Tissue specificity and regulation of the N-terminal diversity of reticulon 3

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Over the last few years, the widely distributed family of reticulons (RTNs) is receiving renewed interest because of the implication of RTN4/Nogo in neurite regeneration. Four genes were identified in mammals and are referred to as RTN1, 2, 3 and the neurite outgrowth inhibitor RTN4/Nogo. In the present paper, we describe the existence of five new isoforms of RTN3 that differ in their N-termini, and analysed their tissue distribution and expression in neurons. We redefined the structure of human and murine *rtn3* genes, and identified two supplementary exons that may generate up to seven putative isoforms arising by alternative splicing or differential promoter usage. We confirmed the presence of five of these isoforms at the mRNA and protein levels, and showed their preferential expression in the central nervous system. We analysed *rtn3* expression in the cerebellum further, and observed

increased levels of several of the RTN3 isoforms during cerebellum development and during *in vitro* maturation of cerebellar granule cells. This pattern of expression paralleled that shown by RTN4/Nogo isoforms. Specifically, RTN3A1 expression was down-regulated upon cell death of cerebellar granule neurons triggered by potassium deprivation. Altogether, our results demonstrate that the *rtn3* gene generates multiple isoforms varying in their N-termini, and that their expression is tightly regulated in neurons. These findings suggest that RTN3 isoforms may contribute, by as yet unknown mechanisms, to neuronal survival and plasticity.

Key words: apoptosis, cerebellum, gene structure, isoform, Nogo, reticulon (RTN).

INTRODUCTION

Reticulons (RTNs) are a family of highly conserved proteins named after their preferred intracellular localization in the endoplasmic reticulum [1,2]. RTNs were initially described as markers of neuroendocrine cells, and their expression patterns were found to be specific for some types of lung cancer. In mammals, four genes (RTN1–4) lead to the expression of multiple isoforms sharing a 188-amino-acid domain localized in the C-terminus, and referred to as the RHD (RTN homology domain). RHD features two potential transmembrane domains and a 66-amino-acid hydrophilic loop, which was called Nogo66 for RTN4/Nogo [1]. Outside of the RHD, RTN proteins do not display evident sequence homologies. RTN1, 2 and 4 are expressed as multiple N-terminal isoforms that are generated by differential promoter usage or alternative splicing [3], while RTN3 has never been studied with regard to the existence of possible variants.

The functions of RTNs are still largely elusive. RTN4/Nogo is the only member of the family that has a well accepted function. RTN4/Nogo exhibits a potent inhibitory effect on neurite outgrowth, and is responsible, at least in part, for the ability of myelin to prevent neuronal regeneration in the central nervous system. In addition, RTN4/Nogo is also known to inhibit 3T3 fibroblast spreading [4–6]. Inhibition of neurite outgrowth by RTN4/Nogo is partially mediated by the Nogo66 loop, which is able to interact with a neuronal-specific GPI (glycosylphosphatidylinositol)-anchored receptor called NgR [7]. NgR is also a receptor for other myelin-derived inhibitory factors [8–10], and its binding to Nogo/RTN4 activates a RhoA-dependent pathway through p75 recruitment [11–13].

Inhibition of neurite outgrowth and fibroblast spreading are functions not common to other RTNs. Indeed, peptides equivalent to the Nogo66 loop in RTN1, 2 or 3 were unable to inhibit neurite outgrowth [5]. On the other hand, that RTNs accumulate specifically in the endoplasmic reticulum strongly suggests an intracellular function linked to this organelle. Interestingly, RTN1 and RTN4/Nogo can interact with proteins of the Bcl-2 family, and regulate both their subcellular localization and activity [14]. Furthermore, RTN4/Nogo appeared to be pro-apoptotic in several cell lines [15], although these findings are still controversial [16]. Whatever the final outcome, it is conceivable to suggest that RTN4/Nogo, and perhaps other RTNs, may be involved in the regulation of cell survival.

To date, most of the attention has been focused on the RHD region of RTNs, and the functions of their isoform-specific N-terminal domains were largely ignored. In this regard, GrandPre et al. [5] showed that the N-terminal domain of Nogo-A, the longest RTN4 isoform, has the ability to prevent fibroblast spreading, and Oertle et al. [17] discovered that two stretches of sequences, called NiR-Δ2 and NiG-Δ20, capable of inhibiting both neurite outgrowth and fibroblast spreading, are indeed located in this domain. N-termini are the least conserved regions of RTNs, and it is thus tempting to speculate that different N-terminal domains may support different functions, localizations or regulations among the various RTN isoforms.

As pointed out above, RTN3 protein is the only RTN known to exist only in one isoform, bearing a very short N-terminal domain and the RHD [18,19]. In the present study, we analysed databases for potential new RTN3 isoforms and describe seven putative RTN3 isoforms, which are mainly expressed in the central nervous

Abbreviations used: Cy3, carbocyanine 3; DMEM, Dulbecco's modified Eagle's medium; EST, expressed sequence tag; FBS, foetal bovine serum; HA, haemagglutinin; HK, high potassium; LK, low potassium; RHD, reticulon homology domain; RT, reverse transcriptase; RTN, reticulon.

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system. We also provide evidence for a physiologically regulated expression of these isoforms in a manner strikingly similar to that observed for the RTN4/Nogo gene. These findings have potential interest for the understanding of the functions of these proteins.

MATERIALS AND METHODS

Animal and human tissue preparation

Mice were of the FVB/N strain. Tissues were immediately frozen in liquid nitrogen and were stored at -80°C until use. Animal manipulation followed current European Union regulations. Human tissues were obtained from three forensic autopsy cases at the Institut de Médecine Légale (Strasbourg, France). Parts of the samples were taken during autopsy by order of the Prosecutor. They included brain, muscle, kidney, heart, spinal cord, spleen and liver, and were immediately frozen at -80°C . Subjects were 25, 32 and 55 years old, and the post-mortem interval between death and autopsy was 82–96 h.

In silico analysis and cloning of RTN3 isoforms

Database mining was performed using BLAST and ALIGN algorithms on the NCBI website (<http://www.ncbi.nlm.nih.gov>). Physical cloning of murine RTN3A1 and RTN3B was performed from total murine cerebellar RNA using the Thermoscript RT (reverse transcriptase)-PCR system (Invitrogen). PCR primers were as follows: RTN3A1-specific forward primer, 5'-ATGGCGGAGTCGTCAGCGGCCAC-3'; RTN3B-specific forward primer, 5'-ATGACTTCCTCCTTTCTTCATC-3'; RTN3A1 and RTN3B reverse primer, 5'-TTATTCTGCCTTTTTTGGCGATTCC-3'.

PCR products were cloned and sequenced in pCR Blunt II vector using the TOPO TA system (Invitrogen). RTN3A1 was subcloned further into pcDNA3 (Invitrogen). In the latter case, an HA (haemagglutinin) tag was inserted by PCR.

RT-PCR

Total RNA ($2\text{ }\mu\text{g}$) was reverse-transcribed using 200 units of MoMuLV RT and $0.5\text{ }\mu\text{g}$ of random primers (Promega) as described in [20]. After PCR amplification, products were separated on a 2% agarose gel and visualized by ethidium bromide staining. We used either G3PDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA levels or 18 S rRNA levels as an internal control [20]. PCR primers were as follows: forward primers: Exon 1, 5'-CCTACGCTCTCTTACCCTTC-3'; Exon 2, 5'-TCCTCTCCTCTCAGCCTGT-3'; Exon 3, 5'-GTCTGGGCCACAGAGTTCAT-3' and 5'-TCCTTCTGTAAATGCTTCTGTCC-3'; Exon 4, 5'-TCTGGCAGCCTTCAGTGTTA-3'; RTN3B-specific, 5'-ATGCTGCTTGGCCTGACTA-3'; internal-splice-site-specific Exon 3, 5'-ATCCCCAAGCAAAACAACAG-3'. Reverse primers: Exon 2, 5'-AAACAAAGGAATCCGCACAC-3'; Exon 3, 5'-CAGAGCCTGAGACCAATTC-3'; Exon 4, 5'-ACATCTCGCCAGAAATCAGA-3'; Exon 5, 5'-CCATCGCAGCATTCATGTAG-3'. Nogo primers were as used elsewhere [20].

Preparation of anti-RTN3 and anti-Nogo antiserum

Two peptides of 15 amino acid residues (CYVGIARDQTKSIVE-CONH₂ and AKIQAKIPGLKRKAE) were chosen from human RTN3A1 and Nogo-A sequences respectively, according to hydrophilicity/hydrophobicity profiles and antigenicity prediction analyses. Both peptides were located in the RHD and did not show potential cross-reactivity with other RTNs, since only two out of the 15 amino acid residues were conserved in these peptides among the different RTN proteins (see Supplementary Figure 1 at

<http://www.BiochemJ.org/bj/385/bj3850125add.htm>). After synthesis and purification by standard protocols, peptides were conjugated to keyhole-limpet haemocyanin, and were injected into two specific pathogen-free rabbits. Immunization was boosted with three subsequent injections at 14, 28 and 56 days. Terminal bleeds were affinity-purified against the immunizing peptide, and were stored as frozen aliquots. The polyclonal antisera were found to react specifically with RTN3 or Nogo proteins, and the specificity of binding to mouse central nervous system extracts was confirmed by the elimination of staining in the presence of excess immunizing peptide (see Figure 3 for RTN3 antiserum).

Western blot

Tissues were homogenized in PBS containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS and 1% protease inhibitor cocktail. Cultured cells were lysed in $120\text{ }\mu\text{l}$ of Laemmli's buffer (125 mM Tris/HCl, pH 6.7, containing 3.3% SDS, 0.7 M 2-mercaptoethanol, 10% glycerol and 0.02% Bromophenol Blue). Extracts were sonicated for 20 s and boiled for 5 min before they were centrifuged at 20000 g for 5 min. Equal amounts of protein, according to the bicinchoninic acid assay, were separated on an SDS/10% polyacrylamide gel, and were electrotransferred further on to nitrocellulose membranes. Equal loading was ensured either by reversible staining with 0.1% Ponceau S in 5% ethanoic (acetic) acid or by staining with an antibody directed against actin as indicated in [21]. Immunostaining was performed with the prepared anti-RTN3 (1/2000 dilution) or anti-Nogo antisera (1/2000 dilution), or with polyclonal anti-calnexin (0.1 mg/l; Chemicon) or anti-(cytochrome *c*) (1 mg/l; Chemicon), and horseradish-peroxidase-conjugated goat anti-rabbit IgG diluted at 1/2000. Finally, membranes were developed by enhanced chemiluminescence detection.

Immunoprecipitation

HA-tagged proteins ($400\text{--}750\text{ }\mu\text{g}$ of whole-cell extract) were incubated with $30\text{ }\mu\text{l}$ of 50% slurry Protein-A-Sepharose beads cross-linked to an anti-HA antibody (12CA5) at 4°C for 3 h. After incubation, samples were washed four times with 1 ml of wash buffer (20 mM Tris/HCl, pH 8, containing 150 mM NaCl, 1 mM EDTA, 0.5% Igepal, 1 mM dithiothreitol and 10% glycerol). Excess liquid was then aspirated, and $35\text{ }\mu\text{l}$ of Laemmli's buffer was added. Samples were heated at 95°C for 3 min, centrifuged at 20000 g for 3 min, and electrophoresed on a SDS/10% polyacrylamide gel. For HA detection, the 16B12 monoclonal anti-HA antibody (Babco) was used at 1/1000 dilution.

Primary culture of cerebellar granule neurons

Cerebellar granule neurons were obtained from 7-day-old mice as described previously [21]. Briefly, after enzymic and mechanical dissociation, cells were plated at a density of 500 cells/mm² on 0.1 mg/ml polyornithine-pre-coated six-well culture dishes and were grown at 37°C in a humidified atmosphere (5% CO₂/95% air). Culture medium for plating was composed of DMEM (Dulbecco's modified Eagle's medium; Life Technology) supplemented with 10% heat-inactivated horse serum (Life Technology), 100 nM insulin, 50 $\mu\text{g}/\text{ml}$ gentamycin and 25 mM KCl. At 48 h after plating, cells were switched to defined medium (called high-potassium, or HK, medium) that consisted of DMEM supplemented with 10 nM insulin, 100 $\mu\text{g}/\text{ml}$ human transferrin, 60 μM putrescine, 30 nM sodium selenite, 50 $\mu\text{g}/\text{ml}$ gentamycin and 25 mM KCl (final [K⁺] = 30 mM). Low-potassium (LK) medium was obtained by omitting KCl (final [K⁺] = 5 mM).

Figure 1 Gene structure and splice variants of human and mouse *rtn3*

(A) Gene structure of human and mouse *rtn3* gene. The nine exons are represented by boxes and the three different promoters are indicated by arrows. (B) Schematic representation of the *rtn3* splice variants. Arrows indicate the translation start sites. For clarity, exon 3.1 is shown in the darker shade of grey. (C) Schematic representation of putative *rtn3* gene products and comparison with two of the major *rtn3* gene products. Amino acids derived from exon 1, 2 and 3 are shown as stippled, black and white respectively. The position of the two putative transmembrane domains in the RHD is shown in dark grey.

Transfection

HEK-293 (human embryonic kidney) cells (A.T.C.C., Manassas, VA, U.S.A.) were maintained in DMEM supplemented with 10% FBS (foetal bovine serum) in 5% CO₂ at 37°C. HEK-293 cells and primary cerebellar granule neurons were transfected using a polyethyleneimine-based protocol as described previously [21]. Cells were grown in six-well plates in DMEM/10% FBS and transfected with 3 µg of DNA overnight, after which medium was replaced.

Subcellular fractionation

Tissues were disrupted using a Dounce-type homogenizer and then centrifuged at 1000 *g* for 10 min to obtain the nuclear pellet. The resulting supernatant was centrifuged further at 13 000 *g* for 15 min to generate the mitochondria-enriched fraction. The resulting supernatant was centrifuged further at 100 000 *g* for 1 h to separate the soluble cytosolic fraction from the insoluble membrane fraction (microsomes). Fractions were subsequently analysed by Western blotting.

Immunocytochemistry

Cell staining was performed using an indirect immunofluorescence technique. Briefly, cells were subsequently incubated with 1% Triton X-100 in PBS for 10 min, and 0.5% Triton X-100 plus 5% BSA in PBS for 30 min. After overnight incubation at 4°C with the indicated antibodies, cells were incubated with Alexa-Fluor-coupled goat anti-mouse IgG diluted 1/500 and Cy3 (carboxyanine 3)-coupled goat anti-rabbit IgG diluted 1/500. Alexa Fluor stain was from Molecular Probes and Cy3 was from Jackson Immunoresearch Laboratories. Immunofluorescent preparations were examined with a laser scanning imaging system (MRC-1024; Bio-Rad) equipped with a 15 mV krypton/argon mixed gas ion laser. A ×100 fluor lens was used. The detection pinhole was set to the minimum to give the narrowest possible optical section. The fluorescence emission of Alexa Fluor and Cy3 was recorded

sequentially by separate photomultipliers and visualized using the Laser Sharp version 2.1 software (Bio-Rad).

RESULTS

In silico analysis of N-terminal exons of human and murine *rtn3* genes

The previously published cDNA for human (GenBank® accession number AF059524) and mouse (GenBank® accession number AB046114) RTN3 consists of a six-exon-long RHD of 188 amino acids and a 49-amino-acid N-terminal domain. We assumed that this sequence (noted herein as RTN3A1) was equivalent to that of Nogo-B, and we searched in databases for ESTs (expressed sequence tags) bearing homologies either with the 5' sequence of the RHD, but not with the N-terminal domain, or with the 3' region of this N-terminal domain, but not with the RHD. We found a number of human or murine ESTs that we used to propose the existence of new isoforms. When these ESTs were aligned against the genomic sequences of human and murine *rtn3* genes, we identified two new exons, 2 and 3, in the large genomic region between the previously known exon 1 and those encoding the RHD (Figure 1A). We also found that exon 2 was skipped in several human ESTs, thus suggesting that this exon can be alternatively spliced, and that exon 3 had two potential 5'-splicing acceptor sites which defined exon 3.1 and exon 3.2 (Table 1 and Figure 1B). Moreover, two ESTs (GenBank® accession numbers AW997618 and AK054345) extending the new exon 3 upstream of the 5'-exon acceptor site were found. These two highly homologous sequences carried a conserved methionine residue downstream of the splice acceptor site, which strongly suggests the existence of an intronic promoter. Finally, we identified intron-exon junctions by comparing genomic and cDNA sequences (Table 1). Based on all these findings, we propose a novel organization of the *rtn3* gene containing nine exons and spanning 78 kb in human and 56 kb in mouse (Figure 1A).

Table 1 Intro/exon junctions in the *rtn3* gene

Nucleotide sequences of the 5' and 3' end of exons (in upper case) and of the donor and acceptor site at the intron/exon junction of the human (upper line) and mouse *RTN3* gene (lower line) as deduced from comparison of the human (GenBank® accession number AP000753) or the murine (GenBank® accession number NT_082892) *rtn3* genomic sequences and ESTs covering the different exons of the *rtn3* gene. Sequences of the intron end are given in lower case, except for the donor and acceptor sites in upper case. All splice sites are according to the consensus rules (GT/AG). Exons and introns are numbered as referred to on Figure 1, and sizes are given. Homologies between human and mouse sequences are highlighted by asterisks.

Exon N°	5' end - Exon size (nt) - 3' end	Intron	5' end - Intron size (kb) - 3' end
1	TGTCCTCG - 264 - CTGTGCGG TCTTTGTG - 328 - G*****	a	GTaagg - 23 - ttctAG ***** - 15 - *****
2	ATTCCTTT - 57 - CTCACAAG ***** - 57 - *****	b.1	between exons 2 and 3.1 GTctgt - 13.8 - atagAG ***ca* - 9.4 - *****
3.1	AGGGATTG - 2431 - CTTCTCAG C***** - 2114 - T***C***	b.2	between exons 2 and 3.2 GTctgt - 14.8 - caacAG ***ca* - 10.3 - *****
3.2	GTGATTGG - 1323 - CTTCTCAG ***C*** - 1151 - T***C***	c	between exons 3.x and 4 GTaacc - 28.9 - tgtcAG ***** - 21.1 - *g****
3.3	TGTGTATA - 2448 - CTTCTCAG ***T*GTG - 2369 - T***C***		
4	TGCACGAT - 209 - CCATTCAA ***** - 209 - *****	d	GTgagt - 2.3 - tggaAG ***** - 2.3 - c****
5	AGCCTACC - 139 - CCTTGAAG G*****T - 139 - *****	e	GTtagt - 0.42 - tcacAG ***** - 0.42 - *****
6	CTGGCTGT - 70 - AATTCTTG ***** - 70 - G*****C*	f	GTaagg - 0.62 - ttacAG ***** - 0.93 - *****
7	CTGAAC TG - 47 - AGTACAAG *C**G*** - 47 - ***T***	g	GTaagc - 2.4 - ttaaAG ***** - 1.2 - *****
8	ACCCAGAT - 59 - GTTGAAAA ***** - 62 - *****	h	GTaagt - 2 - caatAG ***** - 1.9 - *****
9	GATCCAAG - 1123 - GAGAATTC ***** - 1971 - TAACTGCT		3' untranslated region

Nomenclature of the new *RTN3* isoforms and RT-PCR validation

Figure 1(B) represents the organization of the eight isoforms of *RTN3* as deduced from our *in silico* studies. Based on the nomenclature described by Oertle et al. [1], we proceeded as follows: isoforms sharing a common putative promoter were designated with the same letter (A, B or C). Each promoter-specific isoform was numbered from 1 to 4, depending on the exon(s) contained, and further designated a or b, depending on the existence of alternative splicing in exon 3. Thus the previously known *RTN3* was renamed here *RTN3A1*, and additional isoforms bearing the exon 1 were named *RTN3A2*, *RTN3A3* and *RTN3A4*. *RTN3A3* and *RTN3A4* yielded, in addition, two isoforms each (*RTN3A3a* and *b*, and *RTN3A4a* and *b*). The isoform originated from the intronic promoter was named *RTN3B*. Furthermore, during the course of our study, we found evidence (see below and the Discussion section) for the existence of an isoform containing only the RHD, which was named *RTN3C*.

To ascertain the existence of the *in-silico*-identified *RTN3* transcripts, we first physically cloned *RTN3A1* and *RTN3B*, which are two isoforms with a very different amino acid composition in the N-terminal domain (sequences deposited in GenBank® database under the accession numbers AY507126 and AY507127 respectively). We performed further RT-PCR experiments using a large set of isoform-specific primers represented in Figure 2(A). Five out of the eight isoforms proposed were identified in several mouse tissues, and were expressed at a particularly high rate in the central nervous system (Figure 2B and Table 2). We were unable to detect *RTN3A3* (both a and b isoforms) in mouse tissue, which suggests that exon 2 and exon 3 are always spliced together,

Figure 2 Existence of multiple *RTN3* isoforms at the mRNA level

(A) Localization of the primer pairs (designated P1–P10) for RT-PCR on mouse *rtn3* gene. (B) Representative RT-PCR products obtained using mouse spinal cord total RNA as a template, and showing the existence of the following *rtn3* splice variants: *RTN3A1* (A1), *RTN3A2* (A2), *RTN3A4a* (A4a), *RTN3A4b* (A4b) and *RTN3B* (B). As a control, RT was omitted in one of the *RTN3B* reactions, since this primer pair did not allow discrimination between cDNA and genomic DNA. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) amplification was used as a standard. (C) Evidence of an RHD-only isoform in skeletal muscle: semi-quantitative RT-PCR was performed using total RNA from either spinal cord (left) and skeletal muscle (right) and various oligonucleotide pairs spanning the whole *rtn3* exons. G3PDH amplification was conducted in parallel to ensure equal cDNA template in both samples (extreme right). 18 S RNA amplification yielded identical results with those of G3PDH (not shown).

yielding *RTN3A4* rather than *RTN3A3*. Next, we asked whether the new exons 2 and 3 were co-expressed with the RHD. Using forward primers in each of the N-terminal exons and the reverse primer located in the RHD, we showed that exons 1, 2 and 3 are expressed in co-linearity with the RHD (Figure 2C). Interestingly, no signal was observed in skeletal muscle, except for the RHD fragment alone. These findings, along with the absence of any EST overlapping the 5'-end of the exon 4 and the presence of a

Table 2 Tissue distribution of RTN3 isoforms as determined using RT-PCR

RT-PCR was performed as described in Figure 2. + means that a strong signal is detectable after 30 PCR cycles. +/- means that a PCR fragment was barely detectable after 30 cycles of PCR. — means that we did not detect any expression after 30 cycles. Oligonucleotides are described in Figure 2(A).

RTN3 isoform(s)	Spinal cord	Cortex	Cerebellum	Skeletal muscle	Testis	Liver
RTN3A1	+	+	+	—	—	+/-
RTN3A2	+/-	+/-	+/-	—	+/-	+
RTN3A3a	—	—	—	—	—	—
RTN3A4a	+	+	+	—	+/-	+/-
RTN3A3b	—	—	—	—	—	—
RTN3A4b	+	+	+	—	+/-	+/-
RTN3B	+	+	+	—	+/-	+/-
Exon 1 + RHD	+	+	+	—	+/-	+/-
Exon 2 + RHD	+	+	+	—	+/-	+
Exon 3 + RHD	+	+	+	—	+/-	+/-
Exon1 + 2	+	+	+	—	—	+
Exon1 + 3	+	+	+	—	—	—
Exon2 + 3	+	+	+	—	+/-	+/-
Pan-RHD	+	+	+	+	+	+

conserved methionine residue in exon 4, strongly support, as proposed in Figure 1(A), the existence of RTN3C, the RHD-only isoform in this tissue.

Existence of the N-terminal isoforms of RTN3 at the protein level

In order to analyse the expression of the various RTN3 isoforms at the protein level, we generated a polyclonal rabbit antiserum that recognizes the RHD region common to all RTN3 isoforms. To verify the specificity of our antibody, we expressed RTN3A1 in HEK-293 cells as an HA-tagged protein, while an HA-p73 expression vector was used as a negative control. We immunoprecipitated the HA-tagged proteins using an anti-HA antibody cross-linked to Protein-A-Sepharose beads and subjected them to Western blotting using either an anti-HA or an anti-RTN3 antibody. Figure 3(A) shows that the immunoprecipitated HA-tagged RTN3 proteins can be detected by both anti-HA and anti-RTN3 antibodies. In contrast, the HA-p73 proteins were not revealed by the RTN3 antiserum. The right-hand panels of Figure 3(A) show HA-p73 and HA-RTN3 expression in the extracts revealed by both antibodies. The specificity of the anti-RTN3 antibody was strengthened further by the elimination of all the immunoreactive bands detected on a Western blot after pre-adsorption of the RTN3 antiserum with the immunizing peptide (Figure 3C). When these data were compared with those obtained by RT-PCR and the expected molecular masses of RTN3 isoforms (Table 3), we concluded that the band migrating at 95–100 kDa is compatible with the existence of RTN3 isoforms bearing a complete exon 3 (i.e. RTN3A4b and RTN3B), whereas the band migrating at 70 kDa corresponds to RTN3A4a, the isoform with a truncated exon 3. Furthermore, the bands migrating at 24 and 27 kDa fit well

Figure 3 Existence of multiple RTN3 isoforms at the protein level

(A) HA-RTN3A1 and HA-p73 were expressed in HEK-293 cells for 24 h. HA-tagged proteins were immunoprecipitated in the presence of anti-HA (@HA) beads. The precipitates were then separated on an SDS/10% PAGE gel, and HA-tagged proteins were detected with an anti-HA antibody or the RTN3 antiserum (left-hand panels). The two right-hand panels show expression levels in the respective extracts detected by an anti-HA antibody or the RTN3 antiserum. IP, immunoprecipitation; WB, Western blot. Ct, control. (B) Tissue distribution of RTN3 isoforms in murine tissues. For each lane, 20 µg of proteins extracted from the indicated tissues was Western blotted using the same antibody as that in (A). Note the existence of a doublet at approx. 25 kDa (RTN3A1 and A2), the multiple bands between 75 and 100 kDa (high-molecular-mass RTN3), and the prominent 18 kDa band in the skeletal muscle corresponding to the RHD-only isoform RTN3C. (C) Specificity of the antisera raised against RTN3: 20 µg of murine cerebellum was Western blotted using either anti-RTN3 immunopurified antiserum (—, left) or the same antibody pre-incubated for 1 h at room temperature (20 °C) with 100 µg of epitope (+). Note the presence of a 40 kDa specific band (asterisk), which does not correspond to any of the expected molecular masses of RTN3 isoforms described in the present paper. This band is also indicated with an asterisk in (B) and (E). (D) Proteins (20 µg) from a human neuroblastoma cell line (Sh5) were Western blotted for RTN3. Note the occurrence of a 100 kDa band corresponding to RTN3A4b. (E) Tissue distribution of RTN3 isoforms in human autopsy tissues. Proteins (20 µg) extracted from necropsies from the indicated tissues were Western blotted for RTN3. Non-specific immunoreactivity was determined using the same antibody pre-incubated with 100 µg of the epitope (results not shown). Molecular masses are given in kDa in (A), (C), (D) and (E).

Table 3 Expected molecular mass of RTN3 isoforms

RTN3 isoform	Mouse		Human	
	Number of amino acids	Expected molecular mass (kDa)	Number of amino acids	Expected molecular mass (kDa)
RTN3A1	237	25	236	25
RTN3A2	255	28	255	28
RTN3A3a	624	67	677	74
RTN3A3b	945	102	1013	110
RTN3A4a	643	69	696	76
RTN3A4b	964	105	1032	112
RTN3B	882	95	951	105
RTN3C	167	19	167	19

with the expected molecular masses of RTN3A1 and RTN3A2 respectively. Finally, the existence of RTN3C was confirmed at the protein level, since a band migrating at approx. 20 kDa, highly enriched in skeletal muscle, was observed consistent with the RHD-only isoform. It should be also noted that a band migrating at 40 kDa (indicated by an asterisk on blots presented in Figure 3), specifically displaced by epitope pre-adsorption, did not correspond to any of the identified isoforms nor to Nogo-B (see Supplementary Figure 2 at <http://www.BiochemJ.org/bj/385/bj3850125add.htm>), and we have no explanation for this observation.

Tissue-distribution studies revealed that, in mouse, the N-terminal diversity of RTN3 is higher in different regions of the central nervous system than in other tissues, including muscle, liver, kidney, testis and heart (Figure 3B). RTN3 isoforms were also present in human autopsy samples (Figure 3E) and in the human neuroblastoma cell line Sh5 (Figure 3D). It should be emphasized that the tissue distribution that we observed in Western blot experiments was consistent with the pattern of expression previously determined by RT-PCR (Table 2).

Subcellular localization of RTN3 isoforms

In the next step, we looked at the localization of RTN3 at the cell level. To this aim, we compared the immunoreactivity of HA-tagged RTN3A1 in cerebellar granule neurons with that of total RTN3 using confocal microscopy. HA immunoreactivity was observed in specific zones in the cytoplasm in form of a conspicuous semicircular spot (Figure 4A) or a perinuclear halo (Figure 4D). This labelling was also detectable using our pan-RTN3 antiserum (Figures 4B and 4E), and both signals co-localized on the merged images (Figures 4C and 4F). It should be noted that HA-RTN3A1 was also present in neurites, and sometimes accumulated at the neurite terminal (results not shown). These findings suggest that RTN3 location is restricted to particular intracellular organelles rather than being evenly distributed as a cytosolic protein. To confirm these data in cells that have a more prominent cytoplasm than that of cerebellar granule neurons, we used HEK-293 cells, and found the same results (Figures 4G, 4H and 4I).

To gain further insight into this question, we performed subcellular fractionation of adult mouse cerebellum. As illustrated in Figure 4(J), both high- (RTN3A4b/B) and low-molecular-mass (RTN3A1/2) isoforms were enriched in the microsomal fraction, which may explain their intracellular compartmentalization, and corroborates the assigned membrane location for this family of proteins. However, a portion of RTN3A4b/B was also detectable in the cytosol. To better clarify this point, we performed differential solubilization of the microsomal fraction, and followed in parallel

the behaviour of calnexin, a classical endoplasmic reticulum protein. As illustrated in Figure 4(K), calnexin, normally present in the membrane pellet after high-speed centrifugation, was very efficiently extracted after membrane disruption by detergent treatment (1% SDS or 1% Triton X-100). High-salt (0.3 M NaCl) or alkaline (0.1 M Na₂CO₃, pH 11) treatment, known to release membrane-associated, but not membrane-integrated, proteins, hardly modified the location of calnexin towards the soluble fraction, thus showing the nature of calnexin as a true transmembrane protein. As far as RTN3 is concerned, we found that any kind of treatment, whether preventing membrane disruption or not, extracted high-molecular-mass isoforms totally from the microsomal fraction. In contrast, a portion of RTN3A1 remained in the membrane pellet after high-salt or alkaline treatment, and only a stronger solubilizing treatment by detergent was able to release the low-molecular-mass RTN3 isoform from the pellet fraction completely (Figure 4K). In all, these findings mean that RTN3A4b/B is likely to be associated with the membrane through a slight interaction, which may explain the presence of an easily extractable pool of high-molecular-mass isoforms contaminating the cytosolic fraction (Figure 4J). Nevertheless, our results do not formally exclude the existence of a cytosolic pool of RTN3A4b/B. Conversely, RTN3A1 seems to be inserted into the lipid bilayer, thus indicating that different RTN3 variants probably have distinct N-termini-dependent topologies and hence distinct locations.

N-terminal diversity of RTN3 is regulated during neuronal differentiation and programmed cell death

It was shown previously that RTN4/Nogo has a complex pattern of expression in the developing cerebellum [22]. In the present study, we examined the expression of RTN3 isoforms in comparison with that of Nogo proteins, and observed that the levels of both RTN3 and Nogo isoforms increased during cerebellar development in a very similar manner (Figure 5A). Specifically, expression of low- (Nogo-C and RTN3C) and high-molecular-mass (Nogo-A and RTN3A4/B) isoforms increased until adult age, while medium-sized isoforms (Nogo-B and RTN3A1) were more constantly expressed. Consistent with these data, RT-PCR analysis revealed that expression of both RTN3A2 and RTN3A4b was up-regulated between post-natal days 3 and 35 of cerebellum development, whereas that of RTN3A1 remained unchanged. Similarly, levels of Nogo-A and Nogo-C transcripts were highly increased, whereas those of Nogo-B were only modestly augmented (Figure 5B).

In vitro development of cerebellar granule neurons, which are prepared from 7-day-old mice and finalize their differentiation in culture, is a common model to study neuronal apoptosis and plasticity [21]. In the present study, we followed the expression of RTN3 and Nogo up to 6 days of culture, and found that several isoforms, including RTN3A1, RTN3A2, RTN3A4, Nogo-A and Nogo-C, were clearly up-regulated at day 4, when neurons are fully differentiated (Figure 6A). Since this effect occurred at a very specific point, when cells are starved of serum and grown in HK medium, we tested whether the changes observed in RTN3 and Nogo expressions were due to the modification of culture conditions. To this aim, cells were left for 5 days in serum-containing medium or challenged to HK medium from day 3. As shown in Figure 6(B), the change of medium did not affect the increase in RTN3 or Nogo isoform expression at day 4, thus suggesting that the complex rearrangement of RTN3/4 expression is a pre-programmed event tightly associated with neuronal differentiation.

As RTNs have been proposed to be involved in the regulation of apoptosis, we tested whether RTN3 and Nogo expressions were regulated during cerebellar granule neuron apoptosis. Survival of

Figure 4 Subcellular localization of RTN3 isoforms

(A–I) Cerebellar granule neurons (CGN, A–F) or HEK-293 cells (G–I) were transfected with pcDNA3-HA-RTN3A1. Double immunostaining for HA tag (@ HA, green) and RTN3 (@ pan-RTN3, red) were performed and visualized by confocal microscopy. Note that HA and total RTN3 immunoreactive cells, co-localized in the merged images. Scale bar, 10 μ m. (J) Adult mouse cerebellum was subjected to subcellular fractionation as indicated in the Materials and methods section. Aliquots of total, nuclear, mitochondrial, microsomal and cytosolic extracts were analysed by immunoblotting with anti-RTN3 antibody, anti-(cytochrome c) (cyt C; mitochondrial marker) and anti-calnexin (endoplasmic reticulum marker). (K) Microsomes obtained as in (J) were treated for 30 min on ice with a 1/10 volume of extraction buffer (none), 3 M NaCl, 1 M Na₂CO₃, pH 11, 10% SDS or 10% Triton X-100. Subsequent high-speed centrifugation yielded a supernatant (S) and a pellet (P) fraction. Western blot analysis was then performed as in (J).

Figure 5 RTN3 and RTN4/Nogo expression in murine developing cerebellum

(A) Representative Western blot showing RTN3 (upper panel) and Nogo (lower panel) proteins extracted from cerebellum of mice at embryonic day 18 (E18), and post-natal days 1 (P1), 7 (P7) and 35 (P35). Each lane represents an individual mouse. (B) Representative RT-PCR showing the expression levels of various RTN3 and RTN4/Nogo isoforms during murine cerebellum development. Primer pairs were the same as in Figure 2. The linear range of amplification was determined (results not shown). 18 S rRNA was used as a control.

Figure 6 RTN3 and RTN4/Nogo expression in differentiating cerebellar granule neurons

(A) Representative Western blot showing protein levels of RTN3 (upper panel) and Nogo (lower panel) in cerebellar neurons differentiating *in vitro*. The days in culture (D1, D2, ...) are indicated. MD defines the switch to defined medium. NS indicates a non-specific immunoreactive band. Ponceau staining was used as a standard (not shown). (B) Representative Western blot showing protein levels of RTN3 (upper panel) and Nogo (lower panel) in cerebellar neurons differentiating *in vitro* with or without (MD) serum.

cerebellar neurons in culture is dependent on the presence of a high-potassium concentration (30 mM) in the medium. When extracellular potassium is lowered to 5 mM (LK medium), cerebellar neurons die by apoptosis in a caspase-3-dependent manner [21]. We compared the regulation of RTN3 and Nogo obtained with mature and immature neurons that had been subjected to LK culture conditions. Figure 7 shows that expression of RTN3A1, RTN3A2 and RTN3A4 was down-regulated in apoptotic mature neurons. However, this effect was not observed in immature neurons. Similar results on mature neurons were observed for Nogo-C, the low-molecular-mass member of the Nogo family. Furthermore, treatment with the caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone) reversed the effect observed on RTN3A1 and RTN3A2, suggesting that the down-regulation was dependent on the apoptotic process, and was directly or indirectly controlled by caspases.

Figure 7 RTN3 and RTN4/Nogo expression in cerebellar granule neurons undergoing apoptosis

(A) Representative RT-PCR showing the expression levels of various RTN3 and RTN4/Nogo isoforms during cerebellar granule neurons apoptosis. Ct, HK medium. The time (h) of treatment is indicated. Primer pairs were the same as in Figure 2. The linear range of amplification was determined (results not shown). 18 S rRNA was used as a control. (B) Representative Western blot showing RTN3 proteins from cerebellar granule neurons undergoing apoptosis. Treatment with 50 μ M Z-VAD-FMK was performed simultaneously with LK treatment. Blots were stripped and re-probed with an anti-actin antibody used as a standard.

Altogether, these data suggest that RTNs may have a role in the control of neuronal survival, which could also justify the tight control of their expressions observed during neuron maturation.

DISCUSSION

rtn genes share a common structure

In the present paper, we analysed the genomic structure of mouse and human *rtn3* genes, and showed the existence and developmental regulation of multiple new isoforms. These data, together with those concerning the phylogeny of RTNs published recently by Oertle et al. [1], show the extreme conservation of the structure of *rtn* genes in mammals. Indeed, all vertebrate *rtn* genes have six exons encoding the RHD and three to five exons encoding the N-terminal domain (see [23] for a review). The general organization of *rtn3* appears closer to that of *nogo*: both genes possess a long exon 3 preceded by two short exons, which is not the case for *rtn1* and *rtn2* [24]. All RTNs exhibit multiple N-terminal isoforms that are generated by either alternative splicing or differential promoter usage, as recently shown in detail for *nogo* [3]. In the case of *rtn3*, we hypothesize the existence of three alternative promoters for the following reasons: (i) the existence of sequences recognized as promoters by *in silico* algorithms in the genomic regions located upstream of exon 1 and exon 3 (M. De Tapia, unpublished work); (ii) the existence of isoforms bearing the exon 1 (RTN3A) or not (RTN3B), which strongly suggests the existence of two promoters upstream of these exons; and (iii) the detection by RT-PCR and Western blot of a RHD-only isoform highly expressed in skeletal muscle, which strongly suggests that a third promoter, located upstream of exon 4, does exist. Interestingly, promoters with a similar location are also documented for RTN4 and RTN2 [24], thus reinforcing the highly conserved structure of these genes. Each of the *rtn* genes encodes for approx. ten splice variants, three of them being predominant: one encodes a protein of approx. 100 kDa, notably enriched in the central nervous system; a second medium-sized protein of approx. 25–37 kDa that is ubiquitously distributed; and a third short isoform, which is expressed especially in skeletal muscle and bears only the RHD. This pattern of tissue distribution is another conserved feature of RTNs.

***rtn* genes share a common regulated expression**

To our knowledge, Nogo is the only member of the RTN family for which developmental studies exist [22], and no study has compared the expression levels of different RTNs during physiological events. To determine whether RTN3 and Nogo share a common regulation, we focused on three different, but somehow overlapping, events, namely the development of a whole organ, i.e. the cerebellum, the *in vitro* differentiation of progenitors from the granule layer of the cerebellum and the programmed death of these same cells. The pattern of expression of RTN3 that we observed was strikingly similar to that of Nogo not only during cerebellar development, but also during *in vitro* differentiation and cell death. In particular, the up-regulation of low-molecular-mass isoforms of RTN proteins during neuronal differentiation *in vitro*, as that found in the present study, had previously been observed by Hens et al. [25] in the case of RTN1C. These data thus show that the different *rtn* genes are tightly co-regulated. In addition, they provide evidence for a link between the *rtn* gene family and the state of differentiation of neurons, thus suggesting that disturbances in the expression of *rtn* genes may compromise neuronal survival.

Functions carried by the N-terminal domain of RTN proteins

From our data, it seems that the expression of the N-terminal domains of RTNs is carefully regulated during development, which raises the question of the functional significance of such a phenomenon. At present, little is known about the functions of these domains, and not much can be said about their putative functions when their primary sequences are analysed. Indeed, the N-terminal domains of RTNs are divergent in length and do not display any significant similarities with each other. However, such domains share some common conserved features. For example, the N-terminal domains of both murine and human RTNs are particularly acidic (pI between 4.3 and 4.6), and they are unusually rich in serine residues (L. Dupuis, unpublished work). A recent study by Oertle et al. [17] suggested a role in extracellular signalling for the regions NiR- Δ 2 and NiG- Δ 20 located in the N-terminal domain of Nogo-A. In addition, three studies described the generation of different lines of Nogo-knockout mice [26–28], and only the transgenic line knockout for the Nogo-A-specific exon, which encodes NiR- Δ 2 and NiG- Δ 20, appeared more permissive to neurite outgrowth during the entire life span [28]. Conversely, Nogo-RHD-knockout mice either did not show any higher regenerative activity [26] or showed such an activity only in young animals [27]. One can speculate that the RHD of Nogo could have a more prominent action during development, while its N-terminal domain could affect neurite outgrowth in the adult age. Unfortunately, there is no study aimed at determining whether the N-terminal domains of the other RTNs may have such a role. Another hypothesis is that N-terminal domains of RTNs have an intracellular function. Indeed, it was shown that RTNs are able to interact with a variety of proteins, including NIMP (Nogo-interacting mitochondrial protein) [29], glucosylceramide synthase [30], cytoskeletal proteins [31] and proteins of the Bcl-2 family [14]. In this regard, the N-terminal domains of RTNs could regulate these interactions by modifying the subcellular localization of the RHD. It should be noted that, in addition to their typical location in the endoplasmic reticulum, certain RTN isoforms appear associated with other specific compartments in the cell. RTN2C, for instance, is present in the Z-bands of sarcomeres in striated muscles [32], and RTNL-C, the shortest isoform of the unique *rtn* gene in *Caenorhabditis elegans*, has been shown to be partly cytosolic [33]. Our present subcellular fractionation experiments revealed differences between isoforms of RTN3

with regard to their abilities to be chemically extracted from microsomes. Thus low-molecular-mass isoforms are more tightly associated with membranes than high-molecular-mass isoforms. Whether these findings result either from the occurrence of multiple topologies in a single cell or differential protein sorting between different cell types clearly needs further investigation. It should be mentioned that the only available evidence in the case of RTN4/Nogo is rather controversial and that no unique topology has been assigned yet [17,23]. Alternatively, N-terminal domains could be by themselves a molecular binding partner. The occurrence of *in silico* predicted SH (Src homology) 2 and 3 domains in the N-terminal domain of Nogo-A reinforces this hypothesis (L. Dupuis, unpublished work). In addition, the *C. elegans* RTNL-C interacts through its unique N-terminal domain with RME-1 (receptor-mediated endocytosis 1) [33], a protein involved in vesicular trafficking [34]. In all, these data suggest that N-terminal domains may be involved in protein–protein interactions, but evidence for this is still lacking in mammals.

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