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Processing and secretion of the N-terminal domain of α-dystroglycan in cell culture media

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Abstract **α-Dystroglycan (α-DG) plays a crucial role in maintaining the stability of muscle cell membrane. Although it has been shown that the N-terminal domain of α-DG (α-DG-N) is cleaved by a proprotein convertase, its physiological significance remains unclear. We show here that native α-DG-N is secreted by a wide variety of cultured cells into the culture media. The secreted α-DG-N was both *N*- and *O*-glycosylated. Finally, a small amount of α-DG-N was detectable in the normal human serum. These observations indicate that the cleavage of α-DG-N is a widespread event and suggest that the secreted α-DG-N might be transported *via* systemic circulation in vivo.**

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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 and binds to several extracellular matrix proteins including laminin, agrin and perlecan [2–4]. In turn, 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; the N-terminal, mucin-like and C-terminal domains (Fig. 1). The N- and C-terminal domains exhibit globular structure and are connected by the central mucin-like domain which is highly glycosylated by *O*-linked sugar chains [5].

Functional defect of α-DG is implicated in the pathogenesis of several types of congenital muscular dystrophies, in which aberrant glycosylation of α-DG causes severe reduction of its laminin binding activity [6–11]. Aberrant glycosylation of α-DG is also demonstrated in a wide range of carcinoma cells, where it may lead to abnormal cell-extracellular matrix interactions and thus contribute to invasion and metastasis of cancer cells [12–15]. Moreover, some pathogens such as lymphocytic choriomeningitis virus, Lassa fever virus and *Mycobacterium leprae* utilize α-DG as a receptor to invade host cells [16,17].

Recently, it has been demonstrated that the N-terminal domain of α-DG (α-DG-N) interacts with Large, a putative glycosyltransferase mutated in the patients with congenital muscular dystrophy type 1D (MDC1D) [10], and then it is cleaved by a proprotein convertase (PC) called furin [18,19] (Fig. 1). However, the physiological significance of the proteolytic cleavage of α-DG-N remains elusive. In the present study, we generated a specific antibody against α-DG-N and characterized its processing in cultured cells.

2. Materials and methods

2.1. Antibodies

Rabbit polyclonal antibody was generated by immunizing New Zealand white rabbits with a peptide corresponding to the 30 amino acids, 141–170, in the N-terminal domain of human α-DG (NGSHIPQTSSVFSIEVYPEDHSDLQSVRTA). The antibody against α-DG-N was affinity purified using GST-α-DG-N (anti-α-DG-N, AP1528) (Fig. 1). Mouse monoclonal antibody against the sugar chain moieties of α-DG (anti-α-DG-sugar, IIH6), mouse monoclonal antibody against the C-terminal domain of β-DG (anti-β-DG), and rabbit polyclonal antibody against the C-terminal domain of human α-DG (anti-α-DG-C, AP1530) were described previously [2,20]. Affinity isolated rabbit anti-laminin was obtained from Sigma–Aldrich.

2.2. Cell culture

Mouse myoblast, C2C12, African green monkey kidney, COS-7, human breast adenocarcinoma, MCF7 and human prostate carcinoma, DU145 were purchased from American Type Culture Collection. Human cervix carcinoma, HeLa and human embryonic kidney, HEK293 were obtained from Human Science Research Resource Bank. Cells were plated on plastic non-coated or laminin-coated culture dishes (DB Bioscience) and cultured in Dulbecco's modified Eagle's medium, except that HeLa, MCF7 and DU145 were fed in Eagle's minimal essential medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. In the case of MCF7, 0.01 mg/ml of bovine insulin was added to the medium. For some cell lines, medium was replaced by a serum-free one 2 to 4 days before harvesting cells. In some experiments, cells were cultured in the presence of 200 μM of decanoyl-RVKR-CMK (Calbiochem), an inhibitor of PC, for 2 days. The culture medium was concentrated using Amicon Ultra-15 (Millipore) and the cells were lysed in sample buffer (65 mM Tris–HCl, pH 6.9, 3% SDS, 1% β-mercaptoethanol, 115 mM sucrose, 0.0004% Bromophenol blue). Materials were separated by 5–15% SDS–PAGE and then analyzed by Western blotting.

2.3. Lectin chromatography

The culture medium of C2C12 cells was incubated with lectin bound agarose, including concanavalin A (Con A), lentil lectin (LCA), wheat germ agglutinin (WGA), *Ricinus communis* agglutinin 120 RCA120), *Aleuria auranlia* lectin (AAL), peanut agglutinin (PNA), *Maackia*

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Abbreviations: DG, dystroglycan; α-DG-N, N-terminal domain of α-dystroglycan; PC, proprotein convertase

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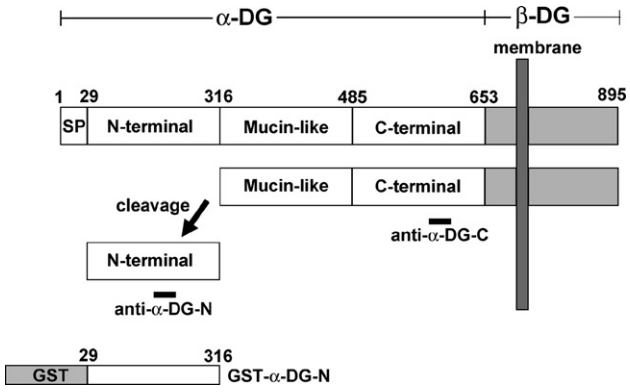

Fig. 1. Schematic representation of human α-DG and GST-α-DG-N fusion protein. Domain structures of α-and β-DG are shown. The N-terminal domain of α-DG is cleaved by a proprotein convertase. Positions of the epitopes for anti-α-DG antibodies are indicated by black bars. SP, signal peptide.

amurensis lectin (MAM), *Sambucus sieboldiana* agglutinin (SSA), phytohemagglutinin-E4, (PHA-E4) (Seikagaku Corporation) and Jacalin (Vector). The void fractions were analyzed by Western blotting using anti-α-DG-N.

2.4. Miscellaneous

For construction of a GST fusion protein, human *DAG1* gene (GenBank accession number NM 004393) corresponding to α-DG-N (amino acid 30–316) was amplified by PCR and subcloned into pGEX-2TK expression vector (GE healthcare) (Fig. 1). GST-α-DG-N fusion protein was expressed by *Escherichia coli* and purified as described previously [21]. Chemical deglycosylation was performed as described elsewhere [2,22]. Western blotting and laminin overlay assay were described previously [11]. Human sera were obtained from the members of the laboratory according to the standard protocol. Albumin concentration in the sera was reduced using ProteoSeek Albumin/IgG Removal Kit (Pierce).

3. Results

3.1. Characterization of the antibody against α-DG-N

We characterized the specificity of the antibody against α-DG-N (anti-α-DG-N) by Western blotting using GST-α-DG-N fusion protein. Anti-α-DG-N detected the 60 kDa band of GST-α-DG-N fusion protein (Fig. 2). Pre-absorption of the antibody by GST-α-DG-N fusion protein or antigen peptide eliminated the 60 kDa band completely (Fig. 2). On the other hand, pre-absorption of the antibody by GST protein or irrelevant control peptide did not eliminate the 60 kDa band (Fig. 2). The 60 kDa band was not detected by anti-α-DG-C, anti-α-DG-sugar, or anti-β-DG, while it was detected by anti-GST antibody. These data confirm the specific recognition of α-DG-N by this antibody.

3.2. Native α-DG-N is secreted into culture media by cultured cells

In the previous report, it was demonstrated that the overexpressed Fc-tagged α-DG was cleaved by a PC [18]. To see if native α-DG-N is cleaved by a PC and released from the cell surface, we analyzed the culture medium of C2C12 cells by Western blotting. Interestingly, anti-α-DG-N detected an intense and broad band with an apparent molecular mass of 37 kDa in the culture medium (Fig. 3). This band was eliminated completely by pre-absorption of the antibody by antigen

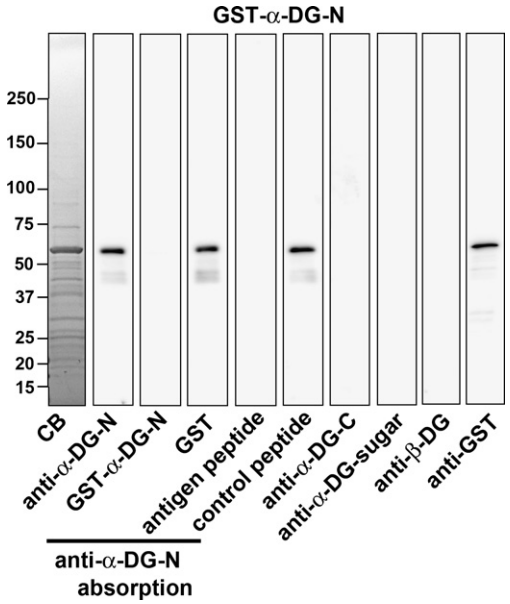

Fig. 2. Anti-α-DG-N recognizes the N-terminal domain of α-DG specifically. Anti-α-DG-N detected the 60 kDa band of GST-α-DG-N fusion protein. Pre-absorption of the antibody by GST-α-DG-N fusion protein or antigen peptide eliminated the 60 kDa band completely. Pre-absorption of the antibody by GST protein or irrelevant control peptide did not eliminate the 60 kDa band. The 60 kDa band was not detected by anti-α-DG-C, anti-α-DG-sugar or anti-β-DG, while it was detected by anti-GST antibody. Molecular mass standards ($D \times 10^3$) are shown on the left. CB, Coomassie blue staining of the gel.

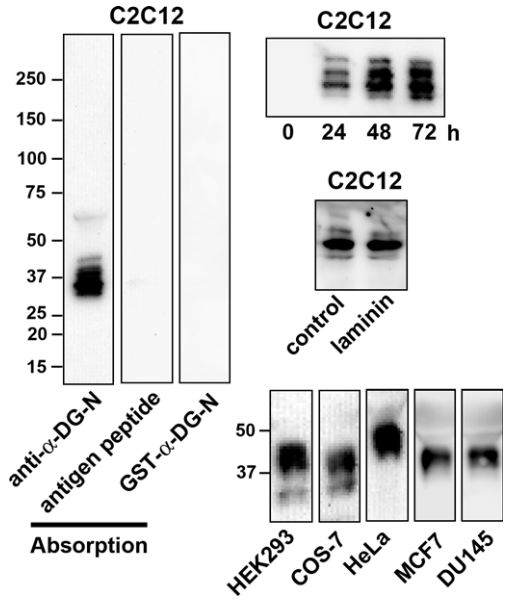

Fig. 3. Native α-DG-N is secreted into culture medium. The culture medium of C2C12 cells was analyzed by Western blotting using anti-α-DG-N. The antibody detected an intense and broad band of α-DG-N with an apparent molecular mass of 37 kDa, which was eliminated completely by pre-absorption of the antibody by antigen peptide or GST-α-DG-N fusion protein. The 37 kDa band increased progressively from 0 to 72 h of cell culture. There was no significant difference in the level of secretion of α-DG-N between the C2C12 cells cultured on the control plastic culture dishes and laminin-coated dishes. α-DG-N was also detected in the culture medium of a wide variety of cell lines, including HEK293, COS-7, HeLa, MCF7 and DU145. Molecular mass standards ($D \times 10^3$) are shown on the left.

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