Mutated WWP1 Induces an Aberrant Expression of Myosin Heavy Chain Gene in C2C12 Skeletal Muscle Cells

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The WW domain containing E3 ubiquitin protein ligase 1 (WWP1), an enzyme to degrade unneeded or damaged proteins, was recently identified as the responsible for chicken muscular dystrophy. Despite of intensive studies on oncogenic characters, the role of WWP1 to muscular diseases has not yet been fully understood. Since it is generally known that the switching of myosin heavy chain (MyHC) isoforms from neonatal isoform to adult one is inhibited in chicken muscular dystrophy, we transfected either of wild and mutated types of WWP1 gene into C2C12 cells to monitor the expression pattern of muscle-differentiation markers including MyHCs by real-time PCR. Excessive WWP1 expression enhanced the expression of the MyHC Ia gene but lowered the expression of the MyHC IIb gene. On the other hand, mutated WWP1 gene transfected into myoblasts was distinct from these cases in that the MyHC gene or genes expression inhibited the normal myoblast differentiation. The present data suggest that WWP1 promotes myoblast differentiation from embryonic into fast twitch phase while a mutation in WWP1 results to retain slow and fast twitch isoforms characteristic of dystrophic fast twitch muscles.

Key words: chicken, gene expression, muscular dystrophy, myosin heavy chain, WWP1


Introduction

The WW domain containing E3 ubiquitin protein ligase 1 (WWP1) is classified into a ubiquitin ligase (E3) which plays an important role in ubiquitin-proteasome pathway (UPP) to degrade unneeded or damaged proteins (Scheffner and Staub, 2007). The WWP1 and similar E3 ligases play important roles in cancer development, bone remodeling and central nervous system regeneration (Chen and Matesic, 2007; Glimcher et al., 2007; Bernassola et al., 2008; Qin et al., 2008).

Recently, we identified the WWP1 gene as a candidate gene responsible for the chicken muscular dystrophy, and the R441Q missense mutation in the WWP1 gene was found to be the cause of muscular dystrophic phenotype (Matsumoto et al., 2007, 2008). It is generally known that myosin is the principal protein of the contractile apparatus in muscle, and myosin diversity is primarily produced by the different expression of multiple isoforms of myosin heavy chain (MyHC) subunits which undergo transition during development in a variety of muscle systems (Schiaffino and Reggiani, 1994). The switching of the adult phenotype in fast muscle is inhibited in chicken muscular dystrophy, resulting in the continued expression of a slow twitch MyHC isoform in adult fast muscles (Bandman, 1985; Bandman and Bennett, 1988; Kaprielian et al., 1991; Tidyman et al., 1997).

The WWP1 gene is expressed strongly in skeletal muscles (Flasza et al., 2002; Komuro et al., 2004; Matsumoto et al., 2009), but the relationship between WWP1 and MyHC proteins has not been examined. To investigate the effects of the overexpression and the expression of the mutated WWP1 gene on the MyHC genes expressions, we transfected the wild and mutated types of the WWP1 gene into C2C12 cells which were myoblasts derived from the mice skeletal muscle. The expression of muscle-differentiation markers, Myogenin (Myog), myogenic differentiation 1 (MyoD) and MyHCs, was analyzed by real-time PCR.
**Materials and Methods**

**Cell Culture**

The C3H murine skeletal muscle cell line C2C12 (CRL-1722) was commercially obtained from the American Type Culture Collection (ATCC), VA, USA. Cells were cultured in the growth medium: Dulbecco’s modified Eagle’s medium (DMEM) (Nissui, Tokyo, Japan) containing 0.2% sodium hydrogen carbonate (Nakalaitesque, Kyoto, Japan), 0.008% kanamycin (Wako, Osaka, Japan) and L-glutamine (10 μg/ml) (Nissui), supplemented with 15% fetal bovine serum (FBS) (Gibco, NY, USA). The cells were cultured in humidified 95% air and 5% CO₂ atmosphere. Differentiation of C2C12 cells was initiated by placing 80% confluent cell cultures in the differentiation medium: DMEM supplemented with 1% FBS.

**Isolation and Mutagenesis of Mouse WWP1 Sequences**

Mouse total RNA was isolated from an ICR strain liver using Sepasol RNAI (Nakalaitesque). The mouse was sacrificed according to the guideline of Animal Experimentation of Kobe University. The cDNA was generated by reverse transcription using oligo (dT) primer and SuperScript III Reverse Transcriptase (Invitrogen, CA, USA). The mouse WWP1 sequence was amplified from the cDNA using primers 5'-ATCGTGTCTTATTCATC-3' (WWP1-full-F) and 5'-GTTCTAGCTGAGATATGATGTA-3' (WWP1-full-R) (NM_177327). The primers were designed to amplify the complete DNA sequence (WT: wild type and mutant type) using the QuikChange site-directed mutagenesis kit (Stratagene) and the following primer:

- **MF**: M-TTCAACCAACAATACCTC-TTCGATCTCTCAG-3' (WWP1-full-F)
- **MR**: 5'-CATAACCCAGAACAACCACC-3' (WWP1-full-R)

**Statistical Analysis**

Values in this paper represent means ± S.D. Differences between two groups were examined for statistical significance using Student’s t test. P value less than 0.05 denoted the presence of a statistically significant difference.

**Results and Discussion**

We transfected the WWP1 gene into C2C12 cells to analyze the expression of the muscle-differentiation markers. To evaluate the vector capacity, WWP1 expression of each
group (pCAGGS-empty; control, pCAGGS-WT; WT and pCAGGS-R436Q-transfected cells; R436Q) was analyzed by RT-PCR using cells at day 0 (Fig. 1A). Although the WT and R436Q groups expressed 3.83 and 5.47 fold higher than control level, clear difference was not observed in the myotube morphology and the proliferation rate of myoblasts (data not shown).

Figure 1B shows the diachronic analysis of the WWPI expression in control cells during myogenic differentiation. Since in vitro myogenesis is completed within a week (Sultan et al., 2006), WWPI expressions analyzed by real-time PCR were made at day 0, 2, 4, 6 and 8. The WWPI gene seems to be expressed stably in each time point, though upward trend was observed in day 2 and 4.

Subsequently, we analyzed the expressions of the muscle-differentiation markers by real-time PCR to access the influence of the WWPI overexpression and WWPI with R436Q mutation on the gene expressions of Myog, MyoD, MyHC 1a and MyHC 1b using C5C12 cells at day 6 (Fig. 2). The former two were analyzed as the markers for early stages (Langlands et al., 1997) and the latter two for later stages of muscle differentiation (Silberstein et al., 1986).

The expressions of Myog in the WT and R436Q groups were 1.04 fold (±0.02) higher and 0.64 fold (±0.26) lower compared to the control group. The R436Q group was significantly lower than other groups. These results indicate that R436Q mutation in the WWPI gene affects early stages of muscle differentiation through the reduction of the Myog expression. There was no clear difference in the MyoD gene expression among three groups.

It was, however, interesting to indicate that the WWPI gene overexpression influenced upon the MyHC genes expression. The MyHCs are among muscle proteins increasing during the course of myogenesis (Silberstein et al., 1986), and are divided into two classes, type I composed of fast twitch fibers and type II of slow twitch fibers (Larsson and Salviati, 1989). The MyHC 1a expression levels in the WT and R436Q groups increased 2.81 (±0.95) and 2.18 (±0.24) fold compared to the control group, respectively.

The expression level of the MyHC 1a gene in the WT group was significantly higher (2.81 fold±0.95) compared to the control group, but the MyHC 1b gene expression was significantly lower (0.26±0.04 fold) in the WT group, indicating that the WWPI promotes to trans-

### Table 1. Primers used for real-time PCR

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**Fig. 1. Exogenous and endogenous expression of WWPI gene in C5C12 cells at day 0.** A) The vector capacity was assessed by RT-PCR method using as internal standard. Each expression of gene in transfection control cells. B) Endogenous expression of the WWPI gene in differentiating control cells. The WWPI expression levels analyzed by real-time PCR were not significantly changed through day 0 to 8. Y-axis indicates relative expression level of WWPI gene to the GAPDH gene expression. Bars indicate standard deviations.
form C5C12 cells into fast twitch characteristics. However, the R436Q-transfected cells persisted in the high expression of both fast MyHC Ia and slow MyHC IIb isoforms compared to the control cells, suggesting retention in slow and fast twitch isoforms characteristic.

One of known proteins interacting with WWP1 is Notch (Flasza et al., 2006), whose ablation in a skeletal muscle results in increased formation of fast twitch fibers and altered fiber type distribution at the expense of slow twitch fibers (Kitamura et al., 2007). The WWP1 might control skeletal muscle fiber types via the regulation of the MyHC genes expression by the Notch signaling. The R436Q-transfected cell group showed the highest MyHC IIb expression (2.80±1.92 fold than control level) among three groups, suggesting that C5C12 cells persisted in the slow twitch character. Taken together, the R436Q mutation in WWP1 gene seems to inhibit the normal fiber type differentiation and to induce atrophy. We assume that the transfection of mutated WWP1 gene into normal chickens will inhibit the switching of the adult phenotype in their fast muscles and exhibit muscular dystrophic phenotypes such as degenerating fibers with many vacuoles in cytoplasm, as observed in muscular dystrophic chickens (Kikuchi et al., 1981; Bandman, 1985). Our results suggest that WWP1 plays an important role in myoblasts’ differentiating process. The mechanism to regulate MyHCs and other related-molecules by WWP1 needs elucidating in the future.

Acknowledgments

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References


