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Abstract To investigate the molecular events controlling myelination of the peripheral nervous system, we compared gene expression of normal mouse sciatic nerves to that of the trembler mouse, whose Schwann cells are blocked in a pre-myelinating phenotype. Using cDNA array, we assessed expression levels of 1176 genes, and we found that delta-like protein (dlk), an epidermal growth factor-like homeotic protein, was expressed in the normal developing nerves, but at a low level in the dysmyelinating mutant trembler. Moreover, dlk expression was down-regulated when myelin protein expression was up-regulated, and no expression was observed in the developing brain. These results suggest that dlk expression is required for Schwann cell acquisition of the myelinating phenotype.

Key words: Differentiation; Myelination; Delta-like protein; Nervous system; Schwann cells; cDNA array

1. Introduction

In the immature, developing peripheral nerve, a large bundle of naked axons becomes encompassed by a single layer of Schwann cells. The establishment of axonal contact triggers Schwann cell proliferation [1,2]. These axons are gradually segregated as the Schwann cells proliferate, and a one-to-one relationship is established between each Schwann cell and the isolated axonal segment to be myelinated [3]. Thereafter, Schwann cells progress from this pre-myelinating phenotype toward a myelinating one. This transition occurs in the first week of postnatal life in the rodent sciatic nerve, and depends upon changes in expression of a whole network of genes. Indeed, peripheral nervous system (PNS) myelin–protein genes are up-regulated, along with several genes encoding enzymes involved in myelin–lipid biosynthesis, and immature Schwann cell genes are down-regulated. This regulation at the level of gene expression involves transcription factors. Among these, Oct-6, Krox-20, and Sox-10 have been shown to participate in the development of the myelinating phenotype of the Schwann cell [4]. Nevertheless, despite extensive studies, the nature of the axonal signals that initiate the myelination process remains elusive. It is most likely that this signal is at least partially mediated by receptors on both the axonal and the Schwann cell membranes, but these molecules have not yet been identified.

In an attempt to identify new genes that are involved in the progression of the Schwann cells from a pre-myelinating toward a myelinating phenotype, we compared the patterns of gene expression in normal and trembler mutant mouse nerves. The autosomal dominant trembler mutation, which affects the peripheral myelin protein of 22 kDa (PMP22) gene, results in an abnormal myelination of the PNS [5]. Indeed, a consequence of this mutation is that the differentiating Schwann cells attain a one-to-one relationship with the axons, but do not progress further towards a myelinating phenotype [6]. Using large-scale cDNA microarrays and Northern blot analyses, we found that the expression of the epidermal growth factor (EGF)-like protein delta-like protein (dlk) may play an important role in Schwann cell differentiation.

2. Materials and methods

2.1. Animals

Normal and heterozygous trembler mice were bred in our University's animal facility. They were on the C57Black background.

2.2. RNA isolation

The mice were sacrificed by cervical rupture at the ages of 5–50 days postnatal. Sciatic nerves from 10–15 age-matched animals were pooled, and RNA was isolated using the guanidinium isothiocyanate/cesium chloride procedure [7]. The integrity of RNAs was assessed by analyzing 18S and 28S ribosomal RNA.

2.3. cDNA array procedures and analyses

Samples of total RNA (5 μg) were used to synthesize [α-^{32}P]dATP cDNA probes for hybridization according to the manufacturer's protocol (Clontech, Palo Alto, CA, USA). The ^{32}P-labeled cDNA probes were further purified by column chromatography (NucTrap® Probe purification Column, Stratagene, CA, USA). The Atlas Mouse 1.2 Array, containing 1176 mouse cDNAs, was purchased from Clontech (Palo Alto, CA, USA). The different probes (3.10^6 cpm/ml) were hybridized overnight (68°C) on separate filter arrays, and washed according to the manufacturer's instructions. After exposure to a PhosphorImager screen (Molecular Dynamics) for 2–4 days, the images were analyzed using the ImageQuaNT software (Molecular Dynamics).

After subtraction of the background, signal values were normalized

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Abbreviations: dlk, delta-like protein; EGF, epidermal growth factor; P0, myelin protein zero; PMP22, peripheral myelin protein of 22 kDa; PNS, peripheral nervous system; STAT, signal transducer and activators of transcription

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by dividing by the mean filter hybridization signal (the expression levels for the majority of the genes remain unchanged between normal and trembler samples). No signals were detected in the spots of plasmid or bacteriophage DNAs, thus confirming the specificity of the hybridization. Four independent experiments were performed with normal samples and two with trembler samples. For each gene studied, the ratio was calculated by dividing the average value obtained for the normal samples ($n=4$) by the average value of the trembler samples ($n=2$).

2.4. Northern blot analysis

The RNA samples were separated in a 1.2% agarose/3% formaldehyde baby gel, transferred to Hybond-N membranes (Amersham Biosciences) in a solution of 20× SSPE, cross-linked to the membrane by UV irradiation (UV Stratalinker 1800, Stratagene, La Jolla, CA, USA) and pre-hybridizations and hybridizations were then carried out using ULTRAhyb solution (Ambion, Austin, TX, USA). Labeling of cDNA probes to a specific activity of 5×10^8 to 2×10^9 cpm/μg was done using a Prime-it II random primer labeling kit (Stratagene). The membranes were exposed in a PhosphorImager cassette, and signals were quantified using ImageQuaNT Software (Molecular Dynamics). The human glyceraldehyde 3-phosphate dehydrogenase cDNA probe was purchased from Clontech. The mouse dlk cDNA fragment was obtained by the RT-PCR technique using normal sciatic nerves cDNA (15 days old), and the following primers (5′–3′): dlk forward, TTC TCT GGA AAG GAC TGC CA, and dlk reverse, AGA GGG GTA CTC TTG TTG AG. The sequences of the primers were chosen from the published sequences of the mouse dlk cDNA [8].

3. Results and discussion

3.1. cDNA arrays

Four distinct RNA samples obtained from 15-day-old normal mouse sciatic nerves and two distinct samples from trembler nerves were used to prepare ^{32}P-labeled cDNA probes, which were then hybridized to individual Atlas Mouse 1.2 Arrays Clontech containing cDNAs for 1176 mouse genes. Of the 1176 genes, around 800 gave a signal above the background value in both types of mice. Approximately 150 genes gave signals above the average hybridization value, and 80 gave a signal above two-fold the average, i.e. around 7% of the 1176 genes studied were expressed at a high level in the sciatic nerves of the trembler and normal mice.

25 genes displayed a differential expression pattern between the control and mutant samples, but only 12 met the required criteria, i.e. they were significantly expressed in normal or trembler samples (expression level twice that of the average hybridization value), and were differentially expressed by a factor greater than 2.5 (Table 1). In total, eight genes were predominantly expressed in the mutant nerves and four in the normal ones.

Among the eight genes expressed in the mutant nerves, three are involved in protein turnover. Cathepsin D is a lysosomal aspartic endopeptidase which is known to be up-regulated during nerve injury [9,10]. Tissue inhibitor of metalloproteinase 3, a protein which regulates extracellular matrix degradation, plays a role in developmental patterning and in the maintenance of specific differentiated tissue. It is over-expressed in a variety of degenerative retinopathies [11–13]. Protease nexin 1, a potent inhibitor of serine proteases, is up-regulated following peripheral nerve lesion [14]. The over-expression of this gene leads to progressive neuronal and motor dysfunction [15]. Two other genes corresponded to the heat shock 86-kDa and 84-kDa proteins, firstly identified as tumor-specific transplantation antigens, which form in vivo the 90-kDa heat shock protein complex [16–18]. We also identified two transcription factors whose expressions are elevated in the mutant nerves. The first was T-box protein 2, a DNA-binding protein that regulates gene expression during embryogenesis [19] and which is highly expressed in primary human breast cancers [20,21]. The second was a member of the signal transducer and activators of transcription (STAT) gene family that are the main mediators in the signal transduction pathways of cytokines. STAT 3 expression has been demonstrated after peripheral nerve injury [22], but this is the first time that the expression of STAT 1 is reported for a peripheral neuropathy. Finally, the last gene encodes the G1/S-specific cyclin D1, which plays a role in the control of cell proliferation, and which was shown to control the mitotic response of Schwann cells in the Wallerian degeneration model of nerve injury [23,24]. In summary, the eight genes over-expressed in the trembler nerve encode proteins which are known to be implicated in abnormal cell proliferation and/or peripheral nerve degeneration, two processes which take place in the trembler PNS [6].

Four genes were predominantly expressed in normal sciatic nerves. As expected, we found the genes encoding the myelin protein zero (P0) and PMP22, whose under-expression in the trembler nerves has been already reported [25,26]. Moreover, the normal/trembler ratios measured in the present study for 15-day-old animals, which were 7.4 for PMP22 and 2.6 for P0, were similar to those previously measured by the Northern blot technique, which were 11 for PMP22 and 3.9 for P0 [26]. This finding validates our screening approach. The third gene encodes the insulin-like growth factor 2. It has previously been shown that this factor supports motoneuron survival, and that it regulates both Schwann cell proliferation and dif-

Table 1
Genes differentially expressed in 15-day-old normal and trembler sciatic nerves

GenBank accession #	Gene/protein name	Normal/trembler ratio[a]
M32240	PMP22	7.4
L12721	dlk	6.0
M14951	Insulin-like growth factor 2	4.2
M62860	P0	2.6
X70296	Protease nexin 1	0.4
X53337	Cathepsin D	0.32
U06924	STAT 1	0.24
L19622	Tissue inhibitor of metalloproteinase 3	0.21
M36829	Heat shock 84-kDa protein	0.18
M36830	Heat shock 86-kDa protein	0.17
S78355	G1/S-specific cyclin D1	0.16
U15566	T-box protein 2	0.12

[a]Ratios were calculated by dividing the average value obtained for the normal samples ($n=4$) by the average value of the trembler samples ($n=2$).

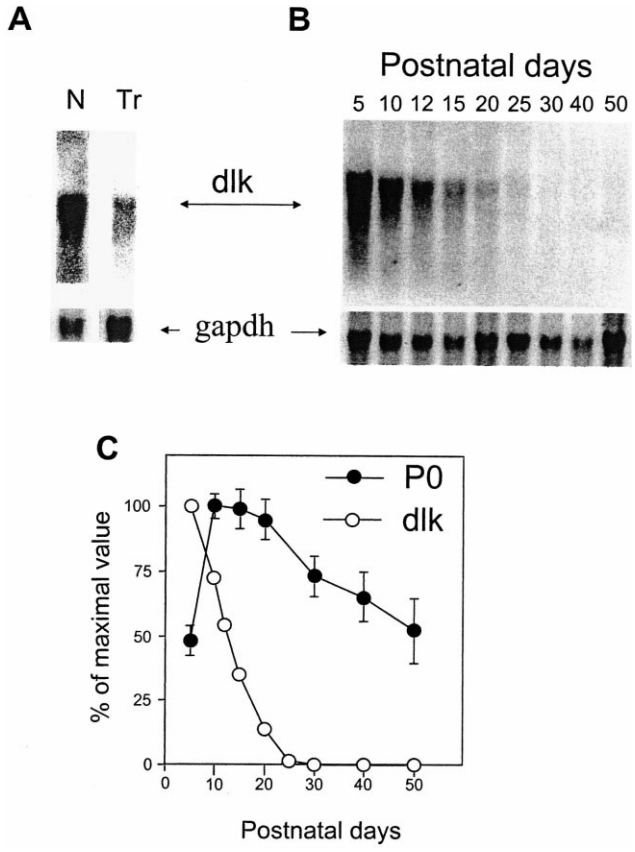

Fig. 1. dlk mRNA expression in mouse sciatic nerves. 5 μg of total RNA were applied to an agarose/formaldehyde gel, blotted and hybridized successively with the dlk and glyceraldehyde 3-phosphate dehydrogenase (gapdh) cDNA probes. A: 15-Day-old normal and trembler samples. B: Samples from normal mouse sciatic nerves. C: dlk and P0 mRNA levels during postnatal development of the mouse sciatic nerve. After correction for RNA levels between lanes using the gapdh results, quantitations were performed using a PhosphorImager. Results are presented as the percentage of the maximal level measured during the developmental period studied. Data are mean values of two experiments for dlk and mean ± S.E.M. (bars) values of five experiments for P0.

ferentiation during nerve regeneration [27–30]. Finally, the most interesting finding is that the gene encoding the dlk, also known as pre-adipocyte factor 1 or adipocyte differentiation inhibitor protein, is predominantly expressed in the normal nerve. This homeotic protein possesses six EGF-like sequences at the extracellular domain, a single transmembrane domain and a short intracellular tail [31]. The EGF-like motifs are highly homologous to the EGF-like motifs of the *Drosophila* homeotic protein Delta, a ligand for the Notch receptor. This homeotic protein is involved in several differentiation processes such as adipogenesis, hematopoiesis and neuroendocrine differentiation [31].

3.2. Expression of dlk

The expression of dlk was further studied using the Northern blot technique. From the blot shown in Fig. 1A, we calculated a normal/trembler ratio of 7, which is to be compared to the ratio of 6 obtained by the cDNA arrays approach, confirming again the reliability of the method used. The developmental expression of dlk mRNA in normal nerve is shown in Fig. 1B. The highest level was measured 5 days after birth. dlk mRNA steady-state levels then gradually declined during the next days to become undetectable at postnatal day 30. Indeed, dlk mRNA expression was inversely correlated to P0 mRNAs expression during sciatic nerve development (Fig. 1C). In addition, the postnatal expression of dlk in the nervous system is specific for the PNS, because the corresponding transcript was not detected in the developing mouse brain during the first weeks of life, a period when central nervous system myelination is initiated (Fig. 2).

3.3. Possible role of dlk during PNS development

dlk has been reported to play an important role during adipocyte differentiation. The corresponding gene is highly expressed upon induction of differentiation in primary rat pre-adipocytes during the first 2 days of the differentiation process. The expression then diminishes during the course of differentiation, and dlk expression is not detected in fully differentiated adipocytes [32,33]. This down-regulation of dlk is needed for complete adipocyte differentiation, because constitutive expression of dlk inhibits adipogenesis [34].

Our results showed that the regulation of dlk expression in the nervous system is myelination-related and PNS-specific. In the peripheral nerve, dlk expression is observed during the early stages of myelinogenesis and is down-regulated once the process is under way, when the genes of the major myelin structural proteins are up-regulated in normal nerves. Moreover, dlk expression is severely reduced in the nerves of the dysmyelinating mutant trembler mouse, in which the terminal differentiation towards a myelinating phenotype is initiated, but blocked at the promyelin stage. Finally, dlk was highly expressed in the developing peripheral nerve, but not in the brain. These results suggest that, as for the adipocytes, dlk expression may be required during the initial stages of Schwann cell differentiation towards a myelinating phenotype, and that its down-regulation could be important in allowing the completion of the myelination program. To investigate further the role of dlk in the myelination process, retroviral

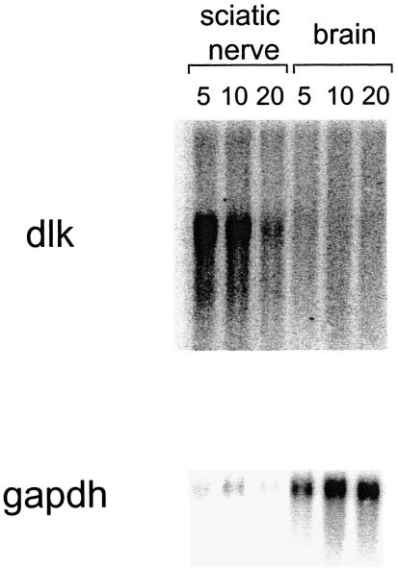

Fig. 2. dlk mRNA in the developing brain and sciatic nerve. 5 μg (sciatic nerve) and 20 μg (brain) of total RNA were applied to an agarose/formaldehyde gel, blotted and hybridized successively with the dlk and glyceraldehyde 3-phosphate dehydrogenase (gapdh) cDNA probes. Lanes are marked according to postnatal age.

vector-infected mouse Schwann cells expressing either sense or antisense dlk mRNAs will be engineered, and their capacity to differentiate into myelin forming cells will be evaluated.

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