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A new monoclonal antibody DAG-6F4 against human alpha-dystroglycan reveals reduced core protein in some, but not all, dystroglycanopathy patients

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Abstract

We generated a novel monoclonal antibody, DAG-6F4, against alpha-dystroglycan which immunolabels the sarcolemma in human muscle biopsies. Its seven amino-acid epitope, PNQRPEL, was identified using phage-displayed peptides and is located immediately after the highly-glycosylated mucin domain of alpha-dystroglycan. On Western blots of recombinant alpha-dystroglycan, epitope accessibility was reduced, but not entirely prevented, by glycosylation. DAG-6F4 immunolabelling was markedly reduced in muscle biopsies from Duchenne muscular dystrophy patients consistent with disruption of the dystroglycan complex. In a range of dystroglycanopathy patients with reduced/altered glycosylation, staining by DAG-6F4 was often less reduced than staining by IIH6 (antibody against the glycan epitope added by LARGE and commonly used to identify glycosylated alpha-dystroglycan). Whereas IIH6 was reduced in all patients, DAG-6F4 was hardly changed in a LARGE patient, less reduced than IIH6 in limb-girdle muscular dystrophy type 2I, but as reduced as IIH6 in some congenital muscular dystrophy patients. Although absence of the LARGE-dependent laminin-binding site appears not to affect alpha-dystroglycan stability at the sarcolemma, the results suggest that further reduction in aDG glycosylation may reduce its stability. These studies suggest that DAG-6F4 may be a useful addition to the antibody repertoire for evaluating the dystroglycan complex in neuromuscular disorders.

Keywords: Alpha-dystroglycan; Beta-dystroglycan; Dystroglycanopathy; Limb-girdle muscular dystrophy; Congenital muscular dystrophy; Dystrophin; glycosylation; Monoclonal antibody; Epitope mapping; Phage-displayed peptides; Fukutin-related protein; DAG-6F4

1. Introduction

The dystrophin-glycoprotein complex (DGC) forms a critical link between the extracellular matrix and the cytoskeleton [1]. In muscle tissue, one function of this complex is to stabilise the muscle membrane and defects in one or more of its components result in various forms of muscular dystrophy [2,3]. Dystroglycan interacts both with proteins in the extracellular matrix [4–6] and with the cytoskeleton via dystrophin [7,8]. The gene for dystroglycan (*DAG1*) encodes both alpha (aDG) and beta (bDG) subunits [4], which are translated from a single mRNA as a propeptide. This is proteolytically cleaved at the Golgi into two proteins which remain associated at the membrane [6]. The primary sequence of aDG predicts a molecular mass of 72 kDa: however, due to extensive post-translational glycosylation, the apparent mass on SDS-PAGE gels is around 156 kDa in skeletal muscle [9]. Extensive O-linked mannosylation of the central mucin domain of aDG mediates binding to basement membrane proteins, including the laminin alpha 2 chain [5], perlecan [10], agrin [11–13], neurexin in the brain [14], pikachurin in the eye [15] and Slit [16] by interaction with laminin LG (laminin globular) domain [17,18]. Defects in the O-glycosylation of aDG are considered to be central to the pathogenesis of a subgroup of limb-girdle and congenital muscular dystrophies (LGMDs and CMDs), the dystroglycanopathies [18–22]. The phenotypic

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severity of the dystroglycanopathies ranges from the severe Walker–Warburg syndrome and muscle–eye–brain disease, to the milder LGMD phenotypes [23].

A considerable restriction to research and diagnostics in this area is the availability of antibodies that can detect aDG reliably. Currently there are two commercial antibodies which have been widely used in a clinical setting, IIH6 and VIA4-1, both of which recognise glycosylated epitopes; IIH6 is thought to recognise the laminin-binding site on aDG [5] and has been used extensively in both research and clinical settings to demonstrate reduced glycosylation of aDG [24,25]. VIA4-1 recognises a glycosylated epitope close to that of IIH6 but cannot functionally block laminin binding [26]. Both antibodies require glycosylation of aDG by LARGE (Like-acetylglucosaminyltransferase) [27]. A reduction or absence of immunolabelling with either of these antibodies in skeletal muscle biopsies is a characteristic feature of the dystroglycanopathy phenotype. Here we describe the properties of a new panel of monoclonal antibodies against core aDG protein.

2. Materials and methods

2.1. Generation of recombinant fragments of aDG

Six recombinant proteins were created from the human DAG1 (NM_004393) cDNA clone (Origene) by PCR cloning using the following primer pairs with restriction sites in bold and underlined:

aDGB-1 -aa312-485
For 5′-GGATCC**GAATTC**AGGCAGATCCATGCTAC Rev 5′-GATG**AAGCTT**GGTGGTGGTGCGAATACG
aDGB-2 -aa501-653
For 5′-GCTATC**GGATCC**AAGAACCATATTGACAGG Rev 5′-GATC**CTCGAG**GGTGATATTCTGCAGGG
aDGB-3 -aa505-600
For 5′-GATATC**GGATCC**GACAGGGTAGATGCCTG Rev 5′-GTA**CTCGAG**GCGCCTGTGGACGTG
Full-length aDGB-5 -aa312-653
For 5′-GGATCC**GAATTC**AGGCAGATCCATGCTAC Rev 5′-GATC**CTCGAG**GGTGATATTCTGCAGGG
aDGB-6 -aa478-543
For 5′-GGATCC**GAATTC**ACTCGTATTCGCACCA Rev 5′-GATG**AAGCTT**CTGCTCCCGCAGTTTCA
aDGB-7 -aa445-511
For 5′-GGATAT**GAATTC**ACGACTCGCAGGCCA Rev 5′-GATG**AAGCTT**GCCAACCCAGGCATCTAG

The PCR products were cloned into pET32a after digestion with EcoRI, BamH1, HindIII or XhoI and the constructs were sequenced to ensure the absence of PCR errors. The pET32a constructs were expressed as thioredoxin fusion proteins in *E. coli* BL21(DE3) by induction with IPTG.

Recombinant proteins DGB-1, -2, -3 and -5 were extracted from inclusion bodies by sequential extraction with increasing concentrations of urea in PBS (0, 2, 4, 6 and 8M). aDGB-1 and -5 were further purified using His-tag columns. Protein concentration was determined by comparison with a BSA loading standard on Coomasie-Blue stained gels. aDGB-6 and -7 were not purified, but used as bacterial extracts for mapping purposes.

2.2. Generation of recombinant glycosylated aDG

DG2L8 protein was purified from a stable HEK293T cell line expressing both murine aDG (aa29-651) cloned into pFUSE-mIgG2A-Fc2, and human LARGE cloned into pCDNA3.1 V5/His (Harrison et al, 2012). The vector adds a human Fc tag. hDG5 protein differs in expressing a truncated form of the human protein (aa29-453). Culture conditions are described elsewhere [28]. For purification, culture medium containing recombinant glycosylated protein was concentrated 20-fold using Vivaspin column concentrators (Vivascience, Hannover) and incubated overnight with protein A-agarose (Invitrogen) at 4 °C. Beads were washed with 1.5 M glycine, 3 M NaCl, pH 9.0, before bound protein was eluted with 0.2 M glycine–HCl, pH 2.8, and neutralised by addition of 1 M Tris–HCl, pH 9.0. For DG-K500-myc^{GFP} protein, murine dystroglycan cDNA (Accession No. X86073) was cloned into the pEGFPN1 Vector (Clontech, USA) and a 10 amino acid myc epitope (EQKLISEEDL) inserted into position K500 [29]. This construct was transiently transfected by electroporation into COS-7 cells for immunofluorescence. For Western blot, EBNA293 cells transiently transfected with DG-K500-myc^{GFP} were lysed with PBS containing 1% Triton X-100 and protease inhibitors (Roche, Switzerland) and centrifuged at 10,000g for 10 min at 4 °C. 1 mg of the supernatant protein extract was incubated with 50 µl magnetic beads conjugated with an anti-myc antibody (Miltenyi Biotec., Germany) for 30 min at room temperature. The adsorbed protein was eluted in 50 µl of SDS sample buffer.

2.3. WGA-enrichment of normal human muscle

Fresh muscle samples were obtained with informed consent and ethical approval from patients with no known muscle disorder undergoing orthopaedic surgery. They were snap frozen in liquid nitrogen and ground to a fine powder. Muscle samples were incubated with protease inhibitors (Sigma, P8340) in a RIPA buffer without SDS (0.5% sodium deoxycholate, 2% NP40 (IGEPAL CA-630), 2 mM EDTA, 300 mM NaCl, 100 mM Tris–HCl, pH 7.4) for 60 minutes at 4 °C. After centrifugation at 10,000g for 10 minutes at 4 °C, the supernatant was added to WGA agarose beads (Vector Labs, Burlingame, CA) and incubated overnight with agitation at 4 °C. Beads were washed 3× with the same RIPA buffer by centrifugation and the WGA-bound protein was eluted with 0.3M N-acetyl-D-glucosamine in 0.5M NaCl–0.05M Tris–HCl pH 7.5 containing Sigma protease inhibitors.

2.4. Production of monoclonal antibodies

BALB/c mice were immunised with bacterial recombinant full-length aDG (fragment 5) purified on a His-Tag column. Monoclonal antibody production was performed as previously described [30]. Spleen cells from hyper-immune BALB/c mice were fused with the Sp2/O mouse myeloma cell line using