Characterization of WWP1 protein expression in skeletal muscle of muscular dystrophy chickens

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A missense mutation in the gene encoding WWP1 was identified as the most promising candidate responsible for chicken muscular dystrophy (MD) by genetic linkage analysis. WWP1 is a HECT-type E3 ubiquitin protein ligase composed of 922 amino acids, which contains 4 tandem WW domains that interact with the proline-rich peptide motifs of target proteins. The missense mutation changes arginine 441 that is located in the centre of the WW domains into glutamine (R441Q), which potentially affects the function of the WWP1 protein. Here, we show that WWP1 is detected as ~130-kDa protein that localizes to various structures, such as the plasma membrane (sarcolemma), sarcoplasmic reticulum, mitochondria and nucleus, in normal chicken skeletal muscle. However, in MD chickens, the mutant WWP1 protein was markedly degraded and was absent in the sarcolemma. These changes were also observed in the muscles of chickens in early pre-pathological states. Moreover, *in vitro* expression analysis showed significant degradation of mutant, but not wild-type WWP1, specifically in myogenic cells. Altogether, our data revealed that the R441Q missense mutation in the WWP1 protein causes degradation and loss of the sarcolemmal localization of WWP1, which may play a role in the pathogenesis of chicken MD.

Keywords: E3 ubiquitin ligase/muscular dystrophy/protein degradation/sarcolemma/WWP1.

Abbreviations: ALD, anterior latissimus dorsi; ATPB, β subunit of ATP synthase; CBB, Coomassie Brilliant Blue; cDNA, complementary DNA; DG, dystroglycan; DM, differentiation medium; GM, growth medium; GST, glutathione *S*-transferase; MBP, maltose-binding protein; MD, muscular dystrophy; NH-413, New Hampshire line 413; NIBS, Nippon Institute for Biological Science; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase; SR, sarcoplasmic reticulum; TBS, Tris-

buffered saline; WBP, WW domain-binding protein; WL-F, White Leghorn-F.

Muscular dystrophy (MD) is a group of genetic disorders characterized by weakening of the skeletal muscles that function to support life activities, and occurs in various animal species, including humans. Naturally occurring MD animals have been important experimental models to investigate molecular mechanisms of MDs, and are expected to provide useful information for the diagnosis of and the development of therapies for the MDs. In the decade after the discovery of the *dystrophin* gene, which is responsible for Duchenne MD (1), many of the causative genes of MD animals were identified. Most of these genes were orthologues of various human MDs, but it is still unknown whether the MD chicken can be an animal model of human MD (2–5).

The MD chicken was identified as chickens with a heavily muscled breast but that were unable to raise their wings (6). In addition to the large width of their breast muscle, microscopic pathological changes were observed in their muscles, such as an increase in the number of nuclei, fibre size variation, vacuolization, degeneration of muscle fibres, and the appearance of ringed fibres, fat deposition and connective tissue (7). Interestingly, these changes were predominantly observed in fast-twitch muscles (pectoralis, posterior latissimus dorsi and biceps) but not in slow-twitch (adductor superficialis) and slow-tonic muscles (anterior latissimus dorsi [ALD], adductor profundus and plantaris) (8, 9). The inheritance pattern of chicken MD was initially reported to be autosomal recessive (*am/am*), but was subsequently reported to be a co-dominant trait due to the observation of dystrophic phenotypes in older heterozygotes (*Am/am*, *am/Am*) (6, 10, 11). Genetic linkage analysis of MD chickens strongly suggested a missense mutation in the *WWP1* gene as the cause of chicken MD (5).

WWP1 is a member of the Nedd4-like family, which consists of nine HECT-type E3 ubiquitin protein ligases characterized by the presence of three functional domains: an amino-terminal C2 domain for lipid binding, a central region containing 2–4 WW domains that are thought to mediate target substrate specificity and a carboxyl-terminal HECT domain for ubiquitin protein ligation (12). WWP1 was identified as a protein that interacts with the proline-rich PY motif of WW domain-binding protein-1 (WBP-1), WBP-2 and atropha-1, which is the gene responsible for dentatorubral

and pallidolusian atrophy (13, 14). Accumulating data from many studies have elucidated the versatile role of WWP1 in a variety of cellular processes, including cell proliferation, survival, apoptosis, bone development, aging and disease (15–17). Northern blot analysis showed a high level of expression of WWP1 transcripts in skeletal and cardiac muscle (14, 18), suggesting that WWP1 plays important roles in the maintenance of these muscles. However, very little is known about WWP1 in skeletal muscle, particularly at the protein level.

In this study, we generated an antibody specific to the WWP1 protein by affinity purification, and found that WWP1 localizes to the sarcolemma and cytoplasmic organelles, including the sarcoplasmic reticulum (SR), mitochondria, and a part of the nucleus in normal chicken pectoralis muscle. However, interestingly, only the sarcolemmal localization of WWP1 was selectively lost in MD chickens. We also showed that significant WWP1 protein degradation occurs in MD chicken muscle. *In vitro* expression analysis indicated that this degradation was due to a single amino acid mutation in WWP1. Our findings provide a molecular basis for understanding the role of WWP1 in chicken MD.

Materials and Methods

Chicken muscles

Frozen muscle block samples of wild-type chickens (White Leghorn-F: WL-F) and MD chickens (New Hampshire line 413: NH-413) were supplied by Nippon Institute for Biological Science (NIBS; Yamanashi, Japan). The NH-413 chicken strain (8) was maintained as a homozygous (*am/am*) dystrophic line at NIBS, and the WL-F strain was established and maintained as closed colonies at NIBS. We confirmed that all the NH-413 muscle samples used in this study had a homozygous missense mutation in the *WWP1* gene, by the genotyping method of Matsumoto *et al.* (5). Handling of tissue samples was carried out in accordance with the guidelines provided by The Experimental Animal Care and Use Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan.

Expression vectors

Artificial DNAs encoding wild-type and mutated chicken full-length WWP1 were synthesized (FASMAC Co., Ltd, Tokyo) and subcloned into the pCAGGS expression vector (19). Codon usage was changed for optimal expression in mammalian cells, resulting in nucleotide sequences with ~70% identity to those of wild-type and mutant chicken WWP1. A c-Myc epitope sequence was inserted immediately before the stop codon to distinguish these proteins from endogenous WWP1 at the protein level.

Antibodies and nuclear staining

An antibody against WWP1 was generated using a recombinant mouse WWP1 fragment (amino acid positions 199–545), excluding the C2 and HECT domains. The mouse WWP1 complementary DNA (cDNA) fragment was amplified from the mouse WWP1 expression vector described previously (20) by polymerase chain reaction using the following oligonucleotide primer sets: one forward primer, 5'-GTT CCC AAC TCC TGC TGT TCA-3' (867–887), and two reverse primers containing a termination codon (TAG) at the 5' terminus, 5'-CTA CCA CCT AAA GCT GCG TTC ATA-3' (1,907–1,887) and 5'-CTA AGC TTC TGT GTT GGT GTT TCC-3' (1,310–1,290), for mouse WWP1 (accession no.: NM_177327). Amplification and cloning of the WWP1 fragment were performed as described previously (21). The 1-kb and 0.4-kb cDNA fragments were subcloned into pGEX-1 λ T (GE Healthcare UK, Ltd, Buckinghamshire, UK) and pMAL-c2 (New England Biolabs, Beverly, MA, USA), respectively. Recombinant proteins were

expressed as fusion proteins with glutathione *S*-transferase (GST) and maltose-binding protein (MBP) in *Escherichia coli* and purified using Glutathione Sepharose and Dextrin Sepharose columns, respectively. The purified GST–WWP1 fusion protein was then used as the antigen for immunization and the antiserum that was subsequently obtained was purified using an affinity Sepharose column coupled with the MBP–WWP1 fusion protein. To confirm the specificity of our antibody with WWP1, the antibody absorption test was performed. The anti-WWP1 antibody was pre-incubated with an excess amount of MBP–WWP1–Sepharose in Tris-buffered saline (TBS) for 1 h at room temperature. After removing the antigen-conjugated Sepharose by centrifugation, the supernatant was used as the primary antibody solution for immunoblotting and immunohistochemical staining.

Mouse monoclonal antibodies against two sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) isoforms, the fast-twitch type (SERCA1; clone CaF1-5C3) and the slow-twitch/cardiac type (SERCA2; clone CaS/C1) for SR labelling, were supplied by the Developmental Studies Hybridoma Bank at the University of Iowa. Mouse monoclonal antibodies against the c-myc epitope tag (4A6) and the anti- β subunit of ATP synthase (ATPB; clone 3D5) for mitochondria labelling were purchased from Millipore (Merck Millipore, MA, USA) and Abcam Japan (Tokyo, Japan), respectively. GelRed dye for nucleus labelling was purchased from Biotium, Inc. (CA, USA).

Histology

Chicken muscle cryosections were subjected to haematoxylin and eosin staining or indirect immunofluorescent staining as described previously (22).

Immunohistochemistry was performed using cryosections (8 μ m) of chicken pectoralis and ALD muscles. Cryosections were mounted on glass slides and fixed in cold acetone. After incubation of the fixed sections in TBS containing 0.1% TritonX-100 for 1 min at room temperature, the sections were washed and equilibrated with TBS. Then, the sections were reacted with the affinity-purified anti-WWP1 rabbit antibody (2 μ g/mL) in TBS containing 2% casein overnight at 4°C, followed by visualization with Alexa488-conjugated anti-rabbit secondary antibody (1:600 dilution) at room temperature for 30 min. The WWP1-stained muscle sections were subsequently incubated with anti-SERCA1, anti-SERCA2 or anti-ATPB mouse monoclonal antibody at room temperature for 1 h, followed by incubation with an Alexa568-conjugated anti-mouse secondary antibody (1:600 dilution). Visualization of the nuclei was performed by incubation of the sections in TBS containing GelRed dye (Biotium, Inc.) (1:10,000 dilution) for 1 min at room temperature. Fluorescence signals on the muscle sections were observed under a confocal laser scanning microscope (Leica TCS-SP5; Leica, Heidelberg, Germany).

Cell culture and transfection

Mouse muscle C2C12 cells were maintained as undifferentiated myoblasts in growth medium (GM: Dulbecco's Modified Eagle Medium [DMEM]/F12 supplemented with 15% foetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in an atmosphere of 5% CO₂ and 95% humidity). Cells were plated onto 12-well plates, and when they had proliferated to 60–70% confluency, the GM was exchanged with fresh medium or switched to differentiation medium (DM: DMEM/F12 supplemented with 2% horse serum, 100 U/ml penicillin and 100 μ g/ml streptomycin). Immediately after the medium exchange, cells were transfected with expression vectors for chicken wild-type or R441Q mutant WWP1 using TransIT-LT1 reagent (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's protocol. Thirty hours later, cells (cultured in GM or DM) were washed with TBS and then treated with heated lysis solution (2% sodium dodecyl sulfate [SDS], 25 mM Tris–HCl, pH 6.8, 192 mM glycine and 15% glycerol). The cell lysates were used for immunoblotting to analyse exogenous WWP1 expression. As a control experiment, the same expression analysis was performed using the Chinese hamster ovary cell line (CHO cells) instead of C2C12.

Immunoblot

Chicken pectoralis and ALD lysates for immunoblot analysis were prepared as follows. Several muscle cryosections were collected in a dry ice-cold Eppendorf tube®, and the appropriate volume of heated lysis solution was added. The tube was then incubated at 100°C for 5

min and centrifuged at $20,000 \times g$ for 10 min. The supernatant was then used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein concentrations of muscle lysates were determined using a protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) with bovine serum albumin as a standard, in an SDS concentration that does not affect the accuracy of the assay system.

SDS-PAGE and protein transfer to polyvinylidene difluoride membranes were performed as described by Laemmli (23) and Kyhse-Anderson (24), respectively. Immunoreactive protein bands were visualized using the chemiluminescence detection system for standard-type Amersham ECL (GE Healthcare UK, Ltd). Immunoblot quantification was performed using densitometry analysis (Multi Gauge version 3.0) (Fujifilm Co., Tokyo, Japan). Signals of full-length WWP1 were quantified by densitometry, and normalized with the expression level of α -actinin that was stained using a mouse monoclonal antibody against chicken sarcomeric α -actinin (25). Molecular sizes of protein bands on SDS-PAGE were estimated with reference to the mobility of BenchMark Protein Ladder (Life Technologies Japan, Tokyo, Japan).

Results

Expression of the WWP1 protein in pectoralis muscle of MD chickens

To analyse WWP1 protein expression, we first raised a rabbit antiserum against the mouse recombinant WWP1 protein. WWP1 is a member of a large family of HECT-type ubiquitin E3 ligases. Members of this protein family share three structurally conserved domains (C2, WW and HECT), and thus to avoid cross-reactivity of the antiserum to the other E3 members, it was affinity purified using a short fragment of WWP1 bearing little resemblance to the other family members (Fig. 1A). The affinity-purified antibody detected a main protein band at ~ 130 kDa and a few smaller bands of <70 kDa in CHO cells that were transfected with an expression vector for c-myc-tagged full-length WWP1 (Fig. 1B). This staining pattern was similar to that obtained using an anti-myc antibody (Fig. 1B). These results clearly indicated that our obtained antibody recognizes chicken full-length WWP1 as a 130-kDa protein on SDS-PAGE, although the molecular weight of WWP1 was theoretically deduced to be about 105 kDa from its primary structure.

We next examined the expression of the WWP1 protein in chicken skeletal muscles (Fig. 1C). The antibody detected a 130-kDa major and 90-kDa minor protein bands in the lysate of wild-type chicken pectoralis muscle. However, in MD chicken muscle, the 130-kDa band was markedly reduced compared with that of wild-type muscle. In contrast, a 90-kDa minor band was more intense than in wild-type muscle. Moreover, an additional small band appeared below 60 kDa. These results indicated that significant degradation of the WWP1 protein occurs in the pectoralis muscle of MD chickens.

We also investigated the localization of the WWP1 protein in chicken skeletal muscles using our affinity-purified antibody. Immunofluorescence microscopy detected WWP1 signals at the plasma membrane (sarcolemma) and in the cytoplasm of muscle fibres in wild-type chicken pectoralis (Fig. 2A), whereas these signals were undetectable in the antibody absorption test performed with the antigen that was originally used for affinity purification, confirming the specificity of our WWP1 antibody (Supplementary

Fig. S1). A high-magnification image of the cytoplasm demonstrated the discontinuous network structure and the many intense dots on or close to it (Fig. 2C). Interestingly, in MD chicken pectoralis, the sarcolemmal signals were much weaker or absent, whereas cytoplasmic signals appeared to be denser than those of wild-type muscle (Fig. 2B and D). Double-staining analysis indicated that the cytoplasmic WWP1 network largely overlapped with the staining of SERCA1 (Fig. 3D–F), whereas the scattered intense dots corresponded to mitochondria that were labelled with an anti-ATPB antibody (Fig. 3G–I). A few relatively large and intense signals in muscle fibres were located in a part of the nucleus, which was stained by GelRed dye (Fig. 3A–C). The same double-staining pattern with organelle markers was shown in the cytoplasm of MD chicken muscle fibres, except that mitochondrial signals were more densely distributed compared with those of wild-type chicken muscle (Fig. 3J–L).

Degradation and localization of the mutant WWP1 protein in chicken pectoralis muscles of pre-pathological MD stages

We observed changes in the localization and instability of the WWP1 protein in MD chicken pectoralis muscle; however, these may be secondary effects caused by the degeneration of muscle fibres. Thus, we examined WWP1 expression in MD muscles in the early pre-pathological state. In our isolated chicken muscle samples, pathological changes were observed in chickens older than 1 month of age, but not in those younger than 3 weeks (Fig. 4A). We confirmed that our anti-WWP1 antibody clearly stained along the sarcolemma of muscle fibres in 1-week-old wild-type chickens as in 3-month-old chickens, whereas very weak or no fluorescent signals were detected along the sarcolemma of the age-matched MD chicken muscle (Fig. 4B). The same results were obtained in muscles of 2-week-old and 3-week-old chickens (data not shown). On the other hand, immunoblot analysis of MD chicken muscle revealed that degradation of WWP1 had already occurred in the pre-pathological younger stage although its level of degradation was slightly milder than that of 3-month-old chickens (Fig. 4C). Quantification of proteins indicated that $\sim 50\%$ of full-length WWP1 was degraded during the pre-pathological stages (1–3 weeks of age) and that 80% was lost during the post-pathological stages (1–3 months of age), suggesting that the 30% disparity in WWP1 degradation may be due to secondary effects of muscle degeneration (Fig. 4D). These data demonstrated that a change in WWP1 protein distribution and degradation precedes the appearance of muscle degeneration in MD chickens.

Expression of the WWP1 protein in slow-tonic ALD muscle

Fast-twitch muscles are predominantly affected in the MD chicken, whereas slow-tonic and slow-twitch muscles are minimally affected (8). We hence examined whether there are any differences in WWP1 expression between slow-tonic ALD and fast-twitch pectoralis

Fig. 1 Expression of the WWP1 protein in chicken pectoralis muscle. (A) Schematic representation of the WWP1 protein. WWP1 is composed of 922 amino acids and arginine 441 is mutated to glutamine (R441Q) in MD chickens. The three functional domains, C2, WW and HECT, are indicated as grey boxes. The black rectangle is a region with low homology to the other HECT-type E3s, which was used for affinity purification of the antibody. (B) Reactivity of the obtained anti-WWP1 antibody. Lysates (2 μ g) of CHO cells expressing exogenous c-myc-tagged chicken WWP1 (WWP1-myc) was subjected to immunoblot analysis. Protein signals were detected by an anti-c-myc monoclonal antibody (Myc) and an affinity-purified anti-WWP1 antibody (WWP1). Total protein on the transferred membrane was visualized by Coomassie Brilliant Blue (CBB) staining. Protein size markers are indicated on the left. (C) Twenty micrograms of wild-type (Wt) and dystrophic (MD) pectoralis muscle (PS) lysate was analysed by immunoblotting using the affinity-purified anti-WWP1 antibody. Arrow and arrowhead indicate the bands representing full-length WWP1 and WWP1 degradation products, respectively.

muscle. Immunofluorescence staining using our anti-WWP1 antibody showed only very weak signals at the sarcolemma in both wild-type and MD chickens, whereas it clearly labelled intramembranous particles and nuclei (Fig. 5A). Double staining with antibodies against SERCA2 or ATPB indicated that the particles were composed of SR and mitochondria (Fig. 5A). On the other hand, immunoblotting of ALD showed a significant decrease in full-length WWP1 in MD chickens compared with wild-type chickens, whereas a number of degradation products were detected in both wild-type and MD chickens (Fig. 5B). Quantification of the protein signals indicated that the expression level of full-length WWP1 in MD chicken ALD was ~40% of that of wild-type chicken ALD (Fig. 5C). This analysis also revealed that WWP1 expression in normal chicken ALD was much lower than that of the pectoralis muscle.

Fig. 2 Localization of WWP1 proteins in chicken pectoralis muscle. Immunofluorescence staining of cryosections from the pectoralis muscle of 3-month-old wild-type (A, C) and MD (B, D) chickens performed using the affinity-purified anti-WWP1 antibody. Panels C and D are magnified images of the two areas inside the white square frames of A and B, respectively. Bars indicate 50 μ m (A, B) and 10 μ m (C, D), respectively.

R441Q missense mutation induces WWP1 degradation

Our analyses using pre-pathological pectoralis and unaffected ALD of MD chickens demonstrated that WWP1 degradation were not secondary effects of muscle degeneration. Thus, we next examined whether the single amino acid change (R441Q) in WWP1 results in its increased degradation using an *in vitro* expression assay (Fig. 6). We transfected an expression vector for the R441Q WWP1 mutant or wild-type WWP1 into C2C12 myogenic cells and examined their protein expression. During the proliferating stage in GM, the exogenous wild-type WWP1 protein was clearly detected as a 130-kDa band, whereas expression of the R441Q mutant was markedly reduced. The same result was obtained in cell culture under a differentiating condition. On the other hand, in CHO cells, no differences were detected in expression levels between wild-type and R441Q WWP1. Downregulation of exogenous mutant WWP1 mRNA was not observed when the expression vector for mutant WWP1 was transfected into C2C12 cells (20). Taken together, our results indicated that the R441Q mutation in WWP1 induced its protein degradation in muscle cells.

Discussion

Here, we presented evidence that the R441Q missense mutation in WWP1 alters its stability in skeletal muscle. We detected full-length WWP1 as a 130-kDa protein on SDS-PAGE in wild-type chicken skeletal muscles, whereas in MD chicken muscles the full-length molecule was markedly degraded (Fig. 1). This

Fig. 3 Localization of WWP1 in the cytoplasm of pectoralis muscle fibres. Double-immunofluorescence staining of cryosections from the pectoralis muscles of a 3-month-old wild-type (A–I) and MD chicken (J–L). Sections were stained for WWP1 (A, D, G, J) or with the following organelle markers: GelRed for nuclei (B), SERCA1 for the sarcoplasmic reticulum (E) and ATPB for mitochondria (H, K). Panels C, F, I and L are merged images of the two left panels. Bars indicate 50 µm (C) and 10 µm (F, I and L), respectively.

degradation in MD chicken muscles was also observed in the pre-pathological early stage in pectoralis muscles and non-affected ALD muscles (Figs 4 and 5). Expression analysis using C2C12 myogenic cells showed that increased WWP1 degradation occurred only when the R441Q mutant was overexpressed, and not when wild-type WWP1 was expressed in the cells (Fig. 6). These data revealed that a single amino acid substitution of arginine 441 to glutamine in WWP1 causes its degradation. Moreover, *in vitro* expression of R441Q mutant WWP1 in CHO non-myogenic cells did not induce its degradation (Fig. 6), indicating the possibility that R441Q-induced degradation occurs specifically in muscle cells.

We clearly showed the localization of WWP1 molecules along the sarcolemma, in the SR and

mitochondria, as well as in a part of the nucleus in wild-type chicken pectoralis muscle (Figs 2 and 3). However, interestingly, only its sarcolemmal localization was lost in the MD chicken pectoralis. This loss of localization was also preceded by the appearance of muscle degeneration, as is the case with WWP1 protein degradation (Fig. 4). This indicated a close association between changes in sarcolemmal localization and WWP1 protein degradation. Arginine 441 is located in the centre of the WW domain cluster that plays a role in the interaction of WWP1 with its target proteins (5, 13, 14). Thus, structural alteration of the WW domain cluster would disrupt the association of WWP1 with the plasma membrane via a particular binding partner, which may then free the protein to be subjected to proteolysis. Another possibility is

Fig. 4 Changes in WWP1 expression in MD chickens in the early non-pathological stage. (A) Pathogenesis of the pectoralis muscle of MD chickens of different ages. Haematoxylin and eosin-stained pectoralis muscle sections of MD chickens at 1, 2 and 3 weeks (w) and 1, 2 and 3 months (m) of age are shown. Bar, 100 μ m. (B) Staining of pectoralis muscle sections of a 1-week-old wild-type (Wt) and MD (MD) chicken using the anti-WWP1 antibody. Bar, 50 μ m. (C) Immunoblot analysis of WWP1 in muscle homogenates from 1-week-old (1 w) and 3-month-old (3 m) wild-type and MD chickens. Arrows and arrowheads indicate full-length WWP1 and WWP1 degradation products, respectively. (D) Quantification of the full-length WWP1 protein from the immunoblots of pectoralis muscles of 1- to 3-week-old (1-3 w; non-pathological stage) and 1 to 3-month-old (1-3 m; pathological stage) MD chickens.

that the structural change in WWP1 would initially cause proteolysis around its WW domains, followed by its release from the sarcolemma. In either case, the decrease in molecular stability of WWP1 by the missense mutation is a possible cause of chicken MD.

Chicken MD was originally reported to be inherited in an autosomal recessive manner with the gene

Fig. 5 Expression of WWP1 in the ALD muscle of MD chickens. (A) Immunofluorescence staining against WWP1 of ALD muscle cryosections from a 3-month-old wild-type (a, b) and MD chicken (c, d). Immunostaining of wild-type ALD sections for WWP1 alone (e, h), and together with SERCA2 (f) or ATPB (i). g and j are merged images of the left two panels. Bars indicate 50 μ m (c) and 10 μ m (d, j). (B) Immunoblotting of wild-type chicken pectoralis (Wt PS) and ALD (Wt ALD) and MD chicken ALD (MD ALD) for WWP1. Twenty micrograms of muscle lysates were subjected to SDS-PAGE. Protein-transferred membranes were stained with CBB and the anti-WWP1 antibody (WWP1). Arrow and arrowheads indicate the bands representing full-length WWP1 and WWP1 degradation products, respectively. (C) Quantification of full-length WWP1 expressed in the (1- to 3-month-old) wild-type (Wt) ALD, MD ALD and wild-type pectoralis muscles.

designated as *am* (6), but subsequent studies have reported the inheritance pattern to be co-dominant or incomplete dominant, because moderate dystrophic symptoms are observed in heterozygous (*am/Am* or *Am/am*) older chickens (10, 11). We showed here

Fig. 6 Expression of the R441Q WWP1 mutant in C2C12 myogenic cells. CHO cells and C2C12 myogenic cells were transfected with expression vectors for wild-type WWP1 (Wt) and the R441Q WWP1 mutant (R441Q). After 30 h of culture in GM or DM following transfection, cells were lysed and protein concentrations were determined. Two micrograms of each lysate was subjected to immunoblotting and expression levels of WWP1 were analysed (right panels). The uniformity of total protein on each transferred membrane is shown by CBB staining (left panels). The arrow and arrowheads indicate full-length WWP1 and its degradation products in C2C12 cells, respectively.

marked degradation of the full-length WWP1 protein in homozygous (*am/am*) MD chickens (Figs 1 and 4). Because degradation is caused by the R441Q mutation, the expression level of the full-length WWP1 protein in heterozygous chicken pectoralis should be in between that of wild-type and homozygous mutant muscle. Expression levels of the full-length WWP1 protein in the chickens appeared to correlate inversely with the severity of their dystrophic phenotype. This is consistent with our hypothesis that a reduction in WWP1 expression by the R441Q mutation is involved in MD. However, the role of the degradation products of mutated WWP1 should also be considered. The amounts of the small degradation products are thought to increase with a decrease in the amount of full-length WWP1 caused by degradation. The protein fragments may work in a dominant-active manner at inappropriate intercellular locations, and this idea is also consistent with the mode of co-dominant inheritance of chicken MD. Moreover, we cannot deny the possibility that the fragments may play roles other than as an E3 ubiquitin ligase. Detailed analyses of the structure and function of the degradation products should lead to the clarification of the molecular mechanism of chicken MD. On the other hand, the expression level of full-length WWP1 in ALD muscle was very low even in normal chicken (Fig. 5C). It was less than that of MD chicken pectoralis muscle (Fig. 4D). Moreover, interestingly, WWP1 is hardly detected at the sarcolemma, not only in MD chickens but also in wild-type chickens (Fig. 5A). WWP1 does not appear to play a large role in the maintenance of slow-twitch and slow-tonic muscle fibres, although it plays a significant role in fast-twitch pectoralis muscle. The other HECT-type E3 members, such as WWP2

and Itch E3 (16), may compensate for the roles of WWP1 in those muscles.

We showed the loss of WWP1 expression from the sarcolemma of MD chicken pectoralis, whereas such a change was not observed in the cytoplasmic region. However, WWP1 signals on the reticulated structures were more intense in MD chicken muscle than in normal chicken muscle fibres (Fig. 2). Immunohistochemical analysis using organelle markers revealed that the modest increase in the WWP1 signal was due to an increase in mitochondria (Fig. 3), which is consistent with previous pathohistological studies. Wilson *et al.* (8) detected an increase in mitochondrial enzyme activities, such as succinic dehydrogenase and NADH dehydrogenase, in the pectoralis muscle of MD chickens by cytochemical staining, whereas Crowe and Baskin (26) demonstrated a significant increase in volume and surface density of mitochondria in dystrophic muscle by stereological analysis. As the level of full-length WWP1 was reduced by its degradation in dystrophic muscle, an increase in its cytoplasmic level should be due to its degradation products associated with the increased mitochondria (Figs 1 and 3). Whether WWP1 is involved in the increase in mitochondria is a point of interest that should be analysed in the future.

Previous biochemical analysis demonstrated hypoglycosylation of dystroglycan (DG) and its decreased binding to laminin in MD chicken muscle (27). In humans, such an impairment of the laminin-binding ability of DG in its extracellular subunit (α -DG) has been known to cause congenital muscular dystrophies, collectively referred to as the dystroglycanopathies, including Fukuyama congenital MD, Walker-Warburg syndrome, and muscle-eye-brain disease (28–30). As the transmembranous DG subunit (β -DG) has a proline-rich motif that can interact with the WW domain of WWP1 via the C-terminus of β -DG (31), the relationship between α -DG hypoglycosylation and the WWP1 mutation has been of interest. Godfrey *et al.* (32) searched for mutations in the human *WWP1* gene in a cohort of 33 dystroglycanopathy patients, but were not able to identify any clear pathogenic mutations. We also attempted to examine the interaction between WWP1 and DG biochemically. In our preliminary analysis using muscle homogenates, DG was not detected in the immunoprecipitate using the anti-WWP1 antibody (data not shown). Therefore, the aberrant glycosylation of DG in MD chicken muscle does not appear to be caused by its direct interaction with R441Q-WWP1.

Northern blot analysis detected two WWP1 transcripts of different sizes in chicken pectoralis muscle (5), suggesting the possible existence of a WWP1 isoform in addition to the 130-kDa full-length form. These transcripts were also expressed in MD chicken pectoralis muscle at slightly lower levels than in wild-type chicken muscle. However, we did not detect any additional protein signals other than the 130-kDa band in the skeletal muscles of both wild-type and MD chickens by immunoblot analysis. The protein from the additional transcript might lack the region containing the epitopes of our antibody, or the transcript may

actually have no structural differences from the full-length transcript in its open reading frames.

In summary, we generated an antibody against the WWP1 E3 protein ubiquitin ligase, which is the most likely candidate responsible for chicken MD, and analysed its protein expression in skeletal muscle tissue. Our results revealed that a missense mutation in the WWP1 gene results in the production of an unstable WWP1 protein. The results from this study should provide new insights towards elucidation of the molecular mechanisms involved in chicken MD.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest

None declared.

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