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Secretion of N-terminal domain of α-dystroglycan in cerebrospinal fluid

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ABSTRACT

α-Dystroglycan (α-DG) plays crucial roles in maintaining the stability of cells. We demonstrated previously that the N-terminal domain of α-DG (α-DG-N) is secreted by cultured cells into the culture medium. In the present study, to clarify its function *in vivo*, we generated a monoclonal antibody against α-DG-N and investigated the secretion of α-DG-N in human cerebrospinal fluid (CSF). Interestingly, we found that a considerable amount of α-DG-N was present in CSF. α-DG-N in CSF was a sialylated glycoprotein with both N- and O-linked glycan. These observations suggest that secreted α-DG-N may be transported via CSF and have yet unidentified effects on the nervous system.

1. Introduction

The dystroglycan complex is composed of two proteins, α- and β-dystroglycan (α- and β-DG), which are encoded by a single gene [1]. α-DG is a highly glycosylated extracellular peripheral membrane protein that binds to several extracellular matrix proteins (including laminin, agrin, and perlecan) [2–4] and synaptic proteins (such as neurexin and pikachurin) [5,6]. The transmembrane protein β-DG anchors α-DG at the extracellular surface of the plasma membrane [2,3]. α-DG is composed of three distinct domains: N-terminal, mucin-like and C-terminal domains. The N- and C-terminal domains are connected by the central mucin-like domain which is highly glycosylated by O-linked glycans [7].

Functional defect of DG is implicated in the pathogenesis of several types of muscular dystrophy, in which defective glycosylation of α-DG results in the severe reduction of its laminin binding activity, leading to the degeneration of muscle cells [8–13]. In addition to skeletal muscle, DG is abundantly expressed in the nervous system and its ablation causes a wide range of structural and functional abnormalities in the brain and peripheral nerves [14,15].

It has been demonstrated that the N-terminal domain of α-DG (α-DG-N) is cleaved by a proprotein convertase (PC), furin [16,17]. However, the physiological significance of this proteolytic cleavage remains unknown. In a previous report, we demonstrated that (1) native α-DG-N is secreted by a wide variety of cultured cells into the culture medium, and (2) a detectable amount of α-DG-N is secreted into human serum [18]. In this study, to further investigate the secretion of α-DG-N *in vivo*, we generated a monoclonal antibody against α-DG-N and assessed its secretion into the cerebrospinal fluid (CSF), a major environmental regulator of the nervous system.

2. Materials and methods

2.1. Antibodies

Mouse monoclonal antibody specific for human α-DG-N (anti-α-DG-N, 1D9) was obtained from BALB/c mice immunized with synthetic peptide corresponding to the 30 amino acids, 141–170, in human α-DG (NGSHIPQTSSVFSIEVYPEDHSDLQSVRTA). Rabbit polyclonal antibody against human α-DG-N (anti-α-DG-N, AP1528), mouse monoclonal antibody against the sugar chain moieties of α-DG (anti-α-DG, IIH6), and mouse monoclonal antibody against the C-terminal domain of b-DG (anti-b-DG, 8D5) were described previously [2,19].

2.2. Cell culture

Immortalized rat Schwann cell, RSC96, was purchased from American Type Culture Collection. Rat pheochromocytoma,

Abbreviations: DG, dystroglycan; α-DG-N, N-terminal domain of α-dystroglycan; CSF, cerebrospinal fluid; PC, proprotein convertase.

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PC12HS, a highly NGF-sensitive clone of PC12, was obtained from Human Science Research Resource Bank. Rat schwannoma cell, RT4, was kindly provided by Drs. A. Asai (University of Tokyo) and Y. Kuchino (National Cancer Center, Tokyo). RSC96 and RT4 cells were plated on plastic culture dishes (DB Bioscience) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. PC12HS cells were plated on a Primaria™ culture flask (DB Bioscience) and fed in RPMI1640 medium with 10% horse serum and 5% fetal bovine serum in the presence or absence of 200 ng/ml of NGF.

2.3. Collection of CSF

CSF was obtained from patients with normal pressure hydrocephalus admitted to the Department of Neurology at Teikyo University Hospital (Tokyo, Japan). All CSF specimens were collected by lumbar puncture with informed consent. Ethical approval for the use of human CSF was obtained from the Teikyo University School of Medicine Ethics Committee (#07–056). No abnormalities were detected in the CSF composition as determined by routine laboratory analyses, including cell count and total protein concentration.

2.4. Miscellaneous

GST–α-DG-N fusion protein was expressed by *Escherichia coli* and purified as described previously [18]. Lectin column chromatography and enzymatic deglycosylation were performed as described elsewhere [18,19]. Western blotting and laminin overlay assay were carried out as described previously [13].

3. Results

3.1. Secretion of native α-DG-N by cultured cells derived from nervous system

In the previous report, we demonstrated that the native α-DG-N is cleaved by PC and secreted into the culture medium by C2C12, HEK293, COS-7, HeLa, MCF7, and DU145 cells [18]. In the present study, we tested if α-DG-N is secreted by other cultured cells, especially those derived from the nervous system. We analyzed the culture medium of RT4 and RSC96 cells, both of which are of Schwann cell origin, and NGF-treated PC12 cells, which is an established differentiation model toward neurons. These culture media were concentrated and examined by Western blotting. Anti-α-DG-N (AP1528) detected intense and broad bands with an apparent molecular mass of 37 kD in these culture media (Fig. 1). The broad bands were composed of multiple bands with slightly different molecular masses (Fig. 1). These results are similar to the previous observation of other cultured cells [18]. Differentiation of PC12 cells toward neurons by NGF treatment did not affect the amount of secretion or molecular mass of α-DG-N (Fig. 1).

3.2. Secretion of α-DG-N into human cerebrospinal fluid (CSF)

Prompted by the result that α-DG-N is secreted in the culture medium by the cultured cells derived from the nervous system, we examined if α-DG-N is secreted into CSF, a fluid that directly surrounds the nervous system. In order to detect α-DG-N with higher specificity, we generated a mouse monoclonal antibody against α-DG-N, 1D9, and characterized its specificity by Western blotting using the GST-α-DG-N fusion protein. 1D9 detected a 60 kD band of GST-α-DG-N fusion protein (Fig. 2A). Pre-absorption of the antibody by GST-α-DG-N fusion protein or antigen peptide eliminated the 60 kD band completely, whereas pre-absorption of the antibody by GST protein or irrelevant control peptide did not (Fig. 2A). These data confirm the specific recognition of α-DG-N by this antibody.

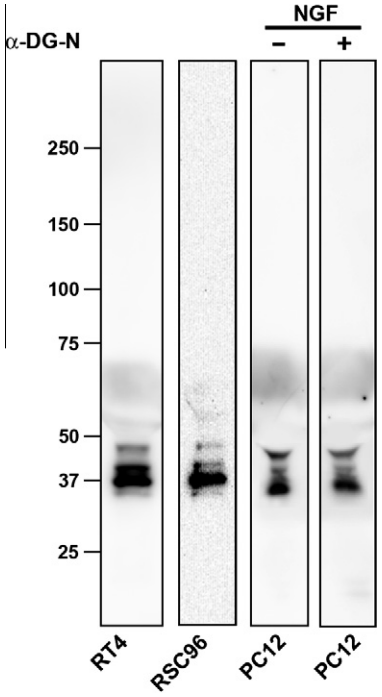

Fig. 1. Native α-DG-N is secreted by cultured cells derived from nervous system into culture medium. The culture medium of RT4, RSC96, and PC12 cells were analyzed by Western blotting using anti-α-DG-N (AP1528). The antibody detected intense and broad bands of α-DG-N with an apparent molecular mass of 37 kD in the cultured medium of these cells. There was no significant difference in the amount of secretion or molecular mass of α-DG-N between NGF-treated and non-treated PC12 cells. NGF−, NGF non-treated; NGF+, NGF-treated. Molecular mass standards ($D \times 10^3$) are shown on the left.

The human CSF specimens were then applied to Western blotting without concentration. Interestingly, anti-α-DG-N (AP1528) detected an intense band with an apparent molecular mass of 40 kD in human CSF (Fig. 2B). The 40 kD band was also detected by the monoclonal antibody, anti-α-DG-N (1D9) (Fig. 2B). This band was not, however, recognized by anti-α-DG (IIH6) nor anti-β-DG (8D5). This 40 kD band did not exhibit laminin binding activity as determined by laminin overlay assay (Fig. 2B). Next, to test the specificity of the polyclonal antibody, anti-α-DG-N (AP1528) was pre-incubated with the GST-α-DG-N fusion protein strip or the antigen peptide. The 40 kD band was eliminated completely by pre-absorption with both the fusion protein and the antigen peptide (Fig. 3). These data confirm the specific recognition of α-DG-N secreted into CSF.

3.3. α-DG-N in CSF is both N- and O-glycosylated

To assess the status of the glycosylation of α-DG-N, we performed the enzymatic deglycosylation of the CSF proteins. Digestion by *N*-glycosidase F decreased the molecular mass of α-DG-N by 3 kD (Fig. 4A). In contrast, digestion by neuraminidase decreased the molecular mass of α-DG-N by 5 kD (Fig. 4A). Finally, digestion by neuraminidase together with O-glycosidase decreased the molecular mass of α-DG-N by 7 kD (Fig. 4A). These data indicate that the α-DG-N in human CSF is a sialylated glycoprotein with both N- and O-linked glycans. We further attempted to characterize the sugar chain moieties of the α-DG-N in CSF by lectin column chromatography. The amount of α-DG-N remaining in