Differential Subcellular Localization of RIC-3 Isoforms and Their Role in Determining 5-HT3 Receptor Composition

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RIC-3 has been identified as a chaperone molecule involved in promoting the functional expression of nicotinic acetylcholine and 5-HT3 receptors in mammalian cells. In this study, we examined the effects of RIC-3a (isoform a) and a truncated isoform (isoform d) on RIC-3 localization, mobility, and aggregation and its effect on 5-HT3 receptor composition in mammalian cells. Human RIC-3a possesses an amino-terminal signal sequence that targets it to the endoplasmic reticulum where it is distributed within the reticular network, often forming large diffuse “slicks” and bright “halo” structures. RIC-3a is highly mobile within and between these compartments. Despite the propensity for RIC-3a to aggregate, its expression enhances the level of surface 5-HT3A (hommeric) receptors. In contrast, RIC-3a exerts an inhibitory action on the surface expression of heteromeric 5-HT3A/B receptors. RIC-3d exhibits an altered subcellular distribution, being localized to the endoplasmic reticulum, large diffuse slicks, tubulo-vesicular structures, and the Golgi. Bidirectional trafficking between the endoplasmic reticulum and Golgi suggests that RIC-3d constitutively cycles between these two compartments. In support of the large coiled-coil domain of RIC-3a being responsible for protein aggregation, RIC-3d, lacking this cytoplasmic domain, does not aggregate or induce the formation of bright aggregates. Regardless of these differences, isoform d is still capable of enhancing homomeric, and inhibiting heteromeric, 5-HT3 receptor expression. Thus, both isoforms of RIC-3 play a role in determining 5-HT3 receptor composition.

The ligand-gated ion channels are critical participants in cellular communication, playing a major role in synaptic transmission. The ligand-gated ion channels include receptors for acetylcholine (nACh), γ-amino-butyric acid type A, serotonin (5-HT), and glycine (the cys-loop superfamily) and N-methyl-d-aspartic acid, kainate, and AMPA (glutamate receptors). A vast array of receptor-interacting proteins have been identified as participating in receptor trafficking and localization (1) and have led to dramatic advances in our knowledge of synaptic architecture and plasticity.

Chaperone molecules play an important role in the intracellular transport of receptors. However, a fundamental question in neurobiology remains: How is receptor biogenesis orchestrated? Despite the fact that receptor composition determines receptor function and their pharmacological repertoire, little information is available regarding how this may be achieved. Specific assembly signals exist that can determine a preference for particular subunit partners (2). However, few selective chaperone molecules have been implicated in the process of receptor biogenesis. General chaperone proteins such as BiP, calnexin, calreticulin, and PDI do operate on the ligand-gated ion channels but offer no specificity. In contrast, molecules such as stargazin (3), PSD-95 (4), 14-3-3 (5), and RIC-3 (6) (7–12) are beginning to offer insight into protein-specific chaperone activity.

Intriguingly, 14-3-3 has been reported to alter the stoichiometry of αβ2 nACh receptors, as a result of the stabilization of α4 subunits (5). Similarly, RIC-3 has been reported to promote the folding, assembly, and surface expression of some (α7) nACh receptors yet inhibit the expression of others (α3β4 and α4β2) when expressed in Xenopus oocytes (7). However, when analyzed in mammalian cells, nACh receptor compositions were either promoted (α7, α8, α3β2, α3β4, α4β2, α4β4) or unaffected (α9, α9α10), with no evidence of inhibition (9, 12). Similarly, RIC-3 has been shown to promote the functional surface expression of 5-HT3A in mammalian cells (10).

The 5-HT3 receptors in the peripheral nervous system are thought to modulate pain and intestinal and cardiovascular functions (13). In the central nervous system, 5-HT3 receptors are important targets for the control of emesis induced by chemotherapy/radiotherapy and have been implicated in schizophrenia (13). The 5-HT3A receptor is capable of functioning as a homomeric ion channel. However, the conductance of these recombinant receptors is too small to be resolved directly (sub-picosiemens) and the receptors do not resemble many native neuronal 5-HT3 receptors (14–15). In contrast, 5-HT3B subunits do not function as homomeric channels but are retained in the endoplasmic reticulum (ER) unless assembled into heteromeric (5-HT3A/B) receptors (16, 17). Electrophysiological analysis suggests that both 5-HT3 receptor types may co-exist within the same neuron (14, 18–20), raising the question of how this may be achieved.
in this study we have investigated the role of RIC-3 isoforms (a and d) (7) on 5-HT3A and 5-HT3A/B receptor expression in mammalian cells. The two RIC-3 isoforms exhibit overlapping, but distinct, localizations between the ER and Golgi. In keeping with a role in receptor biogenesis, these compartments are involved in protein synthesis, assembly, and N-linked glycosylation. Despite differences in RIC-3 isoform localization and the propensity to aggregate, both isoforms promote the surface expression of homomeric receptors and inhibit the formation of heteromeric receptors. Thus, RIC3 can manipulate 5-HT3 receptor composition.

MATERIALS AND METHODS

Cell Culture and Transfection—Simian COS7 cells (ATCC CRL 1651) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 μg/ml streptomycin, and 100 units/ml penicillin in an atmosphere of 5% CO2. Exponentially growing cells were transfected by electroporation (400 V, infinity resistance, 125 μF, Bio-Rad Gene Electropulsers II). 10 μg of DNA was used per transfection (2 × 106 cells), using equimolar ratios of expression constructs (unless otherwise stated). Cells were analyzed 12 to 48 h after transfection.

DNA Constructs—Human 5-HT3A subunit cDNAs were expressed from the mammalian expression vector pGW1 (16). The 5-HT3Amyc and 5-HT3Bmyc have been reported previously (17). 5-HT3A-YFP was generated as reported previously (21). 5-HT3B-CFP was generated by PCR to position CFP immediately downstream of the hemagglutinin epitope (YPY-DVPDYA/SR/MVS K . . . DEL YK/IE/QD SAL). Underlined are residues introduced as the result of the incorporation of restriction sites for cloning purposes. Both 5-HT3A-YFP and 5-HT3B-CFP were capable of assembling into cell surface-expressed receptors as wild-type receptors. Human RIC-3a was a kind gift from M. Treinin (Hebrew University) and used as previously described (10). RIC-3 isoform d and tRIC3 were generated by PCR to generate RIC3 variants terminating with the sequence . . . YILFKLSKGK (RIC3) or . . . YILFKVSR ILLT-ILHQ (isoform d). RIC-3-N and RIC-3-C possessing artificial N-glycosylation sites, AFAKANGGGAGGGG (K80N for RIC-3-N) and LRRKNGSGLE (P364G, Q365S for RIC-3-C), were generated by site-directed mutagenesis. RIC-3-SS-DsRed was generated by replacing the amino-terminal methionine of DsRed with the amino-terminal 33 residues of RIC-3 isoform a. RIC-3-YFP, RIC-3-pHluorin, and RIC-3-DsRed were generated by PCR, cloning the relevant fluorescent protein between residues 30 and 31 of RIC-3. The fidelity of all constructs was verified by DNA sequencing. The addition of these fluorescent probes to the amino terminus of RIC-3 did not alter their subcellular distribution when compared with untagged RIC-3. Furthermore, these chimeras exhibited the same modulatory behavior on 5-HT3 receptors. ER-Red and Golgi-YFP were purchased from Clontech.

Antibodies and Reagents—Rabbit anti-Myc antibodies (Santa Cruz Biotechnology) were used as directed. Antiserum (sheep) to RIC-3 were generated as previously reported (10) and used at 0.5 μg/ml for Western blotting. The secondary horseradish peroxidase-conjugated antibodies were purchased from Perbio and used at 1/5000. Lysotracker-Red (Molecular Probes) and monodansylcadaverine (Sigma) were used as recommended by the supplier.

Quantification of Cell Surface Expression—Transfected COS7 cells were plated into 24-well dishes. Cells were fixed in 3% paraformaldehyde (in phosphate-buffered saline) 12–24 h post-transfection. Cell surface detection was performed in the absence of detergent, and total expression levels were determined following Triton X-100 (0.5%, 15 min) treatment. Briefly, following permeabilization (if required) cells were washed twice in 50 mM NH4Cl (in phosphate-buffered saline) and blocked (5% Marvel, 0.5% bovine serum albumin in phosphate-buffered saline) for 1 h. Receptor expression was detected via the extracellular Myc epitope using rabbit anti-Myc for 1 h, followed by five washes in block, and then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h. Excess antibody was removed by five further washes in block. Horseradish peroxidase levels were assayed using 10 μM Amplex Red (Invitrogen) as the substrate (plus 0.003% H2O2 in sodium phosphate buffer) with excitation at 560 nm and detection at 590 nm after 30 min using a Spectramax Gemini EM plate reader (Molecular Devices).

Immunoprecipitation—COS7 cells were L-methionine starved for 30 min before being labeled with [35S]methionine (0.2 mCi/6-cm dish) (Translabel ICN/Flow) for 4 h. Cells were lysed in 10 mM sodium phosphate buffer containing 5 mM EDTA, 5 mM EGTA, 50 mM sodium fluoride, 50 mM sodium chloride, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 2% Triton X-100, 0.5% deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 10 mg/ml antipain, 10 mg/ml pepstatin, and 0.1 mg/ml aprotinin (lysis buffer). Triton X-114-soluble and -insoluble fractions were produced using 30 °C, 15 min, to precipitate the detergent/membranes and centrifuged at 15,000 × g. The insoluble pellet was washed twice (washed at 4 °C and then precipitated at 30 °C) prior to returning to 4 °C for immunoprecipitation. Immunoprecipitations were performed as described previously (16) and analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography.

Radioisogird Binding—[3H]GR65630 binding was performed on intact and transiently transfected COS7 cells cultured in 24-well plates and analyzed 1 day post-transfection. Mock-transfected cells were used as controls. A saturating concentration (3 nM) of [3H]GR65630 was incubated in sextuplicate for 2 h in binding buffer (135 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 10 mM D(+)-glucose, pH 7.4). Following five washes in binding buffer, cells were solubilized with 0.5% Triton X-100 and counted in a scintillation counter.

Photobleaching Studies—Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching in COS7 cells were carried out using a Zeiss LSM510 confocal imaging system incorporating a heated chamber (35 °C). Optimal conditions for photobleaching were determined using a 488-nm argon laser line (100% power, 20 scans). Post-photobleach imaging was performed using minimal laser excitation to reduce indirect bleaching.
As expected, the RIC-3-SS-DsRed is localized to the ER, as evidenced by the reticular staining pattern (Fig. 1B). To test whether the signal sequence is cleaved, we probed for the secretion of DsRed into the culture medium. RIC-3-SS-DsRed-transfected cells were pulse-labeled with \[^{35}\text{S}]\text{methionine and chased overnight in the absence of radiolabel. DsRed was immunoprecipitated from the medium and cell lysates. To distinguish soluble and membrane-bound DsRed within cells, cells were lysed using Triton X-114. Lysates were separated into soluble and insoluble (membrane) fractions prior to immunoprecipitation. DsRed was detected in both the medium and the soluble cellular fraction, confirming that the signal sequence is cleaved (Fig. 1C). It is not clear why two molecular mass forms (~28-kDa bands) exist, when only one of ~28 kDa is expected, but this finding is reproducible \((n=3)\). It is not likely to be due to the presence/absence of the signal sequence as both forms are detected in the medium and soluble fractions. No sites for \(N\)-glycosylation exist within DsRed, ruling out different glycosylation. Regardless, these results indicate that the amino-terminal region of RIC-3 is capable of functioning as a cleavable signal sequence.

As stated above, RIC-3 has no predicted sites for \(N\)-glycosylation with which to probe the membrane topology of RIC-3. Therefore, we introduced two novel sites by site-directed mutagenesis at positions 80 or 363. These are predicted to reside extracellularly (RIC3-N) or intracellularly (RIC3-C), respectively. A comparison of the molecular mass of each protein, in the presence or absence of tunicamycin (to inhibit \(N\)-glycosylation) reveals that the molecular mass of RIC3-N is increased by ~2–3 kDa, consistent with \(N\)-glycosylation at this engineered site (Fig. 1D, +). Furthermore, this increase in size is lost in the presence of tunicamycin (Fig. 1D, −). In contrast, no changes in the molecular mass of RIC3-C were evident. These findings support the predicted extracellular localization of the pre-TMD1 region (downstream from the signal sequence) and the cytoplasmic localization of the carboxyl terminus of RIC-3.

Controversy also exists with respect to the subcellular localization of RIC-3. The consensus of opinion supports an endoplasmic reticulum localization of RIC-3 isoform a (RIC-3a) \((6, 7, 10, 11, 24)\). However, RIC-3 has been reported in the Golgi (11), within intracellular aggregates (11, 24), and on the cell surface (9). To address this issue, COS7 cells were co-transfected with organelle markers, ER-Red combined with RIC-3-YFP or Golgi-YFP combined with RIC-3-Red (monomeric DsRed). Clearly, RIC-3a is localized predominantly to the ER (Fig. 2A, left panels). Although RIC-3a is not concentrated in the Golgi, it may be present at low levels (11). Interestingly, in other cells RIC-3a is also present within anomalous structures that are not observed in the absence of RIC-3 expression (Fig. 2A, right panels). Two distinct types of structures can be observed: A diffuse slick of low fluorescent intensity (Fig. 2B, thin arrows) and an intensely fluorescent aggregate (Fig. 2B, wide arrows) that appears hollow when imaged using lower exposures (Fig. 2, A and C, and supplemental Fig. S1). Both these structures occur adjacent to the ER (Fig. 2B and supplemental Fig. S2). Similar structures are evident upon the expression of untagged RIC-3a (not shown), eliminating the possibility that protein aggregation is being induced by YFP or DsRed dimerization.

When RIC-3a-YFP is co-expressed with 5-HT3A-CFP, both proteins are observed to co-localize within the ER (not shown) and within both aggregate structures, depending on which...
structures are evident within the cell (Fig. 2C and supplemental Fig. S3). Similarly, when 5-HT3A-CFP, 5-HT3B-YFP, and RIC-3a-Red are co-localized in the same cell, all three proteins are co-localized in the ER and aggregates (Fig. 2C and supplemental Fig. S3). That the 5-HT3 receptors do not accumulate in these structures when expressed in the absence of RIC-3a, combined with the fact that the aggregates are induced by RIC-3a expression, suggests that RIC-3a is able to direct 5-HT3 receptors into these structures. To determine specificity, we investigated the co-expression of the γ-aminobutyric acid type A receptor subunit, γ2Long, which remains unassembled within the ER in the absence of α and β subunits (26). In contrast to the results observed for 5-HT3 receptors, the γ2Long-Red protein did not co-localize to these RIC-3a-YFP-containing structures (Fig. 2C and supplemental Fig. S3).

To address the question of possible cell surface expression of RIC-3a, a fusion incorporating pHluorin, a pH-sensitive variant of GFP that can report on surface labeling when expressed extracellularly was constructed (27). The pHluorin moiety is located within the extracellular (as determined by the introduction of an N-glycosylation site) (Fig. 1D) domain of RIC-3. As such, the pHluorin would be sensitive to pH changes in the extracellular environment but only if RIC-3 were expressed on the cell surface. At an extracellular pH of 8, RIC-3a-pHluorin fluorescence is observed with the ER (pH 7) and aggregates, with no evidence of surface fluorescence (Fig. 2D, left panels). Fluorescence microscopy. In the majority of cells possessing the bright aggregates, these appear relatively static, suggesting that their formation was essentially complete (not shown). However, in cells possessing relatively few aggregates, we were able to capture the process of their formation. In these cases, both the bright aggregates and diffuse sicks are able to form rapidly and exhibit significant mobility (Fig. 3A and supplemental movie S1). Both structures formed ubiquitously throughout the cell with no evidence of any focal point of initiation. Many of the diffuse RIC-3a sicks appear to contain a bright spot of fluorescence, suggesting the existence of a subdomain containing a higher concentration of RIC-3a. Interactions between the diffuse sicks and the bright aggregates were evident in those cells exhibiting high aggregate mobility, with the diffuse sicks being often consumed by the bright aggregates (Fig. 3B and supplemental movie S2).

The progressive aggregation of RIC-3a may serve to concentrate RIC-3a (and associated receptors) for degradation by either lysosomes or proteasomes. We investigated whether the RIC-3a-containing structures co-localize to either of these compartments. No localization of RIC-3a-YFP to lysosomes (lysotracker-Red) or proteasomes (ubiquitin staining) was evident (Fig. 4 and supplemental Fig. S4). Another possibility is that the aggregates are cleared by autophagic vacuoles (28). However, no co-localization of RIC-3-YFP structures with autophagic vacuoles (labeled with monodansylcadaverine) was observed for 5-HT3 receptors, the ER, but not the Golgi, and RIC-3 aggregates are distinct from both compartments. Identical results were observed when RIC-3a-pHluorin was expressed in primary hippocampal neurons (Fig. 2D, right panels). Thus, when expressed recombinantly, RIC3a exists predominantly within the ER and ER-associated structures, with no evidence of surface or endosomal expression. These experiments were performed rapidly (<2 min), eliminating any significant effects of the treatments on protein trafficking.

Given the pleomorphic nature of the RIC-3a aggregates, we investigated the potential for dynamic morphological changes of RIC-3a-YFP and RIC-3a-Red by time-lapse microscopy.
RIC-3 Regulates 5-HT3R Composition

**FIGURE 3.** Time-lapse images of RIC-3a aggregates in COS7 cells. A and B, COS7 cells transfected with RIC-3a-YFP were imaged by fluorescence time-lapse microscopy and selected images shown. A, RIC-3a aggregates form rapidly. B, interplay between small, brightly fluorescent aggregates and large diffuse slicks of low fluorescence. The arrow identifies a large diffuse slick being consumed by a bright aggregate. The fluorescence intensity of the bright aggregate increases concomitantly with the removal of the diffuse slick. Time-lapse movies are provided as supplemental data, supplemental movies 1 (A) and 2 (B). Scale bar, 20 μm.

**FIGURE 4.** RIC-3a protein aggregates do not co-localize with known degradation compartments. COS7 cells expressing RIC-3a-YFP were loaded with lysotracker-Red or monodansylcadaverine (MDC) or processed for immunofluorescence using anti-ubiquitin antibodies and imaged by fluorescence microscopy. Larger merged images can be seen in supplemental Fig. S4. Scale bar, 20 μm.

The short cytoplasmic domain “VSRIILTILHQ” rather than the large coiled-coil domain of RIC-3a (7). Therefore, we investigated the subcellular localization of RIC-3d. RIC-3d-YFP is seen within the ER (ER-Red co-localization) where it is distributed in a diffuse and punctate pattern (Fig. 5B and supplemental Fig. S5). Like the tRIC-3 protein, at lower exposure levels RIC-3d is observed to concentrate within the Golgi (Fig. 5B and supplemental Fig. S5). Similarly, RIC-3d does not induce the formation of bright aggregates. However, it is often present within the diffuse slick-like structures and tubulo-vesicular structures (supplemental movie S3).

As RIC-3d does not induce the formation of bright aggregates, we investigated whether protein aggregates of RIC-3a, but not RIC-3d, exist. We expressed RIC-3a or RIC-3d in COS7 cells and compared the effects of the non-reducible cross-linker dimethylpimelimidate on the molecular mass of the RIC-3 isoforms. A number of apparently nonspecific bands were evident, although the existence of endogenous isoforms of RIC-3 cannot be ruled out. Regardless, in the absence of cross-linking, recombinantly expressed RIC-3a is evident as a band of ~60 kDa, as reported previously (9, 10). Following cross-linking, the 60-kDa band is lost and a high molecular mass smear is observed, consistent with the preservation of RIC-3a protein aggregates (Fig. 5C). RIC-3d migrated at a lower molecular mass of ~15 kDa, in keeping with its reduced polypeptide length. RIC-3d did not exhibit any loss of the 15-kDa band or the formation of high molecular mass species upon protein cross-linking (Fig. 5C). This is consistent with the coiled-coil domain of RIC-3a being responsible for both RIC-3 protein aggregation and the formation of bright aggregates.

To investigate the mobility of both RIC-3 isoforms within and between the ER, diffuse slick, bright aggregates, and Golgi, we performed FRAP experiments. In RIC-3a-YFP-expressing cells, when an area of the ER was photobleached recovery of the short cytoplasmic domain “VSRIILTILHQ” rather than the large coiled-coil domain of RIC-3a (7). Therefore, we investigated the subcellular localization of RIC-3d. RIC-3d-YFP is seen within the ER (ER-Red co-localization) where it is distributed in a diffuse and punctate pattern (Fig. 5B and supplemental Fig. S5). Like the tRIC-3 protein, at lower exposure levels RIC-3d is observed to concentrate within the Golgi (Fig. 5B and supplemental Fig. S5). Similarly, RIC-3d does not induce the formation of bright aggregates. However, it is often present within the diffuse slick-like structures and tubulo-vesicular structures (supplemental movie S3).

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**FIGURE 5.** Subcellular localization of a truncated form RIC-3 and RIC-3 isoform d. A, COS7 cells expressing a truncated RIC-3-DsRed (tRIC3) and Golgi-YFP. B, RIC-3d-YFP and ER-Red (upper panels) or tRIC-3d-DsRed and Golgi-YFP (lower panels) were imaged by fluorescence microscopy. RIC-3 is observed to be concentrated within the Golgi but also exhibits an ER-like staining pattern. Larger merged images can be seen in supplemental Fig. S5. Scale bar, 20 μm. C, COS7 cells expressing RIC3a, RIC3d, or mock-transfected (C) were cross-linked with dimethylpimelimidate; cells were lysed, separated on a 4–20% gradient gel, and probed by Western blotting using anti-RIC-3 antibodies. Specific bands at ~60 and 15 kDa identify RIC-3a and RIC-3d, respectively.
RIC-3 Regulates 5-HT3R Composition

**FIGURE 6.** Fluorescence recovery after photobleaching (FRAP) on RIC-3a. A–D, COS7 cells expressing RIC-3a-YFP were imaged by fluorescence microscopy following 30 bleach scans of defined regions of interest (as indicated). Recovery was monitored within the region bleached for the time indicated and is expressed as a relative value, with 0 being the fluorescence intensity following bleaching (background) and 100% representing maximal recovery. The regions bleached and monitored include a defined region of the ER (A), or diffuse slick (B), or the entire structure for the diffuse slick (C) or bright aggregate (D).

**FIGURE 7.** Photobleaching analysis on RIC-3d. A, COS7 cells expressing RIC-3d-YFP were imaged by FRAP following 30 bleach scans of defined regions of interest (as indicated). Recovery was monitored within the region bleached for the time indicated and is expressed as a relative value, with 0 being the fluorescence intensity following bleaching (background) and 100% representing maximal recovery. B, COS7 cells expressing RIC-3d-YFP were examined following repeated rounds of FRAP to determine the fluorescence loss in photobleaching of RIC-3d in both the Golgi and ER when photobleaching was limited to either the total Golgi or total ER regions. Fluorescence is expressed as fluorescent intensity in the Golgi (black lines) or ER (gray lines).

**Fluorescence occurred with a $t_{1/2} \sim 40$ s, indicating that RIC-3a is mobile within the ER (Fig. 6A). Similarly, RIC-3a is highly mobile within the diffuse slicks and bright aggregates, with recovery from photobleaching occurring very rapidly in both diffuse slicks ($t_{1/2} \sim 10$ s) (Fig. 6B) and bright aggregates ($<3$ s, not shown as full recovery is evident at the first scan following FRAP). Moreover, when an entire structure was photobleached, fluorescence recovery occurred with a $t_{1/2} \sim 80$ s (Fig. 6C, diffuse slicks) and $\sim 20$ s (Fig. 6D, bright aggregates), indicating that delivery to these structures is continuing.

Given the localization of RIC-3d to both the Golgi and ER, we investigated whether the protein traffics dynamically between these two compartments. The distribution of RIC-3d between the ER and Golgi is unchanged following treatment with cycloheximide (100 μg/ml; supplemental Fig. S6). This suggests that the ER localization is a bona fide resident compartment of RIC-3d and not simply the result of ongoing biosynthesis. Following the photobleaching of a section of the ER, recovery of fluorescence occurred with a $t_{1/2} \sim 100$ s (Fig. 7A), confirming that RIC-3d is mobile within the ER. When the entire ER region (apart from the small region interspersed with the Golgi) was photobleached, fluorescence still recovered (in the presence of cycloheximide) with a $t_{1/2} \sim 170$ s. Similarly, when the entire Golgi region was photobleached, fluorescence recovery occurred with a $t_{1/2} \sim 90$ s. Together, these findings suggest that RIC-3d fluorescence may recover by protein recruitment from the other compartment. To address this issue directly, we examined fluorescence loss in photobleaching. FRAP was carried out repeatedly to either the Golgi or an ER region. In each photobleach session, photobleaching followed by fluorescence recovery is seen (Fig. 7B). Following repeated photobleaching of the Golgi, a simultaneous rundown of fluorescence intensity occurred in both the Golgi and ER ($t_{1/2} \sim 2300$ s). Similarly, when a region of the ER was repetitively photobleached, fluorescence intensity decreased in both the ER and Golgi ($t_{1/2} \sim 1800$ s). A control region from a non-photobleached cell (in the same field of view) was monitored to ensure that photobleaching during imaging did not contribute to the observed decreases.

Given the difference in the molecular architecture and subcellular distributions of these two RIC-3 isoforms, it is possible that they may have differential effects on 5-HT3 receptor expression. Therefore, we investigated whether both isoforms exhibit chaperone-like activity on 5-HT3 receptors. RIC-3a has been shown previously to enhance human 5-HT3A receptor surface expression and function in mammalian cells (10). We investigated, therefore, the effects of both isoforms of RIC-3 on the level of homomeric 5-HT3Amyc receptor expression, using the cell enzyme-linked immunosorbent assay approach to quantify cell surface and total receptor expression. The Myc epitope tag on the 5-HT3 receptors is located extracellularly, permitting surface receptor detection (17). When 5-HT3A receptors were co-expressed with either RIC-3 isoform (1:2 cDNA ratio), no significant effect on the total expression levels was observed (Fig. 8A). In contrast, both isoforms of RIC-3 produced a large increase in the level of surface expression of 5-HT3A receptors, as reported previously for the RIC-3a isoform (10).

5-HT3 receptors may also be constructed as heteromeric 5-HT3A/B receptors. In the presence of either RIC-3 isoform, cells expressing 5-HT3A and 5-HT3Bmyc exhibited a pronounced inhibition of cell surface expression, as determined by the Myc epitope on 5-HT3Bmyc (Fig. 8A, B'). The total expression level of 5-HT3A/3B Myc receptors was unaffected...
by the expression of either RIC-3 isoform. Thus, increased receptor instability is not responsible for the inhibitory effect of RIC-3 on heteromeric 5-HT3 receptor surface expression. When the Myc epitope was present on the 5-HT3A subunit, identical results to that observed for 5-HT3A homomers were evident. Thus, in the presence of either isoform of RIC-3 and both subunits of 5-HT3, there is a preferential formation of homomeric 5-HT3A receptors. In keeping with the enhancement of the surface expression of 5-HT3A receptors, both isoforms of RIC-3 enhance the number of ligand binding sites to a similar degree (Fig. 8B).

**DISCUSSION**

5-HT3 receptors are localized to both the central and peripheral nervous systems (20, 29, 30) where they may play roles, for example, in cognition, anxiety and emesis (central nervous system), and gut motility (peripheral nervous system) (13). The pharmacological and physiological characteristics of homomeric and heteromeric 5-HT3 receptors are distinct (29, 31). To date, it is not known how the molecular composition of 5-HT3 receptors is determined or whether it is regulated. Indeed, receptor biogenesis is poorly understood for all members of the ligand-gated ion channels. Although general chaperone proteins such as BiP, calnexin, and protein disulfide isomerase operate on ligand-gated ion channels, they offer no specificity. However, recent discoveries of specialized chaperone molecules, which operate on a restricted subset of ligand-gated ion channels, include PSD-95 (3), stargazin (3), RIC-3 (6–12, 24), and 14-3-3 (5).

In* Xenopus* oocytes, RIC-3a has been shown to be required for functional expression of the a7 nicotinic acetylcholine (nACh) receptor (6, 9, 11, 12, 24). In contrast, it inhibits the expression of a4β2 and a3β4 (7) but has no effect on receptors for γ-aminobutyric acid type A, glutamate, and glycine (6, 7). However, a comprehensive analysis in mammalian cells revealed that RIC-3a could enhance the expression of a7, a8, a3β4, a4β2, and a4β4 but did not affect a9 or a9α10 (12). Thus, it appears that RIC-3a may operate distinctly in amphibian versus mammalian cells, with no evidence of any inhibitory action on nACh receptors in mammalian cells. That human RIC-3a enhances human 5-HT3A expression in COS7 cells (Ref. 10 and this study), whereas human RIC-3a inhibits mouse 5-HT3A expression in *Xenopus* oocytes (7, 11), suggests that RIC-3 function may be tuned to its environment. Perhaps other host-specific factors are involved.

Some discrepancies have been reported on the molecular structure of RIC-3 (6–8, 10, 11). Although *C. elegans* RIC-3 has no amino-terminal signal sequence (6), human RIC-3 is predicted to possess one (11). Our findings support the presence of an amino-terminal-cleavable signal sequence on human RIC-3a (and RIC-3d) that is capable of driving ER targeting. Given the results of this study, human RIC-3a exists as a single pass type I transmembrane protein with an extracellular amino terminus and a cytoplasmic tail. RIC-3a is localized predominantly to the ER, although Golgi localization has also been reported (11). This localization is consistent with its participation in receptor biogenesis and/or transport. This is supported by the ability of RIC-3a to enhance ligand binding and modulate receptor expression (7, 9, 11, 12, 24). Although we see no evidence of the surface expression of RIC-3, it remains possible that in the presence of other receptor subunits RIC-3a may be co-transported to the cell surface, as reported to occur with nACh a7 receptors (9).

In addition to the localization of RIC-3a to the ER, in many cells a variety of anomalous structures have been observed (11, 24). We show that these structures form rapidly (<1 h) and exhibit highly dynamic mobility during their formation. That the bright aggregates appear to consume the larger diffuse sicks suggests a stepwise process is occurring. This is consistent with the observation that following prolonged expression the majority of cells possess only immobile bright aggregates. Nicotinic acetylcholine receptors have been found to co-localize with RIC-3a in these structures (11, 24). Likewise, we show that both 5-HT3A homomers and 5-HT3A/B heteromers are
also localized within these RIC-3a-containing structures. It has been speculated that these structures represent protein aggregates that may explain the inhibitory action of RIC-3a on α4β2 receptors (11, 24). However, it should be noted that in mammalian cells an enhancement of α4β2 expression by RIC-3a is observed (12) in contrast to the inhibition seen in *Xenopus* oocytes (7). Moreover, α7 receptors were also localized to RIC-3a aggregates (11), which are receptors that are widely accepted as being enhanced by RIC-3a expression in both expression systems. Our localization of both homomeric 5-HT3A (enhanced by RIC-3a) and heteromeric 5-HT3A/B (inhibited by RIC-3a) receptors to these structures also argues against such a role for these aggregates. This is supported further by the fact that these structures do not co-localize with any degradation compartments such as lysosomes, proteasomes, or autophagic vacuoles. In addition, the mobility of RIC-3a within these compartments argues against this being a terminal destination.

The fluorescence pattern of the bright aggregates appears to be identical to that of the organized smooth endoplasmic reticulum (OSER) (25). The OSER is produced by the formation of stacked ER membranes as a result of the high expression of molecules capable of low affinity anti-parallel interactions (25). Indeed, the bidirectional transport of proteins between the OSER and ER has been reported to occur (25). Although the OSER may be induced as an artifact of cytoplasmic green fluorescent protein interactions, the fluorescent tags used in this study are within the luminal domain of the ER. Moreover, similar aggregates are observed for wild-type RIC-3a. Despite the similarities between the RIC-3a bright aggregates and the OSER, further studies are required to determine the identity of the diffuse slicks and bright aggregates formed by RIC-3a. The existence of RIC-3a cross-linked species with molecular masses of ~120, 180, and 240 kDa supports the potential for homophilic RIC-3 interactions. A role for the cytoplasmic domain in protein interactions is supported by the observation that it is required for both protein aggregation and the formation of the bright aggregates, but not its modulatory effects on 5-HT3 receptors. Thus, the RIC-3a bright aggregates may result from cytoplasmic interactions between RIC-3a molecules and/or between RIC-3a and other (unidentified) proteins. It remains to be determined whether these RIC-3a aggregates are physiologically relevant or artifacts of overexpression in recombinant systems. Regardless, it will be important to determine what physiological interactions occur within this domain of RIC-3a.

Interestingly, RIC-3d is an isoform of RIC-3 that lacks the coiled-coil domain of RIC-3a and does not induce bright aggregate formation. RIC-3d trafficking between the ER and Golgi is consistent with the existence of mobile tubulo-vesicular structures. Despite this altered subcellular distribution, RIC-3d exhibits the same enhancement of 5-HT3A, and inhibition of 5-HT3A/B, receptor expression observed for RIC-3a. This is consistent with a requirement for the amino-terminal region in the enhancement of the *C. elegans* DEG3 receptor and the inhibition of α4β2 nACh receptor (8, 11), but not the requirement for the cytoplasmic tail in the enhancement of α7 nACh receptors (11, 24). It may be that distinct regions of RIC-3 are responsible for its effects on different receptors. Analogous to the findings of this study, the effect of RIC-3 on DEG3 receptors may be mimicked by altering the ratios of DEG3 to DES2 (8), supporting the hypothesis that RIC-3 expression may determine receptor composition. However, RIC-3 has not yet been shown to exert any inhibitory effects on nACh receptors in mammalian cells (12).

RIC-3 may exert its effects on nACh and 5-HT3 receptors by affecting receptor stability, folding, assembly, or trafficking. Of course, these events are inter-related. We can be more certain regarding the inhibitory effects of RIC-3 on 5-HT3A/B receptors. This does not lead to an increase in receptor instability, as total receptor levels do not decrease upon RIC-3a co-expression despite the observed drop in surface levels.

Given the major impact of RIC-3 on 5-HT3 receptor expression, by promoting homomeric receptors at the expense of heteromeric receptors, it will be important to determine whether RIC-3 expression is regulated. Unfortunately, little is known about the spatial and temporal expression profiles of RIC-3 isoforms or whether its expression may be regulated during physiological or pathological states. Moreover, we do not yet know how the other RIC-3 isoforms (b and c) impact on nACh and 5-HT3 receptor expression or, indeed, the contribution of RIC-3C-E (32).

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