Expression of functional, recombinant α7 nicotinic acetylcholine receptors in several mammalian cell types, including HEK293 cells, has been problematic. We have isolated the recently described human ric-3 cDNA and co-expressed it in Xenopus oocytes and HEK293 cells with the human nicotinic acetylcholine receptor α7 subunit. In addition to confirming the previously reported effect on α7 receptor expression in Xenopus oocytes we demonstrate that ric-3 promotes the formation of functional α7 receptors in mammalian cells, as determined by whole cell patch clamp recording and surface α-bungarotoxin binding. Upon application of 1 mM nicotine, currents were undetectable in HEK293 cells expressing only the α7 subunit. In contrast, co-expression of α7 and ric-3 cDNAs resulted in currents that averaged 42 pA/pF with kinetics similar to those observed in cells expressing endogenous α7 receptors. Immunoprecipitation studies demonstrate that α7 and ric-3 proteins co-localize. Additionally, cell surface labeling with biotin revealed the presence of α7 protein on the plasma membrane of cells lacking ric-3, but surface α-bungarotoxin staining was only observed in cells co-expressing ric-3. Thus, ric-3 appears to be necessary for proper folding and/or assembly of α7 receptors in HEK293 cells.

Nicotinic acetylcholine receptors (nAChRs) are members of the neurotransmitter-gated ion channel superfamily. They are widely expressed in the central and peripheral nervous system where they influence numerous cellular and physiological processes. At least 17 different genes that code for nAChR subunits have been identified (2, 3), and they assemble as pentamers in different combinations to form a diverse set of nAChR subtypes (4, 5). The simplest case is the homopentameric complex such as that formed by the nAChR α7 subunit. The α7 receptor, for which α-bungarotoxin (α-Bgt) is a specific and high affinity antagonist, is one of the most abundant receptor subtypes in the mammalian brain (6, 7). The high Ca$^{2+}$ permeability of the α7 receptor (8) suggests an involvement in the activation of Ca$^{2+}$-dependent events in neurons such as transmitter release, participation in signal transduction, and a variety of modulatory effects (9). In addition, α7 receptors have been implicated in a number of diseases such as schizophrenia, Alzheimers, and Parkinsons disease (1, 10–12).

Heterologous expression of the α7 subunit in Xenopus oocytes results in homooligomeric, α-Bgt-sensitive receptors that activate and inactivate quickly and are highly permeable to Ca$^{2+}$ (8, 13, 14), similar to the properties of α7 nAChRs in neuronal cells. Although there have been reports of successful functional expression in some mammalian cell lines (15–18), measurable levels of functional receptors have been difficult to achieve in multiple cell types and this phenomenon appears to be host-cell dependent (19). The reasons for poor heterologous surface expression in these cells are not well understood. Strategies to increase the number of functional receptors on the cell surface, including alteration of culture conditions (20), the generation of α7-5HT3 chimeras (21), and site-directed mutagenesis (22) have met with some success. However, these strategies have resulted in only a modest increase in the number of functional receptors or the generation of non-native receptors, which are not ideal for drug discovery. Consequently, there is continued interest in identifying cellular factors that influence the expression of functional α7-containing nAChRs.

A screen to identify genes necessary for nAChR function in Caenorhabditis elegans was recently described (23). The search for suppressors of a dominant mutation in the nAChR subunit DEG-3 led to the identification of mutations in ric-3 (resistant to inhibitors of cholinesterase), and subsequent work demonstrated that ric-3 is required for the maturation of multiple nAChRs in oocytes (23). Recent work indicates that ric-3 is a member of a conserved gene family (24). The human homolog (hric3) has diverse effects on co-expressed receptors, including the enhancement of α7-mediated whole cell current amplitudes as well as the reduction of α4β2 and α3β4 currents in oocytes. In this study we further examined the effects of hric3 on α7 receptors. In addition to demonstrating increased current amplitudes when co-expressed with α7 receptors in Xenopus oocytes, we show an association between α7 and hric3 proteins and demonstrate that hric3 promotes the formation of functional α7 receptors on the surface of mammalian cells. We also present evidence that α7 protein can be detected on the surface of HEK293 cells lacking hric3 and those levels do not change significantly in the presence of hric3, thus implicating hric3 as a mediator of folding and/or assembly of nAChR α7 receptors.

**EXPERIMENTAL PROCEDURES**

Isolation of Hric3 Coding Sequence—Sequences encoding the hric3 subunit were isolated by standard PCR techniques. Briefly, total adult brain RNA (Clontech, Palo Alto, CA) was used as the template for first

**This paper is available on line at http://www.jbc.org**
strand cDNA synthesis using random hexamers and a Retroscript kit (Ambion, Austin, TX). An initial set of oligonucleotide primers was designed based on the hric3 sequences contained in the GenBank database (GenBank™ accession number NM_024557). A sense strand 23-mer, TGGCAGCACCCTGAGATGCTAGT (corresponds to hric3 nt –20 to 3), and an antisense 24-mer, CTGGAGAGGAGGTTCCA-TTGG (corresponds to hric3 nt 1142 to 1165), were used in amplification reactions with human adult brain cDNA and KOD Hotstart DNA polymerase (Novagen, Madison, WI). Reactions were performed at 94 °C for 5 min followed by 30 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 1 min and an additional cycle of 72 °C for 7 min. A second sense strand 34-mer, CTCTGGAGGAGTAAAGGATCCACTCCAGT-GCGAG (contains hric3 nt 1 in to 23 preceded by a ribosome binding site and an EcoRI restriction site), and a second antisense strand 34-mer, CCCTGGAGAGGAGGTTCCAATTCAGCATTGCGA(G contains hric3 nt 1111 to 1136 followed by an XhoI site), were used in a nested amplification reaction with the 5′-end primer extended within the 3′-end primer of the nested PCR product was sequenced to confirm its identity.

**PCR—** Total RNA was isolated from cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and first strand cDNA was synthesized as described above. Three primer pairs based on the cloned human sequence and two primer pairs based on partial rat sequences in GenBank (see Table 1) were used in amplification reactions performed at 94 °C for 5 min followed by 30 cycles of 94 °C for 20 s, 58 °C for 20 s, and 72 °C for 60 s. For human templates, primers to glyceraldehyde-3-phosphate dehydrogenase were used in amplification reactions to show the presence of cDNA.

**DNA Constructs and Expression—** For initial expression studies the full-length hric3 cDNA was subcloned into the EcoRI-XhoI sites of pcDNAs1. The (+). For biochemical studies a hric3 cDNA construct containing the hemagglutinin (HA) tag sequence, YPYDVPDYAL, at its COOH terminal was generated by standard PCR techniques. A full-length human α7 sequence was reported previously (25). The insert was subcloned into the EcoRI-XhoI sites of pcDNAs1. For transient expression studies in the BatHam-XhoI sites of pcDNAs/FRT for the development of a stable cell line using the flp-in system (Invitrogen, Carlsbad, CA). A cell line stably expressing the nACHR7 subunit, designated HEK293A, was developed in conjunction with the manufacturer’s instructions. For some biochemical studies an α7 cDNA construct containing the HA epitope tag at a unique HindIII site (nt 82) near the amino terminus of the α7 coding sequence was generated. For transient expression studies in the A7-3 stable cell line cells plated in 10-cm dishes were transfected with a total of 24 μg of a hric3-containing expression plasmid or the bacterial expression plasmid pGEM7z. For expression studies in which both α7 and δ1 subunits were present, co-transfection was performed using the whole cell patch clamp technique. HEK293 cells were transfected with α7 and hric3 DNAs in a 1:1 ratio. All transfusions were performed with Lipofectamine 2000 (Invitrogen) and transfection efficiency, estimated to be 70–90%, monitored by expression of a green fluorescent protein construct. Cells were examined for protein expression 24–48 h after transfection.

**Immunofluorescence Staining—** Mammalian cells expressing nACHR α7 alone, hric3 alone, or nACHR α7 + hric3 were plated on poly-l-lysine-coated glass coverslips (BD Biosciences, Bedford, MA) in a 24-well plate at a density of 1 to 2 × 10⁵ cells/12-mm coverslip. Live images were captured with Alexa Fluor 488-labeled α-Bgt (Molecular Probes) at a 1:50 dilution and a rabbit polyclonal anti-HA antibody (C-20, Santa Cruz Biotechnology) and 50 μg/ml of horseradish peroxidase-linked sheep anti-mouse Ig (Amersham Biosciences) for p42 MAP kinase detection, or with a 1:2000 dilution of horseradish peroxidase-linked goat anti-rabbit Ig (Amersham Biosciences) for Alexa Fluor 594-labeled goat anti-rabbit Ig (Molecular Probes) used at a 1:400 dilution. The primary antibody and α-Bgt were added directly to the growth media or diluted in PBS/bovine serum albumin and allowed to react with the cells for 1 h. Cells were rinsed 3 times and incubated with the secondary antibody for 30 min. Cells were rinsed 4 times with 1× PBS and fixed in 4% paraformaldehyde/PBS for 15 min at room temperature. After a final wash with 1× PBS, coverslips were dried and mounted on glass slides for visualization with a Zeiss LSM510 confocal microscope. In other experiments cells were fixed and permeabilized prior to Ab incubations.

**Xenopus Oocyte Electrophysiology—** Transient transfections for electrophysiological characterization included pCMVCD4, a human CD4 expression plasmid, to permit the identification of transfected cells. Prior to recording, cells were washed with mammalian Ringer’s solution, containing 140 mm NaCl, 5.4 mm KCl, 2 mm CaCl₂, 1 mm MgCl₂, 10 mm HEPES (pH 7.4), and 10 mm Na₂CO₃. A pipette was positioned near the oocyte and a 0.3 M-NaCl solution was applied for 10 min with a 0.1-μl volume. The oocyte was removed, placed in a 140 mm NaCl solution, and applied for 300 ms using a fast application system consisting of a triple-barrel glass pipette attached to an electromechanical switching device (piezo-electric drive, Winston Electronics, Millbrae, CA). The external solution contained (in mM): 120 NaCl, 3 KCl, 2 CaCl₂, 1 MgCl₂, 10 mm HEPES (pH 7.4), and 10 mm Na₂CO₃. The membrane potential of individual H9293 cells was held at –90 mV. Nicotinic was obtained from Sigma. Stock solutions were prepared in water and stored at –20 °C. Nicotine was dissolved in the external solution and applied for 300 ms using a fast application system consisting of a triple-barrel glass pipette attached to an electromechanical switching device (piezo-electric drive, Winston Electronics, Millbrae, CA). The speed of solution exchange between control and nicotine-containing solution, measured as the open-tip response, displayed a time constant of 0.7 ms, with a steady state reached in less than 1.5 ms. Xenopus Oocyte Electrophysiology—Sections of ovary were surgically isolated from anesthetized Xenopus frogs (Nasco, Fort Atkinson, WI). Mature females were anesthetized by immersion in 0.1% tricaine methanesulfonate solution and oocytes were surgically removed. The follicular cell layer was enzymatically removed by gentle shaking with collagenase (Worthington, Type II, 1.7 mg/ml for 90 min, then Sigma, Type II, 1.7 mg/ml for 30 min) in Ca²⁺-free Barth’s solution. Oocyte viability was confirmed by the absence of a high-resistance series circuit (27, with minor changes as indicated below). Oocytes were injected with 50 nl containing 25 ng of α7 in vitro synthesized mRNA. In vitro transcribed hric3 mRNA was injected in a 1:1 ratio when co-injected with rat or human α7. Following injection, oocytes were incubated at 16–19 °C for 4–7 days in Oocyte Ringer-3 medium containing 50% L-15 medium, 100 μg/ml gentamicin, 25 μg/ml tetracycline, 4 mm glutamine, and 30 mM Na₂HEPES (all from Invitrogen), with pH ad-
justed to 7.6 with NaOH. The extracellular recording solution (standard Ringer's) contained (in mM): NaCl (115), KCl (2.5), BaCl₂ (1.8), HEPES (10), atropine (0.001), pH 7.3. Functional expression was examined using the two-electrode voltage clamp technique; membrane potential was held at −70 mV. Concentration-response curves were obtained by normalizing the current responses to varying concentrations of ACh to the maximal current responses to 100 μM ACh.

RESULTS

Hric3 mRNA Is Present in Neuronal Cells and Absent in HEK293 Cells—We amplified and subcloned the hric3 coding sequence from human adult brain RNA using primers based on sequence in the GenBank data base. Sequence analysis indicates that the coding sequence isolated here is identical to GenBank™ accession number XM_219241 except for the absence of a single serine residue (Ser-173 in XM_219241). This polymorphism was described previously (24).

We used PCR to examine hric3 mRNA expression in several cell lines. Primers directed to portions of the hric3 coding sequence (Table I, Fig. 1A) were used to detect transcripts using standard PCR assays. As shown in Fig. 1B, we detected hric3 transcripts in brain tissue and the SH-SY5Y cell line, a human neuronal cell line known to express endogenous α7 receptors (28). In contrast, hric3 transcripts were not detected in HEK293 cells with primer pairs covering the majority of the hric3 coding sequence. A primer pair covering the extreme 3′ end of the coding sequence generated low, but detectable, levels of a fragment of the expected size from HEK293 cells, indicating that low levels of at least a portion of the hric3 transcript may be present in these cells.

We extended our analyses to determine whether ric-3 is expressed in additional cell lines known to express functional α7 receptors. Primers homologous to the rat ric-3 subunit (Table I, Fig. 1C) were again used in standard PCR assays using cDNA generated from rat brain, PC12, and GH4C1 cells. In all cases PCR products of the expected size were detected (Fig. 1D), indicating the presence of ric-transcripts in these cell lines.

α7 Protein Is Detected on the Surface of HEK293 Cells Lacking Hric3—We developed a HEK-based stable cell line constitutively expressing the α7 subunit (A7-3 cells) using the flp-in system. As demonstrated in Fig. 2A, A7-3 cells express α7 protein with a pattern similar to that observed in HEK293 cells transiently transfected with the human α7 subunit. The presence of the doublet at −60 kDa observed in HEK293 cells was similar to the pattern observed with human recombinant α7 expressed in GH4C1 cells² and may reflect differential post-translational processing of the α7 protein. Despite the presence of α7 protein, patch clamp studies failed to identify detectable currents through α7 channels (Fig. 7).

We next examined if the lack of functional expression was because of lack of surface expression of α7 receptors. We biotinylated cell-surface proteins and isolated them by binding to streptavidin-linked agarose beads. The presence of α7 protein was subsequently detected by Western blot analysis. Fig. 2B shows that in the A7-3 cell line a detectable, albeit a proportionally low, level of α7 protein was biotinylated and pulled down with streptavidin beads, indicating its presence on the cell surface. To exclude the possibility of intracellular protein contamination, we also looked for the presence of COX IV, an integral mitochondrial membrane protein, and p42 MAP kinase, a cytosolic protein, in the biotinylation experiment. As demonstrated in Fig. 2B, neither COX IV nor p42 MAP kinase were detected, consistent with the biotinylation of only cell-surface proteins.

α7 and Hric3 Proteins Co-associate—To determine whether hric3 can form a stable complex with the human α7 subunit we performed coimmunoprecipitations using untransfected A7-3 cells, A7-3 cells transiently expressing the hric3-HA fusion protein, or HEK293 cells transiently expressing the hric3-HA protein. Proteins were immunoprecipitated with subunit-specific antibodies from detergent extracts of total membrane fractions. The antibody to the HA epitope tag immunoprecipitated both the α7 subunit and the hric3-HA fusion protein from hric3-transfected A7-3 cells (Fig. 3, samples 1 and 5). There was no signal when using the HA epitope antibody with untransfected A7-3 cell extracts (samples 2 and 6). Conversely, the α7-specific antibody immunoprecipitated both the α7 subunit and hric3 subunits from hric3-transfected A7-3 cells (samples 3 and 7). There was no signal from hric3-transfected HEK293 cells (samples 4 and 8). These results demonstrate that α7 and hric3 are able to form a stable complex in HEK293 cells.

α7 Does Not Alter α7 Expression Levels in HEK293 Cells—We next examined if hric3 co-expression alters the overall expression levels of the α7 subunit or the levels of α7 found on the cell surface of HEK293 cells. Total cell lysates and biotin-labeled surface proteins prepared from cells expressing α7 alone or α7 and hric3 were subjected to Western analysis (Fig. 4A). Lanes 1 and 3 of Fig. 4A indicate that the overall expression levels of α7 protein did not differ significantly when co-expressed with hric3. In addition, we observed no significant difference in the levels of cell-surface α7 when hric3 was co-expressed (Fig. 4A, lanes 2 and 4). Identical results were obtained when both α7 and hric3 were transiently expressed in HEK293 cells (data not shown). These experiments indicate that hric3 does not play a direct role in regulating the trafficking of α7 receptors to the cell surface.

We also used the biotin labeling technique to determine whether hric3 can be detected on the cell surface. The presence of hric3 protein in lane 2 of Fig. 4B indicates that a significant proportion of the hric3 protein expressed in HEK293 cells was...
Co-expression of Ric3 and Nicotinic α7 Receptors

**Fig. 1. Distribution of ric-3 transcripts.** A and C, location of PCR products (PCR1-PCR3 for human and PCR4-PCR5 for rat, respectively) generated from ric-3 coding regions. Coding regions are denoted by shaded rectangles. B and D, PCR assays were performed as described under “Experimental Procedures” and products were separated on 1.5% agarose gels.

**Fig. 2. Immunoblot analysis of a7 expression in HEK293 cells.** A, total a7 expression in HEK293 cells. Total membrane proteins (10 μg/lane) from transiently transfected HEK293 cells and an HEK293-based stable cell line were separated on an 8% gel and immunostained with polyclonal antisera specific for the a7 subunit. B, cell surface expression of the a7 subunit. Biotinylated surface proteins from whole cells were isolated with neutravidin-linked beads and separated on a 4–20% gradient gel. After protein transfer to nitrocellulose the filter was cut horizontally at the 50-kDa marker and below the 36-kDa marker to allow identification of multiple proteins in the same lane. The upper filter was immunostained with a7 antisera, the middle filter was immunostained with P42 MAP kinase antisera, and the lower filter was immunostained with a COX IV mAb. The subunits are denoted with arrows.

**Fig. 3. Physical association of a7 and hric3 subunits.** Anti-a7 or anti-HA epitope antibodies were used to immunoprecipitate proteins from Triton-solubilized cell membrane fractions. For hric3 the immunoprecipitation was performed with hric3-transfected A7-3 cells (samples 1 and 5) or with untransfected A7-3 cells (samples 2 and 6). For a7 the immunoprecipitation was performed with hric3-transfected A7-3 cells (samples 3 and 7) or with hric3-transfected HEK293 cells (samples 4 and 8). Proteins were separated on 8% gels and immunostained with the indicated antibodies. Total membranes from untransfected HEK293 cells or hric3-transfected A7-3 cells (first two lanes of each blot) were included on the gels as references.

experiments indicate that in HEK293 cells hric3 co-expression is necessary for binding of α-Bgt to a7 receptors.

**Hric3 Co-expression Enhances a7-Mediated Currents in Oocytes—**The magnitudes of currents induced by ACh (10 μM to 1 mM) were compared between oocytes injected with rat a7 mRNA alone (25 ng in 50 nl) or with rat a7 and hric3 transcripts (25 ng each, 50 nl total). Oocytes co-injected with rat a7 and hric3 showed larger currents in response to 1 mM ACh (2.9 ± 0.5 μA, mean ± S.D.) than oocytes injected with rat a7 transcript alone (0.68 ± 0.5 μA; p = 0.01, t test, Fig. 6A). ACh-induced currents were also larger in oocytes co-injected with human a7 and hric3 (6.1 ± 2.9 μA) than in those injected with human a7 nAChR alone (2.7 ± 1.3 μA; p < 0.03; data not shown). Despite these changes, the apparent potency of ACh was not significantly different in the two groups: 235 μM (+hric3, Fig. 6B) versus 334 μM (−hric3) for the rat receptor and 198 μM (+hric3) versus 162 μM (−hric3) for the human receptor.

**α7-Mediated Currents in HEK293 Cells in the Presence of Hric3—**Previous efforts to detect functional receptors following transient transfection of α7 nAChRs into HEK293 cells failed to show any detectable currents upon rapid application of agonist, in contrast to the large currents detected in parallel experiments in HEK293 cells transiently transfected with...
α7 expression levels ± hric3 and hric3 surface expression. A, cell surface expression of the α7 subunit. Biotinylated surface proteins from A7-3 cells ± hric3 were isolated with neutravidin-linked beads and separated on a 4–20% gradient gel. Total cell lysate from the same biotinylated cells was run in parallel. After protein transfer to nitrocellulose, the filter was cut horizontally at the 50-kDa marker and below the 36-kDa marker to allow identification of multiple proteins in the same lane. The upper filter was immunostained with α7 antisera, the middle filter was immunostained with p42 MAP kinase antisera, and the lower filter was immunostained with a COX IV mAb. The subunits are denoted with arrows. B, cell surface expression of hric3. Biotinylated surface proteins from A7-3 ± hric3 were processed as in A but the upper filter was immunostained with the HA tag antibody.

Co-expression of Rat α7 nAChR with hric3 in Xenopus Oocytes. Co-expression of Rat α7 nAChR with hric3 in Xenopus Oocytes. A, superimposed traces of current responses to 1 mM ACh applied during the time indicated by the horizontal bar in two different oocytes injected with rat α7 (black trace) or rat α7 + hric3 (gray trace) transcripts. Oocytes were from the same batch and recorded on the same day. B, the potency for ACh derived from the concentration-response curve obtained in oocytes injected with rat α7 + hric3 transcript was 235 μM.

α4β2 or α3β2 nAChR subunits. Similarly, untransfected or mock-transfected A7-3 cells (n = 11) failed to show any detectable inward currents upon application of 1 mM nicotine (see Fig. 7). However, following transient transfection of A7-3 cells with a hric3 expression plasmid, we observed robust, agonist-induced inward currents (Fig. 7). All cells that were transfected, as determined by the binding of CD417-specific beads, showed clear, detectable currents; mean current density = 42 ± 39 pA/pF (n = 12). Nicotine-induced currents displayed very fast kinetics of activation and desensitization (Fig. 7), a hallmark of α7-mediated currents. Thus, hric3 promoted the expression of functional α7 receptors in these cells.

Discussion
There are several examples of the detection of functional, recombinant α7 receptors expressed on the surface of mamma-
For the maturation of sites on the cell surface and that the increase in the number of hnic3. It is conceivable that the increase in current amplitudes seen with receptors when injected only with ric-3. Halevi et al. (24) suggest that ric-3 does not locate inside the cell and plays a role in folding or assembly, processes that take place in the endoplasmic reticulum (ER). In our recombinant expression system, experiments indicate that some of the hnic3 protein expressed is located on the cell surface. Transmembrane prediction algorithms indicate the presence of two transmembrane domains within the hnic3 protein, but such algorithms often cannot distinguish between a signal peptide and a transmembrane domain (32). Indeed, sequence analysis using either SIGNALP2.0, a signal peptide predictor, or Phobius, a relatively new combined transmembrane topology and signal peptide predictor (33), suggests that hnic3 contains a signal peptide. Taken together our data indicate that hnic3 can be found as an integral plasma membrane protein and likely has a single transmembrane domain with its NH2 terminus located on the extracellular surface. Whether ric-3 exerts its effects on nAChRs in the ER or the plasma membrane, the C0OH-terminal coiled-coil domain following the transmembrane domain is predicted to be located within the cytoplasm. Here it may be available to exert its effects directly or bring together α7 subunits and, as yet, unidentified proteins involved in the maturation process.

The list of known accessory/chaperone proteins that mediate folding, assembly, or targeting of nicotinic acetylcholine receptors continues to expand. Calnexin, an ER-resident membrane protein, was found to moderately increase overall expression levels or on the amount of α7 protein on the cell surface. Based on these data it appears that ric3 does not play a direct role in regulating the transport of α7 protein to the cell surface, but more likely facilitates proper folding or assembly of α7 complexes.

The mechanism by which ric-3 exerts its effects on α7 subunits remains to be determined. Halevi et al. (24) suggest that ric-3 is located inside the cell and plays a role in folding or assembly, processes that take place in the endoplasmic reticulum (ER). In our recombinant expression system, experiments indicate that some of the ric3 protein expressed is located on the cell surface. Transmembrane prediction algorithms indicate the presence of two transmembrane domains within the ric3 protein, but such algorithms often cannot distinguish between a signal peptide and a transmembrane domain (32). Indeed, sequence analysis using either SIGNALP2.0, a signal peptide predictor, or Phobius, a relatively new combined transmembrane topology and signal peptide predictor (33), suggests that ric3 contains a signal peptide. Taken together our data indicate that ric3 can be found as an integral plasma membrane protein and likely has a single transmembrane domain with its NH2 terminus located on the extracellular surface. Whether ric3 exerts its effects on nAChRs in the ER or the plasma membrane, the C0OH-terminal coiled-coil domain following the transmembrane domain is predicted to be located within the cytoplasm. Here it may be available to exert its effects directly or bring together α7 subunits and, as yet, unidentified proteins involved in the maturation process.
other entity reported to act as a chaperone protein, appears to play a role in subunit stabilization as it increased the steady state levels and consequently the surface expression levels of the α7 subunit expressed in tsA201 cells (37). Each of these proteins can contribute to nAChR surface expression. Still other proteins, such as lynx1 (38) and the integral membrane protein VILIP-1 (39), have been shown to increase the surface expression levels as well as the functional properties of nAChRs.

In addition to the accessory proteins described above, PDZ-containing proteins of the PSD-95 family have been shown to associate with native α7 receptors in chick ciliary ganglia (40), although their identity was not firmly established. In parallel experiments individual PDZ proteins were tested for their ability to associate with α7 expressed in HEK293 cells. Interestingly, there was no significant association of α7 with any of the PSD95 family members tested. It is possible that splice variants of those members tested or other untested family members associate with α7. Alternatively, the folding/assembly step provided by hric3 may be required for the proper association of α7 with PDZ-containing proteins.

The precise mechanisms by which ion channels fold and assemble are unknown. Ion channel complexity, brought about by their generally large size and requirements for proper subunit composition and stoichiometry, cause their assembly to be a relatively slow and inefficient process (41). Experimental evidence indicates that nAChRs assemble in the ER, but recent evidence suggests that this is an inefficient process (41). Experimental evidence indicates that nAChRs assemble in the ER, but recent evidence suggests that this is not known if these late folding events occur in the ER, but they appear to be required for channel function. The precise role that ric-3 plays in this process, including the location within the cell where it exerts its effects, is unclear and requires further exploration. Nevertheless, robust expression of α7 receptors in non-neuronal cells for high-throughput screening purposes should now be possible with the co-expression of ric-3. Additional characterization of the interactions between ric-3 and nAChRs will further the understanding of the control of nAChR expression and function.

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