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Identification of two *nogo/rtn4* genes and analysis of Nogo-A expression in *Xenopus laevis*☆

Michael Klinger,[a,1] Heike Diekmann,[a,1] Dietmar Heinz,[a] Cordula Hirsch,[a] Sylvia Hannbeck von Hanwehr,[a] Barbara Petrausch,[a] Thomas Oertle,[b] Martin E. Schwab,[b] and Claudia A.O. Stuermer[a,*]

[a] *Department of Biology, University of Konstanz, 78457 Konstanz, Germany*
[b] *Department of Biology, ETH Zurich and Brain Research Institute, University of Zurich, 8057 Zurich, Switzerland*

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Myelin-associated axon growth inhibitors such as Nogo-A/RTN4-A impair axon regeneration in the adult mammalian central nervous system (CNS). Here, we describe the cloning and expression of two independent *Xenopus laevis rtn4* orthologs. As in mammals, alternative transcripts are generated both through differential splicing and promoter usage, giving rise to *Xenopus* nogo-A, -B, -C and to a new isoform, nogo-N/rtn4-N. *Xenopus* is therefore the 'lowest' vertebrate where Nogo-A was identified.

***Xenopus* Nogo-A/RTN4-A is predominantly expressed in the nervous system, whereas the other isoforms mainly occur in non-neuronal tissues. Nogo-A/RTN4-A specific antisera detect the protein in myelinated fiber tracts of the spinal cord, hindbrain, optic nerve, tectum opticum and in isolated oligodendrocytes. In addition, subpopulations of CNS neurons are Nogo-A/RTN4-A positive. This expression pattern is consistent with that observed for rat Nogo-A and suggests similar functions. Nogo-A in *Xenopus* myelin might therefore contribute to the failure of spinal cord regeneration in frogs—a feature that may have evolved during the transition from fish to land vertebrates.**

Introduction

In the adult mammalian and avian central nervous systems (CNS), regeneration of lesioned nerve fibers is prevented by inhibitory proteins that are particularly enriched in myelin and oligodendrocytes (Caroni and Schwab, 1988b), such as myelin-associated glycoprotein (MAG) (McKerracher et al., 1994), oligodendrocyte-myelin glycoprotein (OMgp) (Wang et al., 2002a) and Nogo/RTN4 (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000). Earlier functional studies indicated the presence of related neurite growth inhibitors, in particular the IN-1 antigen, in oligodendrocytes/CNS myelin of *Xenopus* (Lang et al., 1995). The IN-1 antibody has originally been raised against a 250 kDa myelin fraction (Caroni and Schwab, 1988a), and based on its neutralizing activity the bovine Nogo-A ortholog (bNI220) has been purified (Spillmann et al., 1998).

Nogo/RTN4 is a member of the reticulon (RTN) family of proteins (Chen et al., 2000; GrandPré et al., 2000; Prinjha et al., 2000). These proteins share a conserved 188 amino acid (aa) long C-terminal reticulon homology domain (RHD) (Oertle et al., 2003b). The two hydrophobic stretches within the RHD could serve as transmembrane domains for their insertion in endoplasmic reticulum (ER) and plasma membranes (Oertle et al., 2003c; van de Velde et al., 1994). Outside the RHD domain, Nogo/RTN4 and the other three RTN proteins (RTN1-3) have no obvious sequence similarities.

In mammals, the *nogo/rtn4* gene gives rise to different isoforms both through alternative splicing and alternative promoter usage with the three major transcripts known as nogo-A, nogo-B and nogo-C (Oertle et al., 2003a). The largest isoform, Nogo-A/RTN4-A, has been described as a potent neurite growth inhibitor of CNS myelin with two inhibitory sites. A stretch in the Nogo-A-specific region (NiG-Δ20) is strongly inhibitory for neurite outgrowth and cell spreading in vitro (Oertle et al., 2003d). In addition, the loop region between the two C-terminal hydrophobic domains of the RHD (called Nogo-66) has been shown to induce collapse of neuronal growth cones in vitro (GrandPré et al., 2000).

The RTN proteins are evolutionary conserved in eukaryotes (Oertle et al., 2003b), and therefore Nogo-A homologs may exist and impair CNS axon regeneration in amphibians. In frogs, CNS axons fail to regenerate in the spinal cord after metamorphosis (Beattie et al., 1990; Forehand and Farel, 1982). The inability of regeneration correlates with the nonpermissive properties of spinal cord myelin and oligodendrocytes in cocultures with axons, indicating the expression of inhibitory proteins in this region (Lang and Stuermer, 1996; Lang et al., 1995). CNS myelin and oligodendrocytes from the optic nerve and tectum, where amphibian axons do regenerate, were not inhibitory to growing axons in vitro (Lang et al., 1995). Reasons may lie in regional differences concerning

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* Corresponding author. Fax: +49-7531-88 3894.
E-mail address: Claudia.Stuermer@uni-konstanz.de (C.A.O. Stuermer).

[1] Both authors contributed equally.

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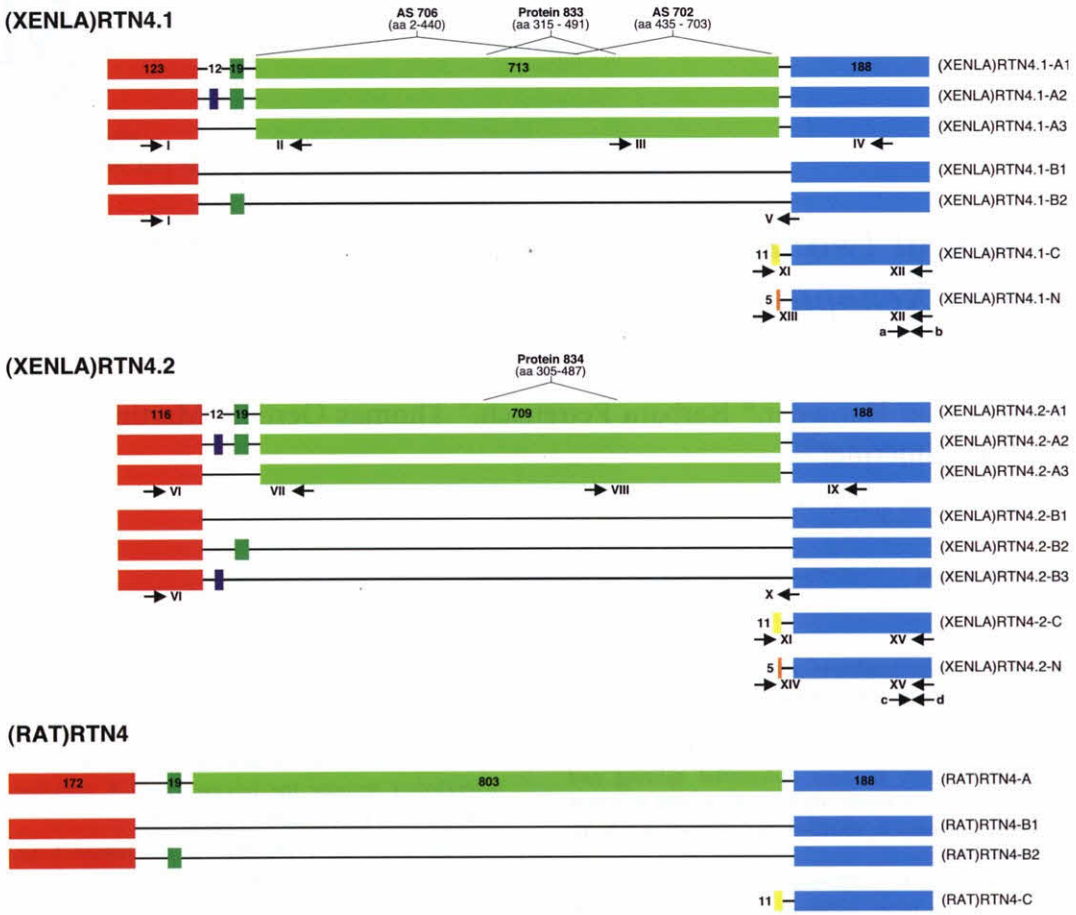

Fig. 1. Schematic representation of *Xenopus* nogo/rtn4 in comparison to rat nogo/rtn4 transcripts. Two *nogo/rtn4* genes were identified in *X. laevis* and the different transcripts were named according to the nomenclature guidelines for reticulon genes ((XENLA)rtn4.1, (XENLA)rtn4.2; see Experimental methods). Their overall structures correspond to mammalian rtn4. Nogo-A/rtn4-A consists of an N-terminal A/B-specific region (red), a central A-specific region (light green) and the RHD (blue). In the B-isoforms, the A-specific region is missing. For both the A- and B-isoforms, multiple minor splice variants exist due to the alternative usage of two (*Xenopus*) or one (mammals) small exons (dark blue and dark green). RTN4-C (yellow) and RTN4-N (orange) are transcribed from alternative promoters, but the RHD is the same as for the -A and -B isoforms. The amino acid length of the respective regions is depicted by Arabic numerals. Localizations of primers are indicated by arrows and Roman numerals (RT-PCR) or lower case letters (genomic PCR). The range of recombinant proteins used for the generation of polyclonal antisera against Nogo-A is outlined with brackets. AS, polyclonal antiserum.

the expression of myelin inhibitors or their subcellular localization, for example, surface exposure in myelin membranes or retention in the ER.

We used conserved sequence motifs of mammalian *nogo*-A to search for *Xenopus nogo/rtn4* homologs. Various transcripts corresponding to the three major isoforms rtn4-A, -B and -C were

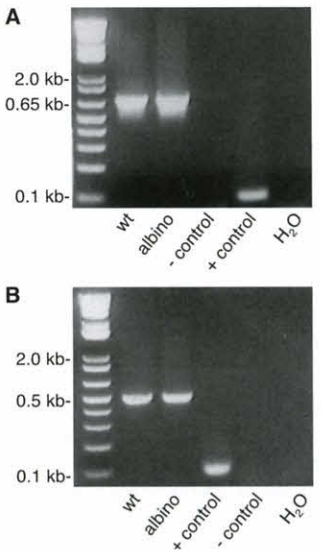

Fig. 2. PCR analysis of *Xenopus* rtn4 introns. The existence of two independent *nogo/rtn4* genes was proven by PCR analysis of intron sizes. Specificity of the used primer pairs for one of the two *rtn4* genes (primers a + b for rtn4.1 and primers c + d for rtn4.2; see Fig. 1) was verified by PCR on cloned plasmid DNA (+control; −control). (A) Primers a + b amplified the expected PCR product of 110 bp only on *rtn4.1* (lane 4) but not on *rtn4.2* (lane 3) plasmid DNA. On two different genomic DNA templates (prepared from either wild-type or albino *X. laevis*), the amplicon was 678 bp (lanes 1 and 2). (B) Primers c + d were specific for rtn4.2 (lane 3) and no amplification was achieved on rtn4.1-negative control plasmid (lane 4). The PCR product on genomic DNA templates was 490 bp (lanes 1 and 2). wt, wild-type.