Export from the Endoplasmic Reticulum of Assembled N-Methyl-d-aspartic Acid Receptors Is Controlled by a Motif in the C Terminus of the NR2 Subunit*

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Functional N-methyl-d-aspartic acid (NMDA) receptors are formed from the assembly of NR1 and NR2 subunits. When expressed alone, the major NR1 splice variant and the NR2 subunits are retained in the endoplasmic reticulum (ER), reflecting a quality control mechanism found in many complex multisubunit proteins to ensure that only fully assembled and properly folded complexes reach the cell surface. Recent studies have identified an RRR motif in the C terminus of the NR1 subunit, which controls the ER retention of the unassembled subunit. Here we investigated the mechanisms controlling the ER retention of the NR2 subunit and the export of the assembled complex from the ER. We found that the Tac chimeras of the C terminus of the NR2B subunit show that an ER retention signal is also present in the NR2B subunit. In assembled complexes, ER retention signals on the individual subunits must be overcome to allow the complex to leave the ER. One common mechanism involves mutual masking of the signals on the individual subunits. Our data do not support such a mechanism for regulating the release of assembled NMDA receptors from the ER. We found that the motif, HLFLY, immediately following transmembrane domain 4 of the NR2 subunit, is required for the assembled complex to exit from the ER. Mutation of this motif allowed the assembly of NR1 and NR2 subunits into a complex that was functional, based on MK-801 binding, but it is retained in the ER. These results are consistent with HLFLY functioning as a signal that is necessary for the release of the assembled functional NMDA receptor complex from the ER.

N-Methyl-d-aspartic acid (NMDA) receptors are multisubunit complexes assembled from three subunit classes, NR1, NR2, and NR3. Co-assembly of NR1 and NR2 subunits is required to form a functional channel, generally considered to be a tetramer containing two NR1 and two NR2 subunits (1). NR3 subunits can assemble with NR1 alone to form a glycine receptor (2), and they also can have a modulatory role on NR1/NR2 complexes by associating in a yet undefined fashion (3). NR1 is a single subunit that can be expressed in eight splice variants, which have distinct trafficking and functional properties. There are four NR2 subunits, NR2A–D, each of which differs in its distribution and developmental expressions. The type of NR2 subunit present in the complex has a major impact on the functional properties of the channel. For example, NR1/NR2A channels display rapidly desensitizing currents and close association with the synapse compared with NR1/NR2B channels (4, 5). Therefore, the subunit composition of NMDA receptors is a major determinant of NMDA receptor-mediated activity and localization in the central nervous system. Several splice variants of NR1 and different subunits of NR2 are present in the same neurons, leading to multiple receptors with different functional and trafficking properties (6). For example, a neuron that expresses NR2A and NR2B subunits can contain three functionally distinct receptors: NR1/NR2A, NR1/NR2B, and NR1/NR2A/NR2B. The process by which an NMDA receptor is assembled from its individual subunits and trafficked to synaptic and extrasynaptic sites remains poorly understood. The major NR1 splice variant (NR1-1) and the NR2 subunits are retained in the endoplasmic reticulum (ER) when expressed alone in heterologous cells, but when expressed together, they assemble to form functional receptors that reach the cell surface (7–10).

ER retention of the NR1 subunit is dependent on the alternative splicing in the C terminus (8–10). The major splice variant NR1-1 is retained in the ER in its unassembled state because of an RRR motif in its C1 cassette. NR1 subunits with other configurations of their C termini, NR1-2, NR1-3, and NR1-4, are not retained either because they lack the C1 cassette and the RRR motif (e.g. NR1-2 and NR1-4) or because they also contain the C2’ cassette (NR1-3). The C2’ cassette has a PDZ binding domain, which can negate the RRR retention motif found in the C1 cassette. When assembled with NR2, the nature of the NR1 splice variant does affect trafficking of the NR1/NR2 complex with complexes containing the NR1-1 splice variant, resulting in reaching the cell surface more slowly than the other splice variants that are not ER-retained (11, 12). The mechanism underlying the ER retention of the NR2 subunit has not been investigated. It is retained in the ER when expressed in the absence of NR1 in heterologous cells (7) and in neurons lacking the NR1 subunit (13). These observations are consistent with NR2 retention caused by a signal in its cytoplasmic domain, similar to that of the NR1 subunit. For other multisubunit complexes in which individual subunits are
mRNA. Two primers (5′-GAGCAATCAGAGGCTCAGC-3′ and 5′-GATCCGGATCTCTGCAGCAAC-3′) were designed and used in PCR cloning of the DNA sequence encoding the C-terminal tail for GABA<sub>A</sub>R1 from TM7 to the end, using the rat brain QUICKLONE cDNA libraries (Clontech) as a template. An XbaI restriction site was introduced at the end of the NR1-1 region in the pCIS-based vector. The XbaI-treated vector was then ligated with XhoI ligase. All point mutations were generated using the QuickChange mutagenesis kit according to the manufacturer’s instructions. All constructs were verified by DNA sequencing.

Expression in Heterologous Cells—Heterologous cells, including HEK 293, COS7, and HeLa cells, were cultured and transfected using the calcium phosphate method or LipofecAMINE 2000 (Invitrogen) according to the manufacturer’s protocols. The NR1 and NR2B receptor subunit clones in the mammalian expression vector pCIS (as described previously (8)). The required portions of the NR2B C terminus were generated by PCR with the XbaI and EcoRV sites engineered at the appropriate positions. The mouse NR2BFLAG construct (17) was used as the background for the mutant NR2B constructs used in this study. Truncations were made by incorporating a stop codon (TGA) in the appropriate place using site-directed mutagenesis (Stratagene). Because antibody binding to the FLAG epitope produced relatively weak staining, this could not be used in the presence of one of the antibodies was used. Cells were plated onto poly-L-lysine coated cell culture plates. Twenty-four h post-transfection, cells were washed and fixed for 5 min in 4% paraformaldehyde/PBS. For surface staining, cells were blocked in a 4% nonfat dried milk/PBS solution for 30 min prior to incubation with the primary antibody for 1 h. For permeabilized staining, the blocking stage was preceded by 5 min of incubation with 0.25% Triton X-100/0.1% Tween 20/PBS. The primary antibodies were diluted in a 1:2000 anti-rabbit or mouse antibodies conjugated to horseradish peroxidase (1:2000). For surface staining, the majority of the antibodies were used. Cells were then washed and fixed for 5 min in 0.5% paraformaldehyde/PBS and stained for another 30 min. The secondary antibody was then added by mixing with 0.5% normal goat serum and 0.1% saponin in PBS followed by incubation with primary and secondary antibodies at room temperature. After incubation on ice for 30 min in the primary antibody, the cells were briefly washed in cold PBS followed by incubation on ice with the secondary antibody for another 30 min. The stained cells were then fixed in 4% paraformaldehyde in PBS and mounted with ProLong Antifade mounting reagent (Molecular Probes). Intracellular labeling of the heterologous cells was performed in a similar manner as surface labeling, except for fixation with 4% paraformaldehyde in PBS before incubation with antibodies. After fixation, cells were treated with 5% normal goat serum and 0.1% saponin in PBS followed by incubation with primary and secondary antibodies at room temperature. Immunoprecipitation—Transiently transfected HEK 293 cells were harvested in ice-cold PBS 24 h after transfection. Cells were collected by centrifugation (1000 × g for 10 min at 4 °C). Cells were lysed using 100 μl of a Dounce glass homogenizer in 50 mM Tris- HCl, 5 mM EDTA, and 5 mM EGTA, pH 7.4, at 4 °C. Lysates were centrifuged at (27,000 × g for 15 min at 4 °C). The pellet was resuspended in a solubilization buffer (50 mM Tris, 0.5 mM NaCl, 5 mM EDTA, 5 mM EGTA, and 1% Triton X-100) containing a mixture of protease inhibitors at 1.5 mg/ml and solubilized by gently agitating for 1 h at 4 °C. After dilution to 1 mg/ml protein in solubilization buffer, the soluble material was collected by centrifugation (100,000 × g for 30 min at 4 °C). Samples of 10% of the total volume were taken from the soluble preparation for later analysis. The remaining supernatant was incubated with either 5 μg of the anti-NR1-C2 antibody or 5 μg of IgG overnight at 4 °C. A 50-μl aliquot of protein A was added and mixed by rotation for 4 h at 4 °C. For experiments using the M2 FLAG-agarose, the pellet was washed three times in solubilization buffer. The precipitated material was eluted from the beads by heating to 75 °C for 5 min in 0.5% sodium dodecyl sulfate and dithiothreitol. Both the samples of the soluble preparation and the precipitated material were analyzed by Western blotting.

Cell Surface ELISA—Cell surface assays using the monoclonal anti-Tac antibody were performed on transfected HeLa cells as described previously (8). For the studies using HEK 293 cells, the following protocol was used. Cells were plated onto poly-D-lysine coated cell culture plates. Twenty-four h post-transfection, cells were washed and fixed for 5 min in 4% paraformaldehyde/PBS. For surface staining, cells were blocked in a 4% nonfat dried milk/PBS solution for 30 min prior to incubation with the primary antibody for 1 h. For permeabilized staining, the blocking stage was preceded by 5 min of incubation with 0.25% Triton X-100/0.1% Tween 20/PBS. The primary antibodies were diluted in a 1:2000 anti-rabbit or mouse antibodies conjugated to horseradish peroxidase (1:2000). After four 10-min washes in 4% milk/PBS, 800 μl of 0.1% BSA solution (Neogen) was added for 25 min. The absorbance was determined within 30 min at 650 nm. The anti-NR2B was used at a final concentration of 15 μg/ml.

Cell Cytotoxicity—HEK 293 cells were transfected with NR1 and NR2B constructs. Twenty hours post-transfection, cell cytotoxicity was determined using the Promega Cytotoxic 96TM cytotoxicity assay according to the manufacturer’s instructions and as described previously (19). Radioligand Binding—[3H]MK-801 binding was performed with cell homogenates prepared from HEK 293 cells transfected with either NR1α1-NR2B2 or NR1α1-NR2B2<sub>αβ2</sub>. The assay was performed in 50 mM Tris citrate, pH 7.1. Aliquots (20 μg) of the cell homogenates were incubated in the presence of 20 mM [3H]MK-801 at room temperature for 2 h. Nonspecific binding was defined as binding of 100 μM thiencyclobexylylperidide. The reactions were terminated by rapid filtration through GF/B filters pretreated with 1% polyethyleneimine followed by three washes with carbonate buffer, pH 7.4, at 4 °C using a Brandel cell harvester.

RESULTS

An ER Retention Domain Is Present in the C Terminus of the NR2B Subunit—NR2B subunits are retained in the ER in heterologous cells (7) and in neurons (13), in the absence of NR1. Previous studies from several laboratories have shown that the NR1-1 splice variant is retained in the ER in the absence of NR2 because of an RXXR retention signal in the C1 cassette of the C-terminal domain. To determine whether a retention signal is also present in the C-terminal domain of NR2B, we constructed chimeras of the NR2B C terminus and the α chain of the interleukin-2 receptor (Tac) (Fig. 1A). Using these chimeras, we could investigate the properties of the NR2B C terminus isolated from the rest of the molecule following an approach used previously for the NR1 subunit (8). Wild type Tac is constitutively expressed on the cell surface, but appending segments of proteins to its cytoplasmic C terminus allows analysis of trafficking information contained within those seg-
ments. Chimeras containing the entire C terminus of NR2B completely eliminated surface staining of Tac, suggesting that a retention signal is present in the C terminus of NR2B (Fig. 1, B and C). Immunocytochemical staining of cells transfected with TacNR2B showed extensive co-localization with ER markers (Fig. 1D). A series of deletions of the TacNR2B construct was made to identify the region of the molecule containing the ER retention information. We have shown previously that Tac chimeras of the distal part of the NR2B C terminus (Ala-1315–Val-1482) are trafficked to the cell surface, indicating the absence of an ER retention signal in this region of the molecule (21). Truncation at His-1119 showed no surface label, whereas truncations at Tyr-1070 and Tyr-985 showed increasing degrees of surface expression (Fig. 1C). Quantification of surface expression using ELISA, in which the data are expressed as the ratio of surface label to total label, showed increases in surface expression in constructs terminated at Tyr-1070 and Tyr-985 (Fig. 1C). An RRR-(1110–1112) motif, which is the motif shown to be responsible for ER retention of NR1-1 as well as other proteins, is found between His-1119 and Tyr-1070; however, mutation of the RRR motif to AAA did not increase surface expression of the full-length TacNR2B construct (data not shown). Two other potential retention signals, KRRK-(1079–1082) and KKR-(1090–1092), are present in this segment, but mutation of these sites, in addition to mutation of the RRR site, did not change surface expression of the TacNR2B construct (data not shown). The graded increase in surface expression with truncations of TacNR2B and the lack of changes in surface expression with elimination of the leading consensus retention motifs suggest that either multiple sites are involved in retention of the NR2B C terminus or that a more complex structure is responsible for the retention.

Export of Assembled NMDA Receptors from the ER Is Not the Result of Interaction of C Termini and Masking of ER Retention Signals on NR1 and NR2—Complex proteins assembled from subunits that contain ER retention signals must use a mechanism to functionally mask the ER retention signals to allow the assembled complex to leave the ER. In some cases, this is done by an interaction of cytoplasmic domains of the subunits that leads to a structural masking of the retention signals. For example, the individual subunits of the ATP-sensitive potassium channels contain ER retention motifs that are masked after the assembly of the subunits, which leads to the correct stoichiometry of the channel (14). Previous studies of the NR1 subunit (8, 9, 10) and our present results on NR2B indicate the presence of ER retention signals on the cytoplasmic C termini of both subunits and suggest that C termini of the two subunits may interact and mutually mask their retention signals. Two approaches were used to determine whether mutual masking is involved. We first investigated whether the cytoplasmic domains of NR1 and NR2 interact. This was carried out using a yeast two-hybrid screen by cloning NR1 and NR2B C termini into the vectors pGBT9 and pACT2 and by co-immunoprecipitation experiments using detergent-solubilized (1% Triton X-100) preparations of HeLa cells transfected with TacNR2B1-644 and TacNR1-1a (8). In neither case was any interaction found (data not shown). These findings suggest that the NR1 and NR2B C termini do not directly interact and tend to rule
out interaction as a mechanism of masking their retention sites. It is possible, however, that masking is not the result of direct interactions of the cytoplasmic domains but, rather, results from an interaction involving other areas of the molecule. Also, an interaction between the two C termini may depend on the assembly of the two subunits to properly position the C-terminal domains for interaction. Thus, in isolation, the C termini may not interact. To address these points, we constructed a chimera of NR1 and the GABA\_B1 subunit. The GABA\_B1 receptor contains two subunits, GABA\_B1\_R1 and GABA\_B1\_R2, which must assemble to form a functional receptor (15, 16). The GABA\_B1\_R1 subunit contains an RXR ER retention motif, and this motif is masked in the assembled receptor by interaction of the two cytoplasmic C termini through a coiled coil interaction. The NR1/GABA\_B1\_R1 chimera contains the cytoplasmic C terminus of GABA\_B1\_R1, which includes the RXR motif (Fig. 2A). When expressed alone, the chimera is not trafficked to the cell surface following a pattern like that of NR1-1 or GABA\_B1, indicating that the retention signal is functioning properly. Previous studies have shown that the RRR motif is the only ER retention signal present on the NR1 molecule, and elimination of the motif allows the unassembled subunit to reach the cell surface (8). If masking is involved in negating ER retention of the NR1/NR2B complex, the NR1/GABA\_B1\_R1 chimera should not be trafficked to the cell surface because the foreign retention signal will not be masked by the NR2B subunit, which does not contain the coiled coil domain necessary for interaction to occur. We found, however, that the chimera assembles with NR2B and reaches the cell surface to form functional receptors (Fig. 2, B and C), suggesting that masking of the retention signals is not required for the assembled NMDA receptor to exit the ER and reach the cell surface.

A Motif in the Proximal C Terminus of the NR2B Subunit Is Required for Cell Surface Expression of NR1/NR2B Receptors—To investigate in more detail the role of the NR2B C terminus in the trafficking of NMDA receptors from the ER, we made a series of truncations of the full-length NR2B subunit and studied the effects on the cell surface expression of the assembled receptor. We found that removal of all but six amino acids of the NR2B C terminus still produced receptors that reached the cell surface when co-expressed with NR1 in heterologous cells (Fig. 3A). These subunits form functional ion channels when expressed in cultured cerebellar granule cells (Fig. 3B). These findings are consistent with those of previous studies (22, 23) that report the formation of functional NMDA receptors on co-expression of the NR1 subunit, with an NR2A subunit that contains only four amino acids of the C terminus. Removal of the entire C terminus (terminated at glutamic acid, immediately after TM4), however, completely eliminated surface expression of the receptor. This suggested that the four amino acid segments following TM4, HLFY, are critical for forming a functional receptor on the cell surface. A truncation made immediately after the transmembrane domain could cause the last transmembrane domain to form improperly and result in a non-functional receptor. To address this point and to investigate more fully the function of this domain of the NR2 subunit, mutations were made in the region immediately following TM4 of the full-length NR2B subunit. Sequential mutation of HLFY led to a loss of functional receptors, based on a cell death assay (Fig. 4A). Mutation of three amino acids, HLF, led to a complete loss of functional receptors. Because a mutation of tyrosine also decreased surface expression, in subsequent studies of the full-length NR2B, the four-amino acid sequence HLFY was mutated to AAAA. Surface expression was also determined using an antibody to an extracellular domain of NR2B. Co-transfection of NR2B\_HLFY\_AAAA with NR1-1 showed that the subunit is not expressed on the cell surface (Fig. 4B) and is retained in the ER (Fig. 4C).

The retention of NR2B\_HLFY\_AAAA could result from a lack of assembly with the NR1 subunit. To address that possibility, co-immunoprecipitation analyses were performed using cells transfected with NR2B\_HLFY\_AAAA and NR1. These studies showed that NR2B\_HLFY\_AAAA as well as NR2BWT assembled with NR1 (Fig. 5A). Therefore, NR2B\_HLFY\_AAAA assembles
with NR1, but the complex remains in the ER. Although NR2B_HLFY-AAAA assembles with NR1, the mutation in the NR2B subunit may lead to the formation of a misfolded subunit that can not form a functional receptor and is retained in the ER as a result of failing the quality control test. The functional characteristics of the complex cannot be determined directly using the standard physiological analysis of receptors on the plasma membrane because it is retained in the ER. Because glycine and glutamate binding are associated with the single subunits NR1 and NR2, respectively, the presence of these sites is not a valid indication of the formation of an assembled receptor complex. MK-801, however, which binds to the open pore of the NMDA receptor, only binds to the assembled receptor (24, 25). MK-801 binding analysis showed significant binding to the ER-retained NR1/NR2B_HLFY-AAAA complex, indicating the appropriate assembly of the subunits into a functional

Fig. 3. Effect of truncation of the NR2B C terminus on cell surface expression. A, COS7 cells were transfected with YFP-NR1-1 and NR2B, with deletions of the C terminus, and total and surface expressions were determined immunocytochemically. Surface labeling was carried out with an antibody to GFP to detect the NR1 subunit. The total represents native YFP fluorescence. For studies using NR2B_6 transfected alone, a GFP-tagged NR2B construct was used. Surface expression was detected with an antibody to GFP, and total expression represents native GFP fluorescence. Surface expression is detected with NR2B C termini with as few as six amino acids, but deletion of the entire C terminus (NR2B_0) resulted in no surface expression when expressed either with NR1 or alone. B, whole cell current measurement shows that NR2B_WT and NR2B_120 (containing 120 amino acids in its C terminus) increased the number of functional receptors on the cell surface when transfected into cultured granule cells, whereas NR2B_6 did not. Cerebellar granule cells were cultured and transfected as described previously (44). Cells were analyzed 48 h after transfection. Data are the mean ± S.E. of at least six cells obtained from two separate transfections. WT, wild type.

Fig. 4. Mutation of the proximal four amino acids of NR2B C terminus (HLFY) inhibited cell surface expression of NR1/NR2B receptors. Mutation of HLFY decreased surface expression in HEK 293 cells transfected with GFP-NR1-1a and NR2B. A, surface expression was quantified using a cell death assay. The histogram shows the percentage of cell death with transfection of NR1-1a and the different NR2B mutants relative to that of wild type NR1-1a/NR2B receptors. The data represent mean (±S.E.) from three independent transfections. B, surface expression was quantified using ELISA and antibodies to an extracellular epitope of NR2B. Data, expressed as arbitrary units, are the mean ± S.E. of three independent experiments. C, NR1-1a/NR2B_AAAA is retained in an intracellular compartment and very closely matches the distribution of calreticulin, an ER marker in transfected HeLa cells. GFP fluorescence was used to localize GFP-NR1-1a/NR2B_AAAA.
receptor complex that is retained in the ER. These results show that a four-amino acid segment of NR2B, HLFY, which immediately follows the fourth transmembrane domain, is not required for assembly with NR1 and formation of a functional receptor but is necessary for the assembled receptor complex to leave the ER.

**DISCUSSION**

Previous studies have shown that co-expression of NR1 and NR2 subunits is required for the formation of functional NMDA receptors on the cell surface (7). The major NR1 splice variant and the NR2 subunits are retained in the ER until assembly into a functional receptor, which is then released from the ER and is trafficked to the cell surface. Such a mechanism of quality control prevents the expression on the cell surface of unassembled or misfolded subunits and is a common step in the assembly process of complex multisubunit proteins (26). Retained subunits that do not assemble because of a lack of a partner or misfolding are degraded; the rapid turnover rate of a large population of NR1 is consistent with this subunit being synthesized in excess of NR2 and degraded in the absence of an assembly partner (27). Although both subunits are retained in the ER, they are processed differently. The excess production of NR1 optimizes the chance that NR2 subunits will find NR1 partners; although most of the NR1 subunits, with a half-life of approximately 1 h, are retained in the ER and degraded, no rapidly degraded pool of NR2 was detected. The optimization of processing NR2 is consistent with its role in determining the major functional characteristics of the receptor. This mechanism ensures that changes in the synthesis of NR2 subunits, such as those that occur during development, are efficiently conveyed through the production of functional complexes that reach the cell surface. The distinct differences between the two subunits would suggest that different mechanisms underlie their ER retention. As shown previously for the NR1 subunit, a Tac chimera containing the complete C terminus of NR2 is retained in the ER, indicating the presence of an ER retention signal (8). Unlike the NR1 subunit, however, in which a single RRR motif is responsible for the retention of the NR1-1 splice variant, the retention of NR2B does not appear to depend on a single motif. In truncation analyses carried out on the TacNR2B constructs, surface expression increased significantly after deletion of His-1119 to Tyr-1070. This segment contains three putative retention signals, KRRK, KKR, and RRR; however, mutating either the RRR site alone or all three sites together in the TacNR2B construct failed to increase surface expression. Additional truncation of the TacNR2B construct to Tyr-985 also significantly increased surface expression. From these results we conclude that sites other than or in addition to the KRRK, KKR, and RRR sites are involved in ER retention of NR2B.

Although ER retention of individual subunits is commonly used to ensure quality control in forming multisubunit protein complexes, the mechanism by which the assembled complex overrides the ER retention of the individual subunits has been studied in only a limited number of cases. Masking of the signals was elegantly described in the case of the ATP-sensitive K⁺ channel α and β subunits (32). A similar mechanism is involved in the GABA<sub>B</sub> receptor in which masking is dependent on the interaction of the R1 and R2 subunits through coiled-coil domains on the C termini (15, 16). Based on these studies, which involve multisubunit complexes, we hypothesized that a similar mechanism controlled NMDA receptors, but neither our truncation studies nor our NR1/GABA<sub>B</sub> receptor chimera studies are consistent with this mechanism. Truncation studies
that showed that functional receptor complexes are formed in the absence of either the NR1 or the NR2 C termini suggest that an interaction between the C termini is not required for ER exit. Our finding that a chimera of NR1 and the C terminus of GABAAβ1R1, which contains an RXR retention motif masked through a coiled coil interaction with GABAAβ2R2, can assemble with NR2 and traffic to the cell surface to form a functional receptor indicates that masking of this site is not required. These results suggest that the NMDA receptor is more likely controlled by a signal that indicates that the assembly process is complete rather than by masking of the retention signals.

Signals that control the export of proteins from the ER have been identified. A DXE motif accelerated the export of vesicular stomatitis virus glycoprotein from the ER (28). The KxR1 channel contains an FCYENE export signal in its cytoplasmic domain (29), and the dopamine D1 receptor has an FXXXFXXXF export motif (30). Our truncation analyses showed that deletion of most of the C terminus has no effect on functional receptors reaching the cell surface. Therefore, export of the assembled receptor from the ER could not be controlled by a signal in the deleted part of the molecule, which included nearly all of the C terminus. We found, however, that the four-amino acid segment HLFY, immediately following TM4, is required for the export of receptors from the ER. NR2B subunits in which HLFY is mutated to AAAA assemble with NR1 and form functional receptors based on MK-801 binding but are retained in the ER. These results are consistent with HLFY serving as a conformationally dependent signal that controls the release of the assembled receptor from the ER. This motif is absent from NR1 but is present in both NR2A and NR2B and differs by a single amino acid, HLVV, in NR2C and NR2D (Fig. 6). The area immediately following TM4 has been shown to be important in the trafficking of NMDA and other glutamate receptors. Tyr-842 of NR2A has been linked to surface regulation of NMDA receptors through its interaction with AP-2 and internalization of the complex following exposure to agonist (22). Because the movement of membrane vesicles from ER to Golgi does not involve clathrin, it is unlikely that retention of NR2BHLFY>AAAA is related to its interaction with AP-2; rather, our results suggest that this area of the molecule is important to the trafficking of the NMDA receptors at more than one step in the secretory pathway. This may be a mechanism common to other glutamate receptors because a 28-amino acid segment immediately following TM4 was shown to be necessary for surface expression of GluR6 (31).

Our findings are consistent with the use of at least two levels of ER retention in the production of NMDA receptors. One level involves the retention of the individual unassembled subunits, and the second level controls ER release of the assembled, functional receptor. Homodimer formation may be the first step in the assembly process (32). Dimers of NR1 can be detected as intermediates in the assembly process, and recent studies show the importance of disulfide bond formation between NR1 subunits in the formation of a functional receptor (33). Dimer formation in the processing of the NR2 subunit remains less clear; however, NR1 and NR2 dimers, if they are formed, are retained in the ER. The second level of ER retention involves the assembled complex. Recent studies suggest that ligand binding and/or channel function may be required for ER exit. Mutations in the ligand binding or the ion pore domain of the Caenorhabditis elegans glutamate receptor GLR-1 causes it to be retained in the ER (34), and mutation of the Q/R site affects ER retention of GluR2 (35, 36). Mutations in the binding site of the kainate receptor subunit GluR6 caused it to be retained in the ER (37). These findings support the notion that an important step in assaying channel quality in the ER involves a demonstration of appropriate ligand binding and/or channel function for some glutamate receptors as well as for receptors of other ligands (38, 39). Because glutamate is ubiquitous, with apparently high levels in the ER based on immunocytochemical studies (40), glutamate would be expected to be available to bind to a receptor in the ER where the binding site would be found. The fact that NR2BHLFY>AAAA can form a functional channel based on MK-801 binding but is not released from the ER indicates that function alone is insufficient for release from the ER. Furthermore, studies involving the truncation of NR1 also would argue against such a mechanism in the case of the NMDA receptor. NR1, truncated immediately N-terminal of TM4, assembles with NR2 to form a non-functional complex that reaches the cell surface, showing that function is not required for ER exit. Schorge and Colquhoun (41) found that NR1 and NR2 truncated immediately before TM4 could form functional receptors if expressed with the TM4 C terminus segments. Therefore, the TM4 tail segments apparently are capable of interacting with the remainder of the subunits to form a functional receptor that can pass the quality control mechanism of the ER.

In summary, our results suggest that interaction through an export signal involving the HLFY motif in some capacity is necessary for the functional NMDA receptor to leave the ER. Although this implies the interaction of a protein to facilitate ER export, our attempts to identify proteins that may interact with this motif failed, using both yeast two-hybrid screens and co-immunoprecipitation of candidate proteins. Conformational changes during assembly can alter exposure of motifs to interacting proteins and cause ER retention or export of proteins. For example, a conformationally dependent motif in a transmembrane domain of the nicotinic receptor is buried in assembled receptors but is exposed on the unassembled subunits, causing ER retention (42).

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