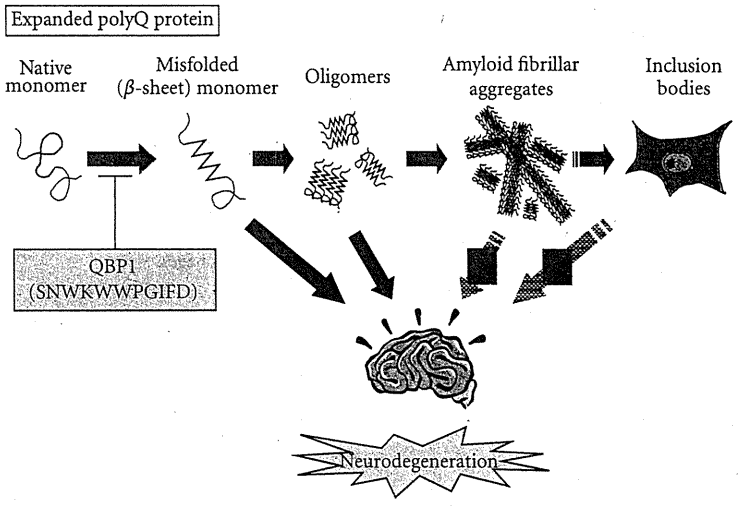

FIGURE 2: Molecular pathogenesis of the polyQ diseases and the therapeutic target of QBP1. Proteins with an expanded polyQ stretch are prone to misfold into a β-sheet dominant structure, leading to their assembly into oligomers and amyloid fibrillar aggregates, followed by their accumulation as inclusion bodies within neurons, eventually resulting in neurodegeneration. The peptide QBP1 inhibits the initial misfolding into a β-sheet dominant structure of the protein by binding to the expanded polyQ stretch, resulting in suppression of polyQ protein aggregation and polyQ-induced neurodegeneration. Question marks indicate structures for which cytotoxicity remains controversial.

expanded polyQ proteins are the most initial events of the pathogenic cascade, and therefore ideal targets since their intervention is expected to lead to the suppression of a broad range of downstream pathogenic events [22, 24, 37, 38]. We therefore aimed towards establishing a therapy targeting misfolding and aggregation of the expanded polyQ protein.

3. Identification of the Aggregation Inhibitor Peptide QBP1

We hypothesized that molecules capable of binding specifically to the expanded polyQ stretch would interfere with its misfolding and aggregation. Identification of the monoclonal antibody 1C2 that selectively binds to the expanded polyQ stretch, probably by recognizing its unique structure [39], prompted us to search for amino acid sequences (domains) or peptides possessing similar properties, which would be more suitable as a drug due to their smaller size and more efficient *in vivo* delivery. We decided to employ phage display screening to identify peptides that bind selectively to the expanded polyQ stretch (Figure 3) [40]. Eleven-amino acid combinatorial peptide libraries expressed on the surface of M13 phage were first screened for their binding to a polyQ62 stretch fused to glutathione *S*-transferase (GST-Q62) by enzyme immunosorbent assay. Phage clones isolated from this first screening were further screened for their selective binding to pathologic length GST-Q62 compared to normal-length GST-Q19. We finally identified six phage clones with greater binding to GST-Q62, and named the encoded peptide sequences polyglutamine binding peptide 1-6 (QBP1-6) (Table 1). Interestingly, most of the peptides were rich in Trp residues, implying that hydrophobic interactions play a role in their binding to the expanded polyQ stretch.

TABLE 1: Polyglutamine binding peptides isolated from phage display screening.

Name	Q62/Q19 binding ratio	Sequence (X_5-fixed-X_5)
QBP1	1.66	Ser-Asn-Trp-Lys-Trp-Trp-Pro-Gly-Ile-Phe-Asp
QBP2	1.31	His-Trp-Trp-Arg-Ser-Trp-Tyr-Ser-Asp-Ser-Val
QBP3	1.30	His-Glu-Trp-His-Trp-Trp-His-Gln-Glu-Ala-Ala
QBP4	1.27	Trp-Gly-Leu-Glu-His-Phe-Ala-Gly-Asn-Lys-Arg
QBP5	1.25	Trp-Trp-Arg-Trp-Asn-Trp-Ala-Thr-Pro-Val-Asp
QBP6	1.23	Trp-His-Asn-Tyr-Phe-His-Trp-Trp-Gln-Asp-Thr
SCR		Trp-Pro-Ile-Trp-Ser-Lys-Gly-Asn-Asp-Trp-Phe

We chose QBP1 (Ser-Asn-Trp-Lys-Trp-Trp-Pro-Gly-Ile-Phe-Asp), which showed the greatest differential binding affinity to pathologic length polyQ compared with normal length polyQ for further analysis.

We first tested our hypothesis that QBP1, a peptide that selectively binds to the expanded polyQ stretch would interfere with polyQ aggregation *in vitro* [40]. We designed thioredoxin-polyQ (thio-polyQ) fusion proteins, and found that thio-polyQ with an expanded polyQ stretch (>40) forms aggregates *in vitro* in a time-, concentration-, and polyQ length-dependent manner, which faithfully mimic the *in vivo* characteristics of disease-causing polyQ proteins. We

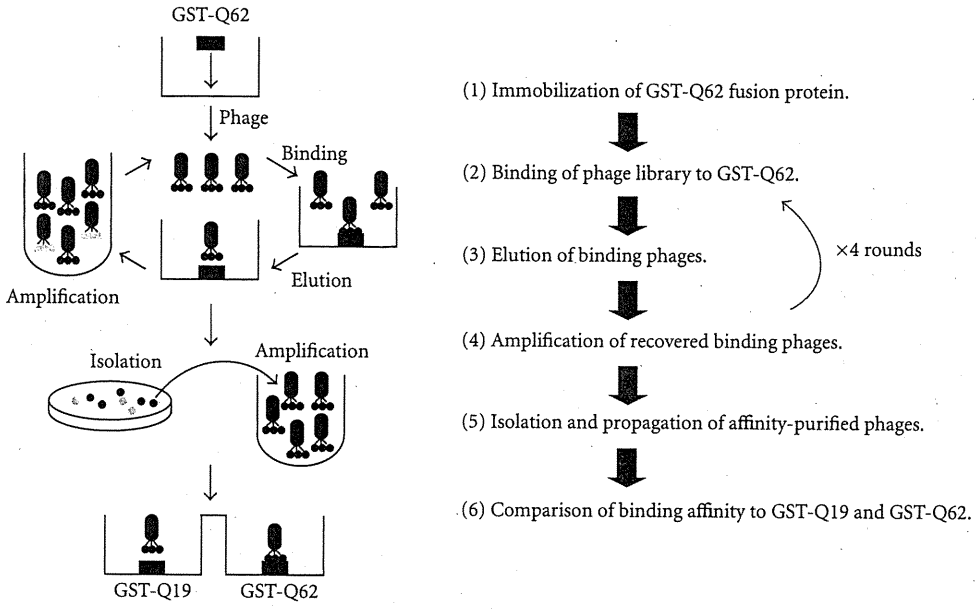

FIGURE 3: Phage display screening strategy for the identification of peptides that selectively bind to the expanded polyQ stretch. Phage libraries expressing random 11-amino acid sequences were first screened for their binding to GST-Q62 via 4 rounds of binding, elution, and amplification. Phage clones isolated from the first screening (350 clones) were further screened for their selective binding to pathologic length GST-Q62 compared to normal-length GST-Q19.

coincubated QBP1 with thio-Q62, and found that QBP1 dramatically inhibits thio-Q62 aggregation in a concentration-dependent manner, showing an almost complete inhibition at a stoichiometry of 3 : 1 (thio-Q62 : QBP1). A scrambled sequence of QBP1 (SCR; Trp-Pro-Ile-Trp-Ser-Lys-Gly-Asn-Asp-Trp-Phe) had no effect on thio-Q62 aggregation. Furthermore, addition of QBP1 after thio-Q62 aggregation has started resulted in inhibition of further aggregate formation, but it could not solubilize the aggregates already formed, suggesting that QBP1 inhibits the earlier stages in the aggregation process of the expanded polyQ protein [41].

4. Mechanism of Action of QBP1

To elucidate the molecular mechanisms by which QBP1 prevents aggregation of the expanded polyQ protein, we have characterized in detail the binding of QBP1 to the expanded polyQ stretch, and analyzed the effect of QBP1 on the conformation of the expanded polyQ protein. To characterize the binding specificities and affinities of QBP1 to the polyQ stretch, we employed the surface plasmon resonance (SPR) technique, which is a highly sensitive method for quantitatively measuring biomolecular interactions [42]. We found that QBP1 binds selectively to the thio-Q62 protein, with an equilibrium dissociation constant (K_d) of 5.7 μM, while it shows significant binding to neither thio-Q0 nor thio-Q19. These results clearly indicate the striking property of QBP1 to specifically recognize and bind to the expanded polyQ stretch, but not the normal length polyQ stretch. We also investigated the relationship between the polyQ binding affinities of QBP1 and its variants and their inhibitory effects on polyQ aggregation. We found a tight correlation between the binding affinities to the expanded polyQ stretch and inhibitory activities on polyQ aggregation of these peptides. Among these, $(QBP1)_2$, a tandem repeat of QBP1 exhibited the greatest binding affinity to thio-Q62 with a K_d value of 0.6 μM. We therefore conclude that binding affinity to the polyQ stretch is a critical determinant of the aggregation inhibitory activity.

Next, we analyzed the effect of QBP1 on the conformation of the expanded thio-polyQ protein [43]. Circular dichroism (CD) analyses revealed that QBP1 inhibits the conformational transition of the thio-Q62 protein to a β-sheet dominant structure. We further demonstrated for the first time that this β-sheet conformational transition of the expanded polyQ protein, which occurs at the level of the monomer before aggregation, causes cytotoxicity. Taken together, we conclude that QBP1 specifically binds to the expanded polyQ protein monomer and inhibits the toxic β-sheet conformational transition, and as a result, also inhibits the downstream aggregation and inclusion body formation (Figure 2).

5. The Therapeutic Effects of QBP1 Expression in Cell Culture Models of the PolyQ Diseases

We also determined whether QBP1 could exert therapeutic effects in cell culture models of the polyQ diseases [40]. Expanded polyQ proteins expressed in cultured cells have been shown to form inclusion bodies and cause cytotoxicity in a time- and polyQ length-dependent manner [44]. We first coexpressed QBP1 fused to cyan fluorescent protein (QBP1-CFP), with various-length polyQ proteins fused to yellow fluorescent protein (polyQ-YFP) in COS-7 cells, and examined

the effect of QBP1 on polyQ inclusion body formation and cytotoxicity. We found a prominent colocalization of QBP1-CFP with polyQ-YFP inclusions, indicating that QBP1 is capable of recognizing the polyQ stretches in cells. Notably, coexpression of QBP1-CFP significantly suppressed polyQ-YFP inclusion body formation, as well as cytotoxicity, and the inhibitory effects were stronger for shorter-length polyQ stretches (Q45 > Q57 > Q81). Furthermore, $(QBP1)_2$-CFP, which has a much higher affinity to the expanded polyQ stretch, exerted an even stronger inhibitory effect on polyQ inclusion body formation, consistent with our *in vitro* aggregation assay results [42].

The expanded polyQ protein is recently believed to form soluble oligomers before microscopically visible insoluble aggregates and inclusion bodies in cells, and these oligomers rather than aggregates or inclusion bodies have been suggested to cause cytotoxicity [24] (Figure 2). We therefore analyzed the effect of QBP1 on polyQ oligomer formation, by using fluorescence correlation spectroscopy (FCS), which is a highly sensitive technique for investigating the dynamics of fluorescent molecules at single molecule sensitivity [45]. We found that the time-dependent decrease in mobility and increase in size of the expanded polyQ-green fluorescent protein (polyQ-GFP) expressed in COS-7 cells, which indicates the formation of slowly moving oligomers, was significantly suppressed by the coexpression of $(QBP1)_2$-CFP [46]. Fluorescence resonance energy transfer (FRET) analyses also confirmed that $(QBP1)_2$ inhibits expanded polyQ oligomer formation in cultured cells [47]. These results are consistent with our *in vitro* observation that QBP1 inhibits the conformational transition of the polyQ protein monomer, which occurs before oligomer and aggregate formation.

6. Therapeutic Effects of QBP1 Expression in Animal Models of the PolyQ Diseases

From a therapeutic viewpoint, it is indispensable to demonstrate the therapeutic effect of QBP1 in *in vivo* disease models. We employed *Drosophila* models to elucidate the therapeutic effects of QBP1 expression on polyQ-induced neurodegeneration, since *Drosophila* models of the polyQ diseases are well established, easy to handle, and suitable for genetic analyses [48]. Transgenic flies expressing an expanded polyQ protein under an eye-specific promoter demonstrate accumulation of polyQ inclusion bodies and degeneration of the eyes. We crossed polyQ expressing flies and $(QBP1)_2$-CFP expressing flies, and found that coexpression of $(QBP1)_2$-CFP significantly suppresses eye degeneration, as well as inclusion body formation. We next examined the effect of $(QBP1)_2$-CFP coexpression on flies expressing the expanded polyQ protein under a panneuronal promoter, which causes premature death due to neurodegeneration. Notably, the median life span of polyQ expressing flies was dramatically improved from 5.5 days to 52 days by coexpression of $(QBP1)_2$-CFP, indicating that QBP1 successfully rescues premature death of the polyQ flies.

TABLE 2: Examples of protein transduction domains.

Name	Origin/design	Sequence
TAT	HIV-1 transactivator	Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg
Antp	*Drosophila* Antennapedia	Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys
VP22	HSV-1 structural protein	Asp-Ala-Ala-Thr-Ala-Thr-Arg-Gly-Arg-Ser-Ala-Ala-Ser-Arg-Pro-Thr-Glu-Arg-Pro-Arg-Ala-Pro-Ala-Arg-Ser-Ala-Ser-Arg-Pro-Arg-Arg-Pro-Val-Asp
Polyarginine	Synthetic	$(Arg)_n$
Transportan	Neuropeptide galanin + wasp peptide mastoparan	Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Lys-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu

These results clearly demonstrate the effectiveness of QBP1 on polyQ-induced neurodegeneration *in vivo*.

7. Therapeutic Effects of Protein Transduction Domain-Mediated Delivery of QBP1

To establish a therapy using QBP1, QBP1 needs to be delivered into affected neurons in the brain, rather than expressed by the crossing of genetically engineered animals. However, as QBP1 is an 11-amino acid peptide, it is too large to cross the cell membrane efficiently and enter cells on its own. To enable the efficient intracellular delivery of QBP1, we utilized protein transduction domains (PTDs), which are peptide sequences capable of penetrating the cell membrane and entering cells. These include the human immunodeficiency virus-1 TAT, *Drosophila* Antennapedia (Antp), herpes simplex virus-1 VP22, and the polyarginines (Table 2). PTDs have indeed been shown to efficiently deliver various biologically active molecules such as peptides, proteins, and nucleic acids into cells [49, 50].

We synthesized QBP1 peptides fused to the TAT or Antp PTD, and confirmed that both of them are efficiently transduced into cells upon addition to the medium of cultured cells, and inhibit inclusion body formation and cytotoxicity of the expanded polyQ protein [51]. To determine whether PTD-QBP1 administration is able to exert therapeutic effects on an *in vivo* model of the polyQ diseases, we first administered Antp-QBP1 to a *Drosophila* polyQ disease model, by adding the peptide into the culture food. Oral administration of Antp-QBP1 remarkably delayed premature death of the polyQ expressing flies compared with the control peptide Antp-SCR. In addition, flies administered with Antp-QBP1 had significantly fewer inclusion bodies compared to the control flies. These results indicated the potential of PTD-mediated delivery of QBP1 as a useful strategy to establish a molecular therapy using QBP1.

We next analyzed the therapeutic effect of Antp-QBP1 administration on a mouse model of the polyQ diseases [52]. Intraperitoneal injection of Antp-QBP1 resulted in a slight improvement of the weight loss in these mice, but did not improve the other phenotypes such as motor dysfunction and premature death. Furthermore, we could not detect a significant suppression of polyQ inclusion body formation by Antp-QBP1 administration in these mice. Although we confirmed the limited delivery of Antp-QBP1 into the mouse brain via intracerebroventricular and intrastriatal injection, we failed to detect a significant amount of Antp-QBP1 delivered in the brain via intraperitoneal injection. These results imply that Antp-QBP1 is unable to efficiently cross the blood-brain barrier (BBB) in mice, which is tighter than in flies.

8. Towards Designing Chemical Analogues of QBP1

Towards developing QBP1 as a therapeutic molecule for the polyQ diseases, we are taking another approach, which is designing low molecular weight chemical analogues of QBP1 with efficient BBB permeability. To design low molecular weight analogues of QBP1, we first determined the essential amino acids required for its activity and pharmacophores of QBP1.

We first synthesized various truncation mutants of QBP1, and tested their activities on polyQ aggregation. We found that truncation of Ser1 and Asn2, or truncation of Asp11 does not affect the inhibitory activity on polyQ aggregation whereas truncation of the N-terminal 4 amino acids (Ser1, Asn2, Trp3, and Lys4), or the C-terminal 2 amino acids (Phe10 and Asp11) results in dramatic loss of activity. These results imply that the aromatic amino acids (Trp3 and Phe10) are required for the activity of QBP1, and we therefore concluded that the central 8 amino acids (Trp-Lys-Trp-Trp-Pro-Gly-Ile-Phe) comprise the minimal active sequence of QBP1 [41]. Since other QBPs that we identified from the combinatorial screening also share Trp/Phe-rich sequences (Table 1), we next investigated the role of the Trp-Lys-Trp-Trp motif of QBP1 for its activity. Although the Trp-Lys-Trp-Trp motif alone is insufficient for inhibiting polyQ aggregation, a tandem repeat of Trp-Lys-Trp-Trp connected by an amino acid spacer was found to be as potent as the original QBP1, suggesting that the Trp-Lys-Trp-Trp motif plays an important role in recognizing the polyQ stretch.

We subsequently performed more comprehensive analyses on all amino acids within the QBP1 sequence by Ala scanning and D-amino acid scanning [53]. Substitutions of Ser1, Asn2, Lys4, Pro7, Gly8, or Asp11 to Ala did not show any significant effects on the activity of QBP1. On the other hand, Ala substitutions of Trp3, Trp5, Trp6, Ile9, or Phe10 led to a dramatic decrease in their activity on polyQ aggregation, indicating that the functional groups of these hydrophobic amino acids are essential for their inhibitory activity. Hence, the hydrophobic property of QBP1 may be important for its interaction with the expanded polyQ stretch. In addition, D-amino acid substitutions revealed that the internal amino acids (Trp3-Ile9) of QBP1 are sensitive to chirality inversion, which probably disrupts the active conformation of QBP1.

Another study using a series of peptide analogues of QBP1 elucidated the role of the Trp residues in the activity of QBP1 [54]. Although N-methylation at the main chain of Trp5 and Trp6, which would lose their potential as main chain hydrogen bond donors, resulted in a substantial loss of activity of QBP1, methylation of the indole nitrogen of these residues did not affect its activity, suggesting that the hydrogen bonding potential of the indole side chains are not necessary for the activity of QBP1.

In order to design chemical analogues of QBP1, it is also indispensable to obtain structural information on the mode of binding of QBP1 to the polyQ stretch. However, due to the high insolubility of the expanded polyQ protein, it has been a challenge to experimentally elucidate the structure of the polyQ stretch at atomic resolution in aqueous solution. Although a molecular dynamics study suggested hydrogen bonding between the amide groups of Ser1 to Gly8 of QBP1 and the main chain carbonyl groups of the polyQ stretch, and the role of the steric hindrance produced by Pro7 to prevent polyQ aggregation [55], there are some inconsistencies with the experimental results described above. Thus, further efforts to elucidate the detailed structure of the QBP1-polyQ complex would provide valuable information for designing chemical QBP1 analogues as a therapeutic molecule for the polyQ diseases.

9. Other Applications of QBP1

Since QBP1 is the only molecule which can distinguish between the expanded and normal length polyQ stretch, it is also useful for specific recognition of the expanded polyQ stretch. Indeed, we have confirmed the colocalization of QBP1 with polyQ inclusions [40], and recently, Raspe et al. also utilized QBP1-CFP to label expanded polyQ peptides within inclusion bodies in cultured cells [56]. These studies raise the possibility that QBP1 could also be developed as an *in vivo* imaging probe for detection of polyQ depositions in the brain.

Bauer et al. also employed QBP1 to recognize expanded polyQ proteins for their specific degradation by chaperone-mediated autophagy (CMA), in which Hsc70 recognizes and delivers substrate proteins to the lysosome for their degradation [57]. Coexpression of a modified QBP1, which was fused with Hsc70-binding motifs, with expanded polyQ proteins accelerated polyQ protein degradation, resulting in suppression of cytotoxicity in cultured cells. They further demonstrated that viral vector-mediated gene therapy of the modified QBP1 decreased polyQ protein aggregation and ameliorated phenotypes such as motor dysfunction and premature death in polyQ disease mice while viral expression of the original QBP1 alone also exhibited a modest therapeutic effect. These results clearly indicate the usefulness of QBP1 as a tool for specific recognition of the expanded polyQ protein.

10. Other Peptides/Proteins that Bind to PolyQ and Inhibit Aggregation

Discovery of QBP1 has facilitated research towards applying various polyQ-binding molecules such as peptides and proteins to prevent misfolding and aggregation of the expanded polyQ protein like as QBP1. Kazantsev et al. designed a bivalent peptide comprised of two normal-length polyQ stretches connected by a spacer, which is expected to bind to the expanded polyQ stretch, and showed that expression of this peptide suppresses polyQ inclusion body formation and cytotoxicity in cell culture and *Drosophila* polyQ disease models [58]. We also designed a normal-length polyQ stretch with a Pro insertion, which disrupts the ordered structure of the polyQ stretch, and showed that this peptide successfully delays polyQ aggregation *in vitro* [59]. However, since these rationally designed peptides contain short polyQ stretches that can be recruited to expanded polyQ aggregates, they have the risk of accelerating polyQ aggregation and enhancing toxicity under certain conditions. Furthermore, the therapeutic effects of these peptides were much weaker compared to QBP1, which is the optimal peptide sequence identified by a combinatorial screening approach for its specific binding affinity to the expanded polyQ stretch, and is the only molecule that has been shown to inhibit the toxic β-sheet conformational transition of the expanded polyQ protein [43].

Several intracellular antibodies, known as intrabodies, which bind to the expanded polyQ protein and inhibit its aggregation have also been identified to date. In 2001, Lecerf et al. identified the intrabody C4 that binds to the N-terminus of huntingtin (htt), the disease-causing protein of Huntington's disease (HD), by phage display library screening [60]. Subsequently, they and other groups further showed that expression of C4 as well as other intrabodies, namely, MW7, V_L12.3, Happ1, and EM48, all of which bind to the polyQ adjacent regions in htt, leads to suppression of htt aggregation and neurodegeneration in cell culture, *Drosophila*, and mouse models of HD [60–67]. The use of intrabodies is an attractive therapeutic approach with regard to their high binding affinity to the disease-causing proteins. However, since the intrabodies identified so far recognize a region in htt other than the polyQ stretch itself, they cannot be applied for the other polyQ diseases, and may cause unfavorable side effects by binding to the wild type htt with a normal polyQ stretch.

11. Perspectives

In this review, we introduced our therapeutic strategy against the polyQ neurodegenerative diseases using QBP1, a peptide sequence that specifically recognizes the expanded polyQ stretch, which we identified from phage display screening. Although we have provided convincing evidence on the potential of QBP1 as a therapy for the polyQ diseases, by demonstrating its ability to inhibit misfolding and aggregation, resulting in suppression of polyQ-induced neurodegeneration *in vivo*, the major problem we are currently facing is its delivery into the brain. Although viral vector-mediated gene therapy may have potential for the delivery of QBP1 into the brain, the difficulty in controlling gene expression, toxicity, and limited delivery within the brain discourage this approach. The success of PTD-mediated delivery of QBP1 and its therapeutic effects in a *Drosophila* model of the polyQ diseases have shed light on the potential of PTDs for *in vivo* delivery of QBP1. Recently, an unconventional secretion signal overlapped with the Antp sequence was identified, which enables secretion from cells in addition to entry into cells via Antp [68], suggesting the potential of identifying or designing novel PTDs with high BBB permeability. Since most therapeutic molecules currently in clinical use are chemical compounds, we believe the most promising approach is to design low molecular weight chemical QBP1 analogues with efficient BBB permeability. Further clarification of the mode of binding of QBP1 to the expanded polyQ stretch and detailed structural analyses of the QBP1-polyQ complex will facilitate the designing of chemical analogues of QBP1 as a potential therapeutic molecule for the polyQ diseases.

Although our work has been focused on the polyQ diseases, our approach could also be applied for a broad range of other neurodegenerative diseases including Alzheimer's disease and Parkinson's disease, which are caused by a common mechanism based on protein misfolding and aggregation. Indeed, various peptides/proteins that inhibit protein aggregation have been reported to exert therapeutic effects in cell culture and animal models of these diseases [69, 70]. We hope that in the near future, aggregation inhibitor peptide-based drugs against protein misfolding neurodegenerative diseases will be developed and bring a cure to patients suffering from these currently intractable neurodegenerative diseases.

Acknowledgments

The authors thank Nobuhiro Fujikake, Takashi Inui, Hironobu Naiki, Yuji Goto, Yasuo Takahashi, Masataka Kinjo, and Osamu Onodera for their helpful discussions, and Yuma Okamoto, Chiyomi Ito, Reiko Sasaki, and Hirokazu Matsushima for their technical assistance. The authors' work on the polyglutamine diseases is supported in part by Grants-in-Aid for Scientific Research on Priority Areas (Advanced Brain Science Project, Research on Pathomechanisms of Brain Disorders, Life of Proteins, Protein Community, Water and Biomolecules, Transportsome, and Intracellular Proteolysis to Y. Nagai), and on Innovative Areas (Synapse and Neurocircuit Pathology to Y. Nagai; Amyloid Propagation to H. A. Popiel) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; by Grants-in-Aid for Scientific Research (B), (C), and Challenging Exploratory Research (to Y. Nagai), for Young Scientists (B; to H. A. Popiel), and a JSPS Postdoctoral Fellowship for Foreign Researchers (to H. A. Popiel), from the Japan Society for the Promotion of Science, Japan; by a Grant-in-Aid for the Research Committee for Ataxic Diseases (to Y. Nagai) from the Ministry of Health, Labor, and Welfare, Japan; by a grant from Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency (to

Y. Nagai); by grants from Takeda Science Foundation, Naito Foundation, AstraZeneca (to Y. Nagai); by a Scholarship for Foreign Nationals in Japan from Ichiro Kanehara Foundation (to H. A. Popiel).

References

[1] J. P. Taylor, J. Hardy, and K. H. Fischbeck, "Toxic proteins in neurodegenerative disease," *Science*, vol. 296, no. 5575, pp. 1991–1995, 2002.

[2] C. Soto, "Unfolding the role of protein misfolding in neurodegenerative diseases," *Nature Reviews Neuroscience*, vol. 4, no. 1, pp. 49–60, 2003.

[3] C. A. Ross and M. A. Poirier, "What is the role of protein aggregation in neurodegeneration," *Nature Reviews Molecular Cell Biology*, vol. 6, no. 11, pp. 891–898, 2005.

[4] J. F. Gusella and M. E. MacDonald, "Molecular genetics: unmasking polyglutamine triggers in neurodegenerative disease," *Nature Reviews Neuroscience*, vol. 1, no. 2, pp. 109–115, 2000.

[5] H. T. Orr and H. Y. Zoghbi, "Trinucleotide repeat disorders," *Annual Review of Neuroscience*, vol. 30, pp. 575–621, 2007.

[6] A. R. La Spada, E. M. Wilson, D. B. Lubahn, A. E. Harding, and K. H. Fischbeck, "Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy," *Nature*, vol. 352, no. 6330, pp. 77–79, 1991.

[7] E. Marcy, M. Christine, P. Mabel et al., "A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes," *Cell*, vol. 72, no. 6, pp. 971–983, 1993.

[8] H. T. Orr, M. Y. Chung, S. Banfi et al., "Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1," *Nature Genetics*, vol. 4, no. 3, pp. 221–226, 1993.

[9] G. Imbert, F. Saudou, G. Yvert et al., "Cloning of the gene for spinooerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats," *Nature Genetics*, vol. 14, no. 3, pp. 285–291, 1996.

[10] S. M. Pulst, A. Nechiporuk, T. Nechiporuk et al., "Moderate expansion of a normally biallelic trinucleotide repeat in spinooerebellar ataxia type 2," *Nature Genetics*, vol. 14, no. 3, pp. 269–276, 1996.

[11] K. Sanpei, H. Takano, S. Igarashi et al., "Identification of the spinocerebellar ataxia type 2 gene using a direct identification of repeat expansion and cloning technique, direct," *Nature Genetics*, vol. 14, no. 3, pp. 277–284, 1996.

[12] Y. Kawaguchi, T. Okamoto, M. Taniwaki et al., "CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1," *Nature Genetics*, vol. 8, no. 3, pp. 221–228, 1994.

[13] O. Zhuchenko, J. Bailey, P. Bonnen et al., "Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the α_{1A}-voltage-dependent calcium channel," *Nature Genetics*, vol. 15, no. 1, pp. 62–69, 1997.

[14] G. David, N. Abbas, G. Stevanin et al., "Cloning of the SCA7 gene reveals a highly unstable CAG repeat expansion," *Nature Genetics*, vol. 17, no. 1, pp. 65–70, 1997.

[15] K. Nakamura, S. Y. Jeong, T. Uchihara et al., "SCA17, a novel autosomal dominant cerebellar ataxia caused by an expanded polyglutamine in TATA-binding protein," *Human Molecular Genetics*, vol. 10, no. 14, pp. 1441–1448, 2001.

[16] R. Koide, T. Ikeuchi, O. Onodera et al., "Unstable expansion of CAG repeat in hereditary dentatorubral- pallidoluysian atrophy (DRPLA)," *Nature Genetics*, vol. 6, no. 1, pp. 9–13, 1994.

[17] S. Nagafuchi, H. Yanagisawa, K. Sato et al., "Dentatorubral and pallidoluysian atrophy expansion of an unstable CAG trinucleotide on chromosome 12p," *Nature Genetics*, vol. 6, no. 1, pp. 14–18, 1994.

[18] L. Mangiarini, K. Sathasivam, M. Seller et al., "Exon I of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice," *Cell*, vol. 87, no. 3, pp. 493–506, 1996.

[19] J. M. Warrick, H. L. Paulson, G. L. Gray-Board et al., "Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in Drosophila," *Cell*, vol. 93, no. 6, pp. 939–949, 1998.

[20] P. W. Faber, J. R. Alter, M. E. Macdonald, and A. C. Hart, "Polyglutamine-mediated dysfunction and apoptotic death of a *Caenorhabditis elegans* sensory neuron," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 1, pp. 179–184, 1999.

[21] J. M. Ordway, S. Tallaksen-Greene, C. A. Gutekunst et al., "Ectopically expressed CAG repeats cause intranuclear inclusions and a progressive late onset neurological phenotype in the mouse," *Cell*, vol. 91, no. 6, pp. 753–763, 1997.

[22] A. Michalik and C. Van Broeckhoven, "Pathogenesis of polyglutamine disorders: aggregation revisited," *Human Molecular Genetics*, vol. 12, no. 2, pp. R173–R186, 2003.

[23] J. Shao and M. I. Diamond, "Polyglutamine diseases: emerging concepts in pathogenesis and therapy," *Human Molecular Genetics*, vol. 16, no. R2, pp. R115–R123, 2007.

[24] Y. Nagai and H. A. Popiel, "Conformational changes and aggregation of expanded polyglutamine proteins as therapeutic targets of the polyglutamine diseases: exposed β-sheet hypothesis," *Current Pharmaceutical Design*, vol. 14, no. 30, pp. 3267–3279, 2008.

[25] P. O. Bauer and N. Nukina, "The pathogenic mechanisms of polyglutamine diseases and current therapeutic strategies," *Journal of Neurochemistry*, vol. 110, no. 6, pp. 1737–1765, 2009.

[26] A. J. Williams and H. L. Paulson, "Polyglutamine neurodegeneration: protein misfolding revisited," *Trends in Neurosciences*, vol. 31, no. 10, pp. 521–528, 2008.

[27] T. Shimohata, T. Nakajima, M. Yamada et al., "Expanded polyglutamine stretches interact with TAF(II)130, interfering with CREB-dependent transcription," *Nature Genetics*, vol. 26, no. 1, pp. 29–36, 2000.

[28] F. C. Nucifora, M. Sasaki, M. F. Peters et al., "Interference by huntingtin and atrophin-1 with CBP-mediated transcription leading to cellular toxicity," *Science*, vol. 291, no. 5512, pp. 2423–2428, 2001.

[29] C. J. Cummings, M. A. Mancini, B. Antalffy, D. B. DeFranco, H. T. Orr, and H. Y. Zoghbi, "Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1," *Nature Genetics*, vol. 19, no. 2, pp. 148–154, 1998.

[30] Y. Chai, S. L. Koppenhafer, N. M. Bonini, and H. L. Paulson, "Analysis of the role of heat shock protein (Hsp) molecular chaperones in polyglutamine disease," *Journal of Neuroscience*, vol. 19, no. 23, pp. 10338–10347, 1999.

[31] Y. Nagai, O. Onodera, J. Chun, W. J. Strittmatter, and J. R. Burke, "Expanded polyglutamine domain proteins bind neurofilament and alter the neurofilament network," *Experimental Neurology*, vol. 155, no. 2, pp. 195–203, 1999.

[32] N. A. Di Prospero and K. H. Fischbeck, "Therapeutics development for triplet repeat expansion diseases," *Nature Reviews Genetics*, vol. 6, no. 10, pp. 756–765, 2005.

[33] J. S. Steffan, L. Bodai, J. Pallos et al., "Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*," *Nature*, vol. 413, no. 6857, pp. 739–743, 2001.

[34] N. F. Bence, R. M. Sampat, and R. R. Kopito, "Impairment of the ubiquitin-proteasome system by protein aggregation," *Science*, vol. 292, no. 5521, pp. 1552–1555, 2001.

[35] S. Gunawardena, L. S. Her, R. G. Brusch et al., "Disruption of axonal transport by loss of huntingtin or expression of pathogenic polyQ proteins in *Drosophila*," *Neuron*, vol. 40, no. 1, pp. 25–40, 2003.

[36] G. Szebenyi, G. A. Morfini, A. Babcock et al., "Neuropathogenic forms of huntingtin and androgen receptor inhibit fast axonal transport," *Neuron*, vol. 40, no. 1, pp. 41–52, 2003.

[37] G. Bates, "Huntingtin aggregation and toxicity in Huntington's disease," *Lancet*, vol. 361, no. 9369, pp. 1642–1644, 2003.

[38] M. Herbst and E. E. Wanker, "Therapeutic approaches to polyglutamine diseases: combating protein misfolding and aggregation," *Current Pharmaceutical Design*, vol. 12, no. 20, pp. 2543–2555, 2006.

[39] Y. Trottier, Y. Lutz, G. Stevanin et al., "Polyglutamine expansion as a pathological epitope in Huntington's disease and four dominant cerebellar ataxias," *Nature*, vol. 378, no. 6555, pp. 403–406, 1995.

[40] Y. Nagai, T. Tucker, H. Ren et al., "Inhibition of polyglutamine protein aggregation and cell death by novel peptides identified by phage display screening," *Journal of Biological Chemistry*, vol. 275, no. 14, pp. 10437–10442, 2000.

[41] H. Ren, Y. Nagai, T. Tucker, W. J. Strittmatter, and J. R. Burke, "Amino acid sequence requirements of Peptides that inhibit polyglutamine-protein aggregation and cell death," *Biochemical and Biophysical Research Communications*, vol. 288, no. 3, pp. 703–710, 2001.

[42] Y. Okamoto, Y. Nagai, N. Fujikake et al., "Surface plasmon resonance characterization of specific binding of polyglutamine aggregation inhibitors to the expanded polyglutamine stretch," *Biochemical and Biophysical Research Communications*, vol. 378, no. 3, pp. 634–639, 2009.

[43] Y. Nagai, T. Inui, H. A. Popiel et al., "A toxic monomeric conformer of the polyglutamine protein," *Nature Structural and Molecular Biology*, vol. 14, no. 4, pp. 332–340, 2007.

[44] O. Onodera, J. R. Burke, S. E. Miller et al., "Oligomerization of expanded-polyglutamine domain fluorescent fusion proteins in cultured mammalian cells," *Biochemical and Biophysical Research Communications*, vol. 238, no. 2, pp. 599–605, 1997.

[45] M. Eigen and R. Rigler, "Sorting single molecules: application to diagnostics and evolutionary biotechnology," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 13, pp. 5740–5747, 1994.

[46] Y. Takahashi, Y. Okamoto, H. A. Popiel et al., "Detection of polyglutamine protein oligomers in cells by fluorescence correlation spectroscopy," *Journal of Biological Chemistry*, vol. 282, no. 33, pp. 24039–24048, 2007.

[47] T. Takahashi, S. Kikuchi, S. Katada, Y. Nagai, M. Nishizawa, and O. Onodera, "Soluble polyglutamine oligomers formed prior to inclusion body formation are cytotoxic," *Human Molecular Genetics*, vol. 17, no. 3, pp. 345–356, 2008.

[48] Y. Nagai, N. Fujikake, K. Ohno et al., "Prevention of polyglutamine oligomerization and neurodegeneration by the peptide inhibitor QBP1 in *Drosophila*," *Human Molecular Genetics*, vol. 12, no. 11, pp. 1253–1259, 2003.

[49] A. Joliot and A. Prochiantz, "Transduction peptides: from technology to physiology," *Nature Cell Biology*, vol. 6, no. 3, pp. 189–196, 2004.

[50] J. S. Wadia and S. F. Dowdy, "Protein transduction technology," *Current Opinion in Biotechnology*, vol. 13, no. 1, pp. 152–156, 2002.

[51] H. A. Popiel, Y. Nagai, N. Fujikake, and T. Toda, "Protein transduction domain-mediated delivery of QBP1 suppresses polyglutamine-induced neurodegeneration *in vivo*," *Molecular Therapy*, vol. 15, no. 2, pp. 303–309, 2007.

[52] H. A. Popiel, Y. Nagai, N. Fujikake, and T. Toda, "Delivery of the aggregate inhibitor peptide QBP1 into the mouse brain using PTDs and its therapeutic effect on polyglutamine disease mice," *Neuroscience Letters*, vol. 449, no. 2, pp. 87–92, 2009.

[53] K. Tomita, H. A. Popiel, Y. Nagai et al., "Structure-activity relationship study on polyglutamine binding peptide QBP1," *Bioorganic and Medicinal Chemistry*, vol. 17, no. 3, pp. 1259–1263, 2009.

[54] L. Hamuro, G. Zhang, T. J. Tucker, C. Self, W. J. Strittmatter, and J. R. Burke, "Optimization of a polyglutamine aggregation inhibitor peptide (QBP1) using a thioflavin T fluorescence assay," *Assay and Drug Development Technologies*, vol. 5, no. 5, pp. 629–636, 2007.

[55] R. S. Armen, B. M. Bernard, R. Day, D. O. Alonso, and V. Daggett, "Characterization of a possible amyloidogenic precursor in glutamine-repeat neurodegenerative diseases," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 38, pp. 13433–13438, 2005.

[56] M. Raspe, J. Gillis, H. Krol et al., "Mimicking proteasomal release of polyglutamine peptides initiates aggregation and toxicity," *Journal of Cell Science*, vol. 122, no. 18, pp. 3262–3271, 2009.

[57] P. O. Bauer, A. Goswami, H. K. Wong et al., "Harnessing chaperone-mediated autophagy for the selective degradation of mutant huntingtin protein," *Nature Biotechnology*, vol. 28, no. 3, pp. 256–263, 2010.

[58] A. Kazantsev, H. A. Walker, N. Slepko et al., "A bivalent Huntingtin binding peptide suppresses polyglutamine aggregation and pathogenesis in *Drosophila*," *Nature Genetics*, vol. 30, no. 4, pp. 367–376, 2002.

[59] H. A. Popiel, Y. Nagai, O. Onodera et al., "Disruption of the toxic conformation of the expanded polyglutamine stretch leads to suppression of aggregate formation and cytotoxicity," *Biochemical and Biophysical Research Communications*, vol. 317, no. 4, pp. 1200–1206, 2004.

[60] J. M. Lecerf, T. L. Shirley, Q. Zhu et al., "Human single-chain Fv intrabodies counteract *in situ* huntingtin aggregation in cellular models of Huntington's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 8, pp. 4764–4769, 2001.

[61] W. J. Wolfgang, T. W. Miller, J. M. Webster et al., "Suppression of Huntington's disease pathology in *Drosophila* by human single-chain Fv antibodies," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 32, pp. 11563–11568, 2005.

[62] A. Snyder-Keller, J. A. McLear, T. Hathorn, and A. Messer, "Early or late-stage anti-N-terminal huntingtin intrabody gene therapy reduces pathological features in B6.HDR6/1 mice," *Journal of Neuropathology and Experimental Neurology*, vol. 69, no. 10, pp. 1078–1085, 2010.

[63] A. Khoshnan, J. Ko, and P. H. Patterson, "Effects of intracellular expression of anti-huntingtin antibodies of various specificities on mutant huntingtin aggregation and toxicity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 2, pp. 1002–1007, 2002.

[64] D. W. Colby, Y. Chu, J. P. Cassady et al., "Potent inhibition of huntingtin aggregation and cytotoxicity by a disulfide bond-free single-domain intracellular antibody," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 51, pp. 17616–17621, 2004.

[65] A. L. Southwell, A. Khoshnan, D. E. Dunn, C. W. Bugg, D. C. Lo, and P. H. Patterson, "Intrabodies binding the proline-rich domains of mutant Huntingtin increase its turnover and reduce neurotoxicity," *Journal of Neuroscience*, vol. 28, no. 36, pp. 9013–9020, 2008.

[66] A. L. Southwell, J. Ko, and P. H. Patterson, "Intrabody gene therapy ameliorates motor, cognitive, and neuropathological symptoms in multiple mouse models of Huntington's disease," *Journal of Neuroscience*, vol. 29, no. 43, pp. 13589–13602, 2009.

[67] C. E. Wang, H. Zhou, J. R. McGuire et al., "Suppression of neuropil aggregates and neurological symptoms by an intracellular antibody implicates the cytoplasmic toxicity of mutant huntingtin," *Journal of Cell Biology*, vol. 181, no. 5, pp. 803–816, 2008.

[68] E. Dupont, A. Prochiantz, and A. Joliot, "Identification of a signal peptide for unconventional secretion," *Journal of Biological Chemistry*, vol. 282, no. 12, pp. 8994–9000, 2007.

[69] A. M. Bodles, O. M. El-Agnaf, B. Greer, D. J. Guthrie, and G. B. Irvine, "Inhibition of fibril formation and toxicity of a fragment of α-synuclein by an N-methylated peptide analogue," *Neuroscience Letters*, vol. 359, no. 1-2, pp. 89–93, 2004.

[70] C. Soto, E. M. Sigurdsson, L. Morelli, R. A. Kumar, E. M. Castaño, and B. Frangione, "β-sheet breaker peptides inhibit fibrillogenesis in a rat brain model of amyloidosis: implications for Alzheimer's therapy," *Nature Medicine*, vol. 4, no. 7, pp. 822–826, 1998.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 11, pp. 8398–8406, March 9, 2012

Mislocalization of Fukutin Protein by Disease-causing Missense Mutations Can Be Rescued with Treatments Directed at Folding Amelioration*[S]

Received for publication, September 6, 2011, and in revised form, January 16, 2012 Published, JBC Papers in Press, January 24, 2012, DOI 10.1074/jbc.M111.300905

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Background: The molecular pathogenesis of fukutin-deficient dystroglycanopathy remains unclear, and no effective treatment is available.
Results: Some disease-causing missense fukutin mutants showed mislocalization in cultured cells, which can be corrected by treatments directed at folding amelioration.
Conclusion: Correction of cellular localization of disease-causing mutants may have a therapeutic benefit.
Significance: A possible therapeutic strategy for fukutin-deficient dystroglycanopathy is proposed based on its molecular pathogenesis.

Fukuyama-type congenital muscular dystrophy (FCMD), the second most common childhood muscular dystrophy in Japan, is caused by alterations in the *fukutin* gene. Mutations in *fukutin* cause abnormal glycosylation of α-dystroglycan, a cell surface laminin receptor; however, the exact function and pathophysiological role of fukutin are unclear. Although the most prevalent mutation in Japan is a founder retrotransposal insertion, point mutations leading to abnormal glycosylation of α-dystroglycan have been reported, both in Japan and elsewhere. To understand better the molecular pathogenesis of fukutin-deficient muscular dystrophies, we constructed 13 disease-causing missense *fukutin* mutations and examined their pathological impact on cellular localization and α-dystroglycan glycosylation. When expressed in C2C12 myoblast cells, wild-type fukutin localizes to the Golgi apparatus, whereas the missense mutants A170E, H172R, H186R, and Y371C instead accumulated in the endoplasmic reticulum. Protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) also mislocalizes when co-expressed with these missense mutants. The results of nocodazole and brefeldin A experiments suggested that these mutant proteins were not transported to the Golgi via the anterograde pathway. Furthermore, we found that low temperature culture or curcumin treatment corrected the subcellular location of these missense mutants. Expression studies using *fukutin*-null mouse embryonic stem cells showed that the activity responsible for generating the laminin-binding glycan of α-dystroglycan was retained in these mutants. Together, our results suggest that some disease-causing missense mutations cause abnormal folding and localization of fukutin protein, and therefore we propose that folding amelioration directed at correcting the cellular localization may provide a therapeutic benefit to glycosylation-deficient muscular dystrophies.

Fukuyama-type congenital muscular dystrophy (FCMD,[2] MIM 253800) is the second most common childhood muscular dystrophy and one of the most prevalent autosomal recessive disorders in the Japanese population. FCMD is clinically characterized by congenital muscular dystrophy in combination with cortical dysgenesis (micropolygyria) and ocular abnormalities (1). We identified *fukutin*, the gene responsible for FCMD, on chromosome 9q31 by linkage analysis and positional cloning (2, 3). Most FCMD-bearing chromosomes have been derived from a single ancestral founder and have a 3-kb retrotransposal insertion in the 3′ noncoding region of the *fukutin* gene. Compound heterozygosity, with both a retrotransposal mutation and a point mutation in *fukutin*, is sometimes seen and generally exhibits more severe pathologies (4, 5). However, a recent report has identified several Japanese patients presenting with mild limb-girdle dystrophy (LGMD2M, MIM 611588) and normal intelligence (6) and who have a retrotransposal mutation and a point mutation in the *fukutin* gene. Outside Japan, *fukutin* mutations have been reported in patients with various phenotypes, from Walker-Warburg syndrome (WWS, MIM 236670) to LGMD (7–13). Overall, the current common understanding is that *fukutin* alterations can give rise to a wide spectrum of phenotypes.

Mutations in *fukutin* cause abnormal glycosylation of the cell surface laminin receptor α-dystroglycan (DG) and reduce its

* This work was supported by the Ministry of Health, Labor, and Welfare of Japan Intramural Research Grant for Neurological and Psychiatric Disorders of National Center of Neurology and Psychiatry 23B-5 and Research on Psychiatric and Neurological Diseases and Mental Health (H20-016), Ministry of Education, Culture, Sports, Science, and Technology of Japan Grant-in-aid for Scientific Research (A) 23249049 (to T. T.), Japan Society for the Promotion of Science Grant-in-aid for Young Scientists (B) 18790220 (to M. T.), Takeda Science Foundation (to M. K.), and Global COE (Centers of Excellence) Program Frontier Biomedical Science Underlying Organelle Network Biology.
[S] This article contains supplemental Tables I–III, Experimental Procedures, and Figs. 1–3.
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[2] The abbreviations used are: FCMD, Fukuyama-type congenital muscular dystrophy; BFA, brefeldin A; DG, dystroglycan; ER, endoplasmic reticulum; FKRP, fukutin-related protein; LGMD, limb-girdle muscular dystrophy; POMGnT1, protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1; POMT1, protein *O*-mannosyltransferase 1; POMT2, protein *O*-mannosyltransferase 2; WWS, Walker-Warburg syndrome.

laminin binding activity (14). The α- and β-DG complex is believed to provide physical strength to the sarcolemma by connecting the basal lamina to the cytoskeleton. Thus, abnormal glycosylation caused by *fukutin* mutations underlies FCMD molecular pathogenesis, but the exact function of fukutin remains unclear. The *fukutin* gene encodes a 461-amino acid protein with a predicted molecular mass of 53.7 kDa (3). Although endogenous fukutin protein has not been detected in cells, likely due to its low abundance, expression studies have proposed that *fukutin* gene product localizes to the Golgi apparatus (15, 16). Fukutin protein contains a transmembrane domain (3, 16), a putative *N*-glycosylation site (3), and a DxD motif that is predicted to modify cell surface glycoproteins or glycolipids (17). Previously, we showed that the transmembrane domain of fukutin binds to the protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1), which is encoded by the responsible gene for muscle-eye-brain disease (MIM 253280) (18), suggesting that fukutin affects the enzymatic activity of POMGnT1 (16). Other regions of the fukutin protein share no sequence homology with known proteins. In addition to FCMD, several other forms of muscular dystrophy are caused by abnormal glycosylation of α-DG; together, these conditions are termed as "dystroglycanopathy." To date, six genes (protein *O*-mannosyltransferase 1 (*POMT1*), protein *O*-mannosyltransferase 2 (*POMT2*), *POMGnT1*, *fukutin*, fukutin-related protein (*FKRP*), and *LARGE*) have been implicated in dystroglycanopathies, and all are thought to be involved in glycosylation of α-DG (19–23). POMGnT1 and the POMT1/2 complex are known to have glycosyltransferase activities directly involved in synthesis of *O*-mannosyl sugar chains on α-DG (18, 24). Quite recently, it has been shown that LARGE can act as a bifunctional glycosyltransferase with both xylosyltransferase and glucuronyltransferase activities (25). On the other hand, the exact function of FKRP is unknown. Yoshida-Moriguchi *et al.* reported that a phosphodiester-linked moiety on *O*-mannose of α-DG is defective in LARGE- or fukutin-deficient dystroglycanopathies (26). This finding suggests that LARGE and fukutin might be involved in the synthesis of the postphosphoryl modification, which is necessary for laminin binding activity.

The precise pathogenic mechanism of FCMD has remained obscure. In this report, to understand molecular pathogenesis of fukutin-deficient muscular dystrophies, we constructed 13 disease-causing missense *fukutin* mutations that have been reported inside and outside Japan (4–6, 9–13, 27, 28) and investigated their pathological roles in fukutin intracellular location. Four mutants (A170E, H172R, H186R, and Y371C) lost their Golgi localization and instead accumulated in the endoplasmic reticulum (ER) when expressed in C2C12 cultured cells. Using *fukutin*-null mouse embryonic stem (ES) cells, we showed that these mutants retain the activity responsible for α-DG glycosylation. Finally, we found that low temperature culture and curcumin treatment are effective in correcting the localization of these missense fukutin mutants.

EXPERIMENTAL PROCEDURES

Reagents—Brefeldin A (BFA) and nocodazole were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Curcumin was purchased from Nacalai Tesque (Kyoto, Japan).

Antibodies used in this study were as follows: monoclonal anti-V5 (Invitrogen), rabbit polyclonal anti-FLAG (Sigma), monoclonal anti-α-DG clone IIH6C4 (Millipore), monoclonal anti-β-DG clone 8D5 (Novocastra Laboratories, Newcastle, UK), monoclonal anti-GM130 (BD Biosciences), monoclonal anti-KDEL antibodies (Stressgen, Victoria, Canada); rabbit polyclonal anti-laminin (Sigma); and goat polyclonal antibody against the C-terminal region of α-DG (AP-074G-C) (29)

Vector Constructions and Site-directed Mutagenesis—For the construction of expression vectors, the coding regions of human *POMGnT1*, human *fukutin*, or human *FKRP* with a FLAG or a V5 epitope at the C terminus were cloned into the pEF1/V5-HisA vector (Invitrogen). Expression vectors encoding 13 different disease-causative missense fukutin mutants were constructed using site-directed mutagenesis. Mutations were confirmed by DNA sequencing.

Cell Culture, Transfection, and Immunofluorescence Detection—Mouse myoblast C2C12 cells were cultured in Dulbecco's modified Eagle Medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and streptomycin (100 μg/ml) (Wako). Mouse ES cells were grown in DMEM with 15% heat-inactivated fetal bovine serum, 100 μM 2-mercaptoethanol, and streptomycin. Targeted disruptions of the *fukutin* gene in ES cells have been described previously (30).

Cell transfection was performed using Effectene (Qiagen) according to the manufacturer's protocol. 48 h after transfection, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and then permeabilized in PBS with 0.5% Triton X-100 (Nacalai Tesque). For immunofluorescence detection, after blocking with 1% BSA (Wako) in PBS at room temperature for 1 h, the cells were first incubated for 90 min with polyclonal anti-FLAG, monoclonal anti-GM130, monoclonal anti-KDEL, or monoclonal anti-V5 antibodies, followed by Alexa Fluor 488-conjugated anti-rabbit IgG and/or Alexa Fluor 546-conjugated anti-mouse IgG (Invitrogen) for 1 h at room temperature. After a final rinse with PBS, cells were observed by fluorescence microscopy using a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany).

For BFA, nocodazole, or curcumin treatment, the cells were incubated with 5 μg/ml BFA or 10 μg/ml nocodazole for 2 h after 48 h of transfection, or 10 μg/ml curcumin for 24 h after 24 h of transfection. For statistical analysis of fukutin cellular localization, cells expressing fukutin were classified into four classes (Golgi localization, Golgi and around localization, dot localization, and ER localization) (see Fig. 6*A*). For statistical analysis of POMGnT1 localization, cells co-expressing fukutin/POMGnT1 were classified into three classes (Golgi localization with fukutin, Golgi localization without fukutin, and ER localization). The number of cells in each class was counted and analyzed using the χ^2 test.

Dystroglycan Preparation—DG was enriched from solubilized mouse ES cells. The ES cells were solubilized in 1 ml of PBS containing 1% Triton X-100 and protease inhibitor mixture (Nacalai Tesque). Solubilized fractions were incubated with 30 μl of wheat germ agglutinin-agarose beads (Vector Laboratories) at 4 °C for 2 h. The beads were washed five times with 1 ml of PBS containing 0.1% Triton X-100 and protease inhibitor

TABLE 1
Disease-causing missense mutations in the *fukutin* gene

Mutation	Other allele	Severity	References
A114T	T286 frame shift	Mild	10
G125S[a]	5370–5842 deletion (3′-UTR)	Severe	11
G125S[a]	F390 frame shift	Severe	11
M133T	3-kb insertion	Typical	28
A170E	Y371C	Typical	13
H172R	3-kb insertion	Typical	27
R179T	3-kb insertion	Mild	6
H186R	Homozygote	Severe	12
R246G	R47 nonsense	Mild	13
C250G	3-kb insertion	Typical	4
W305C	Homozygote	Typical	10
R307Q	Homozygote	Mild	13
R307Q	F390 frame shift	Mild	9
R307Q	N455 frame shift	Mild	9
Q358P	3-kb insertion	Mild	6
Y371C	3-kb insertion	Typical	5
Y371C	A170E	Typical	13

[a] G125S has been registered as a polymorphism (rs_34006675).

mixture, then directly boiled for 5 min in SDS-polyacrylamide gel loading buffer.

SDS-PAGE, Western Blotting, and Laminin Overlay Assay—Cell lysates were dissolved in SDS sample buffer and subjected to SDS-PAGE in 10% gels or 7.5% gels. Gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), probed with anti-FLAG, anti-α-DG core protein (AP-074G-C), anti-α-DG sugar chain (IIH6C4), or anti-β-DG antibodies, and then developed with horseradish peroxidase-conjugated secondary antibody (DAKO, Carpinteria, CA). Blots were processed using ECL plus Western blotting detection system (GE Healthcare) and exposed to Fuji RX-U x-ray film (Fuji Film, Kanagawa, Japan). The laminin overlay assay was performed according to the method of Michele *et al.* (14).

Additional methods are described under supplemental Experimental Procedure.

RESULTS

Mislocalization of Disease-causing Missense Mutant Fukutin Proteins—To examine the cellular location of fukutin proteins containing disease-causing missense mutations, we constructed expression vectors encoding wild-type or mutant fukutin proteins with a FLAG epitope at the C terminus. The missense mutants analyzed in this study have been identified inside and outside Japan (Table 1), and their clinical phenotypes vary from severe WWS-like to mild LGMD-type without mental retardation (Table 1 and Fig. 1). These constructs were transfected into C2C12 myoblast cells, and the cellular localizations of the expressed fukutin proteins were examined by immunofluorescence. Immunofluorescent signals indicated co-localization of the expressed wild-type fukutin with the Golgi apparatus marker GM130 (162/176 cells; Golgi + Golgi and around/total cells) (Fig. 2*A*, *merge*, and supplemental Table I). Nine of the 13 missense mutants (A114T (97/113), G125S (140/144), M133T (109/120), R179T (142/149), R246G (101/110), C250G (124/134), W305C (183/198), R307Q (131/134), and Q358P (174/182)) also co-localized with GM130 (supplemental Table I), indicating that these mutations do not affect the cellular location of fukutin protein. In contrast, the A170E (13/146), H172R (8/145), and H186R (6/141) mutants, as well as the previously reported Y371C (8/128) mutant (16), did not co-localize with GM130 (Fig. 2*A* and supplemental Table I), instead showing co-localization with the ER marker KDEL (Fig. 2*B*). These results indicated that A170E, H172R, H186R, and Y371C aberrantly localize to the ER.

Accumulation of Missense Fukutin Mutants in ER Caused by Impaired Transport to the Golgi—Accumulation of the four mutants in the ER might result from improper cellular trafficking. To determine whether the mutants are not properly transported from the ER to the Golgi or whether they are transported back to the ER after reaching the Golgi, we treated C2C12 cells expressing wild-type fukutin or the four mutants with nocodazole (an inhibitor for retrograde transport from the Golgi to the ER) or BFA (an inhibitor for anterograde transport from the ER to the Golgi). If the four mutants reached the Golgi and then were immediately transported back to the ER, the four mutant proteins should be detected in the Golgi after nocodazole treatment. Immunofluorescent signals indicating ER accumulation of the four mutants were observed following a 2-h incubation with 10 μg/ml nocodazole (Fig. 3*C–F*, *panels 4*). When cells expressing wild-type fukutin were incubated with 5 μg/ml BFA, the wild-type fukutin was detected in the ER (99/107; ER/total cells) (Fig. 3*B*, *panel 6*, and supplemental Table II), as seen in the cells expressing any of the four mutants. These data suggested that failure of proper transport via the anterograde pathway causes mislocalization of the four mutants to the ER.

Correction of Cellular Location of Mutant Fukutin Proteins by Low Temperature Culture—We hypothesized that mislocalization resulted from protein misfolding and therefore examined whether the localization of the four missense mutants could be corrected by folding amelioration. It has been reported that cell culture at low temperature can ameliorate folding and correct the subcellular localization of missense mutant proteins (31). In culture at 37 °C, the four missense fukutin mutants (A170E, H172R, H186R, and Y371C) were co-localized with KDEL (Fig. 4*A*). At low temperature (27.5 °C), the A170E (130/144; Golgi + Golgi and around/total cells), H172R (130/159), and Y371C (102/158) mutants preferentially co-localized with GM130 (Fig. 4*A* and supplemental Table I), indicating that the ER accumulation had decreased and proper localization to the Golgi was restored. Most of the expressed H186R mutant protein, however, remained in the ER (134/155; ER/total cells) at 27.5 °C; only a small proportion of this mutant shifted to the Golgi (Fig. 4*A* and supplemental Table I).

POMGnT1, which has been shown to interact with fukutin and localize to the Golgi (151/156; Golgi with fukutin/total cells) (supplemental Table III) (16), also mislocalized to the ER when co-expressed with the mutants A170E (89/103; ER/total cells), H172R (102/113), H186R (96/111), and Y371C (98/109) (Fig. 4*B*, 37 °C, and supplemental Table III). These results indicated that fukutin mislocalization also affects the cellular location of POMGnT1. Low temperature culture of cells expressing both POMGnT1 and any of the mutants A170E (158/173; Golgi with fukutin/total cells), H172R (103/129), or Y371C (126/192) restored POMGnT1 subcellular localization to the Golgi (Fig. 4*B*, 27.5 °C, and supplemental Table III). When expressed with the H186R mutant, the POMGnT1 localization shifted to the Golgi despite the majority of the H186R mutant remaining in

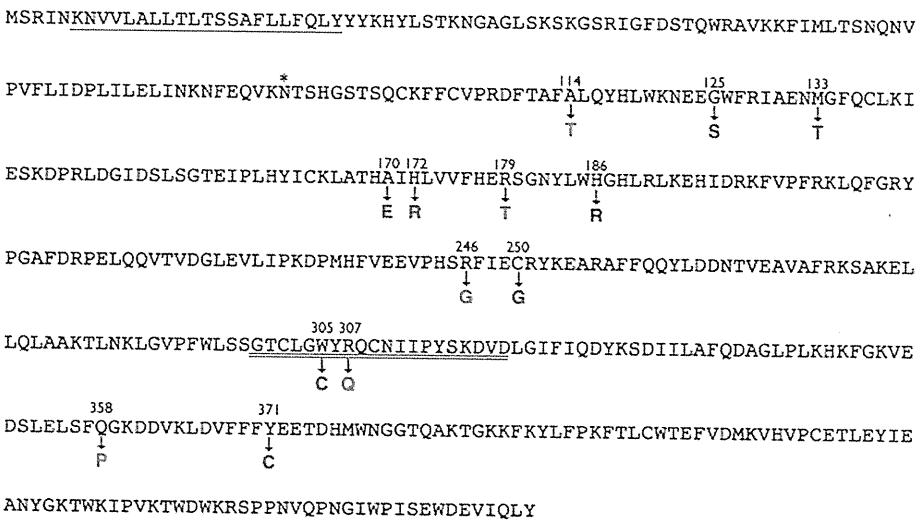


FIGURE 1. **Amino acid sequence of fukutin and location of missense mutations.** Amino acid changes are indicated along with severity of phenotype. *Blue*, mild form (CMD without mental retardation, LGMD, or cardiomyopathy). *Purple*, typical form (FCMD, CMD with mental retardation); *red*, severe form (WWS). *Single underline* indicates the transmembrane domain, the *asterisk* indicates an *N*-glycosylation site, and the *double underline* indicates the DxD motif.

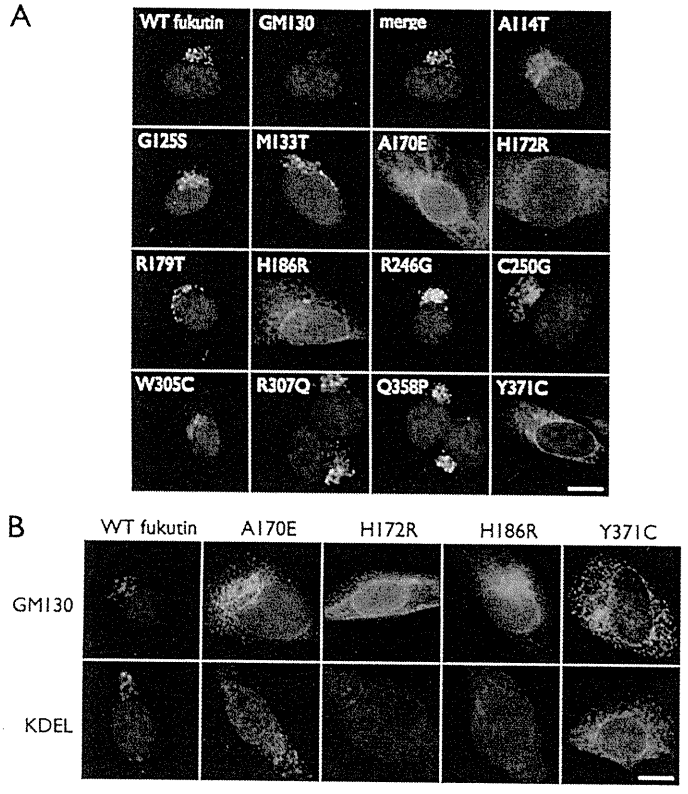


FIGURE 2. **Some disease-causing missense mutations cause abnormal cellular localization of fukutin.** *A*, localization of the FLAG-tagged wild-type or missense fukutin proteins in transfected C2C12 myoblast cultured cells. Cells were double-labeled with anti-FLAG (*green*) and a Golgi marker GM130 (*red*). No co-localization was seen for the missense fukutin mutants A170E, H172R, H186R, and Y371C. Wild-type (*WT fukutin*) and the other nine mutants co-localized with GM130. *B*, mislocalization of missense fukutin mutants to the ER. The fukutin missense mutants A170E, H172R, H186R, and Y371C (*green*) lost their localization to the Golgi (*red*) in transfected C2C12 cultured cells (*upper*). Co-localization of these mutants with the ER marker KDEL (*red*) was seen in the cultures (*lower*). *Blue*, DAPI, indicating the nucleus. *Scale bars*, 10 μm.

the ER (108/141; Golgi without fukutin/total cells) (Fig. 4*B* and supplemental Table III). These data showed that low temperature culture could correct mislocalization of the mutant fukutin proteins, but its effect may depend on the position of missense mutations within the protein.

Residual Function of Fukutin Missense Mutants to Restore α-Dystroglycan Glycosylation—C2C12 cells contain endogenous fukutin, making it difficult to evaluate the α-DG modification activity of exogenously expressed mutant fukutin proteins. Instead, we used *fukutin*-null mouse ES cells (FCMDp182 cell) (30) to examine whether the four missense mutant fukutin proteins retain the activity responsible for α-DG modification. We expressed the four mutants (A170E, H172R, H186R, and Y371C) in FCMDp182 cells (supplemental Fig. 1) and then examined the recovery of glycosylation and laminin binding activity of α-DG. In mock-transfected FCMDp182 cells cultured at either 37 °C or 27.5 °C, α-DG showed no detectable reactivity against the monoclonal IIH6C4 antibody, which recognizes the functionally glycosylated form of α-DG. We also observed hypoglycosylation of α-DG, as indicated by lower molecular mass, and no α-DG laminin binding activity in mock-transfected cells (Fig. 5, *lanes 2* and *10*). When wild-type fukutin was expressed in FCMDp182 cells, we observed restoration of the laminin binding activity and the IIH6C4 reactivity of α-DG, but at much weaker levels than for α-DG in the wild-type mouse ES cell line AB2.2 (*lanes 1* and *3*). This partial restoration and the faint amount of α-DG core protein bands around 100 kDa (the size of α-DG in the AB2.2 cells) may result from low transfection efficiency. Expression of each of the four missense mutants in the FCMDp182 cells restored the IIH6C4 reactivity and the laminin binding activity to levels comparable with those observed in the wild-type fukutin transfectants (*lanes 3–7*). Although these missense mutants preferentially localized to the ER at 37 °C, it is possible that small amounts of the expressed mutants reached the Golgi. To support this interpretation, a report has shown that the mislocalized mutant protein ΔF508 cystic fibrosis transmembrane conductance regulator, which is the most common mutation leading to cystic fibrosis, shows partial function when overexpressed (32). We have not detected restoration of the IIH6C4 reactivity and the laminin binding activity by expression of a mutant fukutin protein with direct substitutions in the DxD motif (D317A/V/D319A) (supplemental Fig. 2). Expression of FKRP, a member

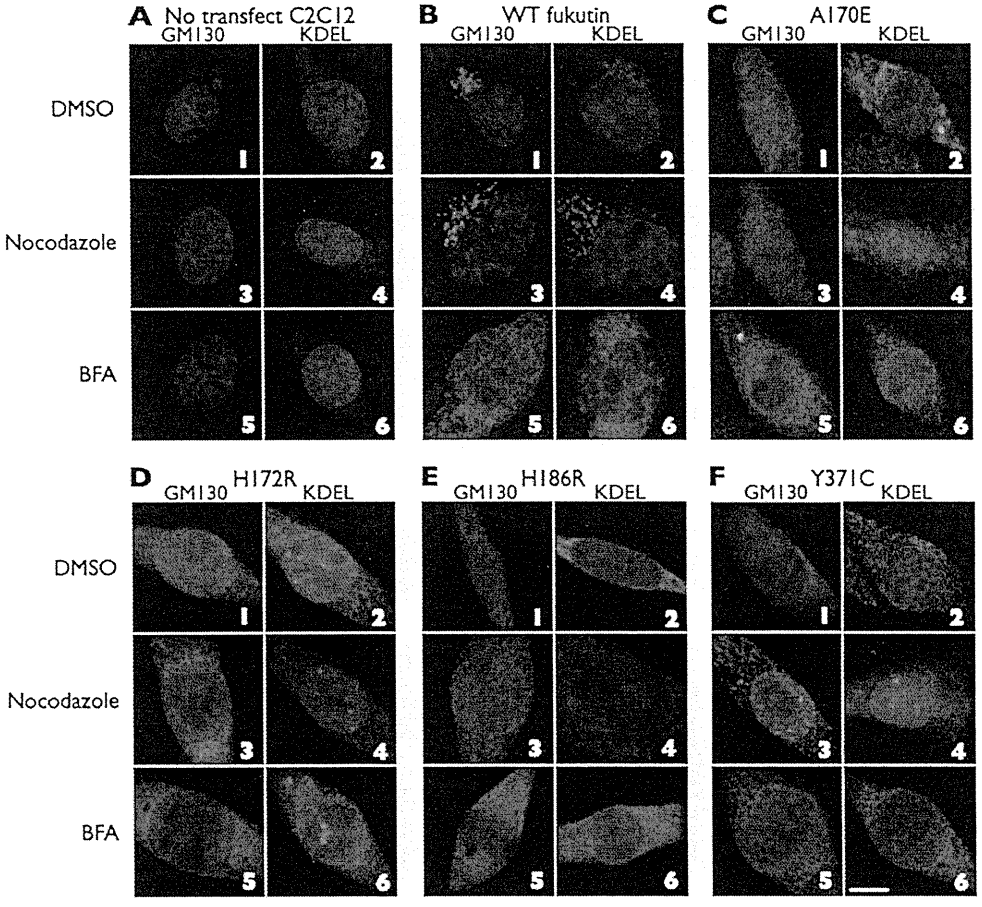


FIGURE 3. **Accumulation of the missense mutants in the ER could be caused by the failure of proper transport via the anterograde pathway.** C2C12 cells without (*A*) or with expression of wild-type fukutin (*B*) or the missense mutants (*C–F*) were incubated with nocodazole or BFA. Nocodazole treatment did not change the cellular localization of the expressed mutant fukutin proteins, whereas BFA treatment shifted wild-type fukutin to the ER. *Red*, GM130 or KDEL; *green*, expressed fukutin proteins; *blue*, DAPI. *Scale bar*, 10 μm.

of the fukutin protein family, in FCMDp182 cells showed no effect on α-DG glycosylation (*lane 8*). We also performed these experiments at low culture temperature (27.5 °C) and obtained similar results (Fig. 5, *lanes 9–16*). The reason for reduced efficiency of glycosylation recovery in low temperature culture is unclear, but the low temperature might affect enzymatic activities involved in the α-DG glycosylation pathway. Because the expression level of each mutant protein was different (Fig. 5, anti-FLAG), it was not possible to simply compare their residual activity. Importantly, however, all four mutants retained the activity responsible for α-DG modification.

Correction of Mislocalization of Missense Fukutin Mutants by Curcumin Treatment—Our results indicated that mislocalization of some missense mutants could cause disease-related abnormal glycosylation of α-DG. Therefore, we next searched for chemicals that could restore proper localization. Curcumin, a nontoxic natural constituent of turmeric spice, has shown the ability to correct misfolding and mislocalization of the ΔF508 mutant (33). We incubated C2C12 cells expressing the four mutants (A170E, H172R, H186R, or Y371C) with 10 μg/ml curcumin at 37 °C for 24 h. Among the four mutants, the A170E mutant showed the greatest benefit from curcumin treatment. In the absence of curcumin, approximately 90% of the cells showed ER localization of the A170E mutant (159/175; ER/total cells), and only a few cells (7/175; Golgi + Golgi and around cells) showed Golgi localization (Fig. 6). Curcumin treatment significantly decreased the ER mislocalization signal of the A170E mutant (69/156; ER/total ells) and increased the Golgi- and Golgi/around signals (72/156; Golgi + Golgi and around cells) ($p = 8.28 \times 10^{-20}$). Although not as dramatic as seen with A170E, the other mutants showed slight beneficial changes in their cellular distributions following curcumin treatment (Fig. 6*B* and supplemental Table II). We also examined glycerol, arginine, and 17-allylaminogeldanamycin for beneficial effects on localization of the mutants, but none was observed (data not shown).

DISCUSSION

In this report, we have demonstrated that some disease-causing missense fukutin mutants lost their Golgi localization in C2C12 cultured cells and that this mislocalization can be corrected by low temperature culture or curcumin treatment. We identified four missense mutants that localized abnormally to the ER. Nocodazole treatment did not alter their ER localization, and low temperature culture shifted three of the four mutants to the Golgi. It is generally recognized that cell culture at low temperature can ameliorate protein misfolding and correct abnormal cellular localization (31). Therefore, we presume that some missense mutants could not be transported to the Golgi via the anterograde pathway because of protein misfold-

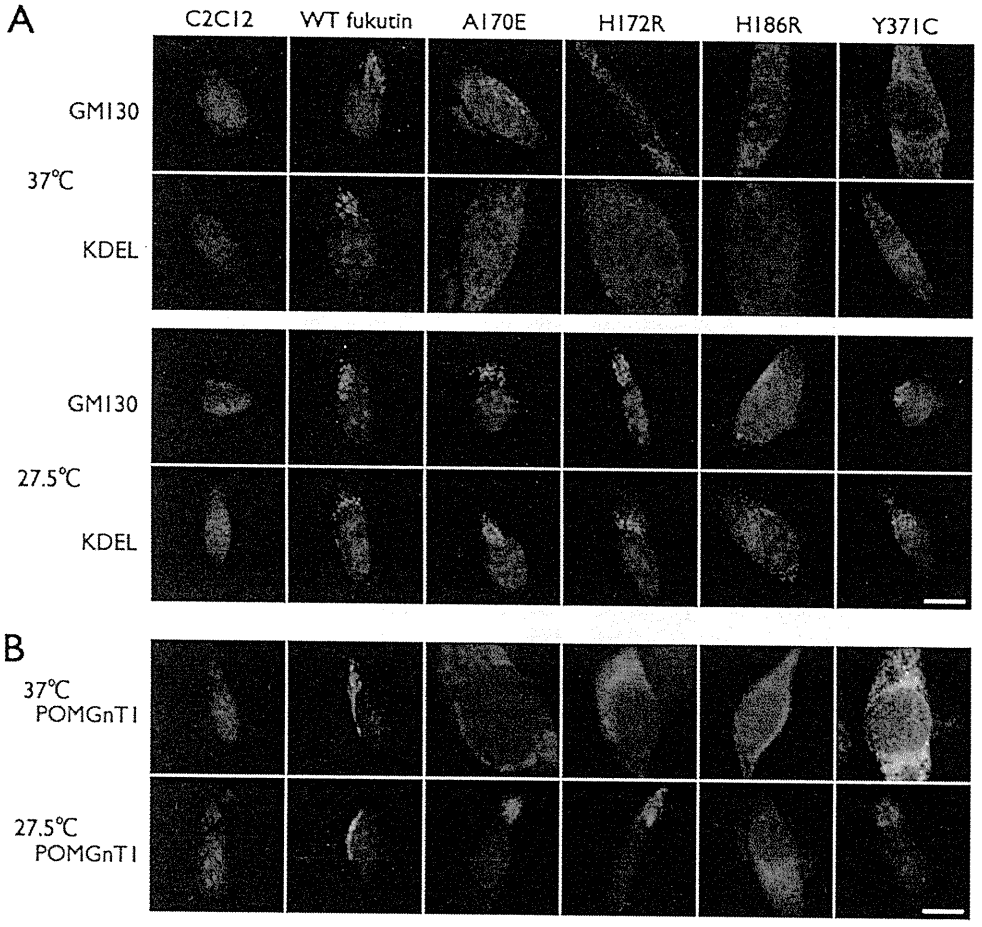


FIGURE 4. **Low temperature culture corrects mislocalization of mutant fukutin proteins.** *A*, effects of low temperature culture on the localization of the missense fukutin mutants. At 37 °C, the missense fukutin mutants (A170E, H172R, H186R, and Y371C, *green*) co-localized with KDEL (ER marker, *red*) in C2C12 cultured cells (*upper*). In contrast, at 27.5 °C, the missense fukutin mutants A170E, H172R, and Y371C lost their co-localization with KDEL and acquired co-localization with GM130 (Golgi marker, *red*) (*lower*). A large amount of the mutant H186R protein retained the ER localization in 27.5 °C culture. *B*, POMGnT1 localization when co-expressed with the missense mutants. POMGnT1 (*red*) mislocalized to the ER when co-expressed with the missense fukutin mutants (A170E, H172R, H186R, or Y371C, *green*) at 37 °C. However, at 27.5 °C, localization of POMGnT1 was restored to the Golgi even when co-expressed with mutant fukutin proteins. *Blue*, DAPI. *Scale bars*, 10 μm.

ing caused by amino acid substitutions. A large amount of the fourth mutant protein, H186R, retained the ER localization even under low temperature conditions (Fig. 4*A*). It has been reported that the patient with the homozygous H186R mutation is affected with WWS (12), which shows more severe pathological features than typical FCMD. The H186R mutation in the *fukutin* gene may affect protein folding severely enough that low temperature conditions cannot correct mislocalization. We reported previously that POMGnT1 interacts with fukutin and co-localizes to the Golgi (16). Our present data show that correction of the mislocalization of the three missense fukutin mutants by low temperature culture was accompanied by correction of POMGnT1 cellular localization (Fig. 4*B*). This POMGnT1 behavior is rational because the transmembrane region of fukutin, through which fukutin binds to POMGnT1 (16), remained intact in these missense fukutin mutants (Fig. 1, *single underline*). Immunoprecipitation experiments confirmed the interaction between each of the missense fukutin mutants and POMGnT1 (supplemental Fig. 3). Most of the H186R mutant remained in the ER at 27.5 °C, but POMGnT1 expressed with the H186R localized to the Golgi. The reason for this result is uncertain, but a possible explanation is that the H186R mutant may have a harmful (dominant negative-like) effect on POMGnT1 localization, and this effect may be ameliorated at low temperature.

The above-mentioned four mutants, which showed abnormal localization to the ER, were identified in patients presenting with a typical or a severe phenotype. However, several of the remaining nine mutants, which showed Golgi localization, were also identified in patients presenting with the typical or the severe phenotype (G125S, M133T, C250G, and W305C) (Table 1). Therefore, the typical or severe phenotypes seem not always to be related to abnormal cellular localization of mutated proteins. Given that FCMD is inherited in an autosomal recessive manner, disease-causing mutations in the *fukutin* gene must lead to loss of function of the fukutin protein. Although the exact function of fukutin is undetermined, these nine mutations may disrupt an important functional domain in the protein. For example, the W305C (10) and R307Q (9, 13) mutations are located in the DxD motif (Fig. 1, *double underline*), which is predicted to be involved in the modification of cell surface glycoproteins or glycolipids (17). These substitutions may disrupt the DxD motif and produce dysfunctional fukutin protein. Five mutations (A114T, R179T, R246G, R307Q, and Q358P) have been identified in patients presenting with mild phenotypes (congenital muscular dystrophy with no

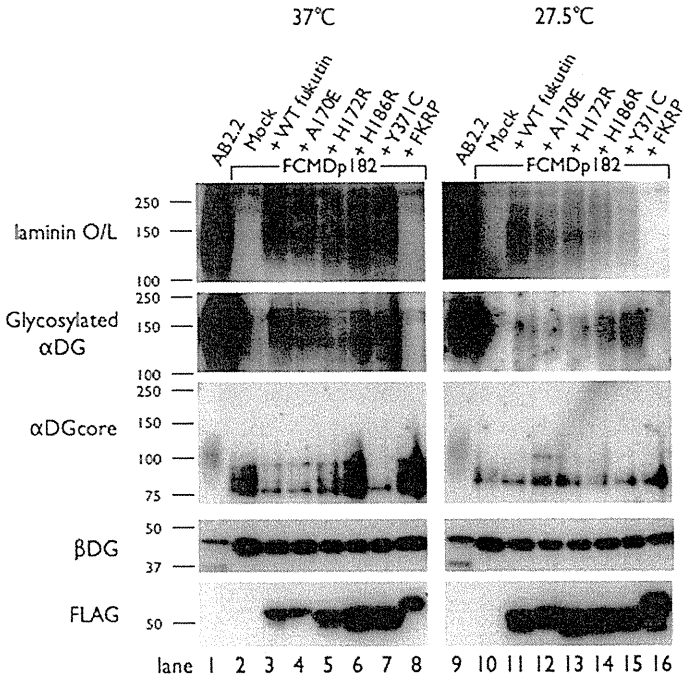
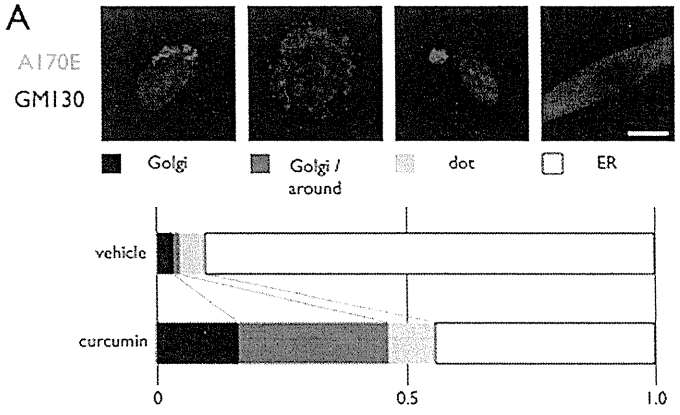


FIGURE 5. **Fukutin missense mutants retain the activity responsible for α-DG glycosylation.** Western blot analysis was performed to detect IIH6C4 (glycosylated α-DG), core α-DG, β-DG, and FLAG tag, and the laminin overlay assay. DG and fukutin proteins were prepared from wild-type mouse ES cells (AB2.2), fukutin-deficient mouse ES cells (FCMDp182), and fukutin- or FKRP-transfected FCMDp182 cells, and cultured at 37 °C and 27.5 °C. FCMDp182 cells expressing any of the mutant fukutin proteins showed levels of glycosylated α-DG signal (IIH6C4-reactivity) and laminin binding activity that were comparable with those observed in FCMDp182 cells expressing wild-type fukutin.



B

	A170E		H172R		H186R		Y371C	
	vehicle	curcumin	vehicle	curcumin	vehicle	curcumin	vehicle	curcumin
Golgi	6	25	1	0	0	0	0	0
Golgi / around	1	47	1	32	0	32	1	11
dot	9	15	1	5	1	5	11	11
ER	159	69	109	102	113	158	115	114
p value	8.28×10^{-20}		1.10×10^{-6}		0.005		0.02	

FIGURE 6. **Curcumin treatment partly corrects mislocalization of the missense fukutin mutants.** *A*, effects of curcumin treatment on the cellular localization of the missense A170E fukutin mutants. C2C12 cells expressing the missense fukutin mutant A170E were cultured in the presence or absence of 10 μg/ml curcumin. Cells were classified into four classes (Golgi localization, Golgi and around localization, dot localization, and ER localization), then counted and statistically analyzed using the χ^2 test. *Red*, GM130; *green*, expressed missense fukutin protein A170E; *blue*, DAPI. *Scale bar*, 10 μm. *B*, statistical analysis of effects of curcumin treatment on the cellular localization of the missense fukutin mutants A170E, H172R, H186R, and Y371C.

mental retardation, LGMD with no mental retardation, or cardiomyopathy) (6, 9, 10, 13). In the present study, these five mutant proteins were localized to the Golgi when expressed in C2C12 cells. In patients with R179T or Q358P mutations, α-DG shows residual reactivity against the monoclonal antibody IIH6C4, which recognizes functionally glycosylated α-DG (6), indicating that these fukutin mutations retain partial function in the DG maturation pathway.

It is of interest that some missense fukutin mutants retain α-DG glycosylation activity and that their mislocalization could be partly corrected by treatments directing at folding amelioration. These observations suggest that drugs capable of correcting the localization might have therapeutic benefits in patients who carry these missense fukutin mutants. Although approximately half of the cells expressing the A170E mutant retained the ER accumulation signals following curcumin treatment (Fig. 6), recent studies have indicated that even partial restoration of α-DG glycosylation can produce therapeutic effects (29). Efforts to identify more efficient folding amelioration reagents may lead to therapeutic strategies.

A large number of missense mutations have been identified in dystroglycanopathy. It has been reported that missense mutations in *POMT1* and *POMGnT1* compromise enzymatic activity in the gene products (18, 34, 35). Disease-causing missense mutations in *POMT2*, *LARGE*, and *FKRP* have been also identified (10, 21–23, 36, 37). Recently, Kawahara *et al.* have reported that expression of some disease-causing missense FKRP mutant proteins in FKRP knock-down zebrafish restores α-DG glycosylation, suggesting a residual FKRP function in these missense mutants (38); however, phenotype improvement depends on the location of the mutation. The FKRP missense mutations C318Y and A455D, which failed to improve the fish phenotype in the report from Kawahara *et al.* (38), were reported to show abnormal cellular localization when expressed in certain cell lines (39). On the other hand, using several different cell lines, Dolatshad *et al.* suggested that a reduced protein (putative enzymatic) function of FKRP rather than protein mislocalization is the primary mechanism of disease (39). Interestingly, Bao *et al.* have reported cells lacking FKRP transcripts but expressing IIH6C4-reactive α-DG (40). This finding may indicate a possibility of a FKRP-independent glycosylation pathway. Alternatively, only a subtle amount of FKRP, even below detectable level by RT-PCR, may be sufficient for α-DG glycosylation. This implies that some missense mutants can restore IIH6C4-reactive α-DG if only a little protein function remains. Of the increasing number of identified disease-causing missense mutations, some may alter the cellular location of the protein, which can be a direct cause of disease. Correction of cellular localization or folding amelioration may have a therapeutic benefit for dystroglycanopathies caused by missense mutations, although the finding from cell culture experiments must be interpreted cautiously when extrapolating to human disease.

In this study, we have used curcumin to correct mislocalization of missense fukutin mutants. It has been reported that curcumin can correct misfolding and mislocalization of cystic fibrosis transmembrane conductance regulator with the ΔF508 mutation (33). Curcumin has also been reported to have protective effects in neurodegenerative diseases by inhibiting protein misfolding and aggregation in Creutzfeldt-Jakob disease and Parkinson disease (41, 42). These studies indicate that curcumin or its derivatives might be new candidate compounds for protein-folding diseases. In addition, the use of pharmacological chaperones to stabilize or promote correct folding of mutant proteins has been shown as a potential therapeutic approach to phenylketonuria, in which more than 500 disease-causing mutations have been identified (43). Our results contribute to a deeper understanding of the molecular pathogenesis of fukutin-deficient muscular dystrophy and have led us to propose a novel therapeutic strategy directed at correction of cellular localization and/or folding amelioration of disease-causing missense mutant proteins.

Acknowledgments—We thank past and present members of the Dr. Toda's laboratory for fruitful discussions and scientific contributions and Dr. Jennifer Logan for help in editing the manuscript.

REFERENCES

1. Fukuyama, Y., Osawa, M., and Suzuki, H. (1981) Congenital progressive muscular dystrophy of the Fukuyama type: clinical, genetic and pathological considerations. *Brain Dev.* **3,** 1–29
2. Toda, T., Segawa, M., Nomura, Y., Nonaka, I., Masuda, K., Ishihara, T., Sakai, M., Tomita, I., Origuchi, Y., Ohno, K., Misugi, N., Sasaki, Y., Takada, K., Kawai, M., Otani, K., Murakami, T., Saito, K., Fukuyama, Y., Shimizu, T., Kanazawa, I., and Nakamura, Y. (1993) Localization of a gene for Fukuyama-type congenital muscular dystrophy to chromosome 9q31–33. *Nat. Genet.* **5,** 283–286
3. Kobayashi, K., Nakahori, Y., Miyake, M., Matsumura, K., Kondo-Iida, E., Nomura, Y., Segawa, M., Yoshioka, M., Saito, K., Osawa, M., Hamano, K., Sakakihara, Y., Nonaka, I., Nakagome, Y., Kanazawa, I., Nakamura, Y., Tokunaga, K., and Toda, T. (1998) An ancient retrotransposal insertion causes Fukuyama-type congenital muscular dystrophy. *Nature* **394,** 388–392
4. Kondo-Iida, E., Kobayashi, K., Watanabe, M., Sasaki, J., Kumagai, T., Koide, H., Saito, K., Osawa, M., Nakamura, Y., and Toda, T. (1999) Novel mutations and genotype-phenotype relationships in 107 families with Fukuyama-type congenital muscular dystrophy (FCMD). *Hum. Mol. Genet.* **8,** 2303–2309
5. Kobayashi, K., Sasaki, J., Kondo-Iida, E., Fukuda, Y., Kinoshita, M., Sunada, Y., Nakamura, Y., and Toda, T. (2001) Structural organization, complete genomic sequences and mutational analyses of the Fukuyama-type congenital muscular dystrophy gene, fukutin. *FEBS Lett.* **489,** 192–196
6. Murakami, T., Hayashi, Y. K., Noguchi, S., Ogawa, M., Nonaka, I., Tanabe, Y., Ogino, M., Takada, F., Eriguchi, M., Kotooka, N., Campbell, K. P., Osawa, M., and Nishino, I. (2006) Fukutin gene mutations cause dilated cardiomyopathy with minimal muscle weakness. *Ann. Neurol.* **60,** 597–602
7. Silan, F., Yoshioka, M., Kobayashi, K., Simsek, E., Tunc, M., Alper, M., Cam, M., Guven, A., Fukuda, Y., Kinoshita, M., Kocabay, K., and Toda, T. (2003) A new mutation of the fukutin gene in a non-Japanese patient. *Ann. Neurol.* **53,** 392–396
8. de Bernabé, D. B., van Bokhoven, H., van Beusekom, E., Van den Akker, W., Kant, S., Dobyns, W. B., Cormand, B., Currier, S., Hamel, B., Talim, B., Topaloglu, H., and Brunner, H. G. (2003) A homozygous nonsense mutation in the fukutin gene causes a Walker-Warburg syndrome phenotype. *J. Med. Genet.* **40,** 845–848
9. Godfrey, C., Escolar, D., Brockington, M., Clement, E. M., Mein, R., Jimenez-Mallebrera, C., Torelli, S., Feng, L., Brown, S. C., Sewry, C. A., Rutherford, M., Shapira, Y., Abbs, S., and Muntoni, F. (2006) Fukutin gene mutations in steroid-responsive limb girdle muscular dystrophy. *Ann. Neurol.* **60,** 603–610
10. Godfrey, C., Clement, E., Mein, R., Brockington, M., Smith, J., Talim, B., Straub, V., Robb, S., Quinlivan, R., Feng, L., Jimenez-Mallebrera, C., Mercuri, E., Manzur, A. Y., Kinali, M., Torelli, S., Brown, S. C., Sewry, C. A., Bushby, K., Topaloglu, H., North, K., Abbs, S., and Muntoni, F. (2007) Refining genotype phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan. *Brain* **130,** 2725–2735
11. Cotarelo, R. P., Valero, M. C., Prados, B., Peña, A., Rodríguez, L., Fano, O., Marco, J. J., Martínez-Frías, M. L., and Cruces, J. (2008) Two new patients bearing mutations in the fukutin gene confirm the relevance of this gene in Walker-Warburg syndrome. *Clin. Genet.* **73,** 139–145
12. Manzini, M. C., Gleason, D., Chang, B. S., Hill, R. S., Barry, B. J., Partlow, J. N., Poduri, A., Currier, S., Galvin-Parton, P., Shapiro, L. R., Schmidt, K., Davis, J. G., Basel-Vanagaite, L., Seidahmed, M. Z., Salih, M. A., Dobyns, W. B., and Walsh, C. A. (2008) Ethnically diverse causes of Walker-Warburg syndrome (WWS): FCMD mutations are a more common cause of WWS outside of the Middle East. *Hum. Mutat.* **29,** E231-E241
13. Vuillaumier-Barrot, S., Quijano-Roy, S., Bouchet-Seraphin, C., Maugenre, S., Peudenier, S., Van den Bergh, P., Marcorelles, P., Avila-Smirnow, D., Chelbi, M., Romero, N. B., Carlier, R. Y., Estournet, B., Guicheney, P., and Seta, N. (2009) Four Caucasian patients with mutations in the fukutin gene and variable clinical phenotype. *Neuromuscul. Disord.* **19,** 182–188
14. Michele, D. E., Barresi, R., Kanagawa, M., Saito, F., Cohn, R. D., Satz, J. S., Dollar, J., Nishino, I., Kelley, R. I., Somer, H., Straub, V., Mathews, K. D., Moore, S. A., and Campbell, K. P. (2002) Post-translational disruption of dystroglycan-ligand interactions in congenital muscular dystrophies. *Nature* **418,** 417–422
15. Matsumoto, H., Noguchi, S., Sugie, K., Ogawa, M., Murayama, K., Hayashi, Y. K., and Nishino, I. (2004) Subcellular localization of fukutin and fukutin-related protein in muscle cells. *J. Biochem.* **135,** 709–712
16. Xiong, H., Kobayashi, K., Tachikawa, M., Manya, H., Takeda, S., Chiyonobu, T., Fujikake, N., Wang, F., Nishimoto, A., Morris, G. E., Nagai, Y., Kanagawa, M., Endo, T., and Toda, T. (2006) Molecular interaction between fukutin and POMGnT1 in the glycosylation pathway of α-dystroglycan. *Biochem. Biophys. Res. Commun.* **350,** 935–941
17. Aravind, L., and Koonin, E. V. (1999) The fukutin protein family: predicted enzymes modifying cell surface molecules. *Curr. Biol.* **9,** R836–837
18. Yoshida, A., Kobayashi, K., Manya, H., Taniguchi, K., Kano, H., Mizuno, M., Inazu, T., Mitsuhashi, H., Takahashi, S., Takeuchi, M., Herrmann, R., Straub, V., Talim, B., Voit, T., Topaloglu, H., Toda, T., and Endo, T. (2001) Muscular dystrophy and neuronal migration disorder caused by mutations in a glycosyltransferase, POMGnT1. *Dev. Cell* **1,** 717–724
19. Beltrán-Valero de Bernabé, D., Currier, S., Steinbrecher, A., Celli, J., van Beusekom, E., van der Zwaag, B., Kayserili, H., Merlini, L., Chitayat, D., Dobyns, W. B., Cormand, B., Lehesjoki, A. E., Cruces, J., Voit, T., Walsh, C. A., van Bokhoven, H., and Brunner, H. G. (2002) Mutations in the *O*-mannosyltransferase gene POMT1 give rise to the severe neuronal migration disorder Walker-Warburg syndrome. *Am. J. Hum. Genet.* **71,** 1033–1043
20. van Reeuwijk, J., Janssen, M., van den Elzen, C., Beltran-Valero de Bernabé, D., Sabatelli, P., Merlini, L., Boon, M., Scheffer, H., Brockington, M., Muntoni, F., Huynen, M. A., Verrips, A., Walsh, C. A., Barth, P. G., Brunner, H. G., and van Bokhoven, H. (2005) POMT2 mutations cause α-dystroglycan hypoglycosylation and Walker-Warburg syndrome. *J. Med. Genet.* **42,** 907–912
21. Brockington, M., Blake, D. J., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Ponting, C. P., Estournet, B., Romero, N. B., Mercuri, E., Voit, T., Sewry, C. A., Guicheney, P., and Muntoni, F. (2001) Mutations in the fukutin-related protein gene (FKRP) cause a form of congenital muscular dystrophy with secondary laminin α2 deficiency and abnormal glycosylation of α-dystroglycan. *Am. J. Hum. Genet.* **69,** 1198–1209
22. Brockington, M., Yuva, Y., Prandini, P., Brown, S. C., Torelli, S., Benson, M. A., Herrmann, R., Anderson, L. V., Bashir, R., Burgunder, J. M., Fallet, S., Romero, N., Fardeau, M., Straub, V., Storey, G., Pollitt, C., Richard, I.,

Sewry, C. A., Bushby, K., Voit, T., Blake, D. J., and Muntoni, F. (2001) Mutations in the fukutin-related protein gene (FKRP) identify limb girdle muscular dystrophy 2I as a milder allelic variant of congenital muscular dystrophy MDC1C. *Hum. Mol. Genet.* **10,** 2851–2859

23. Longman, C., Brockington, M., Torelli, S., Jimenez-Mallebrera, C., Kennedy, C., Khalil, N., Feng, L., Saran, R. K., Voit, T., Merlini, L., Sewry, C. A., Brown, S. C., and Muntoni, F. (2003) Mutations in the human LARGE gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α-dystroglycan. *Hum. Mol. Genet.* **12,** 2853–2861
24. Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U., and Endo, T. (2004) Demonstration of mammalian protein *O*-mannosyltransferase activity: co-expression of POMT1 and POMT2 required for enzymatic activity. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 500–505
25. Inamori, K., Yoshida-Moriguchi, T., Hara, Y., Anderson, M. E., Yu, L., and Campbell, K. P. (2012) Dystroglycan function requires xylosyl- and glucuronyltransferase activities of LARGE. *Science* **335,** 93–96
26. Yoshida-Moriguchi, T., Yu, L., Stalnaker, S. H., Davis, S., Kunz, S., Madson, M., Oldstone, M. B., Schachter, H., Wells, L., and Campbell, K. P. (2010) O-mannosyl phosphorylation of α-dystroglycan is required for laminin binding. *Science* **327,** 88–92
27. Matsumoto, H., Hayashi, Y. K., Kim, D. S., Ogawa, M., Murakami, T., Noguchi, S., Nonaka, I., Nakazawa, T., Matsuo, T., Futagami, S., Campbell, K. P., and Nishino, I. (2005) Congenital muscular dystrophy with glycosylation defects of α-dystroglycan in Japan. *Neuromuscul. Disord.* **15,** 342–348
28. Yoshioka, M., Higuchi, Y., Fujii, T., Aiba, H., and Toda, T. (2008) Seizure-genotype relationship in Fukuyama-type congenital muscular dystrophy. *Brain Dev.* **30,** 59–67
29. Kanagawa, M., Nishimoto, A., Chiyonobu, T., Takeda, S., Miyagoe-Suzuki, Y., Wang, F., Fujikake, N., Taniguchi, M., Lu, Z., Tachikawa, M., Nagai, Y., Tashiro, F., Miyazaki, J., Tajima, Y., Takeda, S., Endo, T., Kobayashi, K., Campbell, K. P., and Toda, T. (2009) Residual laminin binding activity and enhanced dystroglycan glycosylation by LARGE in novel model mice to dystroglycanopathy. *Hum. Mol. Genet.* **18,** 621–631
30. Takeda, S., Kondo, M., Sasaki, J., Kurahashi, H., Kano, H., Arai, K., Misaki, K., Fukui, T., Kobayashi, K., Tachikawa, M., Imamura, M., Nakamura, Y., Shimizu, T., Murakami, T., Sunada, Y., Fujikado, T., Matsumura, K., Terashima, T., and Toda, T. (2003) Fukutin is required for maintenance of muscle integrity, cortical histiogenesis, and normal eye development. *Hum. Mol. Genet.* **12,** 1449–1459
31. Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E., and Welsh, M. J. (1992) Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature* **358,** 761–764
32. Cheng, S. H., Fang, S. L., Zabner, J., Marshall, J., Piraino, S., Schiavi, S. C., Jefferson, D. M., Welsh, M. J., and Smith, A. E. (1995) Functional activation of the cystic fibrosis trafficking mutant ΔF508-CFTR by overexpression. *Am. J. Physiol.* **268,** L615–624
33. Egan, M. E., Pearson, M., Weiner, S. A., Rajendran, V., Rubin, D., Glöckner-Pagel, J., Canny, S., Du, K., Lukacs, G. L., and Caplan, M. J. (2004) Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. *Science* **304,** 600–602
34. Manya, H., Sakai, K., Kobayashi, K., Taniguchi, K., Kawakita, M., Toda, T., and Endo, T. (2003) Loss-of-function of an *N*-acetylglucosaminyltransferase, POMGnT1, in muscle-eye-brain disease. *Biochem. Biophys. Res. Commun.* **306,** 93–97
35. Akasaka-Manya, K., Manya, H., and Endo, T. (2004) Mutations of the *POMT1* gene found in patients with Walker-Warburg syndrome lead to a defect of protein *O*-mannosylation. *Biochem. Biophys. Res. Commun.* **325,** 75–79
36. Brockington, M., Torelli, S., Prandini, P., Boito, C., Dolatshad, N. F., Longman, C., Brown, S. C., and Muntoni, F. (2005) Localization and functional analysis of the LARGE family of glycosyltransferases: significance for muscular dystrophy. *Hum. Mol. Genet.* **14,** 657–665
37. Keramaris-Vrantsis, E., Lu, P. J., Doran, T., Zillmer, A., Ashar, J., Esapa, C. T., Benson, M. A., Blake, D. J., Rosenfeld, J., and Lu, Q. L. (2007) Fukutin-related protein localizes to the Golgi apparatus and mutations lead to mislocalization in muscle *in vivo*. *Muscle Nerve* **36,** 455–465
38. Kawahara, G., Guyon, J. R., Nakamura, Y., and Kunkel, L. M. (2010) Zebrafish models for human FKRP muscular dystrophies. *Hum. Mol. Genet.* **19,** 623–633
39. Dolatshad, N. F., Brockington, M., Torelli, S., Skordis, L., Wever, U., Wells, D. J., Muntoni, F., and Brown, S. C. (2005) Mutated fukutin-related protein (FKRP) localizes as wild type in differentiated muscle cells. *Exp. Cell Res.* **309,** 370–378
40. Bao, X., Kobayashi, M., Hatakeyama, S., Angata, K., Gullberg, D., Nakayama, J., Fukuda, M. N., and Fukuda, M. (2009) Tumor suppressor function of laminin-binding α-dystroglycan requires a distinct β3-*N*-acetylglucosaminyltransferase. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 12109–12114
41. Hafner-Bratkovic, I., Gaspersic, J., Smid, L. M., Bresjanac, M., and Jerala, R. (2008) Curcumin binds to the α-helical intermediate and to the amyloid form of prion protein: a new mechanism for the inhibition of PrP(Sc) accumulation. *J. Neurochem.* **104,** 1553–1564
42. Ono, K., and Yamada, M. (2006) Antioxidant compounds have potent anti-fibrillogenic and fibril-destabilizing effects for α-synuclein fibrils *in vitro*. *J. Neurochem.* **97,** 105–115
43. Pey, A. L., Ying, M., Cremades, N., Velazquez-Campoy, A., Scherer, T., Thöny, B., Sancho, J., and Martinez, A. (2008) Identification of pharmacological chaperones as potential therapeutic agents to treat phenylketonuria. *J. Clin. Invest.* **118,** 2858–2867

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 12, pp. 9560–9567, March 16, 2012

Absence of Post-phosphoryl Modification in Dystroglycanopathy Mouse Models and Wild-type Tissues Expressing Non-laminin Binding Form of α-Dystroglycan*[S]

Received for publication, June 14, 2011, and in revised form, January 13, 2012 Published, JBC Papers in Press, January 23, 2012, DOI 10.1074/jbc.M111.271767

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Background: The biosynthetic pathway for the ligand-binding moiety of α-dystroglycan, defects in which cause dystroglycanopathy, remains unclear.
Results: The phosphodiester-linked moiety on *O*-mannose is absent in dystroglycanopathy models and in wild-type lung and testis.
Conclusion: Post-phosphoryl modification is a key determinant of the functional expression of α-dystroglycan as a laminin receptor.
Significance: This work expands our understanding of the molecular mechanism of a unique post-translational modification.

α-Dystroglycan (α-DG) is a membrane-associated glycoprotein that interacts with several extracellular matrix proteins, including laminin and agrin. Aberrant glycosylation of α-DG disrupts its interaction with ligands and causes a certain type of muscular dystrophy commonly referred to as dystroglycanopathy. It has been reported that a unique *O*-mannosyl tetrasaccharide (Neu5Ac-α2,3-Gal-β1,4-GlcNAc-β1,2-Man) and a phosphodiester-linked modification on *O*-mannose play important roles in the laminin binding activity of α-DG. In this study, we use several dystroglycanopathy mouse models to demonstrate that, in addition to fukutin and LARGE, FKRP (fukutin-related protein) is also involved in the post-phosphoryl modification of *O*-mannose on α-DG. Furthermore, we have found that the glycosylation status of α-DG in lung and testis is minimally affected by defects in *fukutin*, *LARGE*, or *FKRP*. α-DG prepared from wild-type lung- or testis-derived cells lacks the post-phosphoryl moiety and shows little laminin-binding activity. These results show that FKRP is involved in post-phosphoryl modification rather than in *O*-mannosyl tetrasaccharide synthesis. Our data also demonstrate that post-phosphoryl modification not only plays critical roles in the pathogenesis of dystroglycanopathy but also is a key determinant of α-DG functional expression as a laminin receptor in normal tissues and cells.

Dystroglycan (DG),[2] a cell surface receptor for several extracellular matrix proteins, plays important roles in various tissues (1). DG consists of a heavily glycosylated extracellular α subunit (α-DG) and a transmembrane β subunit (β-DG). α-DG and β-DG are encoded by a single gene and post-translationally cleaved to generate the two subunits (2). α-DG binds to extracellular proteins such as laminin, agrin, perlecan, neurexin, and pikachurin (2–7). β-DG anchors α-DG at the cell surface and binds intracellularly to dystrophin, which in turn binds to the actin cytoskeleton. Thus, α/β-DG functions as a molecular axis, connecting the extracellular matrix with the cytoskeleton across the plasma membrane (1).

O-Glycosylation of α-DG is necessary for its interaction with ligands, and genetic disruption of the glycosylation pathway for DG is associated with a group of muscular dystrophies known as "dystroglycanopathy" (8–10). Six genes (*POMT1*, *POMT2*, *POMGnT1*, *fukutin*, *FKRP*, and *LARGE*) have been identified as causative genes for dystroglycanopathy. A common biochemical characteristic of these disorders is abnormal glycosylation and reduced laminin-binding activity of α-DG; however, the precise glycan structure required for α-DG ligand binding is not completely determined. Two unique *O*-mannosyl modifications have been identified in α-DG: an *O*-mannosyl tetrasaccharide (Neu5Ac-α2,3-Gal-β1,4-GlcNAc-β1,2-Man) (11), and

* This work was supported by Ministry of Health, Labor, and Welfare of Japan Intramural Research Grant (23B-5) for Neurological and Mental Disorders and The Research on Psychiatric and Neurological Diseases and Mental Health H20-016 (to T. T.), Grant-in-aid for Scientific Research (A) 23249049 (to T. T.) and a Grant-in-aid for Young Scientists (B) 21790318 (to M. K.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Takeda Science Foundation (to M. K.).
[S] This article contains supplemental Fig. 1.
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[2] The abbreviations used are: DG, dystroglycan; IMAC, immobilized metal affinity chromatography; MW, molecular weight; HFaq, aqueous hydrofluoric acid.

a phosphodiester-linked branch structure present at the C6 hydroxyl residue of *O*-mannose (12). The *O*-mannosyl tetrasaccharide was first identified on peripheral nerve α-DG (11). The initial mannose transferred to Ser/Thr residues on the α-DG polypeptide backbone is catalyzed by the POMT1/POMT2 complex (13). Mutations in *POMT1* and *POMT2* were originally identified as causative for Walker-Warburg syndrome (14, 15). *POMGnT1*, established as a causative gene for muscle-eye-brain disease, encodes a glycosyltransferase that transfers GlcNAc to *O*-mannose on α-DG (16). In these disorders, α-DG lacks laminin-binding activity (17); therefore, the tetrasaccharide plays an important role in the post-translational maturation of α-DG as a laminin receptor. On the other hand, recent studies have suggested that the Neu5Ac-α2,3-Gal-β1,4-GlcNAc branch on *O*-mannose *per se* is not likely the laminin-binding glycan of α-DG (12, 18).

fukutin was originally identified as the causative gene for Fukuyama-type congenital muscular dystrophy (19), and *FKRP* was identified as the causative gene for both MDC1C (congenital muscular dystrophy type 1C) (20) and LGMD2I (limb-girdle muscular dystrophy type 2I) (21). The precise function of fukutin and FKRP is still uncertain. Mutation of *LARGE* causes muscular dystrophy in the spontaneous *Large*^{myd} mouse model (22) and in human congenital muscular dystrophy type 1D (23). Recently, a phosphodiester-linked modification on an *O*-mannose was identified (12). It was shown that α-DG in *fukutin*-mutated Fukuyama-type congenital muscular dystrophy and *Large*^{myd} muscle cells exhibits defective post-phosphoryl modification on the *O*-mannose, suggesting that this phosphorylated branch serves as the laminin-binding moiety. To explore the role of phosphorylated *O*-mannose in functional α-DG ligand-binding and in other forms of dystroglycanopathy, we have investigated α-DG glycosylation in several dystroglycanopathy mouse models.

EXPERIMENTAL PROCEDURES

Cell Culture—TM3 and CHL cell lines were purchased from European collection of cell cultures and the RIKEN BioResource Center, respectively. TM3 cells were cultured in Ham's F12/DMEM (1:1) containing 5% horse serum and 2.5% fetal bovine serum. CHL cells were cultured in DMEM containing 10% fetal bovine serum. Expression vectors for LARGE were constructed by cloning human *LARGE* with a V5 tag into pcDNA vectors (24). Transfection was carried out using Lipofectamine 2000 (Invitrogen) for CHL and TM3 cells according to the manufacturer's instructions. Transfected cells were grown at 37 °C and harvested at 48 h after transfection. The transfected cells were solubilized in TBS with 1% Triton X-100. Samples were centrifuged at 15,000 rpm for 10 min at 4 °C. Supernatants were collected, and protein concentrations were measured by Lowry methods, using BSA as a standard.

Animals—Large^{myd} mice were obtained from The Jackson Laboratory. Generation of FKRP-neo-P448L knock-in mice, Hp/− mice, and *POMGnT1*-deficient mice has been described previously (25–27). Mice were maintained in accordance with the animal care guidelines of Kobe University. All animal studies using FKRP-neo-P448L knock-in mice were approved by the Institutional Animal Care and Use Committee of the Carolinas Medical Center.

Protein Enrichment—Frozen tissue samples were solubilized in TBS (pH 7.4) with 1% Triton X-100. The solubilized materials were incubated with wheat germ agglutinin beads, and the DG-enriched fraction was then eluted with 0.3 M *N*-acetyl-D-glucosamine in TBS containing 0.1% Triton X-100. For the immobilized metal affinity chromatography (IMAC)-binding assay, aqueous hydrofluoric acid (HFaq) treatment, and deglycosylation assay, the DG-enriched fractions were diluted in 0.25% CHAPS/water (w/v) and then desalted and concentrated using Amicon-ultra filters (Millipore).

IMAC-binding Assay—Samples were diluted in a solution containing 250 mM acetic acid, 30% acetonitrile, and 0.15% CHAPS and incubated with PHOS-Select iron affinity gel (Sigma) at room temperature for 0.5 h. Bound materials were directly eluted with SDS-loading buffer. Equal ratios of the void and the bound samples were used for Western blot analysis.

HFaq Treatment—Samples were incubated with 48% aqueous hydrofluoric acid (Wako) on ice for 12 h. Control samples were incubated with water instead of hydrofluoric acid. After removal of the reagents under a stream of nitrogen gas, residues were dissolved with SDS-loading buffer for Western blot analysis.

Deglycosylation Assay—Glycopeptidase F (peptide-*N*-glycosidase; Wako), α-2 (3, 6, 8, 9) neuraminidase (Calbiochem), β1–4 galactosidase (New England Biolabs), β-*N*-acetyl-hexosaminidase (Seikagaku Corp.), and *O*-glycosidase (Roche Applied Science) were used according to the manufacturer's protocol.

Antibodies—Antibodies used for Western blotting were mouse monoclonal antibody IIH6 against glycosylated α-DG (Millipore) and goat polyclonal antibody against the C-terminal domain of the α-DG polypeptide (AP-074G-C) (26).

Laminin and Agrin Overlay Assays—Recombinant mouse laminin LG4–5 domains of laminin α1 and laminin α2 chains fused to Fc tags were recovered from the cell culture media using protein A beads (28). Recombinant agrin was purchased from R&D Systems. Laminin and agrin overlay assays were performed as described previously (26).

RT-PCR Analysis—Total RNA was isolated from wild-type mouse testis and TM3 cells using the RNeasy Plus mini kit (Qiagen) and converted to cDNA using Superscript III reverse transcriptase (Invitrogen). The forward and reverse primers used in gene amplification were as follows: *Large* (5′-TCAATCTTCTGCGAAACGTG-3′ and 5′-TCCAACATTGACAGCAGCTC-3′), *POMT1* (5′-CGGGTCTCTTGTTCCTGTG-3′ and 5′-AGTGACTGAGCACGCGCATA-3′), *POMT2* (5′-CGGAACCTGCACAGTCACTA-3′ and 5′-AATCCGCCAGAAGTCATTTG-3′), *POMGnT1* (5′-CCAAGGGGTATCTCCACAGA-3′ and 5′-GGTCCTCTTCCAGAACCACA-3′), *fukutin* (5′-CGCACTGCAGTATCACCTGT-3′ and 5′-AAGTGGATGGCATGAGTGGT-3′), *FKRP* (5′-CTTCTGTCCCGCTTCAGTTC-3′ and 5′-AACCAGAGAGAGCCCAGTCA-3′), *β3GnT1* (5′-TTCAATCGAATCAGCCAGGTA-3′ and 5′-TCCTCAATTCTCCATCATCCA-3′), GAPDH (5′-CGTAGACAAAATGGTGAAGG-3′ and 5′-GTTGTCATGGAT-

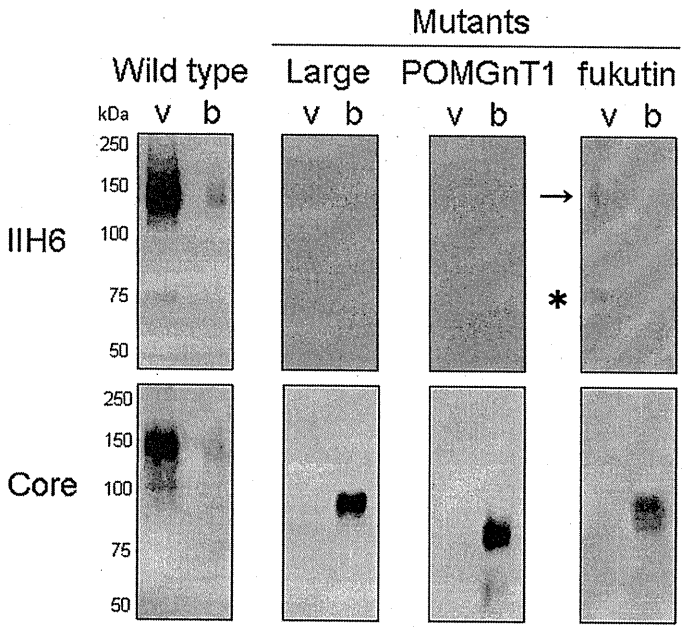

FIGURE 1. **Defects of post-phosphoryl modification in the dystroglycanopathy models.** α-DG-enriched samples from skeletal muscle of wild-type, *Large*^{myd}, *POMGnT1*-deficient, and *fukutin*-deficient Hp/− mice were subjected to IMAC beads. The void (*v*) and bound (*b*) fractions were collected. An *arrow* indicates the IIH6-positive intact α-DG in the Hp/− mice. An *asterisk* indicates a background signal that is not specific for IIH6 antibody.

GACCTTGG-3′), and *DAG1* (5′-ACCAAAGCACCCATCAC-CAG-3′ and 5′-GTTCCCACCCAGGCATCTAC-3′).

RESULTS

Defects of Post-phosphoryl Modification in FKRP-deficient Mice—To examine whether dystroglycanopathy models share a common defect in the post-phosphoryl modification of α-DG, we performed an IMAC bead-binding assay. IMAC beads bind to monoester-linked, but not diester-linked, phosphorylated compounds, and it has been shown that α-DG with defects in post-phosphoryl modification binds to IMAC beads (12). First, we used *Large*^{myd} mice (22), genetically engineered *POMGnT1* knock-out mice (27), and transgenic Hp/− knock-in mice carrying the retrotransposal insertion in *fukutin* (26). α-DG in skeletal muscle tissues from these mutant mice was not properly glycosylated, as indicated by the loss of reactivity against the monoclonal antibody IIH6 (Fig. 1, *upper panel*). The hypoglycosylated α-DG has a lower molecular weight (MW) that can only be detected by the DG core antibody (Fig. 1, *lower panel*). IIH6 antibody reacts with the laminin-binding glycans present in α-DG (17). Hypoglycosylated α-DG was captured by the IMAC beads, indicating that the monoester-linked phosphate residues do not undergo further modification. A small portion of α-DG with a MW of 150,000 in the Hp/− mice (Fig. 1, *arrow*) did not bind to the IMAC beads and showed reactivity against the IIH6 antibody, which might be due to the residual activity of fukutin in the Hp/− mice (26). These data support the observations made in muscle-eye-brain disease and Fukuyama-type congenital muscular dystrophy patients' cells and skeletal muscle biopsies (12).

We next examined whether FKRP is also involved in the post-phosphoryl modification of α-DG. Consistent with previous observations, α-DG from the skeletal muscle of homozygous FKRP-neo-P448L knock-in mice (FKRP-P448L mice) was aberrantly glycosylated, as indicated by the loss of IIH6 reactivity (25). The hypoglycosylated α-DG, showing a lower MW of 90,000 compared with wild-type α-DG at 150,000, bound to the IMAC beads (Fig. 2*A*, *lower panel*). In brain tissue, IIH6-positive α-DG shows a MW of 100,000, whereas hypoglycosylated α-DG shows a MW of 70,000. As was the case in skeletal muscle, hypoglycosylated α-DG from the homozygous mouse bound to IMAC beads (Fig. 2*A*). It has been reported that treatment with cold HFaq cleaves the phosphodiester linker in α-DG (12). After HFaq treatment, the MW of α-DG was reduced to ~90,000, and α-DG lost IIH6-reactivity (Fig. 2*B*, *left panel*). In contrast to the mature α-DG from heterozygous controls, the hypoglycosylated α-DG from homozygous FKRP-P448L muscle showed almost no change in MW after the HFaq treatment (Fig. 2*B*, *right panel*). Treatment with several mixtures of glycosidase predicted to remove *N*-glycan, mucin type *O*-glycan, and the trisaccharide at the non-reducing end of the Neu5Ac-α2,3-Gal-β1,4-GlcNAc-β1,2-Man glycan (12, 18) generated stepwise decreases in the MW of α-DG through multi-step digestions (Fig. 2*C*). These results indicate for the first time that FKRP is involved in the post-phosphoryl modification of α-DG rather than in the synthesis of the Neu5Ac-α2,3-Gal-β1,4-GlcNAc-β1,2-Man glycan. This concept is supported by the previous observation that neither POMT1/2 nor POMGnT1 activity was reduced in lymphoblast cells from patients with *FKRP* mutations (29). Overall, our results establish and confirm that a defect in post-phosphoryl modification on *O*-mannose is a common biochemical characteristic in dystroglycanopathy caused by mutations in *LARGE*, *POMGnT1*, *fukutin*, and *FKRP*.

Post-phosphoryl Moiety of α-dystroglycan Is Absent in Lung and Testis—We have demonstrated that disruption of *Large*, *fukutin*, or *FKRP* decreases the MW of α-DG in skeletal muscle and brain due to the lack of post-phosphoryl modification. It is known that the MW of α-DG and its reactivity to the monoclonal antibody IIH6 vary among different tissues (1, 30). We hypothesized that the low MW of α-DG in some tissues may result from the lack of post-phosphoryl modification and/or the Neu5Ac-α2,3-Gal-β1,4-GlcNAc-β1,2-Man glycan. Several tissues from dystroglycanopathy model mice were therefore investigated. We found that the decreases in the MW of α-DG were relatively minor in lung and very scarce in testis from FKRP-P448L mice and Hp/− mice when compared with litter controls (Fig. 3*A*). Minor changes in α-DG MW in the lung and testis of *Large*-deficient mice have been also observed elsewhere (30), supporting our findings. On the other hand, α-DG in lung and testis from *POMGnT1*-deficient mice clearly shows a lower MW compared with litter heterozygous controls and other mutant mouse strains (Fig. 3*B*). These results suggested that the GlcNAc-β1,2 branch on *O*-mannose is present in wild-type α-DG in lung and testis, but post-phosphoryl modification is absent.

We examined these tissues in wild-type mice using an IMAC bead-binding assay and HFaq treatment. In Fig. 4*A*, Western blot analysis of wild-type tissues showed that α-DG in testis has a MW of 90,000 and was not recognized by the IIH6 antibody,