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β-dystroglycan is regulated by a balance between WWP1-mediated degradation and protection from WWP1 by dystrophin and utrophin

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ABSTRACT

Dystroglycan is a ubiquitous membrane protein that functions as a mechanical connection between the extracellular matrix and cytoskeleton. In skeletal muscle, dystroglycan plays an indispensable role in regulating muscle regeneration; a malfunction in dystroglycan is associated with muscular dystrophy. The regulation of dystroglycan stability is poorly understood. Here, we report that WWP1, a member of NEDD4 E3 ubiquitin ligase family, promotes ubiquitination and subsequent degradation of β-dystroglycan. Our results indicate that dystrophin and utrophin protect β-dystroglycan from WWP1-mediated degradation by competing with WWP1 for the shared binding site at the cytosolic tail of β-dystroglycan. In addition, we show that a missense mutation (arginine 440 to glutamine) in WWP1—which is known to cause muscular dystrophy in chickens—increases the ubiquitin ligase-mediated ubiquitination of both β-dystroglycan and WWP1. The R440Q missense mutation in WWP1 decreases HECT domain-mediated intramolecular interactions to relieve autoinhibition of the enzyme. Our results provide new insight into the regulation of β-dystroglycan degradation by WWP1 and other Nedd4 family members and improves our understanding of dystroglycan-related disorders.

1. Introduction

Dystroglycan (DG) is a ubiquitously expressed cell adhesion protein that links the extracellular matrix to the cytoskeleton [1–4]. In skeletal muscle, DG is a key component of the dystrophin-glycoprotein complex (DGC) known to play an important role in maintaining the structural and functional stability of muscle fibers [4–7]. DG is composed of α- and β-subunits derived from a single gene product following proteolytic cleavage [8]. β-DG, a transmembrane protein, interacts with dystrophin on the cytoplasmic side, which in turn binds to actin filaments [9]. α-DG is a highly glycosylated extracellular protein, which forms a complex with β-DG and binds to extracellular matrix proteins, such as laminin [10–12], agrin [13,14], and perlecan [11]. In addition to DG and dystrophin, the skeletal muscle DGC contains syntrophins [15,16], dystrobrevins [17], sarcoglycans [18], and sarcospan [18,19]. Mutations affecting DGC components underlie various types of congenital muscular dystrophies [5,20–22]. The best known examples come from dystrophinopathy such as Duchenne and Becker muscular dystrophies (DMD and BMD) caused by mutations in the human dystrophin gene [5,7,20,22]. Malfunction of DG is also associated with the development of muscular dystrophies [23–25]. O-linked glycosylation of α-DG is essential for its function as an extracellular matrix molecule receptor [26,27]. Abnormal glycosylation of α-DG is responsible for forms of dystroglyconopathy such as muscle-eye-brain disease, Walker-Warburg syndrome, and Fukuyama congenital muscular dystrophy, which are all caused by mutations in various glycosyltransferase genes [28–32]. Targeted disruption of the DG gene results in embryonic lethality, due to the disorganization of the basement membranes [33–35]. In non-muscle cells where dystrophin is not expressed, the dystrophin homologue utrophin associates with DG connecting the actin cytoskeleton to the extracellular matrix [36–40].

The cytoplasmic tail of β-DG contains WW domain-binding PPXY motifs required for its interaction with dystrophin and utrophin [41–43]. Structural and biochemical studies on the interaction between the dystrophin C-terminal region and the cytoplasmic tail of β-DG have revealed that the most C-terminal PPXY motif primarily binds to dystrophin WW domain. Residues preceding the PPXY motif interact with one of the EF-hands of dystrophin, providing additional specificity in the interaction [42,44]. Recent studies using in vivo assays of Drosophila cell polarity, however, have indicated that a second, internal PPXY motif of β-DG may also mediate the interaction with dystrophin and support dystroglycan function when the extreme C-terminal PPXY motif is mutated [45,46].

It has been reported that DG protein levels are decreased in the

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muscles of DMD patients [47] and mdx mice [48], while DG mRNA levels are not affected by a decrease in dystrophin levels [49]. Furthermore, proteasome inhibitors stabilize DG and dystrophin in animal models of DMD (eg, mdx mice [50,51] and sapje zebrafish [52,53], [54]), and in DMD and BMD patient muscle biopsies [55]. In addition, tyrosine phosphorylation of β-DG within the most C-terminal PPXY motif affects its association with dystrophin [56] and utrophin [57], and preventing tyrosine phosphorylation protects β-DG from degradation [53,58]. Thus, cellular levels of DG appear to be regulated by a poorly understood process of protein degradation.

Recently, a missense mutation within WWP1 has been shown to cause muscular dystrophy in chickens classified as having a type of dystroglyconopathy [59]. Interestingly, WWP1 does not belong to the glycosyltransferase family like other dystroglyconopathy-causing genes, but rather to the E3 ubiquitin ligase group. WWP1 is involved in the regulation of a variety of cellular process (e.g., signal transduction [60–62], transcription [63–65], protein trafficking [66,67] and apoptosis [68,69]) through its interaction with PPXY motif-containing and PPXY motif-absent substrates. The exact substrate(s) of WWP1 leading to the onset of chicken muscular dystrophy have yet to be identified. WWP1 contains a C2 domain, four WW domains, and a HECT domain (from the N- to C-terminus). WWP1 belongs to, and shares a similar architecture with other members of the NEDD4 family [70]. The enzymatic activities of NEDD4 family members are tightly regulated and kept mostly in an inactive state to prevent unnecessary degradation of substrates and enzymes themselves [71,72]. Thus, WW-HECT and C2-HECT interactions are auto-inhibitory [73], and this inhibition can be relieved in several ways, including phosphorylation [70] and binding to adaptors [74] or phospholipids [75,76].

As part of our effort to identify novel substrates of WWP1, we have used a recently devised method based on proximity-dependent biotin labeling [77]. In this study, we identified β-DG as a substrate of WWP1. We found that the muscular dystrophy-causing mutation of WWP1 renders the enzyme hyperactive by relieving autoinhibition.

2. Materials and methods

2.1. Plasmids

WWP1, DAG1 (full-length dystroglycan precursor), WWP2, SMURF2, NEDD4L, and Dystrophin cDNAs were purchased from DNASU Plasmid Repository (Tempe, AZ, USA). The DNA fragments corresponding to the coding sequences of WWP1, WWP2, SMURF2, and NEDD4L were amplified by PCR with gene-specific primers and inserted into the expression vectors, pcDNA3.1 (Invitrogen) or pYR vectors, previously reported [77], to generate the constructs capable of expressing proteins with the N-terminal FLAG or HA-tag. The coding sequence of WWP1 was also subcloned into another expression vector encoding N-terminally FLAG-tagged BirA as previously described [77]. The coding sequence of DAG1 was subcloned into C-terminally FLAG or HA-tagged pcDNA3.1 or pYR vectors. WWP1 deletion mutants ($WWP1_{C2\text{-}WW,\ HECT,}$ WWP1ΔHECT) and DAG1 deletion mutants (DAG1 ΔC7 (1-888a.a), ΔC35 (1-860a.a), ΔC68 (1-827a.a), and ΔC112 (1-783a.a)) were generated by PCR amplification with appropriate primers and separately subcloned into expression vectors. The constructs of pGEX-6p-WWP1 and pET21b-$WWP1_{HECT}$ were generated from these vectors. The point mutants (R440Q and C890A of WWP1, and Y831G, Y892G, and Y831/892G of DAG1) were generated by using a QuickChange Site-directed mutagenesis kit according to the manufacturer's protocol (Agilent). Information for all primers used are provided in Supplemental Table 1. The DG-K500myc^{GFP} was a kind gift from Andrea Brancaccio (UCSC, Rome) [78]. Mini-dystrophin gene was generated by PCR using the previously published primers (Δ3990; nucleotides 1–1668, 7270–7410, 8059–10,227, and 11,047–11,058 [79]) followed by insertion into the C-terminally Myc/His-tagged pcDNA3.1 vector. SMURF1 and NEDD4 cDNAs were purchased from Addgene (Cambridge, MA, USA) and pCMV-ITCH and pCMV-ITCH C890A constructs were obtained from Shanghai institutes for biological science. The coding sequences of these genes were amplified by PCR using specific primers and inserted into the N-terminally FLAG-tagged pcDNA3.1 or pYR vectors.

2.2. Cell culture and transfection

HeLa and C2C12 cells were cultured in Dulbecco's modified Eagle medium (WelGENE) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin in the incubator at 37 °C with 5% CO_2. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or polyethylenimine (Sigma, St Louis, MO, USA) was used to transiently transfect HeLa cells and C2C12 cells. Cycloheximide (CHX) and MG132 were purchased from Sigma and Cayman Chemical Company, respectively.

2.3. Affinity purification of biotinylated proteins and ubiquitinated-peptides

Purification of ubiquitinated proteins was carried out by the affinity chromatography using biotin as described previously [77]. Briefly, HeLa cells were transfected with the constructs expressing FLAG-BirA-WWP1 and AP-HA-Ub. 48 h later, the cells were treated with 50 μM Biotin and 25 μM MG132 for 4 h, harvested, and lysed in the homogenization buffer (0.25 M sucrose, 10 mM HEPES, pH 7.4, 1 mM EDTA, 10 mM PMSF, 10 mM NEM). Cell lysates were centrifuged for 1 h at 28,000*g* to separate the membrane-enriched and the cytosolic fractions. The membrane-enriched fraction was subsequently solubilized in lysis buffer (2% SDS, 250 mM NaCl, 50 mM Tris-Cl, pH 7.4), incubated with anti-FLAG M2 agarose beads, and separated from the lysate by centrifugation. The lysates containing biotinylated proteins were immunoprecipitated with Streptavidin agarose (Sigma) overnight at 4 °C. The beads were then washed in the washing buffers and sequentially treated with buffers to achieve reduction with DTT, alkylation by IAA, and trypsinization as described previously [77]. Finally, the trypsinized peptides were purified using a Ubiquitin Branch Motif (K-ε-GG) kit (Cell Signaling, Danvers, Waltham, MA, USA) according to the manufacturer's protocol and analyzed further.

2.4. Mass spectrometry

The purified ubiquitinated-peptides were analyzed by nanoelectrospray LC–MS/MS on a LTQ Orbitrap Velos (Thermo Fisher Scientific Inc., MA, USA) as described previously [77].

2.5. RNA interference

SiRNAs for the control, human-specific WWP1, ITCH, and Utrophin were synthesized (Bioneer, Korea). The siRNA sequences for human WWP1 [80], ITCH [81] and Utrophin [82] have been previously described. The siRNAs were transfected with Lipofectamine RNAimax (Invitrogen) according to the manufacturer's instructions.

2.6. Reverse transcription and quantitative real-time PCR

Total RNAs from cells were obtained using Trizol reagent according to the manufacturer's instructions (Invitrogen). Muscle tissues were obtained from C57BL/6 mice and frozen in liquid nitrogen. Frozen muscle tissues were homogenized using nitrogen-cooled grinding bowl and total RNAs were isolated using Trizol reagent. Total RNA was subjected to Quantitative real-time PCR (qRT-PCR) following cDNA synthesis using M-MLV reverse transcriptase kit (Promega, USA). The qRT-PCR was performed using SYBR Premix Ex Taq II (Takara, Japan) and CFX Real-Time system (Bio-Rad, USA). The relative mRNA levels were determined based on the expression of GAPDH. Information for all primers for qRT-PCR are provided in Supplemental Table 2. The qRT-