Two subunits of the 5-hydroxytryptamine type 3 (5-HT₃) have been identified (5-HT₃A and 5-HT₃B) that assemble into homomeric (5-HT₃ₐ) and heteromeric (5-HT₃ₐ+5-HT₃₇) complexes. Unassembled 5-HT₃B subunits are efficiently retained within the cell. In this study, we address the mechanism controlling the release of 5-HT₃B from the endoplasmic reticulum (ER). An analysis of chimeric 5-HT₃ₐ receptor(R)-5-HT₃₈R constructs suggests the presence of elements downstream of the first transmembrane domain of 5-HT₃B subunits that inhibit cell surface expression. To investigate this possibility, truncated 5-HT₃₈B subunits were constructed and assessed for their ability to access the cell surface in COS-7 and ts201 cells. Using this approach, we have identified the presence of an ER retention signal located within the first cytoplasmic loop between transmembrane domains I and II of 5-HT₃B. Truncation of this signal (CRAR) into the homologous region of 5-HT₃ₐ results in the ER retention of this subunit until rescued by co-expression of 5-HT₃A and 5-HT₃B, 5-HT₃₈B is rescued and expressed on the cell surface as a heteromeric complex with a channel conductance similar to that of most native receptors (9). Electrophysiological studies suggest that both homomeric and heteromeric 5-HT₃ₐRs may co-exist within the same neuron (6, 8, 10, 11).

The 5-HT₃Rs belong to the superfamily of transmitter-gated ion channels that includes the nicotinic acetylcholine, GABAₐ, and glycine receptors. The structural relationship of the 5-HT₃Rs to the other members of this group suggests that their assembly may involve similar post-translational events (12). The assembly of GABAₐ receptors is directed by specific assembly signals within the amino-terminal extracellular domain (13–20). The export of receptors from the ER represents a critical checkpoint for surface expression, with quality control within the lumen of the ER performed by the chaperone proteins BiP, calnexin, and protein disulfide isomerase (9, 21, 22). Interaction with such proteins can cause the intracellular retention of immature proteins by virtue of ER retention signals within the chaperone molecules. In addition, cytoplasmically exposed ER retention signals within the cargo proteins themselves have been identified as elements that control protein export from the ER (23–27). A unifying mechanism for the regulation of ER export by these cytoplasmically localized signals is not known. However, a role for COPI and COPII recruitment of proteins into ER transport vesicles has been postulated (Refs. 28–31, but see Ref. 24).

To investigate the mechanisms involved in the ER retention of the 5-HT₃₈R, we have examined the assembly and surface expression of 5-HT₃ₐ-5-HT₃₇ subunit chimeras and truncation mutants of 5-HT₃₇. We demonstrate that 5-HT₃₇ possesses an ER retention signal capable of preventing homomeric cell surface expression. This “CRAR” signal requires masking by subunit interactions with the 5-HT₃ₐ subunit, regardless of whether it is present natively in the 5-HT₃₇ subunit or recombinantly within the homologous position in 5-HT₃ₐ. In addition, the export of 5-HT₃₇ from the ER appears to require the masking or exposure of other signals downstream from this ER retention signal.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Simian COS-7 cells (ATCC CRL 1651) and mammalian embryonic kidney tsA201 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100
The protocol above was followed to construct the 5-HT<sub>3</sub>α-HA cDNA, using the following primers: 5'-GAGAACCATGCTTACTGGCCCTATGAAAGCTGCCAGAAGCAGGGA-3' and 5'-GTTGAAGGGAATCCCATATGGCGTCAAAAG-3'. The amplified products were digested with HindIII and XhoI and ligated between the HindIII and XhoI sites of pCDM8.

The amplified products and HA epitope (EQKLISEEDL), and the epitope tag sequence is tagged using the Myc or hemagglutinin (HA) epitopes. These cDNAs, at equimolar ratios of expression constructs. Cells were analyzed 12–48 h after transfection. DNA was introduced into tsA201 cells by lipofection, using the Effectene reagent (Qiagen) according to the manufacturer’s instructions. Cells were analyzed 40–44 h after lipofection.

The chimera 5-HT<sub>3</sub>B-HA was tagged using the Myc or hemagglutinin (HA) epitopes. These cDNAs, at equimolar ratios of expression constructs. Cells were analyzed 12–48 h after transfection. DNA was introduced into tsA201 cells by lipofection, using the Effectene reagent (Qiagen) according to the manufacturer’s instructions. Cells were analyzed 40–44 h after lipofection.

The subunit assembly of 5-HT<sub>3</sub>Rs was examined by heterologous expression of 5-HT<sub>3</sub>A or 5-HT<sub>3</sub>B, 5-HT<sub>3</sub>A-Myc/5-HT<sub>3</sub>B-Myc, and 5-HT<sub>3</sub>B-Myc/5-HT<sub>3</sub>A subunit cDNAs in COS-7 cells. To facilitate biochemical and morphological analyses, 5-HT<sub>3</sub>R subunits were tagged with the Myc epitope (EQKLISEEDL), and the epitope tag sequence is tagged using the Myc or hemagglutinin (HA) epitopes. These cDNAs, at equimolar ratios of expression constructs. Cells were analyzed 12–48 h after transfection. DNA was introduced into tsA201 cells by lipofection, using the Effectene reagent (Qiagen) according to the manufacturer’s instructions. Cells were analyzed 40–44 h after lipofection.

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The subcellular distribution of the tagged 5-HT₃Rs expressed in COS-7 cells was determined by immunofluorescence and confocal microscopy. As shown previously (9), 5-HT₃A-Myc when expressed alone and assessed for Myc immunofluorescence revealed surface labeling, as determined by secondary antibody conjugated to Alexa Fluor 488, in the absence of detergent (surface). The same cells were then permeabilized and processed as above, using secondary antibodies conjugated to Alexa Fluor 568 (intracellular). The scale bar represents 20 microns. COS-7 cells expressing the 5-HT₃B-Myc, the chimeric 5-HT₃Rs, 5-HT₃A-Myc-5-HT₃B-Myc (3A-B) and 5-HT₃A-Myc-5-HT₃B-Myc (3B-A) were probed with antibodies against the Myc epitope, followed by secondary antibodies conjugated to Alexa Fluor 488, in the absence of detergent (surface). The same cells were then permeabilized and processed as above, using secondary antibodies conjugated to Alexa Fluor 568 (intracellular). The scale bar represents 20 microns. COS-7 cells expressing 5-HT₃B-Myc (3B), 5-HT₃A-Myc-5-HT₃B-Myc (3A-B) and 5-HT₃A-Myc-5-HT₃B-Myc (3B-A) were probed with antibodies against the Myc epitope, followed by secondary antibodies conjugated to Alexa Fluor 568 (intracellular). The scale bar represents 20 microns.

To probe for the existence of regions influencing cell surface expression within the 5-HT₃B-Myc subunit, a series of truncated 5-HT₃B subunits was constructed. Subunits were truncated after the transmembrane domain I, between transmembrane domain I and II (following residue 267), after transmembrane domain II, and between transmembrane domains III and IV (following residues 349, 369, 380, 393, 411, and 418) (Fig. 2A). The rationale for this approach is that if inhibitory signals exist within 5-HT₃B and are responsible for ER retention, then once all such signals are removed the resulting protein will be released from the ER and expressed on the cell surface. To confirm the fidelity of the truncated constructs, COS-7-transfected cells were [³⁵S]methionine-labeled and immunoprecipitated using 9E10 antibodies. The approximate molecular masses of the polypeptide backbones of the truncations are expected to be: 28.8 kDa (TM-I-stop), 29.4 kDa (267-stop), 31.8 kDa (TM-II-stop), 38.4 kDa (349-stop), 40.6 kDa (369-stop), 41.8 kDa (380-stop), 43.2 kDa (393-stop), 45.2 kDa (411-stop), 46 kDa (418-stop), and 48.5 kDa (full-length). In addition, 5-HT₃B-Myc has five potential N-linked glycosylation sites, each expected to contribute ~2–3 kDa to the molecular mass. The apparent molecular mass of the 5-HT₃B-Myc-truncated subunits (Fig. 2B) is consistent with the presence of multiple sites for N-linked glycosylation and the absence of degradation products, as observed previously (9).

When expressed in COS-7 cells, truncations of 5-HT₃B at positions 418, 411, 393, 380, 369, 349, and TMII all had no effect on ER retention and did not result in surface expression (Fig. 2C, lower panels). In contrast, when 5-HT₃B was truncated to residue 267, found between TM-I and TM-II, surface expression was observed. Similarly, a further truncation of 5-HT₃B such that the construct terminated at TMII, also resulted in cell surface expression. Quantification by a cell-based enzyme-linked immunosorbent assay confirmed the results observed by immunofluorescence. No significant cell surface detection of 5-HT₃B or when the construct was truncated to TMII was observed. In contrast, a significant enhancement of cell surface receptors (>14-fold, compared with TMII truncation) could be detected for the TMII truncation. These findings suggest the existence of an ER retention signal within 5-HT₃B within the first cytoplasmic domain, between TMII and TMII. Interestingly, this cytoplasmic domain possesses a potential ER retention motif “RAR” analogous to those found in other receptors (23, 25, 26, 34).

Comparison of the homologous regions between 5-HT₃A and 5-HT₃B (Fig. 3A) revealed that these subunits differ in only four residues incorporating the RAR motif. To assess the capacity of this putative motif to function in ER retention we generated a mutant 5-HT₃A-R containing CRAR in place of SGER, termed 5-HT₃A(CRAR). In keeping with a role in ER retention, this signal is capable of preventing the cell surface expression of 5-HT₃A(CRAR) (Fig. 3B). To eliminate the possibility that the
mutant receptor subunit was unable to correctly fold and assemble, the 5-HT3AR(CRAR) was co-expressed with wild-type 5-HT3AR. Under these circumstances the 5-HT3AR(CRAR) was rescued from the ER in a similar manner to the wild-type 5-HT3BR subunit. Furthermore, the 5-HT3AR(CRAR) could not be rescued by wild-type 5-HT3BR (not shown). To assess the role of this ER retention signal within 5-HT3BR, a reciprocal mutation was constructed, yielding 5-HT3BR(SGER). The subcellular distribution of this construct was indistinguishable from wild-type 5-HT3BR, in that it was efficiently retained within the ER when expressed alone yet could be rescued upon the co-expression of 5-HT3AR, but not 5-HT3BR (Fig. 3B).

The failure of the 5-HT3AR(SGER) to access the cell surface might be explained by the existence of other ER retention signals. To address this issue, the 5-HT3BR(SGER) construct was used to generate truncations at residue 270 and TMII. The truncation of 5-HT3BR(SGER) at residue 270 (immediately following SGER) resulted in cell surface expression (Fig. 3C). Furthermore, and in direct contrast to the findings of the 5-HT3AR (Fig. 2C), when the 5-HT3BR(SGER) was truncated at TMII significant cell surface expression was observed (Fig. 3C). These findings confirm the ability of the RAR signal to function as an ER retention signal in both 5-HT3AR and 5-HT3BR.

In an attempt to understand how the identified ER retention signal might operate, we sought to determine whether a direct masking of the ER retention signal might occur between the two homologous domains in 5-HT3AR(SGER) and 5-HT3BR(CRAR) subunits. To address this possibility, 5-HT3AR(CRAR) and 5-HT3BR(SGER) were co-expressed and assessed for cell surface expression. Our findings indicate that these two subunits are
To examine whether the 5-HT3BTMI-II construct is masking the ER retention signal(s) and promoting cell surface expression of 5-HT3B-HA, these constructs were co-expressed in COS-7 cells and precipitated via the 5-HT3ATMI-II fragment (9E10) or the full-length subunit fragment (12CA5). Significant interactions (Fig. 4A) are observed when the immunoprecipitation is performed using the 12CA5 antibody directed against the full-length subunits. Much less dramatic, yet still evident, is an interaction detected via the 9E10 antibody against the subunit fragment, 5-HT3BTMI-II. Although these results do not prove direct subunit interactions are required for the masking of the ER retention signal, it does show that subunit interactions occur within this region. Identical results were observed when the immunoprecipitation is performed using the 9E10 antibody against the subunit fragment, 5-HT3BTMI-II. 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identity (and significance) of the other co-purifying proteins is unknown.

**DISCUSSION**

To date, two subunits of the human 5-HT_{3}Rs have been cloned, 5-HT_{3}A (4, 5) and 5-HT_{3}B (5, 8). 5-HT_{3}A is capable of forming functional homomeric receptors exhibiting a very low single channel conductance (sub-picosiemens) (5, 35, 36). In contrast, recombinant 5-HT_{3}B does not form functional ion channels (5) because of a failure to exit the ER and reach the cell surface (9). Upon co-expression of the human 5-HT_{3}A and 5-HT_{3}B subunits, receptors with a large (16 pS) single channel conductance, low relative permeability to calcium ions, and a current-voltage relationship similar to that observed for many neuronal 5-HT_{3}Rs are produced (5). From these observations, it is evident that 5-HT_{3}B expression is regulated by processes that control receptor assembly. Such regulation could occur at several stages, including subunit oligomerization and export from the ER. Consistent with the failure of the 5-HT_{3}B subunit to produce functional receptors (5, 8), we have shown previously (9) that the 5-HT_{3}B subunit does not reach the cell surface but is efficiently retained within the ER as identified by the characteristic “lace-work” reticular staining pattern.

There are two possible explanations for the localization of 5-HT_{3}B in the ER (unless rescued by the 5-HT_{3}A subunit). First, the 5-HT_{3}B may not be capable of attaining the appropriate tertiary or quaternary structure unless co-assembled with 5-HT_{3}A. In this scenario, the 5-HT_{3}B would remain bound to ER chaperone proteins, BiP and calnexin (9, 21), and be retained within this compartment by virtue of the ER retention signals residing on these chaperone molecules. By analogy with the GABA_{A} receptors, the most likely failure to achieve a functional maturity is the lack of appropriate assembly signals within the large extracellular amino terminus (13–20). A second possibility is the exposure of cytoplasmic ER retention signals that actively retain the subunit within the ER until masked by the assembly with another subunit, interactions with other proteins, or phosphorylation (23, 24, 26, 28, 29, 34, 37, 38).

To discriminate between these possibilities, the subcellular distribution of 5-HT_{3}R chimeras was investigated. Interestingly, when the extracellular region of 5-HT_{3}A was fused (beyond TMI) to the downstream (including both cytoplasmic) regions of 5-HT_{3}B (5-HT_{3}A/5-HT_{3}B), no cell surface expression was observed. This is despite the presence of all the predicted extracellular assembly signals likely to be required for the formation of 5-HT_{3}A homomeric receptors. Thus, other regions (provided by 5-HT_{3}B) may be lacking that are also required for surface expression. Alternatively, inhibitory regions within the 5-HT_{3}B sequence may be present. Analysis of the 5-HT_{3}A/5-HT_{3}A chimera that revealed significant cell surface expression supported the latter possibility. Thus the apparent lack of ligand-binding sites evident on 5-HT_{3}B (9) does not limit subunit folding and release from the ER. Indeed, this chimera is capable of assembling into high molecular weight oligomers, indicative of assembly. The results with these chimeras suggest that the failure of 5-HT_{3}B to exit the ER and reach the cell surface is not because of a lack of appropriate assembly signals or protein misfolding. We determined, therefore, to assess the potential for the existence of inhibitory sequences, such as ER retention signals, within 5-HT_{3}B.

The rationale for this approach was that if 5-HT_{3}B were progressively truncated, all ER retention signals would be removed. If successful, this approach would identify the most amino-terminal ER retention signals if multiple signals exist. Consistent with this hypothesis, progressively truncated 5-HT_{3}B constructs remained ER-localized until the truncation reached the region between TMI-II. This truncation mutant (5-HT_{3}B-stop267) and a further truncation (at TMI) were efficiently expressed on the cell surface. Interestingly, a potential arginine-based (RXX) ER retention signal homologous to that identified in other proteins (23, 25, 26, 34) is present within this region of 5-HT_{3}B, but not of 5-HT_{3}A.

In support of a role of this signal as an ER retention signal within the environment of a 5-HT_{3}R, when CRAR is transferred to 5-HT_{3}A homomeric cell surface receptors are no longer observed. Like 5-HT_{3}B, the 5-HT_{3}A(CRAR) mutant can be rescued by wild-type 5-HT_{3}A, but not 5-HT_{3}B, and expressed on the cell surface, suggesting that protein misfolding is not responsible but that this signal may function to retain the 5-HT_{3}R subunit within the ER until masked. To identify whether the CRAR signal functions similarly within its native 5-HT_{3}B subunit, we generated the reciprocal mutation, 5-HT_{3}B(SGER). This mutant was retained in the ER and could be rescued by the co-expression of 5-HT_{3}A, but not 5-HT_{3}B, as observed for the wild-type 5-HT_{3}B subunit, implicating the presence of other ER retention signals. To provide direct evidence that the CRAR signal functions within 5-HT_{3}B to retain the subunit within the ER, new truncation mutants using 5-HT_{3}B(SGER) as the template were generated. Termination of 5-HT_{3}B(SGER) following the SGER signal, or after TMII, resulted in cell surface expression of 5-HT_{3}B(SGER) (Fig. 3). This is in contrast to the identical TMII truncation generated on the wild-type 5-HT_{3}B (Fig. 2). Thus, the CRAR does function as an ER retention signal within 5-HT_{3}B, at least in the TMII-truncated version of 5-HT_{3}B. Furthermore, it demonstrates that the SGER homologous sequence in 5-HT_{3}A does not function as an ER retention signal that may have been masked by intra-molecular or inter-molecular homomeric subunit interactions.

To determine whether the SGER (5-HT_{3}A) and the CRAR (5-HT_{3}B) might cooperate by direct binding to mask the presence of the ER retention signal in heteromeric receptors, the distribution of both mutant (5-HT_{3}A(SGER) and 5-HT_{3}B(CRAR)) subunits when co-expressed was examined. As observed when expressed alone (Fig. 3), no cell surface expression is evident, implying that a different mode of masking is operating. This is supported by the use of 5-HT_{3}R chimeras (Fig. 4). Paradoxically, the 5-HT_{3}B/5-HT_{3}A chimera can access the cell surface despite the presence of the CRAR signal. The most likely explanation for this discrepancy is that the major intracellular loop of 5-HT_{3}A is responsible for the masking of the CRAR signal. No evidence of a significant interaction with endogenous proteins was evident upon immunoprecipitation with the TMI-II constructs or wild-type 5-HT_{3}Rs. However, this is not surprising because such interactions may be of low affinity or transient. Furthermore, although dilyssine ER retention signals appear to interact with components of COPI, RXX motifs do not appear to do so (24), suggesting a different mechanism may be operating. Indeed, a COPI-independent route between the Golgi and the ER also exists (40). In support of a masking interaction involving the TMI-II region of 5-HT_{3}Rs, constructs expressing this domain are capable of interacting with wild-type 5-HT_{3}A and 5-HT_{3}B subunits when co-expressed within COS-7 cells. That the mutation of this ER retention signal in 5-HT_{3}B to the homologous region of 5-HT_{3}A does not prevent ER retention implies the existence of multiple ER retention signals. Alternatively, 5-HT_{3}B may lack essential forward signals necessary for export from the ER (29, 30, 38, 41–43). We are currently investigating the presence of other ER transport signals within the 5-HT_{3}Rs.

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