Muscular Dystrophies Due to Glycosylation Defects

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Summary: In the last few years, muscular dystrophies due to reduced glycosylation of alpha-dystroglycan (ADG) have emerged as a common group of conditions, now referred to as dystroglycanopathies. Mutations in six genes (*POMT1*, *POMT2*, *POMGnT1*, *Fukutin*, *FKRP* and *LARGE*) have so far been identified in patients with a dystroglycanopathy. Allelic mutations in each of these genes can result in a wide spectrum of clinical conditions, ranging from severe congenital onset with associated structural brain malformations (Walker Warburg syndrome; muscle-eye-brain disease; Fukuyama muscular dystrophy; congenital muscular dystrophy type 1D) to a relatively milder congenital variant with no brain involvement (congenital muscular dystrophy type 1C), and to limb-girdle muscular dystrophy (LGMD) type 2 variants with onset in childhood or adult life (LGMD2I, LGMD2L, and LGMD2N).

ADG is a peripheral membrane protein that undergoes multiple and complex glycosylation steps to regulate its ability to effectively interact with extracellular matrix proteins, such as laminin, agrin, and perlecan.

Although the precise composition of the glycans present on ADG are not known, it has been demonstrated that the forced overexpression of *LARGE*, or its paralog *LARGE2*, is capable of increasing the glycosylation of ADG in normal cells. In addition, its overexpression is capable of restoring dystroglycan glycosylation and laminin binding properties in primary cell cultures of patients affected by different genetically defined dystroglycanopathy variants.

These observations suggest that there could be a role for therapeutic strategies to overcome the glycosylation defect in these conditions via the overexpression of *LARGE*. **Key Words:** Alpha dystroglycan, glycosylation, O-mannosylation, laminin binding, pharmacological upregulation.

INTRODUCTION

Mutations in structural muscle proteins are a major cause of muscular dystrophies (MDs), including Duchenne MD and various limb-girdle muscular dystrophy (LGMD) variants. However, it has become evident that a number of congenital muscular dystrophy (CMD) and LGMD variants are associated with mutations in proteins that are either putative or demonstrated glycosyltransferases.^{1–4}

These conditions include four severe forms of CMD with severe structural brain involvement and variable associated eye abnormalities: 1) Walker-Warburg syndrome (WWS) OMIM 236670, 2) muscle-eye-brain disease (MEB) OMIM 253280, 3) Fukuyama CMD (FCMD) OMIM 253800, and 4) congenital muscular dystrophy type 1D (MDC1D) OMIM 608840. Another CMD variant, MDC1C (OMIM

606612), is usually not associated with brain involvement, and the same applies to a milder form of limb-girdle muscular dystrophy (LGMD2I) OMIM 607155.^{5,6} Other LGMD variants (such as LGMD2L and LGMD2N) have also been described.^{7,8} Mutations in six genes have been identified in this group of disorders: *protein-O-mannosyl transferase 1* (*POMT1*) OMIM 607423; *protein-O-mannosyl transferase 2* (*POMT2*) OMIM 607439; *protein-O-mannose β 1,2-N-acetylglucosaminyltransferase 1* (*POMGnT1*) OMIM 606822; *Fukutin* (OMIM 607440); *Fukutin-related protein* (*FKRP*) OMIM 606596; and *acetylglucosaminyl-transferase-like protein* (*LARGE*) OMIM 603590.^{5,9–13} Of these six genes, the one most frequently seen in the Caucasian population is *FKRP*. Although this was the first gene to be associated with an extremely wide range of clinical severity, more recent data suggest this is a common theme for mutations in the remaining genes.¹⁴ A characteristic and diagnostic feature of all of these MD variants is their association with abnormalities in the glycosylation of α -dystroglycan (ADG), and this has resulted in the term “dystroglycanopathies” being applied to this group of disorders.

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DYSTROGLYCAN AND ITS GLYCOSYLATION

Dystroglycan was originally identified as a component of the dystrophin-glycoprotein complex (DGC) present at the sarcolemma of skeletal muscle, although it is now clear that it has a much wider tissue distribution. Dystroglycan is encoded by the *DAG1* gene, which encodes a precursor protein that is post-translationally cleaved into α and β subunits. The full function of the DGC is unclear, although by binding laminins, perlecan, and neurexin, it forms a structural link between the extracellular matrix and the actin associated cytoskeleton.^{15,16} The α subunit has globular N- and C-terminal domains separated by a central mucin-rich domain. The N-terminal domain appears to be cleaved by a convertase-like activity. The predicted molecular mass of α -dystroglycan is 76 kDa, but on immunoblotting it ranges widely from 120 kDa (brain) to 156 kDa (skeletal muscle), most likely due to tissue and developmentally regulated patterns of *O*-glycosylation within the mucin rich domain.¹⁷ Differential glycosylation of ADG confers tissue-specific functional variability.¹⁸ Structural analysis of the sugars decorating α -dystroglycan is limited. One structure that has received attention is the *O*-mannosyl linked oligosaccharide Neu5Ac(α 2-3)Gal(β 1-4)GlcNAc(β 1-2)Man(α 1-O)-S/T, a class of glycan found on only a very few mammalian proteins, and apparently accounting between 30 and 50% of the total mass of α -dystroglycan. In addition, the core 1 *O*-linked structure Gal(β 1-3)GalNAc(α 1-O-S/T) is abundant, although its role in α -dystroglycan function is unclear.^{19,20–22} Other glycan structures that are derivatives of the *O*-linked mannose structure have also been identified. These include the CT carbohydrate antigen, which is restricted to the neuromuscular junction,²³ the HNK-1 carbohydrate antigen,²⁴ and the Lewis X antigen.²¹

Dystroglycan plays an important role in the organization of the actin cytoskeleton and laminins during the development of basement membranes in muscle and nonmuscle tissues.²⁵ In turn, the presence of functional laminin- α 2 is necessary to organize dystroglycan, integrin, dystrophin, and spectrin at the sarcolemma and costameres.^{26–28} Laminin- α 2 binding to ADG is mediated by the C-terminal globular LG domains (LG4-5) in the laminin molecule and the *O*-linked carbohydrate moieties in the mucin-like domain of ADG.²⁹ The carbohydrate-specific anti-ADG antibody IIH6 can competitively inhibit laminin binding, and as such its epitope is believed to represent a functional laminin binding site.³⁰

Disruption of the laminin- α 2-ADG axis, and more generally the interaction between ADG and its other ligands, has severe consequences for muscle and brain function and structure (FIG. 1A). In particular, the defects in ADG glycosylation appear to cause perturbation of the basement membrane, and this is believed to play a central role for these muscle and CNS pathologies.^{31–33} The reduction in ADG glycosylation that can be observed with antibodies,

such as IIH6 or VIA4-1 that recognize glycosylated epitopes, is now used as a key diagnostic tool in the dystroglycanopathies.³⁴

Role of dystroglycanopathy genes in ADG glycosylation

Of the six proteins identified so far that underlie the dystroglycanopathies, three are clearly involved in biosynthesis of *O*-mannosyl linked oligosaccharide: *POMT1*, *POMT2*, and *POMGnT1*.^{11,35,36} *POMT1* is essential for the first step in *O*-mannosyl glycan synthesis. *POMT2* is a second *O*-mannosyltransferase that shows an expression pattern in adults that overlaps with *POMT1*. Both *POMT1* and *POMT2* form a complex that confers the full *O*-mannosyltransferase activity.^{36,37} *POMGnT1* is a *O*-mannose β -1,2-*N*-acetylglucosaminyltransferase and catalyzes the transfer of *N*-acetylglucosamine to *O*-mannose.¹¹

The function of the remaining three genes (*Fukutin*, *FKRP* and *LARGE*) is still unclear.^{34,38–40} The only functional information available for *FKRP* suggests that it is localized predominantly in the Golgi^{41–43} or the rough endoplasmic reticulum (ER),⁴⁴ although an association with the sarcolemmal dystrophin-associated glycoprotein complex has also been recently suggested.⁴⁵ The mechanism of disease in *FKRP*-deficient patients is not entirely clear; although some authors have reported endoplasmic reticulum retention of mutant *FKRP* proteins,⁴⁶ we have not been able to confirm these findings.^{42,43} No clear information is available on the function of *fukutin*; this protein is expressed in the Golgi^{41,44} and recent data have suggested an interaction between *fukutin* and *POMGnT1*. In addition, reduced *POMGnT1* enzymatic activity in the *fukutin*-deficient mouse model supports the concept of a functional complex between these enzymes.³⁸ The *LARGE* protein, also localized in the Golgi, is unusual in that it is predicted to contain two putative catalytic domains.⁴⁷ Mutational analysis suggests that both domains are required for its biochemical function.⁴⁰ *LARGE* is ubiquitously expressed, with highest levels in heart, brain, and skeletal muscle.⁴⁸ Research from the Campbell group has shown that the N-terminal domain of ADG interacts directly with *LARGE* and this is a requirement for its normal glycosylation (FIG. 1B).⁴⁹ In fact, although the N terminal domain of ADG is cleaved in the mature protein, there is evidence that *LARGE* uses this domain to recognize dystroglycan as a substrate.⁴⁹

More recent work performed predominantly by the Campbell group⁵⁰ has clearly demonstrated that the forced overexpression of *LARGE* affects the post-translational modification of ADG by inducing the synthesis of species that are enriched in glycans recognized by the IIH6 antigen.^{40,48,50}

The *LARGE* induced hyperglycosylation of ADG is followed by increased laminin binding, in keeping with

FIG. 1. A: The glycosylation of α -dystroglycan (ADG) is heterogenous. Some carbohydrate structures (green and black circles) bind extracellular matrix molecules such as laminin, whereas others have other functions (blue circles). In patients with a dystroglycanopathy, the laminin-binding glycans are either absent or reduced. **B:** The overexpression of LARGE activates an alternative pathway to synthesize laminin-binding glycans (pink and black circles) that results in hyperglycosylation that can circumvent the genetic defects in dystroglycanopathy patients.

the notion that IIH6 recognizes a functional laminin-binding epitope.^{40,48,50} A closely related homologue of LARGE, *glycosyltransferase-like 1B (GYTL1B)* or *LARGE2*,⁴⁸ has been shown by our group and others to also induce ADG hyperglycosylation.⁴⁰ LARGE2 originated from LARGE by an ancient duplication event, but

despite their sequence similarities, the two putative enzymes differ in their pattern of expression. Although LARGE is expressed in a range of adult tissues, with highest levels in brain, heart, skeletal muscle, stomach, bladder, and pancreas, LARGE2 is virtually absent in skeletal muscle and heart, but confined to placenta, adult

and fetal kidney, pancreas, and prostate and salivary glands.⁴⁸

OVEREXPRESSING LARGE AS A THERAPEUTIC STRATEGY FOR DYSTROGLYCANOPATHIES

Transfection of wild-type adult mouse muscle with a human recombinant adenovirus that expresses *LARGE* under the control of a cytomegalovirus promoter was unexpectedly found to result in robust ADG hyperglycosylation.⁵⁰ This was apparently not associated with any detrimental muscle pathology up to 1 month after transfection. A significant further finding was the demonstration that the forced expression of *LARGE* was capable of inducing the synthesis of the IIH6 antigen also in primary cell cultures derived from patients with dystroglycanopathies in whom the *O*-mannosylation pathway is disrupted; for example, in POMT1- and POMGnT1-deficient patients.⁵⁰ This important observation indirectly confirms that *LARGE* may exert its action via the modification of N-glycans and/or mucin O-glycans, rather than on the O-mannosyl-linked sugars.⁵¹ Therefore, the data point toward the existence of dual, concentration-dependent functions of *LARGE*. At physiological concentrations, *LARGE* may be involved in the *O*-mannosylation pathway of ADG in skeletal muscle, whereas its forced expression may activate alternative glycosylation pathways of ADG. This alternative glycan mimics the *O*-mannose glycan in its ability to bind the extracellular matrix proteins associated with ADG, and therefore, it seems capable of bypassing defects in patients with a dystroglycanopathy.⁴ If this hypothesis is correct, the upregulation of *LARGE* could represent an effective therapeutic strategy for patients with dystroglycanopathies. This could be particularly valuable for patients affected with the common, relatively mild, LGMD2I variant. These patients have only a modest decrease in ADG glycosylation, and as such, they would be particularly amenable to therapeutic interventions.³⁴ Indeed, it is hoped that even a small increase in ADG glycosylation might have a tangible effect in ameliorating their disease course.

HOW TO ACHIEVE FORCED EXPRESSION OF LARGE, AND UNANSWERED QUESTIONS

The work originally performed by the Campbell group⁵⁰ used an adenoviral vector; despite the efficiency of the skeletal muscle transfection using viral vectors, there are well-known limitations of performing a bodywide gene delivery to skeletal muscle using this approach. The finding that *LARGE* is intact in the vast majority of dystroglycanopathy patients (only three patients with *LARGE* mutations have been reported so far, worldwide) may

suggest that strategies aimed at modulating the expression or the activity of *LARGE* could be a viable option. The concept is similar to that being used to upregulate fetal hemoglobin and utrophin for the treatment of thelasmia⁵² and DMD,⁵³ respectively. This could be achieved either by the identification of compounds active on the promoter of *LARGE* (and/or *LARGE2*); or with compounds that would affect the recycling of *LARGE* (and/or *LARGE2*) from the Golgi. Different possibilities are represented by the identification of pharmacological agents able to affect the transcription of *LARGE* or drugs aimed at modulating the recycling of *LARGE*, so that the concentrations of *LARGE* at the Golgi levels are increased.

For these options to become viable, the following questions need to be addressed:

1. *Is the upregulation of LARGE safe?* Data from the Campbell group⁵⁰ indicate that muscle-specific expression of *LARGE* up to 1 month is not associated with any significant pathology. However, it will be important to determine safety over longer periods of observation. For example, it will be important to assess if the addition of the IIH6 epitope could mask other functional ADG epitopes, such as HNK, CT antigen, and the Lewis X. Therefore, it will be important to assess if the forced expression of *LARGE* in tissues other than muscle is safe. This is particularly relevant to drug-induced upregulation of *LARGE* expression. To answer these questions, we are generating transgenic mice, which express a human *LARGE* transgene. Preliminary data of a transgenic line suggests that the long-term overexpression of *LARGE* is not associated with any detrimental effects (Brockington et al., in preparation).
2. *Which levels of LARGE are required to induce the synthesis of the alternative glycans on the nonmannosylated structures of ADG?* This is an important question, as it will have a bearing on the likelihood of successfully using a small molecule upregulation approach. Indeed, it is more likely that this strategy will be successful if the levels of the molecule required to synthesize the alternative glycans are not extremely elevated. To answer this question, we have generated a stably-transfected cell line system in which *LARGE* is under the control of an inducible promoter. Using this cellular system we can titrate the levels of *LARGE* upregulation and measure the effect on IIH6 synthesis (Brockington et al., in preparation).
3. *Will an already established muscle pathology respond adequately to the proposed intervention?* Dystroglycan plays a role in satellite-cell function,⁵⁴ and the congenital onset of muscle pathol-

ogy in many patients suggests that prenatal muscle formation is affected in dystroglycanopathies. Although this might not be an obstacle for conditions, such as LGMD2I, the issue of efficacy of such an approach in more severe variants needs to be assessed.

4. If *LARGE* upregulation was achieved in the CNS, would it be realistic to expect improved brain function in the CMD variants with associated central nervous system involvement? Although the structural brain defects (i.e., cobblestone lissencephalic changes)² are unlikely to be affected by any post-natal intervention, ADG plays also plays a role in synaptic function. Dystroglycan-deficient mice have severely blunted hippocampal long-term potentiation with electrophysiologic characterization indicating a role for dystroglycan in learning and memory.⁵⁵ Therefore, it is possible to hypothesize at least some effect of dystroglycan “re-glycosylation” on brain function in these patients.

Animal models of dystroglycanopathies are currently being studied by us and others to answer these very questions.

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