localized (18).

To gain insight into the function of the N-terminal domain in the AMPA receptors, we have examined the properties of GluR-D (GluR4) receptors carrying N-terminal deletions. We report that, surprisingly, homomeric AMPA receptors that lack the entire X domain form functional glutamate-gated channels in transfected HEK 293 cells. A closer analysis of the properties of the mutant channels shows that the assembly of homomorphic receptors, transport to cell surface, ligand binding, agonist-triggered channel activation, and rapid desensitization can occur normally in the complete absence of the X domain.

MATERIALS AND METHODS

DNA Constructs—Standard molecular biological techniques were used to clone the cDNA encoding residues 22–902 of rat GluR-D (flip isoform; residues 1–21 code for the signal peptide; Ref. 19) into a derivative of pcDNA3.1(−) (Invitrogen), which carries a viral signal peptide followed by an N-terminal FLAG epitope (6). All N-terminal deletion constructs were built on this vector (wild-type GluR-D). The deletion mutants, created by PCR methodology, were used to replace the native region in wild-type GluR-D by appropriate restriction enzyme digest. All PCR-generated constructs were verified by sequencing.

Expression in Insect Cells—The recombinant baculovirus for the expression of GluR-D 4492 was prepared by using Bac-to-Bac system (Invitrogen) according to manufacturer’s instructions. High Five insect cells (Invitrogen) cultured in T75 flasks in serum-free SF-900II medium
were infected with recombinant baculoviruses for FLAG-tagged full-length GluR-D (11) and Δ402 and harvested 48 h later.

**Ligand Binding Analysis**—Preparation of membranes, determination of [3H]AMPA (specific activity 60.0 Ci/mmol; PerkinElmer Life Sciences) binding by using a filtration assay, and displacement experiments using unlabeled compounds were performed as described previously (11). Ligand binding data were analyzed by using the PRISM nonlinear curve-fitting software (GraphPad Inc.).

**Expression in HEK 293 Cells and Immunofluorescence Staining**—HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 2 mM l-glutamine and 1% penicillin-streptomycin solution. Immediately prior to transfection they were replated at a density of 2 x 10^5 cells/ml. Transfection was done by using the calcium phosphate method, and the medium was changed the following day and the cells harvested 40 h following transfection. For patch clamp experiments the cells were cotransfected with a plasmid encoding green fluorescent protein (pEGFP-C1, Clontech) for visualization with epifluorescence microscopy.

For immunofluorescence, cells were fixed in 3% paraformaldehyde, and preblocked with 3% goat serum. For total staining, the cells were additionally incubated in 0.05% Triton X-100 in phosphate-buffered saline prior to blocking step. Cells were labeled with monoclonal anti-FLAG antibody (M2, Sigma) followed by Cy3-conjugated anti-mouse IgG secondary antibody (Jackson Laboratories; 7 μg/ml). Cells were examined using an Olympus Provis AX70 epifluorescence microscope and pictures collected by a Photometrics SenSys air-cooled CCD camera using Image ProPlus software.

**Quantitative Immunolabeling**—Transfected HEK 293 cells were plated in 96-well tissue culture plates coated with Matrigel (BD Biosciences) at a density of 4 x 10^3 cells per well. Cells were fixed 40 h post-transfection by either 3% paraformaldehyde in phosphate-buffered saline (for surface labeling) or by 100% methanol at -20 °C (for total labeling). Following incubation in 3% goat serum to block nonspecific binding, the cells were labeled with monoclonal anti-FLAG antibody (M1; 100 ng per well) for 1 h at 37 °C. Cells were washed three times with 1% goat serum, and incubated with Eu³⁺–conjugated anti-mouse IgG (Wallac; 25 ng per well) for 1 h at 37 °C. Thereafter, the cells were washed as above, rinsed with Tris-buffered saline, and Enhancement solution (Wallac; 100 μl per well) was added to form the fluorescent Eu³⁺–chelate. The samples were measured on a Wallac VICTOR² instrument using excitation filter at 340 nm and emission filter at 615 nm settings and a delay time of 400 μs. The fluorescence values (recorded as cpm) for the surface and total labeling were corrected for the non-specific background obtained from HEK 293 cells transfected for expression of non-FLAG-tagged GluR-D.

**Electrophysiology**—Glutamate and AMPA currents were recorded from HEK 293 cells 48 h after transfection using the outside-out configuration of the patch clamp technique. Recording pipettes had a resistance of 3–5 MΩ when filled with a solution containing (in mM): potassium-glucuronate, 110; NaCl, 2.5; Heps, 10; EGTA, 10; pH 7.3 (adjusted with KOH). The patches were continuously superfused with the extracellular solution containing (in mM): MgCl₂, 1; NaCl, 150; KCl, 2.5; CaCl₂, 2; Heps, 10; pH 7.35 (adjusted with NaOH). Glutamate, AMPA, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were directly dissolved in extracellular solution. Cyclothiazide was added from a stock solution. All compounds were from Tocris Cookson.

Rapid applications of agonists were made using the SP-77B perfusion fast system with a narrow-mouthed theta tube (Warner Instrument Corporation). Rise time of glutamate currents was less than 1 ms. No currents were observed in untransfected cells or cells transfected with a nonrelevant cDNA construct. The currents were recorded using an EPC-9 patch clamp with Pulse and Pulsefit software (HEKA Electronics). The desensitization rate constant (τₙ) was determined by fitting a single exponential to the decaying component of currents elicited by 5 mM glutamate. Concentration-response relationships were fitted with a logistic equation using Michaelis Origin software. The data were compared using Student's t test. All data are given as mean ± S.E.

**RESULTS**

To gain insight into how the N-terminal X domain contributes to the properties of AMPA receptors, we analyzed receptors mutated to eliminate all or parts of this domain. N-terminally FLAG-tagged GluR-D (flip isoform) and mutants lacking increasing lengths of the N-terminal segment (residues 22–541; signal peptide: 1–21; Fig. 1A) of GluR-D were transiently expressed in transfected HEK 293 cells. Expression of the mutant proteins was verified by immunoblotting, which showed the presence of FLAG-immunoreactive bands with electrophoretic mobilities consistent with the sizes inferred for the deletion constructs from the sequence and predicted glycosylation status (Fig. 1B). The expression levels of the N-terminal deletion constructs Δ162, Δ403, and Δ366 were roughly equivalent to that of the full-length GluR-D, but the two smallest constructs, Δ402, lacking the entire X domain, and Δ541, which lacked the whole N-terminal extracellular domain (i.e. both the X domain and the ligand-binding S1 segment), were expressed often at a somewhat lower level (Fig. 1B).

The subcellular distribution of the deletion mutants was determined by immunofluorescence microscopy. Under permeabilized conditions, all constructs showed intracellular staining by anti-FLAG antibody (Fig. 2). In contrast, only two constructs, the full-length GluR-D and Δ402, devoid of the entire X domain, were expressed on cell surface as indicated by specific immunostaining under nonpermeabilizing conditions (Fig. 2). Both stainings had a finely punctate appearance that was evenly distributed over the cell membrane. These findings imply that deletions within (the predicted) structural domains (X and S1S2) of the receptor, unlike a complete deletion of the X domain, block the transport of the receptor to cell surface, possibly due to incorrect folding. The latter possibility is consistent with our finding that none of the deletion mutants was stained by a monoclonal antibody Fab22, which recognizes a conformation-sensitive epitope within the X domain of GluR-D (20), although cells expressing the full-length GluR-D were
intensely stained both intracellularly and on cell surface (results not shown).

To complement the immunofluorescence studies, we used europium-labeled secondary antibody to quantify the relative amounts of FLAG-tagged full-length GluR-D and Δ402 present on cell surface. Time-resolved fluorescence measurements indicated that 84 ± 14% (mean of surface labeling/total labeling ± S.D.; n = 4) of the full-length receptor and 66 ± 13% of Δ402 (n = 4) were accessible to Eu³⁺-labeled anti-mouse IgG in fixed cells under nonpermeabilizing conditions; the slight decrease is statistically not significant (in nonpaired t test). No significant surface labeling was obtained with receptor constructs that were negative in immunofluorescence microscopy (results not shown). The expression level of Δ402 was similar (105 ± 8%) to that of the full-length GluR-D as determined by europium labeling.

Next, we wished to determine whether the presence or absence of the X domain has any influence on the interaction of the receptor with agonists and antagonist as determined by radioligand binding experiments. To obtain larger amounts of receptor protein to facilitate more accurate radioligand binding measurements, we used the baculovirus expression system to produce full-length GluR-D and GluR-D Δ402 as 110-kDa and 50-kDa FLAG-immunoreactive species (Fig. 3A). Consistent with results obtained with the soluble extracellular domains of GluR-D (11), deletion of the X domain did not have any significant effect on the binding affinity of [³H]AMPA (GluR-D: Kᵣ 42 ± 6 nM, n = 4; Δ402: Kᵣ 45 ± 3 nM, n = 3; Fig. 3B). The expression level of Δ402 in High Five cells was consistently higher than that of the full-length receptors. The Bₘₐₓ determined for [³H]AMPA binding in the membrane preparations was 12.1 pmol/mg of protein for GluR-D (n = 4), whereas for Δ402 membranes a Bₘₐₓ value of 45.3 ± 4.5 pmol/mg of protein (n = 3) was obtained. No differences between GluR-D and Δ402 membranes were observed in the relative abilities of the unlabeled agonists (glutamate and kainate), and the unlabeled antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX) to inhibit [³H]AMPA binding. The apparent affinities of unlabeled glutamate (GluR-D: Kᵣ 0.20 ± 0.06 μM, n = 4; Δ402: Kᵣ 0.26 ± 0.05 μM, n = 4), kainate (GluR-D: Kᵣ 2.10 ± 0.14 μM, n = 4; Δ402: Kᵣ 3.5 ± 1.8 μM, n = 3), or 6,7-dinitroquinoxaline-2,3-dione, a competitive antagonist (GluR-D: Kᵣ 0.55 ± 0.18 μM, n = 4; Δ402: Kᵣ 0.42 ± 0.16 μM, n = 4) (Fig. 3C) are as indicated. These findings indicate that the presence or absence of the X domain in the native receptor does not have any significant effect on the interaction of agonists and competitive antagonists with the ligand binding domain.

The unaltered membrane localization and ligand binding properties of Δ402 suggested the possibility that functional receptors may be formed by GluR-D subunits lacking the X domain. To investigate this, outside-out patches were pulled from HEK 293 cells transiently expressing the full-length GluR-D and Δ402. As expected, both L-glutamate and AMPA triggered rapidly desensitizing currents in GluR-D-containing patches. Surprisingly, however, highly similar agonist responses were recorded from the Δ402-containing patches (Fig. 4A). The glutamate sensitivity of Δ402 channels was also similar to that of wild-type channels (Fig. 4A). However, further analysis revealed a slight change in desensitization kinetics:

![Fig. 2. Immunofluorescent staining of HEK 293 cells expressing GluR-D constructs.](image)

![Fig. 3. Deletion of the X domain does not change ligand binding properties of GluR-D.](image)
The rate of desensitization of glutamate responses was somewhat slower for the Δ402 channels (τdes, 4.52 ± 0.31 ms, n = 5) than for GluR-D (3.47 ± 0.24 ms, n = 6; p = 0.024; Fig. 4B). Responses of Δ402 channels to 1 mM glutamate were also fully blocked by the specific AMPA receptor antagonist CNQX (100 μM; Fig. 4C), consistent with its unchanged antagonist binding characteristics.

Finally, we studied the effect of cyclothiazide, an allosteric modulator of AMPA receptor desensitization, on GluR-D and Δ402. By using a filtration assay, we measured the inhibitory effect of cyclothiazide on [3H]AMPA binding to GluR-D and to Δ402 expressed on insect cell membranes. As shown in Fig. 5A, cyclothiazide caused a concentration-dependent inhibition of [3H]AMPA binding on both GluR-D (IC50 = 11.1 ± 1.0 μM, n = 4) and Δ402 (IC50 = 10.3 ± 1.9 μM; n = 4) receptors. The IC50 values and the level of maximal inhibition (~50%) are close to the previously reported value for the flip isoform of GluR-D (16 μM, ~60%; Ref. 21). These findings demonstrate that the cyclothiazide binding site, and the structures mediating its allosteric effect on agonist binding, are present in the deletion construct as well. We then studied the effect of cyclothiazide current responses of GluR-D and Δ402 channels expressed in HEK 293 cells. Consistent with the ligand binding data, glutamate responses recorded from outside-out patches indicated that cyclothiazide removed desensitization of glutamate currents of GluR-D and Δ402 channels in a similar manner (Fig. 5B). These findings clearly show that the Δ402 construct contains a functional cyclothiazide binding site, thus demonstrating that the X domain is not a critical participant in the allosteric transitions involved in homomeric GluR-D receptor desensitization or its regulation by cyclothiazide.

DISCUSSION

Based on biochemical, cell biological, and electrophysiological analyses of native and recombinant iGluR, a number of different activities or potential physiological roles for the N-terminal X domain have been suggested. Our present analysis on the properties of GluR-DΔ402—402 receptors clearly exclude an essential role of the X domain in the assembly of homomeric channels, transport to cell surface, channel gating, rapid desensitization, and allosteric potentiation by cyclothiazide in AMPA receptors. It must be noted, however, that our study focuses on one AMPA receptor subunit, and the specific functions of the X domain are likely to vary among receptor subunits, and possibly even among subunits. In the following, we discuss the possible functions of the N-terminal domain of GluR-D AMPA receptor in the light of the present results.

Channel Gating—Interestingly, the N-terminal domain is not present in the small kainate-binding proteins expressed in avian and amphibian species, which are otherwise homologous to iGluR subunits. These proteins do not seem to have a capacity to form active ligand-gated channels, even though their M1–M3 segment is able to mediate an ion conductance when artificially inserted into AMPA or kainate receptor subunits (22). These findings raise the possibility that the N-terminal domain might be essential for channel activity, possibly by participating in the conformational coupling between the ligand-binding domain and the channel domain. The identification of a prokaryotic glutamate-sensitive potassium channel ("GluR0"), which is distantly related to the core of iGluR sub-
units and has no X domain, demonstrates that a direct coupling between the ligand-binding S1-S2 domain and the ion channel gate can take place without an obligatory involvement of an N-terminal domain (23). The overall sequence similarity between the prokaryotic channel and eukaryotic iGluR is fairly low, however, and even the ligand binding mechanism employed by GluR0 seems to profoundly differ that observed for the AMPA receptor (24). Therefore, it is entirely possible that the mechanism of ligand-triggered activation of the GluR0 channels is also fundamentally different. However, our present results convincingly demonstrate that in an eukaryotic AMPA receptor, the neurotransmitter-binding domain can directly couple to and regulate the ion channel without an involvement of the N-terminal domain.

Desensitization and Allosteric Modulation—Several studies have implicated the N-terminal domain of NMDA receptor subunits as a determinant or direct participant in some forms of desensitization. Glycine-independent desensitization of NMDA receptors was assigned to two regions in the NR2A subunit, the X domain and an area within the S1 segment (13, 17). Moreover, participation of the N-terminal domain in the regulation of NMDA receptor channel function by allosteric modulators, including Zn2+ ions (14, 16), ifenprodil (8, 15, 16), spermine (8), protons (25), and redox agents (26), has been demonstrated. In some cases, this modulation has been shown to involve an direct allosteric interaction between the N-terminal domain and the agonist-binding domain (16).

So far, no studies have indicated any direct engagement of the X domain with allosteric modulation or desensitization of AMPA receptors. Indeed, mutagenesis experiments on AMPA receptors have localized structural determinants responsible for the rapid desensitization and its modulation by allosteric ligands exclusively in the S1 and S2 segments (27–29). Our present results do not exclude the possibility that the X domain can participate in the fine-tuning of channel responses, as shown by the minor difference in desensitization rates observed between the Δ402 and full-length GluR-D constructs. However, the presence of rapid desensitization in the Δ402 channels together with removal of desensitization by cyclothiazide of show that the N-terminal domain is not an obligatory participant in these processes. This is also consistent with a recent model for AMPA receptor desensitization, inspired and supported by high resolution structures of S1S2-ligand complexes, proposing that destabilization of a dimer interface between agonist-binding domains of neighboring subunits may underlie desensitization (23, 30, 31).

Assembly—Several studies have suggested a role for the X domain in the assembly of multimeric channels (10, 12, 32, 33). Furthermore, experiments performed with separately expressed extracellular domains of GluR-D subunit indicate that the X domain is able to form dimers, consistent with a role in the assembly (11). A recent study suggests that the X domain participates in early dimerization of subunits, whereas the transmembrane part and the S2 segment are important in the formation of the functional tetramer assembly (12). Our current findings show that functional homomeric AMPA receptors can assemble from subunits lacking the entire X domain. Furthermore, our data actually suggest that homomeric Δ402 receptors assemble at least as efficiently as wild-type GluR-D. These results are consistent with a role in the formation of the functional tetramer assembly (12).

Conclusion—We conclude that the N-terminal X domain does not play a critical role in the formation of active glutamate-gated channels in homomeric GluR-D AMPA receptors. Furthermore, the ligand binding, allosteric transitions involved in the rapid activation and desensitization of AMPA receptor channel responses, as well as its regulation by cyclothiazide, can take place in the complete absence of the X domain.

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