The Membrane Topology of RTN3 and Its Effect on Binding of RTN3 to BACE1

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Reticulon 3 (RTN3) has recently been shown to modulate Alzheimer BACE1 activity and to play a role in the formation of dystrophic neurites present in Alzheimer brains. Despite the functional importance of this protein in Alzheimer disease pathogenesis, the functional correlation to the structural domain of RTN3 remained unclear. RTN3 has two long transmembrane domains, but its membrane topology was not known. We report here that the first transmembrane domain dictates membrane integration and its membrane topology. RTN3 adopts a ω-shape structure with two ends facing the cytosolic side. Subtle changes in RTN3 membrane topology can disrupt its binding to BACE1 and its inhibitory effects on BACE1 activity. Thus, the determination of RTN3 membrane topology may provide an important structural basis for our understanding of its cellular functions.

Reticulon (RTN) proteins are a group of membrane-bound proteins that are largely localized in the endoplasmic reticulum (ER) (1, 2), perhaps more restricted to the tubular ER (3). RTNs exist in plants, fungi, and animals (4). In mammalian genomes, four independent reticulon genes, retn1 to retn4, have been identified to encode a large number of gene products (1, 2). All RTNs are composed of a variable length of N-terminal domain, which is completely divergent among RTN members, and a highly conserved RTN homology domain (RHD) that is unique to this family of proteins. Within the RHD, there exist two long stretches (28–36 amino acids) of hydrophobic residues, and each is expected to embed RTNs in the membrane.

The divergent N-terminal domain among RTNs potentially allows each RTN member to exert its specific function. RTN4, or its more popular acronym Nogo, is identified as a critical inhibitory molecule in axonal growth and regeneration (5, 6). Recently, reticulon proteins (including Nogo) have been shown to interact with β-secretase, also known as β-site APP-cleaving enzyme 1 (BACE1) (7–10), and this interaction negatively modulates the enzymatic activity of BACE1 (11–13). Among the RTNs, we have paid particular attention to RTN3 because of its strong inhibitory effects on BACE1 activity and high expression by neurons. RTN3 is localized not only in the ER compartment but also in the Golgi (11, 14), axons, dendrites, and growth cone (15). The localization of RTN3 in the neuritic region suggests its potential role in neuritic functions. Most recently, we have demonstrated that RTN3 also plays a role in the formation of dystrophic neurites in Alzheimer brains (15).

Because of the important role of RTN3 in Alzheimer pathogenesis, we set out to study the structure and function of RTN3. We report herein that the first transmembrane stretch or domain (TM1) is important for the membrane insertion of RTN3. Disruption of the TM1 of RTN3 results in misfolding of the mutant protein and failure to integrate the protein into the membrane. We also show that RTN3 adopts a ω-shape membrane topology with both the N- and C-terminal ends facing the cytosolic side. Knowledge from this study will be applicable to the structural explanation of RTN3 in its known cellular functions.

EXPERIMENTAL PROCEDURES

Reagents—Monoclonal antibody 9E10 recognizing Myc epitope was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-Xpress tag antibody was purchased from Invitrogen. Polyclonal antibodies R454 and R458 were raised to recognize the residues 2–15 and 217–233 of RTN3, respectively; the specificity of the antibody has been characterized previously (11). Antibody R461 recognizes the N-terminal residue 2–28 of RTN4B (11).

Cell Lines—Human human embryonic kidney 293 (HEK-293) cells were maintained at 37 °C in a humidified, 5% CO2 controlled atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and gluten. HM cells were previously established by stably expressing HA-tagged BACE1 in HEK-293 parental cells (17).

Site-directed Mutagenesis—Mutations of RTN3 were performed by using either the QuikChange site-directed mutagenesis kit according to the provided procedure (Stratagene, Palo Alto, CA) or PCR amplification. Deletion of the RTN3 loop region was achieved by PCR amplification of two fragments and then ligation of two fragments that share a common restriction site in the middle. All constructs were validated by double strand DNA sequencing. The RTN3 cDNA sequence in GenBankTM (gi 4091867) (18) was used for comparison.

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2 The abbreviations used are: RTN, reticulon; ER, endoplasmic reticulum; RHD, RTN homology domain; BACE, β-site APP-cleaving enzyme; HEK, human embryonic kidney; HA, hemagglutinin; CPMM, canine pancreatic microsomal preparation; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; TM, transmembrane.

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Preparation of Microsomes and Proteinase K Digestion—Iso-
lation of microsomes from the indicated cells was performed as
described (19) with minor modifications. Confluent cells (5 × 10⁶)
were harvested and washed with 15 ml of ice-cold buffer
containing 25 mM Tris, pH 7.4, 150 mM NaCl, and 15% glycerol.
The cell pellets were suspended in 0.6 ml of the same cold buffer
containing protease inhibitor mixtures (Roche Applied Sci-
ence). The plasma membranes were disrupted by sonication,
and the homogenates were centrifuged at 6800 rpm at 4 °C for
15 min. The supernatant was transferred to another tube for
centrifugation at 85,000 rpm for 20 min at 4 °C. The pellets after
this centrifugation consisted of mainly the microsomes. Micro-
somes are small vesicles derived from the fragmented ER pro-
duced when cells are homogenized.

Microsomal protein concentrations were adjusted to 10
mg/ml. For protease digestion, 2 μl of proteinase K (1 mg/ml)
was added to a 10-μl aliquot of freshly prepared microsomes in
the presence or absence of 0.5% CHAPS as the final concentra-
tion. The reactions were carried out on ice for 2 h and termi-
nated by adding phenylmethylsulfonyl fluoride to a final concen-
tration of 3 mmol/liter. After protease digestion, the microsomes
were washed three times with the above buffer containing 3 mmol/
liter of PMSF, and the pellets were dissolved in SDS sample buffer
and subjected to SDS-PAGE analysis.

In Vitro Transcription and Translation—The coupled in
vitro transcription and translation were performed according
to the TNT Quick-coupled Transcription/Translation proce-
dures described in the manufacturer’s manual (Promega,
Madison, WI). Biotinylated lysine was used for tracking the
translation product by utilizing the Transcend Non-Radioactive Translation Detection System (Promega). Canine pancreatic microsomal mem-
branes (kit Y4041) were also pur-
chased from Promega for the
translation of membrane-bound
proteins. After the translation was
terminated, transmembrane pro-
teins were separated from the soluble proteins by the floatation exper-
iment as described previously (20). This procedure can separate the
integrated membrane proteins from the non-integrated membrane-as-
associated proteins.

Immunoprecipitation and Western Blot Assay—Cells were first
grown in Dulbecco’s modified Eagle’s medium for 24 h in 10-mm
plates to ~70% confluence and then transfected with the indicated
expression constructs. After being cultured for 48 h, cells were lysed
and equal amounts of lysates were used for immunoprecipitation with
monoclonal antibody specific to HA tag overnight. The extensively
washed immunoprecipitates were

RESULTS
Co-translational Insertion of RTN3 into the Membrane—All
RTNs possess two transmembrane domains but no recogniz-
able signal peptide sequence (2). It is possible that the signal
peptide sequence in RTNs may be concealed within the two
putative transmembrane domains. Sequence comparison
reveals low sequence identity of these two domains between
vertebrate and invertebrate RTNs (2), which implies that a
structural feature, but not the sequence motif, may potentially
aid in targeting RTNs to the membrane. To determine how
RTN3 is integrated into the membrane and its membrane
topology, we first performed an in vitro translation of wild type
RTN3. On the Western blot, one major translated product cor-
responding to RTN3 was detected by using horseradish perox-
ide-conjugated streptavidin and further verified by using
antibody R454 that is specific to the N terminus of RTN3 (Fig.
1). When canine pancreatic microsomal preparation (CPMM)
was included during the translational reaction, significantly
more RTN3 protein was produced using the same amount of plasmid DNA and the translated RTN3 was partitioned into the
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membrane fraction (Fig. 1). This suggests that RTN3 is inserted into the lipid bilayer when CPM is present. However, if CPM was added after the translational reaction was terminated, the synthesized RTN3 stayed in the soluble fraction (visible in fraction S upon longer exposure), similar to the condition with no CPM added (Fig. 1). Clearly, insertion of RTN3 into the lipid bilayer is a co-translational event.

The TM1 of RTN3 Determines Membrane Integration—To determine which domain is important for governing the membrane integration of RTN3, we generated RTN3 variants with truncation either at the N- or C-terminal region, and all these mutant constructs would express N-terminal Xpress tag. Deletion of the first 61 amino acids in R3-ΔN61 did not markedly affect the integration of RTN3 into the membrane (Fig. 2B). However, deletion of an additional 36 residues, which includes removal of the majority of the residues spanning the TM1, caused a significant reduction of R3-ΔN97 in association with the membrane fraction, while the translation of this RTN3 variant was not affected (Fig. 2B). This indicates that the TM1 is important for the proper integration of RTN3 into the membrane. To further verify this, we converted L71L72 to K71K72 by site-directed mutagenesis, and this replacement yielded the R3-L71K/L72K mutant that significantly shortened the first hydrophobic stretch. R3-L71K/L72K was translated in a way similar to the wild type RTN3 but not integrated into the membrane (Fig. 2B) and not visible in the membrane fraction even after a longer exposure. These results confirmed that the TM1 is responsible for the membrane integration.

Deletion of the C-terminal domain including the entire second transmembrane domain (TM2) had no effect on the integration of RTN3 in the membranes (Fig. 2B). Thus, our results show that the TM1 determines membrane integration.

The Orientation of the N-terminal Domain of RTN3—The computational prediction of RTN3 membrane topology suggests that the orientation of the RTN3 N-terminal domain was likely bilateral with an almost equal possibility of facing the axoplasmic or cytoplasmic side, whereas the C-terminal domain is more likely to face the intracellular or ER/Golgi cytoplasmic side (Fig. 3). The loop region of RTN3 is predicted to have a slightly higher possibility of facing the intracellular/cytoplasmic side.

To determine the exact membrane topology of RTN3, we first performed protease K digestion of the in vitro translated RTN3 variants. We found that the epitope recognized by either antibody R454 or R458 was lost after treatment of the translated RTN3 with protease K (Fig. 4), suggesting that both the N- and C-terminal residues were exposed to digestion by protease K without a rupture of CPM. The translated product would be completely degraded upon prolonged treatment with protease K.

To determine the membrane topology of RTN3 in cells, we purified microsomes from either parental HEK-293 cells or the same cell line transfected with N-terminal-tagged full-length RTN3 (Ax-RTN3). Endogenous RTN3 from HEK-293 microsomes migrated near 25 kDa, whereas an Xpress-tagged RTN3 (Ax-RTN3) migrated near 27 kDa due to the addition of the tag sequences (Fig. 5A). Treatment with protease K completely removed the R454-recognizable epitope (Fig. 5A) or the N-terminal Xpress tag (data not shown) even when the microsomal membranes were still intact, consistent with the results in Fig. 4. Thus, the undetectable RTN3 N-terminal epitopes should be attributable to the complete digestion of the N-terminal residues by protease K. Therefore, the N-terminal domain of RTN3 has to face the intracellular or cytosolic side.

The Orientation of the C-terminal Domain of RTN3—Assignment of the C-terminal orientation was complicated because of the discrepancy in results between Figs. 4 and 5. Three smaller fragments (17, 13, and 11 kDa) recognizable by the
C-terminal antibody R458 were produced when the microsomal membranes were not even ruptured (Fig. 5A). The same treatment of the microsomes from cells transfected with Ax-RTN3 showed identical cleavage patterns although these smaller fragments in transfected samples were significantly stronger in intensity. Clearly, the processing of both endogenous RTN3 and Xpress-tagged RTN3 was identical.

We noticed the N terminus of caveolin-1 on the same blot was lost only by ~50% (Fig. 5B) even though the N terminus of caveolin-1 is supposed to be exposed for digestion. Caveolin-1 adopts a hairpin topology with both ends facing the cytosolic side of the plasma membrane (21, 22). If the membranes were ruptured by 0.5% CHAPS, caveolin-1 and the RTN3 fragments would be further digested by proteinase K (Fig. 5). Hence, this partial digestion of caveolin-1 is perhaps related to the fact that its N terminus was in close contact with the lipid bilayer as suggested (22).

To distinguish whether the C-terminal end is actually inaccessible to digestion or just protected from digestion by proteinase K, we transfected a C-terminal Myc-tagged RTN3 in HEK-293 cells and performed the same treatment with proteinase K. Interestingly, the Myc epitope was undetectable even without rupture of the microsomal membranes (Fig. 6A), suggesting that the C-terminal extreme end is accessible to digestion.

Alternatively, we performed another commonly employed microsomal digestion by trypsin. The C-terminal tail of RTN3 has several positive charged residues that are cleavable by trypsin. Microsomes were prepared from either HEK-293 cells or a stable cell line (HM cell line) that expresses HA-tagged BACE1 as described previously (17). We found that the C-terminal end of RTN3 was completely digested by trypsin even in the absence of CHAPS (Fig. 6B, upper panel), suggesting that this end is definitely exposed to digestion by trypsin.

On the same blot, we probed with antibodies recognizing BACE1, a known type I transmembrane protein with the C terminus facing the intracellular/cytoplasmic side. Interestingly, the C terminus of BACE1 could only be completely digested by proteinase K, not by trypsin (Fig. 6, lower panel). As expected, the luminal BACE1 N-terminal fragments were largely protected from digestion by both proteinase K and trypsin. Therefore, it seems not uncommon that the same exposed residues can be subjected to differential digestions by different enzymes. Considering the above results altogether, we conclude that the C-terminal end of RTN3 also faces the intracellular/cytoplasmic side.

**The Orientation of the Loop Region of RTN3**—According to the sequence features, we have previously predicted several potential membrane topologies (2). The ori-

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**FIGURE 4. Digestion of the translated RTN3 by proteinase K.** Both the C-terminal Myc-tagged RTN3 and N-terminal Xpress-tagged RTN3 were produced in vitro by use of the TNT transcription/translation system. Digestion of the products was performed either in the absence (−) or presence (+) of proteinase K for 1 h.

**FIGURE 5. Assignment of the orientation of the N terminus of RTN3 on the microsomes.** A, microsomal membranes were prepared from HEK-293 cells and treated with proteinase K either in the absence or presence of 0.5% CHAPS. The R454-recognizable epitope was lost whereas three R458-recognizable fragments were produced after the digestion. Under the RTN3-overexpressed condition, these fragments were not completely abolished after rupture of the membranes by CHAPS. B, the same blot was reprobed with antibody recognizing the N terminus of caveolin-1. The antibody recognizes an unknown band that was not digested even after the membrane was ruptured.
entation of the loop region determines which of the predicted membrane topologies of RTN3 might be adopted in cells, and we now narrowed possible membrane topologies down to four, as depicted in Fig. 7A. Based on the following observations, we mapped the loop region of RTN3 to face the intracellular/cytosolic side.

First, we reasoned that, if the loop regions would face the luminal side as depicted in Topo-1 and Topo-2, the loop residues should not be exposed for digestion by proteinase K when the microsomal membranes were not ruptured. If the loop residues were not digested, only the 17-kDa fragment would be observed after proteinase K treatment because this is the remaining fragment after the removal of the exposed N-terminal residues (Fig. 7C). The finding of additional fragments after digestion of RTN3 by proteinase K suggested that the topology of Topo-1 or Topo-2 was unlikely.

Second, we inspected the sequence within the loop region and found that residues 124–144 were relatively hydrophobic and this feature is unique to RTN3 and not to the other RTNs (2). Potentially, these hydrophobic residues might adopt a conformation similar to the Topo-4 as illustrated (Fig. 7, A and C). If RTN3 adopted a Topo-4 conformation, removal of the first 124 residues would produce a fragment with ~12.8 kDa remaining and the removal of the first 148 or so residues would leave a fragment near 10 kDa (Fig. 7C). Indeed, we found that digestion of RTN3 by proteinase K produced a digestion pattern similar to the wild type RTN3 if there were no rupture of the membranes. This, on the other hand, confirmed that TM1, but not TM2, governs membrane integration. However, after rupture of the membrane with 5% CHAPS, an extra band near 7 kDa was protected from digestion by proteinase K (Fig. 7B). Apparently, the mutation of Leu-190 and Ile-191 to Lys-190 and Lys-190 increased the positive charges of this 7-kDa fragment and potentially enhanced interaction of the tail with the negatively charged lipid bilayer. This perhaps explained why the C terminus of RTN3 was partially inaccessible to digestion by proteinase K.

**Disruption of TM1 and TM2 Hydrophobic Stretches Affects the Stability of RTN3 and Its Interaction with BACE1**—To confirm that TM1 governs the insertion of RTN3 into the lipid bilayer and topology in cells, we transfected an equal amount of mutant constructs in HM cells and examined their expression. Disruption of the TM1 hydrophobic stretch significantly reduced expression of the mutant proteins (see lanes R3-D16N79, R3-L171K/L72K, and R3-I87K/L88K in Fig. 8A). It appeared that the mutant RTN3 proteins would be degraded in cells if they failed to be inserted into the lipid bilayer. Converting F178N179 to K178K179 did not affect the stability of R3-F178K/N179K (Fig. 8A). However, converting L190/I191 to K190/K191 would affect the stability of R3-L190K/I191K in cells.

Importantly, we found that these mutants failed to interact with BACE1 even after the expression of each construct in HM cells was normalized by abnormally increasing expression of these poorly expressed RTN3 mutants (Fig. 8B). Despite the observation that the expression of R3-F178K/N179K was not affected (Fig. 8A), the binding of R3-F178K/N179K to BACE1 also appeared impaired, suggesting that this mutation causes a change in its conformation. We also found that the above membrane-perturbed RTN3 mutants failed to inhibit the production of Aβ whereas wild type RTN3 would (data not shown). Thus, proper integration of RTN3 into the lipid bilayer as well as optimal conformation is important for the binding of RTN3 to BACE1.

**DISCUSSION**

RTN proteins are characterized as a group of membrane-bound proteins that share a highly conserved C-terminal RHD (1, 2, 23). RHD is comprised of two hydrophobic stretches separated by a 66-amino acid loop followed by a short C-terminal
Although the functional role of RTNs is not completely understood yet, the emerging results indicate that an individual member of the RTN family can inhibit axonal regeneration (24–26), modulate Alzheimer/H9252-secretase activity (11–13), regulate vascular homeostasis and remodeling (27), participate in the formation of dystrophic neurites in Alzheimer disease (15), etc. Because of the importance of RTN3 in many cellular processes, it is important to understand the relationship between the structure and function. In this study, we focus on the investigation of membrane topology of RTN3, a protein known to play an important role in modulating BACE1 activity and neuritic function.

We have previously speculated that the RHD determines the membrane topology of RTNs (2). Surprisingly, RTN3 adopted a different membrane topology compared with Nogo. A recent study based on scanning by cysteine modification of mutated Nogo-C suggests that Nogo-C/RTN4-C mutants form a hairpin structure with the N- and C termini facing the axoplasmic side (3). Several other studies suggest that Nogo may adopt a horseshoe topology with the N terminus facing the axoplasmic side and the C terminus facing the intracellular side (24, 28). Our study indicates that RTN3 forms a w-shaped structure with the N- and C termini of RTN3 facing the intracellular/cytosolic side. These studies suggest that the RHD alone is not sufficient to dictate the membrane topology of RTNs.

To understand this discrepancy further, we used TM prediction software to compare the probabilities of additional RTN variants. We found that the sequences in the N-terminal domain, in addition to the loop sequence as discussed in Fig. 7B, could also affect the membrane topology. For example, Nogo-A is predicted to adopt the hairpin structure with both ends facing the axoplasmic side, but Nogo-B likely adopts an orientation with the N-terminal end facing both sides (supplemental Fig. S2, A and B). The difference between these two isoforms is that Nogo-B lacks 890 amino acids encoded by exons 2 and 3 (2). Therefore, it is not surprising that RTN3 can adopt a different membrane topology from the other RTN members. We also speculate that the cellular environments or the interaction of RTNs with their partners may potentially influence their membrane topology, although this remains to be verified. Noticeably, the N terminus of Nogo is experimentally found to adopt a conformation with its N-terminal domain facing the intracellular/cytosolic side as well as the axoplasmic side (28). We indeed found that the Nogo-B (RTN4B) N-terminal epitope recognized by antibody R461 was lost upon digestion by protease K (supplemental Fig. S2C), suggesting that the N terminus of RTN4B faces the cytoplasmic side.

The results in this study suggest that the TM1 governs the membrane integration and topology of RTN3. Because the mutation of RTN3 in the TM1 region failed to integrate the mutants into the membrane, these mutants are perhaps misfolded and degraded in cells. We could barely detect RTN3 TM1 mutants

FIGURE 7. Determination of RTN3 membrane topology. A, the potential membrane topologies of RTN3 were predicted based on the knowledge that both the N- and C termini face the cytosolic side. B, digestion of RTN3 and its mutants in microsomes by proteinase K. The indicated constructs were expressed in HEK-293 cells. C, the potential degraded products are illustrated with expected size and molecular mass. Among the C-terminal 36 residues, there exist nine positive charged Lys or Arg residues.
by Western analysis if equal amounts of expression constructs were transfected in HEK-293 cells (Fig. 8A). Failure to detect these mutants under the normal transfection condition was not due to the defects in the expression construct, because they could be normally translated in the in vitro transcription/translation system (Fig. 2B).

TM2 is predicted to break into two hydrophobic stretches, residues 149−171 and 176−198 (Fig. 3). Although the TM2 domain is not required for the integration of RTN3 in the membrane, disruption of the TM2 domain can affect the proper folding of RTN3. For example, residues Phe-178 and Asn-179 are located in a region predicted to be in close proximity to a short loop (residue 172−175), and converting these two residues to Lys-178 and Lys-179 would lengthen this short loop and shorten the latter hydrophobic stretch. Interestingly, this R3-F178K/N179K mutant failed to interact with BACE1 even though its expression in cells was not altered (Fig. 8B). Likely, the conformation of the lengthened C-terminal tail was folded differently and the binding domain is buried. Similarly, R3-TM2L190K/191K would form only one hydrophobic stretch between the residues 165−187, but the highly positively charged C-terminal tail may increase the interaction with the lipid bilayer. This change clearly also suppresses R3-TM2L190K/191K from interacting with BACE1.

The determination of RTN3 topology explains the interaction between RTN3 and BACE1. We have recently mapped the residues near the C-terminal tail of RTN3 that are responsible for its interaction with BACE1 (12). Similarly, a short C-terminal stretch more proximal to the TM domain of BACE1 is responsible for the binding of BACE1 to RTN3. Because both C-terminal tails face the cytosolic side, the same topological orientation makes this binding compatible. As discussed previ-
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