A reticular rhapsody: phylogenetic evolution and nomenclature of the *RTN/Nogo* gene family¹

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ABSTRACT Reticulon (*RTN*) genes code for a family of proteins relatively recently described in higher vertebrates. The four known mammalian paralogues (*RTN1*, -2, -3, and -4/*Nogo*) have homologous carboxyl termini with two characteristic large hydrophobic regions. Except for *RTN4-A/Nogo-A*, thought to be an inhibitor for neurite outgrowth, restricting the regenerative capabilities of the mammalian CNS after injury, the functions of other family members are largely unknown. The overall occurrence of *RTNs* in different phyla and the evolution of the *RTN* gene family have hitherto not been analyzed. Here we expound data showing that the *RTN* family has arisen during early eukaryotic evolution potentially concerted to the establishment of the endomembrane system. Over 250 reticulon-like (*RTNL*) genes were identified in deeply diverging eukaryotes, fungi, plants, and animals. A systematic nomenclature for all identified family members is introduced. The analysis of exon-intron arrangements and of protein homologies allowed us to isolate key steps in the history of these genes. Our data corroborate the hypothesis that present *RTNs* evolved from an intron-rich reticulon ancestor mainly by the loss of different introns in diverse phyla. We also present evidence that the exceptionally large *RTN4-A*-specific exon 3, which harbors a potent neurite growth inhibitory region, may have arisen de novo ~350 MYA during transition to land vertebrates. These data emphasize on the one hand the universal role of reticulons in the eukaryotic system and on the other hand the acquisition of putative new functions through acquirement of novel amino-terminal exons.—Oertle, T., Klinger, M., Stuermer, C. A. O., Schwab, M. E. A reticular rhapsody: phylogenetic evolution and nomenclature of the *RTN/Nogo* gene family *FASEB J.* 17, 1238–1247 (2003)

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THE *RTNs* are a family of proteins, typically 200–1200 amino acids (aa) in length, described relatively recently in higher vertebrates (1–4). They all share a carboxyl-terminal 150–201 aa reticulon homology domain (RHD; Pfam PF02453) comprising two large hydrophobic regions with an ~66 aa loop in between. *RTN1* was the first identified member (5) and is expressed in the

central nervous system (CNS) as well as in neuroendocrine cells (1, 6). The functions of *RTN1*, *RTN2*, and *RTN3* are unknown, yet all (including *RTN4/Nogo*) are enriched in endoplasmic reticulum membranes (1, 4, 7).

RTN proteins are ubiquitously expressed in vertebrates and have recently been described in *Caenorhabditis elegans* (8). Their distribution among other metazoan phyla is still entirely undefined.

The fourth vertebrate family member, *RTN4/Nogo*, gives rise to different isoforms through alternative splicing and alternative promoter usage (9). The largest isoform, *Nogo-A*, has been described as a potent neurite outgrowth inhibitor of CNS myelin (4, 10, 11). In line with the more oligodendrocyte-restricted expression of *Nogo-A* (12), we have identified a stretch in the *Nogo-A*-specific region (NiG-Δ20, aa 544–725) that is strongly inhibitory for neurite outgrowth and cell spreading (13, 14). The loop region between the two carboxyl-terminal hydrophobic domains of the RHD (called *Nogo-66*) has been shown to induce collapse of neuronal growth cones (10) through interaction with a GPI-anchored receptor, NgR (15, 16), which simultaneously serves as a receptor for the myelin proteins OMgp (17) and MAG (18, 19). Since *Nogo-66* is present in all known *Nogo* splice variants, one would anticipate they should all be restrictive for regenerative axons. *Nogo-B*, for instance, has a rather ubiquitous expression pattern (12, 20, 21) that is not compatible with earlier findings showing that adult CNS myelin is particularly enriched in neurite growth inhibitory factors (22). Because the adult CNS of lower vertebrates is able to regenerate after a lesion (23, 24), inhibitory components being largely absent (25, 26), a closer phylogenetic analysis of the *nogo* gene may elucidate the key evolutionary steps from lower to higher vertebrates required to give birth to its unique growth inhibitory function. In addition to the neurite out-

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growth inhibitory character of Nogo, Nogo-B is thought to play a role in apoptosis, particularly in cancer cells (20, 21), although some of the evidence is disputed (27).

We describe here the identification of >250 RTN and RTNL genes in eukaryotes. We present a systematic nomenclature for all known members of the RTN family. Knowledge of the biological role of a shared protein in one organism can certainly illuminate, and often provide strong evidence of, its role in other organisms. Our analysis is intended not only to elucidate the evolutionary relationships between RTNs, but also to give insight into those residues that play critical roles for RTN structure and function.

MATERIALS AND METHODS

Sequences

Sequences were obtained from GenBank databases (Supplementary Tables). From ESTs, contiguous sequences were established using SeqManTMII (DnaStar) and noncomputational analyses. Exon-intron structures were examined by comparing genomic sequences against cDNA sequences. Where possible, the GT-AG rule of splice donor and acceptor sites was respected.

Nomenclature

The RTN family nomenclature was performed based on the homology of the sequences (see Results). The suggested nomenclature has been approved by the HUGO Gene Nomenclature Committee, the Mouse Genomic Nomenclature Committee, the Zebrafish Nomenclature Committee, the *Saccharomyces* Genome Database, and the *Schizosaccharomyces pombe* Genome Project.

Sequence analysis

Hydrophobicity plots were created using DAS (<http://www.sbc.su.se/~miklos/DAS>), TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), TMPred (http://www.ch.embnet.org/software/TMPRED_form.html), and TopPred (<http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html>) using the KD, GVH, and GES scale. A conservation plot (inverted Wu-Kabat variability plot) was calculated manually based on the protein sequences aligned with ClustalW (28) and plotted using Prism. Sequence logos have been generated using GENIO/logo (<http://genio.informatik.uni-stuttgart.de/GENIO/logo/logo.cgi>). Bootstrap proportions and Neighbor-Joining Trees of protein sequences have been calculated using ClustalX (v.1.8), with the default parameters and ClustalW. Cladograms and trees have been drawn using Tree-View (v.1.6).

RESULTS AND DISCUSSION

In silico and physical cloning of RTN and RTNL proteins

Using the sequences of known RTN proteins, i.e., their carboxyl-terminal RHD, we searched the public databases for homologous proteins, mRNAs, genomic sequences, and ESTs. We physically cloned RTN members from *R. norvegicus*, *B. taurus*, *G. gallus*, *X. laevis*, *C. auratus*, and *D. rerio* cDNA. From a total of 81 mRNA sequences, 48 genomic sequences, ~500 genomic traces, and ~7500 ESTs, we constructed contiguous strings encoding the entire or partial sequences of 351 mRNA/proteins and isoforms arising from 272 bona fide independent genes from 94 different species (Suppl. Tables). Homologous genes could neither be identified in archaeal or bacterial species nor in the microsporid *Encephalitozoon cuniculi*.

These results show that RTN-like genes are present in most eukarya and particularly in plants, where multiple duplication events led to an array of RTN paralogues in the same plant species (see Suppl. Table F).

Nomenclature

The reticulon gene family is based on a cellular component ontology, i.e., proteins are considered to be members of the family if they contain at least part of an identifiable RHD (Pfam PF02453, <http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF02453>) characterized by two large (>30 aa) hydrophobic regions and an ~60 aa spacer between the two regions (see Fig. 5A). The two hydrophobic stretches are responsible for the association of RTN proteins to membranes, in particular those of the endoplasmic reticulum (27). The “functional” motif of RTNs and RTN-like proteins is therefore the RHD. A standardization of the RTN nomenclature should help in the identification and understanding of new gene products and facilitate communication between scientists in different disciplines. We suggest that “RTN” serves as the gene symbol for chordate reticulons and “RTNL” for homologous proteins in nonchordate taxa. RTN-like genes in nonchordate metazoans are designated as RTNL, in fungi as RTNLA, in plants as RTNLB, and in protists as RTNLC (Fig. 1; see Supporting Material on <http://www.fasebj.org> for further details on the nomenclature guidelines).

These data are the basis for the construction of a consistent expandable RTN nomenclature.

Phylogenetic analysis

On the basis of the alignment of the RHD sequences from 226 distinct genes and 80 different species, a phylogenetic tree of the reticulon family has been inferred. There is an excellent relationship between the clustering pattern of members in the family tree and taxonomic classification (Fig. 1). Similar to reticulons, many endomembrane-associated genes have been

Figure 1. Tree diagram representing taxonomic distribution of the RTN gene family based on alignment of the reticulon homology domain. RTNL genes are found in most eukaryotic kingdoms from protists to animals.

found in most eukaryotic genomes but are absent in bacteria (29).

In the following we will discuss the different subfamilies. First, we will present the RTN genes and their splice isoforms. Second, important evolutionary steps that happened within a subfamily are discussed. Third, we will briefly review known functional data of these RTN genes (e.g., protein interaction partners), with the exception of the vertebrate reticulons, where a large body of publications already covers these facets. Finally, evolutionary features of the entire gene family as well as highly conserved residues will be exposed.

Chordate RTN subfamily

There are four major vertebrate reticulon paralogs: the previously identified *rtn1*, *rtn2*, *rtn3*, and *rtn4/nogo* genes (Fig. 2A). All paralogs in fish, amphibians, and mammals have six carboxyl-terminal exons encoding the RHD (Fig. 2C, see Fig. 6; M. Klinger et al., unpublished results).

Mammalian *rtn1* is formed by at least 10 exons and can give rise to 3 isoforms (RTN1-A, -B, and -C) by alternative promoter usage (Fig. 2C; Suppl. Table A; 30). *Rtn1* has been duplicated probably in euteleosts (generating the paralog *rtn5*).

There are three known RTN2 mRNA variants (2) derived from 11 different exons (Fig. 2C). Our analysis has revealed three additional minor splice variants in humans (Suppl. Table A). RTN2 is the most divergent

of the mammalian tetralogues, with 36 nucleotide insertions in exon 11.

The nine exons of human *rtn3* can give rise to at least five isoforms (Fig. 2C, Suppl. Table A), from which only RTN3-A1 has so far been described in the literature (3, 7). As previously published (3), a RTN3 pseudogene exists in humans on chromosome 4. Since no intron sequences are present, this pseudogene represents a retronon of the RTN3-A1 transcript (Suppl. Fig. 1).

Human *rtn4* is formed of 14 exons that can give rise to at least 10 splice variants (Fig. 2C, Suppl. Table A; 9). There is good evidence that *rtn4* has been duplicated in *X. laevis* (M. Klinger et al., unpublished results) while being present as a single gene in *S. tropicalis*, in conformity with the ancestral diploid karyotype of the genus.

In the urochordate *C. intestinalis* we were able to ascertain only a single RTN gene, which can give rise to at least six different isoforms originating from alternative splicing and the use of three alternative promoters (Fig. 2C; Suppl. Table A). The RHD of *C. intestinalis* is encoded by only four exons.

Vertebrate RTN1-4 are present on four different chromosomal loci, have conserved intron-exon boundaries and intron phasing of the RHD-encoding region (1-2-0-1-0-2), and are more similar to each other than to invertebrate RTN members. The presence of a single bona fide RTN gene in invertebrates and the presence of the vertebrate tetralogues are in accordance with the hypothesis of genome quadruplication in amphioxus-like animals ~760-530 MYA before the cyclostome/gnathostome split (31, 32). A reservation remains for the origin of RTN2. Indeed, we were not able to identify any nonmammalian RTN2 sequences. This could mean that 1) RTN2 is a recent duplication during the evolution of mammals followed by rapid sequence divergence or 2) RTN2 is present as a pseudogene (or has been secondarily lost) in lower vertebrates and has been retained or exempted in mammals.

Between the four vertebrate paralogs, no homology of the amino-terminal exons could be identified. The divergence of the amino-terminal exons within the same orthologs is also higher compared with the RHD exons, demonstrating nonhomogeneous evolution of the genes (Fig. 2B). Homologous ESTs to the amino-terminal exons of *rtn1* could be identified down to *D. rerio*, whereas ESTs homologous to the amino-terminal exon 1 of *nogo* were found in *S. tropicalis* and *X. laevis* (Fig. 2B). Homologous exons to the huge 2400 bp long, Nogo-A-specific exon 3 that encodes the neurite outgrowth and cell spreading inhibitory region of this protein could be found in *X. laevis*, but not in fish (Fig. 2B). Thus, some of the amino-terminal exons seem to have arisen ~360 MYA during the transition to land vertebrates or have not yet been identified in the fish genomes. However, there are also more recent gains of exons, e.g., exon 1F of the *rtn4/nogo* gene has been newly acquired in primates after their divergence from rodents (100 MYA) (9).

These results demonstrate that all vertebrate RTN paralogs originated by gene duplication from an

Figure 2. RTN genes in chordates. A) Cladogram displaying the different vertebrate RHDs. RTN1, -3, and -4/Nogo are the largest groups, with orthologs from fish to mammals. RTN2 genes have been identified only in mammals. RTN1 and RTN3 have been duplicated in euteleosts giving rise to the

ancestor with the same RHD gene structure (see below).

Insect RTN-like subfamily

Two RTN-like genes can be found in the *D. melanogaster* genome. The *rtnl1* gene is formed of 12 exons that can give rise to at least 11 splice variants (Fig. 3A, Suppl. Table B). The RHD is formed by four carboxyl-terminal exons that are conserved in the *Anopheles gambiae* ortholog (Fig. 3A).

A *rtnl2* gene (formerly tropomyosin-like gene) has not been identified in the *Anopheles* genome and no ESTs originating from *rtnl2* could be found in public databases. Since the gene is in a single exon (thus lacking the typical reticulon exon-intron structure of insects) and its homology is relatively low to the other insect homologues, we speculate that this is a retroviral insertion with pseudogene character.

(DROME)Rtnl1 was trapped using a GFP-carried P1 element (33) (it has not been recognized as a reticulon protein by the authors and was called G9). The given insertion point of the transposon would be exon 1D, according to our data, and could potentially be spliced into the -A, -B, -C isoforms of the gene. Based on these data these transcripts are expressed ubiquitously in early embryonic development and become CNS specific late in embryogenesis (34). The fusion protein is localized to the ER (33) and during cell division is in a ring-like structure that surrounds the spindle rotating with it during neuroblasts division (34).

Nematode RTN-like subfamily

In the *C. elegans* genome, a single RTN-like gene could be identified with 11 exons giving rise to at least 5 isoforms originating from 3 alternative promoter sites (Fig. 3B). Orthologous RTNL sequences were identified in a number of other nematodes (Suppl. Fig. 2b; Suppl. Table C). The RHD is formed by three carboxyl-terminal exons (Fig. 3B).

(CAEEL)RTNL-C (formerly nRTN-C) has been

RTN5 and RTN7 paralogues, respectively. The RTN6 paralogues present in euteleosts have relatively low homology to the other reticulon members. Based on cladistic analysis, it is probably derived from a duplication of the RTN4/Nogo gene. Nogo has been duplicated recently in *X. laevis*. Numbers indicate the bootstrap (BS) values (for 1000 replicate data sets). The tree is rooted taking the urochordate RHD as an outgroup. B) Cladogram of the amino-terminal exons of RTN1-A and RTN4-A. Note the higher nucleotide substitution rates in comparison to the RHD, proving nonhomogeneous evolution of the reticulon genes. Numbers indicate BS values (1000 replicates). C) Exon-intron structure of the urochordate RTN gene and the four main mammalian RTN genes. Exons coding for the RHD are indicated in black, in gray those coding for the paralogue-specific amino termini, and in white boxes the UTRs. Arrows indicate the translation start sites. Numbers designate intron phasing. Exon sizes are matched according to the indicated line.

Figure 3. RTNL genes in non-chordate animals. A) Exon-intron structure of the RTNL gene of *Anopheles* and *Drosophila*. B) Exon-intron structure of the RTNL gene of *Caenorhabditis*. Exon/intron representation as in Fig. 2C.

shown to be associated with ER membranes (8). The protein is detectable almost exclusively during the late gastrulation stage of the embryo whereas mRNA is present in developing gonads and oocytes of larvae (8). The amino-terminal region specific for RTNL-C interacts with RME-1 (8), a protein localized to the endocytic recycling compartment (ERC) and involved in the exit of proteins from ERC to the plasma membrane or to the *trans*-Golgi network (35).

Platyhelminth RTN-like subfamily

We were able to identify RTNL ESTs in *S. mansoni*, a parasitic digenetic trematode belonging to the family of blood flukes (Suppl. Table D).

Although some vaccines against schistosomiasis are currently in human trials, none promises high efficacy so that the need for the identification of effective antigens for vaccination is strong (36). Since (LEIIN) RTNLC has been successfully used as a vaccine against visceral leishmaniasis in hamster models (see below), it would be of interest to envision vaccination with (SCHMA)RTNL against schistosomiasis in order to lower the T_H2 response of chronic infection.

Fungi RTN-like subfamily (RTNLA)

Despite other reports (8), RTN-like proteins could be identified in the genome of *Saccharomyces cerevisiae* as well as of *S. pombe* (Fig. 1, Suppl. Fig. 2C). In both cases single, intron-less genes encode the proteins (see Fig. 6). *S. cerevisiae* is the only fungus where we identified two separate genes, suggestive of a separate gene duplication event consistent with previous findings (37). Orthologs were identified in different fungi, but the divergence between them is very high (Suppl. Fig. 2C, Suppl. Table E). The homology between (YEAST)RTN1 and (SCHPO)rtn is only ~25%, which is comparable to the homology between (YEAST)RTN1 and vertebrate reticulons. This is not uncommon (38); there is evidence that yeasts are generally fast-evolving organisms (39) and that *S. cerevisiae* and *S. pombe* had already diverged 420-330 MYA (38).

Overexpression of (SCHPO)rtn (formerly *cwl1*+) has been shown to reduce the cell wall content (~20%),

inhibit cell separation after division in the absence of thiamine, and cause cell lysis in the absence of sorbitol (40).

Fission and budding yeasts are viable and have no obvious phenotype after gene disruption (40, 41).

(YEAST)RTN2 is induced by the G-protein regulator Sst2 probably via a 5' stress response element in its promoter region (42) and is generally up-regulated in response to stressors (<http://www.transcriptome.ens.fr/ymgv/>).

It has been shown that (YEAST)RTN1 interacts with YOR285w and YDL089w, two genes of unknown function (43). Furthermore, RTN1 has been shown to interact with the H3/H4 histone acetyltransferase HAT2 (YEL056w), a yeast homologue of the human retinoblastoma binding protein 4 (44). (YEAST)RTN2 interacts with the inositol-1,4,5-trisphosphate 5-phosphatase INP54, with the osmosensor SHO1, with karyopherin KAP104, with SEC27 involved in the ER-to-Golgi protein trafficking, and with YMR110c, an aldehyde dehydrogenase type III homologue (43, 44).

In conclusion, our data present not only new members of the RTN family but also new interaction partners pointing to possible functions.

Plant RTN-like subfamily (RTNLB)

In plants we were able to identify >180 members of the RTNLB subfamily (Suppl. Fig. 3; Suppl. Table F). *A. thaliana* has at least 15 RTNLB genes with conserved exon-intron structure and intron phasing (Fig. 4). The RHD domain for *Arabidopsis* and *Oryza sativa* RTNLB genes is usually formed by four exons (generally exon 2-5) and the conserved intron phasing is 1-2-0-1. We have, however, identified some exceptions. In (*ARATH*)*rtnlb5* intron IV is absent whereas in (*ARATH*)*rtnlb12* and (*ARATH*)*rtnlb13* intron III is absent and the two exons are fused. In (*ARATH*)*rtnlb6* exons 3 and 4 are nonhomologous to the other paralogues, i.e., not encoding a RHD domain. The phasing of intron III in this gene is indeed different and therefore might represent an example of exon shuffling. (*ARATH*)*rtnlb14* lacks the last exon and (*ARATH*)*rtnl15* lacks the first two exons of the RHD (Fig. 4). At least in two of the *A. thaliana* RTNLB genes (10 and 11) there is an additional,

Figure 4. RTNLB genes in plants. Exon-intron structure of the RTNLB genes identified in *Arabidopsis thaliana* and *Oryza sativa*. Exon/intron representation as in Fig. 2C.

noncoding exon located 3' to the four RHD encoding ones. The phylogenetically most ancient group is probably represented by the *rtnlb10-12/18* orthologs, since they are the closest homologues of the fern *C. richardii* (*rtnlb34-35*) and the moss *P. patens* (*rtnlb36-40*) reticulons (Fig. 1; Suppl. Fig. 3).

The majority of the RTNLB genes have a single amino-terminal exon with relatively low homology between the members.

RTN-like proteins in early branching eukaryotes (RTNLC)

Besides the three eukaryotic kingdoms of metazoans, fungi, and plants, we have identified RTNL genes in euglenozoa (*Leishmania infantum* and *major*), pelobiontida (*Mastigamoeba balamuthi*), and mycetozoa (*Dictyostelium discoideum*) (Fig. 1; Suppl. Table G). Of these three subfamilies, (DICDI)RTNLC and (MASBA)RTNLC have the closest relationship in concordance with the literature (45). It is noteworthy that *Mastigamoeba* lack recognizable Golgi bodies. (LEIMA)RTNLC is encoded by a single, intron-less exon whereas (DICDI)RTNLC is encoded by three exons (see Fig. 5).

(LEIIN)RTNLC (formerly papLe22) has recently been described as a protein that potentially aggravates visceral leishmaniasis, stimulates IL-10 release from patient-derived peripheral blood mononuclear cells in vitro, and is found in nuclear fractions of the promastigote form (46). The protein itself as well as anti-RTNLC antibodies have been detected in the serum of patients and specific IgG titers correlated with the clinical status of the patients (46). Intramuscular vacci-

nation in hamster models with RTNLC cDNA down-regulated the T_H2 response after *Leishmania* infection and reduced episodes of *Leishmania* circulation by 50% (47).

Evolution of the intron-exon structure

Intron loss or gain represents critical steps in the divergence of expression patterns of individual gene family members, since introns are known to host regulatory elements. Intron loss can occur through an mRNA intermediate mechanism as processed retro-transcripts (48) or can be due to direct genomic deletion (49). Figure 5 shows the conservation of the intron-exon structure and intron phasing in different phyla. The most parsimonious scenario would propose an intron-rich reticulon ancestor ("Proto-Reticulon") in early eukaryotes and multiple independent intron losses in distantly related lineages. The ancient exon-intron structure of the proto-reticulon would still be conserved in most plant genes and has been reacquired by urochordates after intron losses (Fig. 5). Our proposal would best fit with the intermediate view postulating that introns arose at the initiation of multicellularity. In different phyla, extensive intron loss therefore has to be assumed. However, two episodes of intron gain also have to be claimed in our model. A first phase 2 intron insertion occurred around the time of the fungi/metazoan split. It is probable that the second insertion of the phase 0 intron antedates the tetraploidization event before the fish/tetrapode split (~500 MYA; ref 50) but took place after the urochordata-cranialia split (Fig. 5). The absence of introns in

Figure 6. Hyperconserved regions within the reticulon homology domain. *A*) Hydrophobicity plot of the vertebrate RHD consensus sequence. The graph is an overlay of the plots calculated with the three TopPred2 scales (GvH, KD, GES) and with the algorithm based on TMbase offered by TMpred. *B*) Conservation plot of the RHD of all chordate members. Amino acids are displayed for the residues that are 100% conserved. The amino acids that are conserved in *all* known RTNL proteins are indicated by arrows. Amino acid types conserved in most of the eukaryotic RTNL proteins are indicated by an arrowhead (ψ =hydrophobic residue; ϕ =aromatic residue). *C*) Sequence logos of vertebrate 66-loop region. NEP1-40 is the peptide shown to bind to NgR, particularly via residues 1-10 and 30-33, as indicated by dotted lines. Peptide4/NEP31-55 has been shown to be a relatively weak inducer of growth cone collapse despite its lack of binding affinity to NgR. This region is thought to be mainly responsible for the observed inhibitory properties of Nogo-66. Logos for hv-Nogo-66 (10 sequences), lv-Nogo-66 (3 sequences), Nogo-66 (13 sequences), RTN1-66 (9 sequences), RTN2-66 (4 sequences), and RTN3-66 (10 sequences) are shown (hv=higher vertebrates, i.e., amphibian to mammalian sequences; lv=lower vertebrates, i.e., fish sequences).

would allow us to spot key mutations responsible for the putative gain of inhibitory activity (Fig. 6C).

Evolution of Nogo/RTN4 in vertebrates

Concerning the evolution of RTN4/Nogo as a relevant inhibitor for neurite outgrowth, it is of interest to

consider whether this function agrees with the data gathered in the present study. Nogo-66, which is part of the RHD, and NgR (M. Klinger et al., unpublished results) are both present down to teleosts, where regeneration does occur. The Nogo-66 region is also present in all known Nogo splice variants and therefore has an almost ubiquitous tissue distribution that strictu sensu does not fit with the postulated constraint of inhibitors to the CNS. Sequence comparison did not permit us to identify key evolutionary mutations that could have allowed Nogo-66 to become a potent inhibitory site in higher vertebrates, although the overall variability of course does not exclude an evolutionary gain of function (Fig. 6). Future studies will show whether the orthologous fish Nogo-66 displays binding properties to and inhibitory activity via NgR and its fish ortholog or not. In contrast, the Nogo-A-specific 2400 bp long exon 3 seems to appear during the transition to land vertebrates. Both NiR- Δ 2 and NiG- Δ 20, shown to be inhibitory for neurite outgrowth and cell spreading (14), so far have no homologous counterpart in fish and are present with weak similarity in amphibians. This would be in concordance with the observation that the lack of regeneration during phylogeny was acquired during amphibian evolution and ontogenetically after metamorphosis (55). However, based on ESTs, the amphibian Nogo-A orthologs are expressed in embryos and tadpoles and not only after metamorphosis, similar to the situation in mammals, where Nogo-A is present in the CNS before myelination and thus before the CNS-specific lack of axonal regeneration. Developmental regulation of the membrane topology and surface expression of Nogo-A might explain some of the discrepancies, but more in-depth studies are needed on this issue. If this exceptionally long exon originated de novo in amphibians or was acquired by horizontal gene transfer from a prokaryotic or viral entity cannot be decided at this time.

FINAL REMARKS

This study demonstrates that the evolution of the RTN genes, probably arising during early eukaryote origin from an intron-rich ancestor, has been inhomogeneous. There is no evidence for evolutionary pressure on the highly variable amino termini of all subfamilies, in contrast to the conserved RHD. This is suggestive of a basal function of the common RHD, a function that might be shared by most of the identified members in the distinct taxa. Since the collective feature of RTNs is the association with ER membranes and because the RHD seems to be restricted to the eukaryotic world, we might speculate that the evolution of the RTNs was concomitant with the development of the endomembrane system $\sim$ 1.7 billion years ago and its function is related to it. Based on this hypothesis, the in-depth study of simple model organisms might provide important insight into the role of reticulons in other eukarya, including plants and vertebrates, where the functions

of most members are still elusive.

FJ

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