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THE CELLULAR DISTRIBUTION OF THE Wld^s CHIMERIC PROTEIN AND ITS CONSTITUENT PROTEINS IN THE CNS

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Abstract—The C57BL/Wld^s mouse is a mutant strain of mouse that shows greatly slowed Wallerian degeneration both in the central and peripheral nervous system. Using immunohistochemistry, immunofluorescence and Western blotting, we have investigated the distribution of the chimeric Wld^s protein and its different components in neurons of the CNS of Wld^s mice and wild-type C57BL/6J mice. The expression of the Wld^s protein is restricted to the nucleus in Wld^s mice. Wld^s was not detected in axons. The Wld^s mice express both the normal and chimeric forms of ubiquitination factor E4 (Ube 4b) and nicotinamide mononucleotide adenylyltransferase-1 (Nmnat-1). The normal forms were expressed both in the cytoplasm and the nuclei of neurons in Wld^s mice and wild-type mice, and were also present in the axon. The normal form of Ube4b, mono- and poly-ubiquitin and IκBα, a substrate of Ube4b, were not differentially expressed in Wld^s mice compared with wild-type mice. However, the expression of both the normal and mutant forms of Nmnat-1 was higher in the nuclei of Wld^s mice compared with wild-type mice. Therefore, axon protection in Wld^s mice does not appear to be controlled by expression of Wld^s protein in the axons per se and also is unlikely to be related to the different activity of Ube4b either in general ubiquitination or toward this particular substrate. The increased Nmnat-1 activity in the nucleus of Wld^s mice compared with wild-type mice seems to be a significant factor in the axon protection. It is not known whether the expression of the Nmnat-1 in the axon is significant. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: WldS protein, Ube4b, Nmnat-1, mouse, axotomy, NF-kappaB inhibitor alpha, Wallerian degeneration.

Wallerian degeneration, the degeneration of the distal stump of an injured axon, occurs in traumatic and neurological disorders as diverse as spinal cord injury and multiple sclerosis (Dal Canto and Gurney, 1995; Zhang et al., 1996; Coleman and Perry, 2002), but the molecular events are poorly understood. Until recently, it was believed that following axon injury, the degeneration of the distal fragment came about because the axon was separated from the cell body and the axon withered as a consequence of a lack of trophic support from the cell body (Finn et al., 2000). In addition, an overload in calcium at the site of injury activates calcium-dependent proteases that degrade the cytoskeleton (Lopachin and Lehning, 1997; Stys and Jiang, 2002). Available evidence suggests that Wallerian degeneration differs from apoptosis, as it does not involve caspase activation (Finn et al., 2000; Sievers et al., 2003), and is not inhibited by bcl-2 (Sagot et al., 1995). The slow Wallerian degeneration mutant mouse, C57BL/Wld^s, shows extremely delayed axon degeneration of both peripheral and central nerve fibers after axotomy (Perry et al., 1991; Lunn et al., 1989). In contrast to wild-type mice, the distal portion of a transected Wld^s axon remains viable and able to conduct action potentials for up to 2 weeks (Lunn et al., 1989). This remarkable property is a consequence of an autosomal dominant mutation, is intrinsic to the axon and does not depend on macrophages or Schwann cells (Perry et al., 1990; Glass et al., 1993; Buckmaster et al., 1995). The normal axons must therefore contain the biochemical machinery which, when triggered by injurious molecules, will initiate an autodestructive process that results in the rapid degradation of the axon. Furthermore, there must be natural inhibitors of this degeneration pathway that prevent its activation in the uninjured axon.

The Wld^s gene has recently been identified (Conforti et al., 2000). It encodes an in-frame fusion protein consisting of the N-terminal 70 amino acids of the ubiquitination factor E4 (Ube4b) with the entire coding sequence of nicotinamide mononucleotide adenylyltransferase-1 (Nmnat-1) (Conforti et al., 2000; Mack et al., 2001).

Nmnat-1 is an essential enzyme in all organisms, because it catalyzes a key step of nicotinamide adenine dinucleotide (NAD^+) synthesis (Hughes et al., 1983; Gerdes et al., 2002). However, the NAD^+ levels in neural tissue of Wld^s mice are similar to those of wild-type mice in the steady state (Mack et al., 2001). Therefore, it has been suggested that axon protection in Wld^s mice is mediated by the mutation in Ube4b rather than by Nmnat-1.

There is evidence that the Wld^s gene product is expressed in the nucleus *in vivo* (Samsam et al., 2003; Sajadi et al., 2004; Mack et al., 2001) and one study reports of its presence in the neurites in primary culture (Wang et al., 2001). To further our aim of understanding how the Wld^s protein slows axon degeneration, we studied the expression of the chimeric protein Wld^s and its constituent components Ube4b and Nmnat-1 in wild-type and Wld^s mice.

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Abbreviations: MPT, mitochondrial permeability transition pore; NAD, nicotinamide adenine dinucleotide; Nmnat-1, nicotinamide mononucleotide adenylyltransferase-1; PARP, poly (ADP-ribose) polymerase; SDS, sodium dodecyl sulfate; TTBS, 50 mm Tris, 150 mm NaCl, 0.1% Tween-20, pH 8; Ube4b, ubiquitination factor E4; UCHL-1, ubiquitin-C-terminal hydrolase 1; UPS, ubiquitin-proteasome system Wld^s, slow Wallerian degeneration.

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Table 1. List of antibodies used in this study

Antibody	Source	Type	Specificity	Working dilutions			Fixation
				LM	LSCM	WB	
N-70	Dr. Coleman	Rabbit pAb	N-terminus of Ube4b	1:100			B
W18	Dr. Coleman	Rabbit pAb	Connection part of Wld^s protein	1:1000	1:500	1:500	B
183	Dr. Coleman	Rabbit pAb	C-terminus of Nmnat-1	1:1000		1:500	F and B
Ube4b	Serotec	Mouse mAb	C-terminal of Ube4b	1:1000		1:1000	F
Ufd2a	Dr. Nakayama	Rabbit pAb	Residues 129–142 of Ube4b	1:1000			B
sc-371	Santa Cruz	Rabbit pAb	C-terminus of IκBα	1:500		1:500	F and B
sc-8404	Santa Cruz	Mouse mAb	Phosphorylated Ser-32 of IκBα	1:100		1:100	F and B
NeuN	Boehringer-Mannheim	Mouse mAb	Neuronal nuclei		1:2000	1:2000	B
GFAP	Serotec	Mouse mAb	Glial fibrillar acidic protein		1:800		B
F4/80	Serotec	Mouse mAb	Microglia and macrophage marker		1:200		
Fk2	Affinity	Mouse mAb	Mono- and polyubiquitinated species	1:5000		1:5000	F and B
Ubiquitin	DAKO	Rabbit pAb	Free and conjugated ubiquitin	1:2000	1:1000		F and B
UCHL-1	Affinity	Rabbit pAb	Ubiquitin C-terminal hydrolase	1:5000			F and B
PW8155	Affinity	Rabbit pAb	20S Proteasome α/β subunit	1:3000			F and B
APP	Zymed	Mouse mAb	Amyloid protein precursor		1:200		B

B, Bouin's fixation; F, 10% formalin fixation; LM, light microscope; LSCM, laser scanning confocal microscopy; WB, Western blot.

In addition we have studied the expression of components of the ubiquitin-proteasome system (UPS) and IκBα, a substrate of Ube4b in the CNS between these two strains of mice.

EXPERIMENTAL PROCEDURES

Reagents and antibodies

The antibodies used in this study are shown in Table 1. The specificity of the antibodies raised against the Wld^s protein and its constituents is shown in Fig. 1. Unless otherwise stated, all chemicals were purchased from Merck (Poole, Dorset, UK) and were of AnalaR grade.

Animals

Male C57BL/Wld^s and C57BL/6J mice, aged 4–6 weeks, were obtained from Harlan-OLAC (Bicester, Oxfordshire, UK). All animals were housed under standard conditions with pelleted food and water available *ad libitum*. The animals were anesthetized with hypnorm (JANSSEN-CILAG Ltd., Saunderton, UK; 0.25 mg fluanisone per 20 g body weight) and midazolam (CP Pharmaceuticals Ltd., Wrexham, UK; 0.25 mg per 20 g body weight), and subjected to spinal cord lesions. After surgery the animals were kept in a thermostatically regulated heating chamber until fully recovered. All the experimental animals were cared for in strict accordance with The Animals (Scientific Procedures) Act, 1986, under a Home Office license. All efforts were made to minimize both the suffering and the numbers of animals used.

Spinal cord lesion

A dorsal hemi-transection of the spinal cord was produced at the T8 thoracic level as previously described (Schnell et al., 1999). The skin overlying the vertebral column was incised, the muscles detached from the vertebrae and under a surgical microscope, a partial laminectomy was performed, exposing the dorsum of the spinal cord. The dura was cut longitudinally and the dorsal columns were exposed. Iridectomy scissors were used to lesion one dorsal half of the spinal cord, thereby completely transecting the dorsal columns and corticospinal tract. The muscles and skin were reapposed with sutures and the animals were allowed to recover.

Brain lesion

The animals were anesthetized with i.p. avertin (100 μl/5 g) and held in a stereotaxic frame. The skin overlying the skull was incised and a craniotomy was created at the following coordinates: bregma +1.0 mm, lateral 1.5 mm. A size 11 sterile scalpel blade

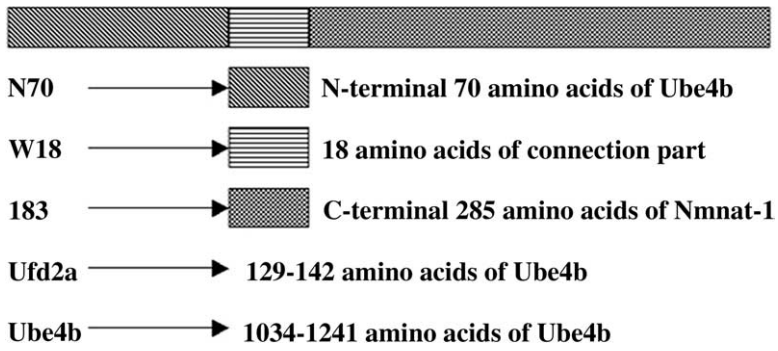

Fig. 1. Schematic diagram of the Wld^s protein and the specificity of the different antibodies.