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# Transient activation of an adaptor protein, disabled-2, in rat spinal cord injury

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ABSTRACT

We previously reported that disabled-2 (Dab-2), a cytosolic adaptor protein, was expressed in inflammatory and glial cells in the central nervous system (CNS) in experimental autoimmune encephalomyelitis and cerebral cryoinjury. Here, to determine the pattern of Dab-2 expression in a clip compression-induced rat spinal cord injury (SCI) model, the protein level and localization of Dab-2 in the spinal cord were investigated in rats with SCI using Western blotting and immunohistochemistry. Western blotting revealed that the expression of both the 75- and 100-kDa isoforms of Dab-2 peaked significantly in the spinal cord after clip compression injury 7 days post-injury compared to sham controls, and declined slightly thereafter. Immunohistochemistry revealed weak Dab-2 immunostaining in some neurons, glial cells, and ependymal cells in the spinal cords of the control animals, compared to staining in the macrophages and reactive astrocytes in lesions of the SCI animals. Overall, these findings suggest that both isoforms of Dab-2 are transiently upregulated in response to SCI and that the increased expression of Dab-2 is associated with the early activation of macrophages and astrogliosis in the course of CNS inflammation.


## Introduction

Spinal cord injury (SCI) includes primary mechanical injury and secondary damage induced by acute inflammatory responses (Jung et al., 2003; Moon et al., 2004). The neuropathological outcome of SCI is characterized by edema, axonal degeneration, inflammatory cell infiltration, and fibronectin exudation through the damaged blood–brain barrier (Jung et al., 2003; Prewitt et al., 1997). In the early stages of SCI, macrophages clear damaged resident cells from the central nervous system (CNS) *via* increased lysosomal activity; they have proinflammatory roles involving the expression and release of cytokines, chemokines, and adhesion molecules (Moon et al., 2004, 2008; Mueller et al., 2006). In addition to these classical proinflammatory macrophages, alternatively activated macrophages are involved in the recovery of injured areas *via* the secretion of anti-inflammatory mediators, including transforming growth factor (TGF)-β (Jeong et al., 2014; Shin et al., 2013).

Disabled-2 (Dab-2), a cytosolic adaptor protein belonging to the Disabled gene family, plays an important role in cell signaling, cell migration, oncogenesis, and development (Ezratty et al., 2009; Hocevar et al., 2001). Dab-2 is expressed widely in various tissues, including the brain (Cheung et al., 2008; Huang et al., 2007), ovary (Fazili et al., 1999), intestine (Vazquez-Carretero et al., 2011), and kidney (Morris et al., 2002). Dab-2 interacts with several proteins, including clathrin and low-density lipoprotein family receptors (Morris and Cooper, 2001), and it inhibits Wnt/β-catenin signaling by binding low-density lipoprotein receptor-related protein 6 and promoting its internalization through clathrin (Jiang et al., 2012).

Dab-2 has two major alternatively spliced isoforms, originally identified as p96 and p67 in mice, corresponding to polypeptides with molecular masses of 96 and 67 kDa, respectively (Cho et al., 2000; Xu et al., 1995). Previously, we found that Dab-2 expression was increased significantly in activated macrophages following cryogenic brain injury in rats (Moon et al., 2005) and rat experimental autoimmune encephalomyelitis (EAE) (Ahn et al., 2011). In addition, Dab-2 has been identified as a mitogen-responsive phosphoprotein in the cell signaling pathway induced by colony-stimulating factor (Xu et al., 1995, 1996), which was found in

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macrophages and astrocytes after SCI (Buss et al., 2008; Tyor et al., 2002).

Of the two Dab-2 isoforms, the short isoform (67 kDa) is associated with cell positioning and structure formation during embryogenesis (Yang et al., 2002), suggesting that it is upregulated in lesions in injured adults, as many embryonic proteins, including nestin and vimentin, have been identified in CNS lesions, including SCI (Kim et al., 2003), head injury (Moon et al., 2004), EAE (Shin et al., 2003), and scrapie-infected brain (Jin et al., 2004). The spatiotemporal expression of Dab-2 variants, including the short and long isoforms, remains to be studied in a rat SCI model. To further understand the potential role of Dab-2 in rat SCI, we investigated temporal changes in the expression and localization of Dab-2 using Western blot analysis and immunohistochemistry.

## Materials and methods

### Animals

Male Sprague-Dawley rats (200–250 g each) were studied (Daehan Biolink, Cheongju, Korea). All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Jeju National University.

### Antibodies

The purified mouse anti-Dab-2/p96 antibody (catalog number 610465; BD Transduction Laboratories, San Jose, CA, USA) used for Western blotting in this study recognizes both the short and long isoforms of rat Dab-2, with approximate molecular masses of 75 and 100 kDa, respectively (Romero et al., 2007). Rabbit polyclonal anti-Dab-2 (H-110; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies were used for immunohistochemistry. The following antibodies were also used: monoclonal anti-β-actin antibodies (Sigma-Aldrich, St. Louis, MO, USA), mouse anti-rat macrophage (ED1) antibodies (Serotec, London, UK), and anti-glial fibrillary acidic protein (GFAP) antibodies (Sigma-Aldrich).

### Surgical procedure

The surgical procedure used to induce clip compression injury was performed as reported previously (Kim et al., 2003; Moon et al., 2004; Shin, 2007; Shin et al., 2013). The animals were anesthetized with chloral hydrate (Fluka, Buchs, Switzerland; 375 mg/kg body weight, intraperitoneal injection) and subjected to a laminectomy at T9/T10. Immediately following the laminectomy, the spinal cord was compressed with a vascular clip (Stoelting, Wood Dale, IL, USA) that was applied vertically to the exposed spinal cord at an occlusion pressure of 15–20 g for 1 min. After compression, the muscles and skin layers were closed. In sham-operated control rats, only the laminectomy was performed. After surgery, bladder emptying was assisted by massage in the spinally injured rats at least twice daily.

### Tissue sampling

For Western blotting, rats were sacrificed under ether anesthesia 0 (sham control), 1, 4, 7, and 14 days post-injury ($n = 5$ per time point). An approximately 1-cm-long segment of the spinal cord, including the SCI epicenter at T9/T10, was harvested and frozen.

For immunohistochemistry, three sham-operated control rats, and five rats on days 1, 4, 7, and 14 post-surgery were perfused through the left ventricle with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2). After perfusion, a section of the spinal cord located approximately 1 cm cranial and caudal to the core lesion was dissected and fixed.

### Western blotting

The spinal cords were homogenized in lysis buffer containing 40 mM Tris–HCl (pH 7.4), 120 mM NaCl, and 0.1% Nonidet P-40 (polyoxyethylene [9] *p*-*t*-octyl phenol) supplemented with the protease inhibitors leupeptin (0.5 μg/mL), phenylmethanesulfonyl fluoride (1 mM), and aprotinin (5 μg/mL). After a 60-min incubation in an ice bath, the homogenates were centrifuged at 14,000 rpm for 20 min and the supernatants were harvested. Equal amounts of protein (60 μg/20 μL) were loaded in each lane of polyacrylamide gels and electrophoresed under denaturing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the proteins were electrotransferred to nitrocellulose transfer membranes (Schleicher and Schuell, Keene, NH, USA). The residual membrane binding sites were blocked by incubation with 5% non-fat milk in Tris-buffered saline (TBS; 10 mM Tris–HCl, pH 7.4, and 150 mM NaCl) for 1 h at room temperature (RT). Subsequently, the membrane was incubated for 2 h at RT with purified mouse anti-Dab-2/p96 antibodies diluted 1:2000 (BD Biosciences, Franklin Lakes, NJ, USA). The membranes were washed three times in TBS containing 0.1% Tween 20 before incubation with horseradish peroxidase-conjugated anti-mouse IgG (diluted 1:2000; Vector, Burlingame, CA) for 1 h at RT. Immunoreactive bands were visualized by chemiluminescence using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). After imaging, the membranes were stripped and reprobed with a monoclonal antibody to β-actin (Sigma-Aldrich) using a protocol similar to that described above. The signals on the immunoblots were quantified with a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA). The optical density (OD; per $mm^2$) of each band was measured and the density ratio relative to the β-actin band was compared using ImageJ software (NIH, Bethesda, MD, USA); the values are presented as means ± SEM. The data were analyzed using a one-way analysis of variance followed by the Student–Newman–Keuls *post hoc* test for multiple comparisons. In all cases, $P < 0.05$ was considered statistically significant.

### Immunohistochemistry

The spinal cords were removed, immersed in the same fixative for 24 h, and processed for embedding in paraffin. Sections (5 μm) of paraffin-embedded spinal cord were deparaffinized and heated with citrate buffer (0.01 M, pH 6.0) in a microwave oven for 3 min. Then, they were incubated in 0.3% hydrogen peroxide in methyl alcohol for 20 min to block endogenous peroxidase activity. After three washes with PBS, the sections were incubated with 10% normal goat serum (ABC Elite kit; Vector) diluted in PBS, and then incubated with rabbit polyclonal anti-Dab-2 antibodies for 1 h at RT. After three more washes in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (1:200 dilution; Vector) for 45 min at RT. After three additional washes in PBS, the sections were incubated with avidin-biotin peroxidase complex (Vector), prepared according to the manufacturer's instructions, for 45 min at RT. The peroxidase reaction was developed using a diaminobenzidine substrate kit (Vector), prepared according to the manufacturer's instructions, for 5 min.

To examine Dab-2 expression, we used a double-immunofluorescence procedure. First, paraffin sections, prepared as described above, were reacted with primary rabbit anti-Dab-2 antibodies overnight at 4 °C, followed by tetramethyl rhodamine isothiocyanate-labeled goat anti-rabbit IgG (Sigma-Aldrich) for 1 h at RT. Then, the slides were incubated with a second primary reagent, either ED1 or anti-GFAP, overnight at 4 °C and subsequently reacted with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Sigma-Aldrich). The