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α -Dystroglycan hypoglycosylation affects cell migration by influencing β -dystroglycan membrane clustering and filopodia length: a multiscale confocal microscopy analysis.

V. Palmieri^{1#}, M. Bozzi^{2#}, G. Signorino², M. Papi¹, M. De Spirito¹, A. Brancaccio^{3,4}, G. Maulucci^{1*}, F. Sciandra^{3*}

¹*Istituto di Fisica, Università Cattolica del Sacro Cuore, Rome Italy*

²*Istituto di Biochimica e Biochimica Clinica Università Cattolica del Sacro Cuore, Rome Italy*

³*Istituto di Chimica del Riconoscimento Molecolare (ICRM) - CNR c/o Università Cattolica del Sacro Cuore, Rome Italy*

⁴*School of Biochemistry, University of Bristol, Biomedical Sciences Building, Bristol (UK)*

These authors contributed equally to this work

* Corresponding and senior authors

Abstract

Dystroglycan (DG) serves as an adhesion complex linking the actin cytoskeleton to the extracellular matrix. DG is encoded by a single gene as a precursor, which is constitutively cleaved to form the α - and β -DG subunits. α -DG is a peripheral protein characterized by an extensive glycosylation that is essential to bind laminin and other extracellular matrix proteins, while β -DG binds the cytoskeleton proteins. The functional properties of DG depend on the correct glycosylation of α -DG and on the cross-talk between the two subunits. A reduction of α -DG glycosylation has been observed in muscular dystrophy and cancer while the inhibition of the interaction between α - and β -DG is associated to aberrant post-translational processing of the complex. Here we used confocal microscopy based techniques to get insights into the influence of α -DG glycosylation on the functional properties of the β -DG, and its effects on cell migration. We used epithelial cells transfected with wild-type and with a mutated DG harboring the mutation T190M that has been recently associated to dystroglycanopathy. We found that α -DG hypoglycosylation, together with an increased protein instability, reduces the membrane dynamics of the β -subunit and its clustering within the actin-rich domains, influencing cell migration and spontaneous cell movement. These results contribute to give novel insights into the involvement of aberrant glycosylation of DG in the developing of muscular dystrophy and tumor metastasis.

Keywords: Dystroglycan, dystroglycanopathies, extracellular matrix, cytoskeleton, glycosylation, fluorescence recovery after photobleaching, confocal microscopy

Abbreviations: FRAP, fluorescence recovery after photobleaching; DG, Dystroglycan; ERK, extracellular-signal-related kinase; MAPK, mitogen-activated protein kinase; LARGE,

acetylglucosaminyltransferase-like; ECM, extracellular matrix; GFP, Green Fluorescent protein; ICQ, intensity correlation quotient; M2, Manders fractional index; DMEM, Dulbecco Modified Eagle Medium.

1. Introduction

Dystroglycan (DG) is a ubiquitously expressed cell adhesion complex composed by two interacting subunits, α - and β -DG, raised from a post-transductional cleavage of a single precursor. α -DG is a peripheral protein characterized by a dumbbell-structure formed by two globular N- and C-terminal domains separated by a mucin like region rich in N- and O-glycans and O-mannosyl glycans [1]. α -DG interacts with the extracellular matrix (ECM) components laminin, agrin, perlecan, and neurexin and retains the contact with the plasma membrane interacting non-covalently with the N-terminal region of β -DG, a transmembrane protein [2]. The C-terminal domain of β -DG is a short unfolded cytoplasmatic tail that binds dystrophin and utrophin, which in turn bind to the actin cytoskeleton (Fig. 1A). DG represents a bridge between the ECM and the cytoskeleton and plays a variety of functions during morphogenesis and in adult tissues [2]. Indeed, DG has a crucial role in maintaining muscle integrity during the continuous contraction-relaxation cycles [3, 4]. DG is also implicated in the structure and function of the central nervous system [5], in the myelination of peripheral nerves [6], in the epithelial morphogenesis and cell polarization [7-9].

The functional properties of DG largely depend on the extensive glycosylation of the α -subunit and on the correct cross-talk between the two subunits. In fact, correctly glycosylated α -DG interacts with high affinity with the laminin globular (LG)-containing ECM molecules providing the cell adhesion and the transduction of signals through the extracellular region of β -DG from outside to inside [10]. Inside the cell, β -DG serves as a scaffold for various proteins involved in signaling transduction, such as the adapter protein Gbr2 and the kinases ERK (extracellular-signal-related kinase) and MAPK (mitogen-activated protein kinase) [11, 12] (Fig. 1A). Cell adhesion to laminin can induce the tyrosine phosphorylation of β -DG followed by a loss of association with dystrophin or utrophin modulating the cytoskeletal architecture [13]. In particular, the β -DG interaction with the cytoskeleton occurs specifically at the filopodia, the actin-rich structures that function as cell sensors of the local microenvironment during cell adhesion, cell migration, cell morphology and polarity [14].

Given the importance of carbohydrates residues for the α -DG functional properties, aberrant glycosylation of α -DG is a hallmark of a group of neuromuscular diseases collectively termed dystroglycanopathies characterized by different phenotypic severities that range from the most devastating in Walker-Warburg syndrome to the less severe and late-onset in limb-girdle muscular dystrophies. Several

genes have been identified to be responsible for dystroglycanopathies that encode for enzymes and glycosyltransferases involved in the addition and modification of O-mannosyl glycans within the mucin-like region of α -DG [15]. In the most severe forms of dystroglycanopathies, also the peripheral and the central nervous system are compromised. The hypoglycosylation of α -DG results in aberrant neurons migration, brain malformations and peripheral dysmyelination, features that are recapitulated by the brain-specific and peripheral nervous system-specific knock-out mice [5, 6]. Recently, the first missense mutation (T192M) within the DG gene had been identified in a patient affected by a mild form of dystroglycanopathy associated to cognitive impairment [16]. The mutation hits an O-mannosyl-glycosylation site located within the N-terminal domain of α -DG and, influencing the overall flexibility and stability of the protein domain [17], perturbs the interaction between α -DG and LARGE, a putative glycosyltransferase which participates in post-phosphoryl-glycosylation of α -DG [16]. Consequently, the missense mutation severely reduces α -DG glycosylation and its ability to bind to laminin [16]. The functional modification of the mutant α -DG was evaluated in cultured myoblasts by Western blotting with an antibody (IIH6) that recognizes the glycosylated form of α -DG and by laminin-overlay assay, showing a band-shift of the mutant α -DG compared to the wild-type [16]. A *knock-in* mouse, harboring the mutation T190M, the murine counterpart of the human mutation, recapitulated the muscular dystrophy phenotype observed in the patient as a consequence of impaired α -DG post-translational modification [16]. Possibly, similar defects in α -DG glycosylation have been reported in tumors of epithelial origin, including breast, colon, cervix, and prostate cancers [18, 19]. In fact, the aberrant α -DG glycosylation leads to the disruption of the ECM-cytoskeleton interactions and consequently to the loss of cell adhesion and polarity thus favoring migration and invasiveness.

The binding between laminin and α -DG triggers the essential signals to the cell for the reorganization of cortical cytoskeletal components [20]. The interaction between the two DG subunits constitutes a control point for the modulation of these DG ligand-binding properties [21, 22]. In fact, the expression of an uncleavable mutant is associated to α -DG hyperglycosylation and muscular dystrophy in transgenic mice [22, 23]. Although α -DG glycosylation levels are known to be important to connect the ECM to intracellular actin and to elicit intracellular signal transduction, the influence of α -DG glycosylation on the stability and functional properties of the β -subunit is poorly investigated. Confocal fluorescent imaging, unraveling biological mechanisms at a multiscale level, allows establishing correlations and casual relationships between protein dynamics at the submicrometric scale and large scale phenotypic changes occurring at the micrometric scale. In this context, using epithelial cells transfected with wild-type DG and the hypoglycosylated DG mutant, namely DG^{T190M} [16], we investigated the influence played by the level of α -DG glycosylation on 1) β -DG clustering in actin-rich domains by image

analysis and colocalization techniques; 2) β -DG membrane dynamics by means of fluorescence recovery after photobleaching (FRAP) at a molecular level, and 3) cell migration by cell tracking techniques.

2. Materials and Methods

2.1 DNA manipulations

The full-length cDNA encoding for the murine DAG1 was cloned in the pEGFP vector as described elsewhere [24] and the point mutation T190M was introduced using the QuikChange site-directed mutagenesis kit (Stratagene, Cedar Creek, TX, USA) and the following primers:

Forward: 5'-CCAGTGACTGTCCTTATGGTGATTCTGGATGCT-3'

Reverse: 5'-AGCATCCAGAATCACCCATAAGGACAGTCACTGG-3'

Both constructs also contain a myc-tag inserted within the C-terminal domain of α -DG [24].

The mutation was verified by direct DNA sequencing.

2.2 Cell culture, transfection and Western blot

293-Ebna cells were cultured in glass bottom dishes (Ibidi) for 24 h in DMEM supplemented with antibiotics and 10 % (v/v) fetal calf serum. Cells were also cultured on 10nM laminin-111 coated dishes (Sigma). Cells were transfected with 2 μ g of DG^{WT} and DG^{T190M} constructs using the calcium phosphate method as described elsewhere [24]. After 24 h, 1 μ g/ml of Brefeldin A was added to the medium and after 1h transfected cells were analysed for cell-tracking or FRAP.

24h after transfection, cells were collected and lysed in lysis buffer (PBS, 1% Triton-X100) containing a proteases inhibitors cocktail (Roche). 20 μ g of total protein extracts were separated in SDS-PAGE using a 4-15% gradient gel. For western blots, proteins were then transferred to nitrocellulose and probed with different primary antibodies: anti-myc-HRP (Miltenyi Biotec, diluted 1:5000), monoclonal anti α -DG I1H6 (Millipore, diluted 1:100) and monoclonal anti- β -DG 43-DAG (Leica Biosystem, diluted 1:50). After several washes, nitrocellulose membranes were probed with anti-mouse secondary antibodies and the blots were then developed using the luminol-based ECL system.

2.3 α -DG and actin staining and Spinning Disk Confocal Microscopy

24h after transfection, transfected cells were fixed with 4% paraformaldehyde, blocked with PBS 1% BSA and incubated with mouse anti-myc antibody (Sigma) diluted in PBS for 1h. Secondary goat anti-mouse antibody conjugated to Alexa-Fluor647 (Life Technologies) was then applied at a 1:400 dilution. After several washes in PBS, the cells were finally mounted in Vectashield containing DAPI (Vector Laboratories) for nuclear counterstain. For actin staining, fixed cells were permeabilized in PBS containing

0.1 % Triton-X100 for 10 min followed by incubation with rhodamine-conjugated phalloidin (Life Technologies). After several washes with PBS, cells were mounted and imaged with a multichannel white light source with DAPI, GFP or Rhodamine filter settings on a CARV II Spinning-Disk Microscope (Crisel Instruments, Rome, Italy) by using a 60X oil immersion objective. Z-stacks have been acquired for each cell. Background values were subtracted with ImageJ software (NIH) as previously reported [25]. Cell protrusions analysis was performed on Z projections of each cell by using Filodetect, a software for detecting, counting and measuring the length of filopodia [26]. The number of filopodia per cell has been normalized to the cell area to account for different dimensions and/or spreading of cells. Finally, the parameters that have been analysed were the Number of Filopodia per cell area (N_F) and the Filopodia length (F_L). At least 20 cells per sample were analyzed.

2.4 Colocalization analysis

Colocalization of DG with actin was quantified by using intensity correlation quotient (ICQ) [27] and Manders fractional index M_2 , which gives the fraction of β -DG which colocalizes with actin [28].

Manders' Colocalization Coefficients (MCC) metrics are widely used in biological microscopy. For two probes, denoted as R and G , M_2 represents the fraction of G in compartments containing R . This coefficient is calculated as:

$$M_2 = \frac{\sum_i G_{i,colocal}}{\sum_i G_i}$$

where $G_{i,colocal} = G_i$ if $R_i > 0$ and $G_{i,colocal} = 0$ if $R_i = 0$

Analysis was performed by the ImageJ (NIH) plugin 'Coloc2', by strictly following preprocessing guidelines found in [28].

2.5 Fluorescence Recovery After Photobleaching (FRAP)

FRAP experiments were performed on a Confocal Microscope (Leica SP2, Leica Microsystems, Germany) using a 63X oil immersion objective (NA 1.4). Cells were kept at 37°C at 5% CO₂ in a stage incubator (OKOLAB, Italy). Cells were excited at 476 nm wavelength with an Ar/Kr laser and emission was recorded in the range 500-550 nm. Bleaching was performed with a circular spot using the same excitation/emission settings. Fluorescence recovery was monitored in the bleached region, and the whole cell at 3.265 seconds time intervals, over a period of at least 150 seconds. Acquisitions at longer time points were performed to check the stability of the plateau of the recovery curve. 10 separate FRAPs were performed and then averaged to generate a single FRAP curve for each sample. To enable a correct normalization of the data, cells with comparable GFP intensity were selected for FRAP experiments.

Normalization of curves has been performed as explained elsewhere [29]. Each fluorescence signal acquired in the bleached region ($F_{\text{bleach}[t]}$) has been normalized for background fluorescence ($F_{\text{bgd}[t]}$) and unbleached membrane ($F_{\text{ctr}[t]}$) to obtain the normalized Recovery Curve $R_{\text{norm}[t]}$

$$R_{\text{norm}[t]} = \frac{(F_{\text{bleach}[t]} - F_{\text{bgd}[t]})}{(F_{\text{ctr}[t]} - F_{\text{bgd}[t]})}. \quad [1]$$

In order to superimpose recovery curves from different experiments, a further correction to set postbleach intensities (F_0) to 0 has been done by calculating the fractional fluorescence recovery curve (R):

$$R = \frac{R_{\text{norm}[t]} - F_0}{1 - F_0}. \quad [2]$$

We analyzed the recovery in terms of single-class of binding sites described by the following chemical equation [30]:



Where β_{free} represents unbound β -DG-GFP (free proteins), α_{free} represents specific β -DG-GFP binding sites of α -DG, $\alpha\beta$ represents bound α/β -DG-GFP complexes [FS], and k_{on} and k_{off} are the on- and off-rates, respectively (Fig.4A).

We assumed that i) the system had reached the equilibrium before photobleaching and ii) α -DG belongs to a large extracellular complex relatively immobile, at least in the time scale of FRAP experiments. Moreover, since diffusion is very fast compared both to binding and to the timescale of the FRAP measurement (see Supplemental Fig. S1), free molecules instantly equilibrate after the bleach, so that diffusion is not detected in the FRAP recovery. This particular scenario is known as reaction-dominant model [30], and FRAP curves can be fitted by using Origin Software (Microcal) with the following exponential equation:

$$R = (1 - r)(1 - C_{\text{eq}}e^{-k_{\text{off}}t}) \quad [4]$$

where r is the immobile fraction, C_{eq} is the fraction of bound molecules, k_{off} is the unbinding rate constant and t is time.

The association constant K_a has been derived as follows [29]

$$K_a = \frac{C_{\text{eq}}}{1 - C_{\text{eq}}}. \quad [5]$$

2.6 Cell-tracking analysis

Cell tracking experiments were performed as previously described [31, 32]. Briefly, 293-Ebna cells were cultured on glass bottom dishes (Ibidi GmbH) in the presence or absence of laminin. Displacement of transfected cells was followed by exciting DG-GFP at $\lambda=476$ nm and recording emission between 500 and 600 nm. Migration was evaluated and analysed by using the ImageJ software plugin ‘Particle Tracker’

and 'Chemotaxis and migration tool'. On average 40 cells were tracked per experiment over a time period of 45 min. The time interval between consecutive frames was 2 min.

2.7 Statistical analysis

For all experiments mean \pm SD values were determined (samples from n=20 to 50) and utilized for two-tailed Student's t-test analysis. Values of $p < 0.05$ were considered significant.

3 Results

3.1 Characterization of the hypoglycosylated DG mutant T190M expressed in 293-Ebna cells

To study the effects of the glycosylation of α -DG on β -DG membrane dynamics and actin-rich filopodia formation in epithelial cells, we compared wild type DG (DG^{WT}) with a hypoglycosylated mutant, the DG^{T190M}, which has been recently associated to a primary dystroglycanopathy [16]

As epithelial cell reference system, we used the 293-Ebna line, which is derived from human embryonic kidney. Cells were transfected with full-length DG^{WT} and DG^{T190M} constructs respectively, cloned in the pEGFP-N1 vector, which allows expressing DG with a green fluorescent protein (GFP) fused at the C-terminus of β -DG (Fig. 1A). Moreover, a myc-tag was inserted after K498, within the C-terminal domain of α -DG to better visualize the α -subunit expressed in transfected cells by Western-blot and immunofluorescence [24] (Fig. 1A).

The endogenous α -DG expressed in 293-Ebna cells is characterized by a molecular mass of about 120 kDa and in Western-blot appears as a broad band when stained with IIH6 antibody, which recognized its glycosylated modifications (Fig. 1B). The α -DG^{WT} expressed in transfected 293-Ebna cells was under the recognition sensitivity of the IIH6 antibody however, when detected with an antibody directed against the myc-tag, showed a similar band patterns as the endogenous protein indicating the correct glycosylation of the overexpressed DG (Fig. 1B). In Western-blot the mutated α -subunit showed a clear band-shift compared to DG^{WT} due to its reduced glycosylation (Fig. 1B); moreover, DG^{T190M} was correctly cleaved in α and β subunits (Fig. 1B) [16]. The transfection lead to a stronger expression of the DG constructs than the endogenous DG (Fig. 1B).

Confocal microscopy analysis of transfected 293-Ebna cells did not show any relevant differences between the DG^{WT} and DG^{T190M}. In fact, the α - and β -subunits were properly targeted at the plasma membrane and along filopodial protrusions (Fig. 2A). Some spots throughout the cytoplasm indicated active GFP-tagged proteins synthesis.

3.2 α -DG hypoglycosylation reduces filopodia elongation and β -DG clustering within the filopodia

To better address the role of the α -DG glycosylation on the cytoskeleton, we first considered the induction of filopodia in cells transfected with DG^{WT} and DG^{T190M} in the presence or in the absence of laminin. In fact, β -DG has the ability to interact directly with F-actin at the level of the filopodia, which represent actin rich structures similar to spikes that are important for cell adhesion and migration and that are induced by DG expression [14, 33, 34] (Fig. 2A). To characterize potential differences in the induction of filopodia in cells expressing wild-type and hypoglycosylated α -DG, the number of filopodia per cell area (N_F) (Fig. 2B) and filopodia length (F_L) (Fig. 2C) were assessed. While no significant differences in the N_F were visible between DG^{WT} and DG^{T190M} when cells were cultured both on glass and on laminin, F_L was significantly higher in cells transfected with DG^{WT} and cultured on laminin (+ 16%). To monitor variations in the spatial distributions of DG^{WT} and DG^{T190M} in relation with actin distribution in the presence or in the absence of laminin, colocalization of DG within actin-rich domains and filopodia has been evaluated by means of simultaneous confocal imaging of DG-GFP constructs and actin. As expected, actin (pseudocoloured in red in Fig. 3A) was mainly localized throughout the cell cortex, also known as the actin cortex or actomyosin cortex, a specialized layer of cytoplasmatic proteins located on the inner face of the cell periphery [35]. The distribution of actin within the cortex was not homogeneous, since it was mainly clustered in some regions and in filopodia. Colocalization of DG constructs with actin was present both when the cell are grown on laminin or on glass (Fig. 3A).

To establish quantitative differences, a colocalization analysis of DG and actin along the filopodia by using intensity correlation quotient (ICQ) was performed [27]. The ICQ is a widely used dimensionless index varying from 0.5 (co-localisation) to -0.5 (exclusion), while random staining and images impeded by noise give a value close to zero [27]. While no significant differences in the ICQ were visible between DG^{WT} cultured on glass and DG^{T190M} when cells were cultured on glass or laminin (0.16 ± 0.04), ICQ was significantly higher in cells transfected with DG^{WT} cultured on laminin (0.19 ± 0.04) (Fig.3B). To discern if this higher localization was induced simply by the overall higher DG^{WT} expression or indeed by a specific clusterization process, the Manders fractional index M_2 , which gives the fraction of β -DG which colocalizes with actin [28], was retrieved. Indeed M_2 was significantly higher in cells transfected with DG^{WT} cultured on laminin, indicating a clustering of this protein in filopodia and actin-rich structures (Fig.3C) (0.563 ± 0.101 with laminin, 0.454 ± 0.059 without laminin).

3.3 α -DG Glycosylation influences β -DG binding to α -DG and actin

The influence of α -DG glycosylation on β -DG lateral membrane mobility was investigated using fluorescence recovery after photobleaching (FRAP) on live cells transfected with wild-type and mutated DG, in the presence or in the absence of laminin substrate [36]. β -DG is involved in two kind of interactions (Fig. 4A): inside the cells, β -DG forms an immobile cluster with actin cytoskeleton (R fraction);

extracellularly, β -DG interacts with α -DG, which in turn binds the ECM. FRAP experiments unable us to monitor the exchange between unbound and α -DG-bound β -DG.

In Fig. 4B, representative images of a typical FRAP experiment of cells transfected with DG^{WT} and DG^{T190M} are reported, in which the pre-bleach, the post bleach and the final recovery phases are shown. In Fig. 4C and 4D, FRAP curves of the β -subunit of respectively DG^{WT} and DG^{T190M} in the absence (black squares) and in the presence of laminin substrate (red squares) are reported. A pure-diffusion regime interpretation for these data has been excluded because in such a case the recovery of the DG, calculated on the expected mass of the protein (Supplementary Figure S1), would have been much faster with respect to the observed timescale of FRAP recovery in our experimental curves. Therefore, to analyze our FRAP experiments we used equation (4) which provided consistent fits for all curves (Fig. 4). From the curves reported in Fig. 4C- D, a main recovery process is visible, as well as the presence of an immobile fraction (R) of fluorescent molecules [36]. In our assumption, the immobile fraction (R) of the β -subunit of DG represents the fraction of protein clustered in filopodia and actin-rich domain. The presence of laminin caused a 75% increase of the immobile fraction R of the DG^{WT} compared to the β -DG expressed in cells grown on uncoated plates (Fig. 4E). Conversely, laminin did not influence the membrane dynamics of the β -subunit of DG^{T190M} (0.28 ± 0.08) (Fig. 4E). However, the immobile fraction of the β -subunit is higher in cells transfected with DG^{T190M} compared to the cells expressing the DG^{WT} and grown in the absence of laminin (0.19 ± 0.06) (Fig.4E).

Using the Eq. 5 we derived the K_a for the association between α and β -DG (Fig. 4F). When the glycosylated α -subunit of DG^{WT} is bound to laminin, the affinity for β -DG (6.47 ± 2.29) is 18% higher than the one measured when the cells were grown on uncoated plates (5.49 ± 0.62), thus stabilizing the α/β -DG interaction. Conversely, laminin has a less evident effect on the interaction between the mutated α -DG and the β -subunit (4.59 ± 0.99) (Fig.4F).

The assumption that R represents the fraction of protein associated within filopodia and actin-rich domains is enforced by its linear relationship with M_2 (see section 3.4), which is an indicator of β -DG clustering in these actin domains (Fig.4G).

3.4 Glycosylation of DG affects cells migration on laminin substrate

Results of cell tracking experiments performed in the presence or in the absence of laminin substrate are shown in Fig.5. In Fig.5A displacements of cells are shown in a two dimensional spatial plot. In Fig. 5B recovered speed values from the maps are reported. The absence of laminin induced an overall decrease of cell speed, in particular the decrease in cell speed is -31% for β -DG^{WT} ($p < 0.0001$) and -25% for β -DG^{T190M} cells ($p = 0.024$). On laminin substrate, compared to β -DG^{WT}, β -DG^{T190M} cells displayed a marked reduction of cell migration (-55% in respect to β -DG^{WT} $p < 0.0001$) with the respect of wild-type

construct, indicating that the mutation associated with muscular dystrophy impaired the cell migration properties influencing its interaction with laminin.

Discussion

DG is translated as a single precursor and processed into α - and β -subunit by a proteolytic cleavage. The mature and highly glycosylated α -DG binds to the ECM molecules, including laminin, neurexin, and agrin, and remains associated to the plasma membrane interacting with the transmembrane β -subunit that in turn binds actin. The non-covalent interaction between α and β -DG is crucial for the proper post-translational maturation and functions of DG [37]. Indeed, the expression of an uncleavable mutant is associated to α -DG hyperglycosylation and muscular dystrophy in transgenic mice [22, 23]. The influence of α -DG glycosylation in modulating the cross-talk with the β -subunit and the functional properties of β -DG is still largely unknown. In particular, β -DG plays an important role in the dynamic of the actin cytoskeleton, recruiting ezrin and the regulatory elements of the Rho GTPase signaling pathway to the cell membrane, thereby helping the formation of filopodia and microvilli that are membrane protrusions important for adhesion [10, 12, 14, 38, 39]. Here, we have analyzed the influence of α -DG glycosylation on the stability of the β -subunit, and on the ability of β -DG to modulate filopodia formation and ultimately cell migration in 293-Ebna epithelial cells transfected with wild-type and the hypoglycosylated DG harboring the mutation T190M, the first primary defect identified within the DG gene associated to muscular dystrophy [16].

The heterologous cell system we used provides a simplified system to study the functional properties of β -DG. In fact, while in muscle cells, DG is the integral part of the dystrophin-glycoprotein complex (DGC), a group of peripheral, membrane and cytoskeletal proteins that links dystrophin to ECM, in human fetal kidney epithelial 293-Ebna cells, sarcoglycans and sarcospan are not present and in addition, α -DG displays a reduced glycosylation level [40]. In 293-Ebna cells, sarcoglycans are limited to the ϵ -sarcoglycan that does not directly interact with β -DG [40].

We found that in 293-Ebna cells, the number of filopodia is independent from the presence of laminin, as already observed in oligodendrocytes and fibroblasts [14, 38] (Fig. 2A). Indeed, cells transfected with the hypoglycosylated DG produced the same number of membrane protrusions as the cells transfected with the DG^{WT} (Fig.2B). However, we found that α -DG glycosylation has a significant effect on the filopodia length, which is increased in cells grown in the presence of laminin and transfected with DG^{WT} with respect to cell grown in the absence of laminin and to cells transfected with DG^{T190M} (+16%) (Fig. 2C). This is an indication that the interaction between α -DG and laminin can trigger an increased clustering of the β -subunit on actin domains, directing actin remodeling and potentiating membrane protrusions. Accordingly,

in cells transfected with DG^{T190M} the β -DG clustering is significantly reduced compared to the cells transfected with DG^{WT} (Fig. 3 A, B and C). These results are in agreement with, and expand upon, that of Colognato et al. [20] and Cohen et al. [41] who showed that the interaction between α -DG and laminin induces the polymerization of the laminin/DG network on cell surfaces and a reorganization of the cortical cytoskeleton elements.

This scenario was further supported by FRAP experiments (Fig. 4 B and C). Indeed, laminin strongly increases the immobile fraction of β -DG when the β -subunit is interacting with a fully glycosylated α -DG (+75%) (Fig. 4D). Therefore, the binding between laminin and α -DG strongly influenced the fraction of the β -subunit clustered within the actin domains and not available for the exchange within the bleached area. Conversely, when β -DG interacts with the aberrant glycosylated α -DG, its membrane clustering is not influenced by the presence of laminin (Fig. 4D). Unexpectedly, the immobile fraction R of the β -subunit expressed in cells transfected with DG^{T190M} is higher (0.28 ± 0.08) compared to that of cells grown in the absence of laminin and transfected with DG^{WT} (0.19 ± 0.06). On the basis of the crystallographic studies, revealing a slight lower structural stability of the α -DG N-terminal domain carrying the point mutation T190M with respect to its wild-type counterpart [17], it can be argued that the DG^{T190M} may undergo to a partial aggregation, that accounts for its higher immobile fraction R (Fig. 4D). However, the induction of β -DG-clustering is suppressed in response to laminin: the mutated α -DG is not able to influence, throughout its direct interaction, the β -DG binding with the cytoskeleton (Fig. 3B, C and Fig. 4). The linear relationship between the immobile fraction R of β -DG and the colocalization with actin (Fig.4G) reinforces the notion that unclustered DG^{WT} can be trapped in actin-rich clusters in response to laminin [41]. Conversely, our data indicate that aberrant glycosylation of α -DG not only influences its laminin binding but also the functional plasticity of the β -subunit.

We had already shown, using *in vitro* binding assays between recombinant α - and β -DG proteins, that the non-covalent interaction between α - and β -DG is not directly dependent on α -DG glycosylation [37]. This observation was indirectly confirmed by the analysis of skeletal muscle biopsies of patients affected by dystroglycanopathies in which the hypoglycosylated α -DG still localized at the sarcolemma [42]. Indeed, here we showed that the hypoglycosylation of α -DG only slightly weakens the interaction between the two DG subunits compared to the DG^{WT} (5.49 ± 0.62 and 6.47 ± 2.29 , respectively) whereas it influences the signals transduction through the DG complex. (Fig. 4F). In fact, the hypoglycosylation, together with the intrinsic structural instability of α -DG^{T190M} [17], reducing the capability of laminin to modulate the β -DG clustering, cannot accomplish the filopodia elongation process (Fig. 2 and Fig. 3).

We have finally shown that one of the direct effects of the α -DG glycosylation-induced β -subunit clustering and cytoskeletal reorganization is the modulation of cell migration on laminin substrate. Representative displacements of cells are shown in Fig. 5A. It is evident how the area fraction covered by

the displacement of cells overexpressing DG^{WT} is definitely higher with respect to cells transfected with mutant DG. Moreover, cells overexpressing DG^{WT} showed an increased speed on laminin compared to cells grown on glass and to cells transfected with DG^{T190M}. (Fig. 5B). These results are in line with previous works that show that DG overexpression modulated the ability of cells to spread on laminin substrate [43], and conversely inhibition of DG function decreased cell attachment [44]. The speed of spontaneous cell movement is in direct correlation with the K_a for the association between α and β -DG (fig.5C), reinforcing the observation that glycosylation of the α -subunit modulates cells migration, not only via the interaction with laminin, but also throughout the actin cytoskeleton remodeling induced by the cross-talk with the β -subunit.

The outlined connection between glycosylation of α -DG and the cytoskeletal rearrangements driven by the β -subunit, and its effect on cell migration, may have therefore a central role in dystroglycanopathies: abnormal glycosylation of α -DG is associated to muscular dystrophy and central nervous system defects due to its inability to bind not only laminin but also agrin and neurexin [15]. In particular, the T190M patient is affected by a relatively mild limb-girdle muscular dystrophy probably because the mutated α -DG still displays a residual laminin-binding capacity (Fig. 2) [16]. However, the increased instability of the mutated α -subunit negatively influences this interaction that is apparently too weak to promote the formation of stable DG/actin clusters (Fig. 3). Moreover, the severe cognitive impairment observed in the patient is not accompanied by the structural abnormalities in the brain characterizing the most severe dystroglycanopathies such as disarray of cerebral cortical layering and aberrant migration of granule cells [5]. Accordingly, our results suggest that the DG^{T190M} is still able to form cellular protrusions that are important for the cell migration and morphological differentiation (Fig. 2B). However, on the basis of our results, it can be hypothesized that the hypoglycosylated α -DG would be unable to modulate the β -DG membrane- clustering that is necessary to establish correct synaptic functions (Fig.3) [38].

Conclusions

Using a multi-scale, confocal microscopy based analysis, we have shown how altered α -DG glycosylation not only lead to the weakening of the ECM ligand binding but also to an alteration of the cytoskeletal architecture. The β -DG interacting with a hypoglycosylated α -DG is characterized by an increased rigidity that may inhibit structural responses to external stimuli (Fig. 6). The molecular mechanism we have proposed has a profound consequence on cell migration and cell spontaneous movement: future characterization of the elements that control the dynamic behavior of DG will provide novel insights into this molecular mechanism and its involvement in dystroglycanopathies and cancer progression.

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Figure legends

Fig. 1

A) A schematic representation of the domain organization of DG. The α -subunit has a dumbbell-like structure with the N-terminal and C-terminal globular domains separated by a highly glycosylated mucin-like region. The mutation T190M hits the N-terminal domain and a myc-tag is inserted at the K498 of the C-terminal domain. The β -subunit is a transmembrane protein whose cytoplasmic domain contains multiple consensus sequences for proteins-proteins interactions. The RKKRK sequence at juxtamembrane region binds ERM family proteins and ERK whereas the PPxY sequence at the cytoplasmic domain binds WW, SH2- and SH3-domains containing proteins.

B) Biochemical analysis of DG^{WT} and DG^{T190M} in transfected 293-Ebna cells. 293-Ebna cells expressed an endogenous α -DG that, when overloaded on the gel (> than 30 μ g), is recognized in Western-blot as a smeared band of 120 KDa apparent molecular weight by IIIH6 antibody, which binds to a carbohydrate epitope within the mucin-like region. The α -DG^{WT} expressed in transfected 293-Ebna cells and detected with an antibody directed against the myc-tag shows a similar band patterns as the endogenous protein indicating the correct glycosylation of the overexpressed DG. α -DG^{T190M} shows a band-shift compared to DG^{WT} due to the reduced glycosylation. DG^{WT} and DG^{T190M} are correctly cleaved liberating the β -DG-GFP subunit, detected with 43-DAG antibody, which is more abundant compared to the endogenous β -DG (asterisk). NT: not transfected cells.

Fig.2 Glycosylation of α -DG augments filopodia length.

(A) In cells transfected with DG^{WT} and DG^{T190M}, both in the presence or in the absence of laminin, α and β -subunits are localized at the plasma membrane and along the filopodia. α -DG is stained with an anti-myc antibody (red) and β -DG is fused to EGFP (green). Nuclei were counterstained with DAPI (blue)

(B) Number of Filopodia per cell area (N_F) and (C) filopodia length (F_L). No significant (ns) differences in the N_F were visible between DG^{WT} and DG^{T190M}, when cells are cultured both on glass and on laminin. F_L is significantly higher in cells transfected with DG^{WT} and cultured on laminin (+ 16%). Data represent mean \pm SD of 3 independent experiments (20 cells per sample).

Fig.3 Glycosylation induces β -DG clustering within filopodia.

(A) Colocalization of DG and actin within actin-rich domains and filopodia has been evaluated by means of confocal imaging of cells transfected with DG^{WT} and DG^{T190M} DG (in green) in the presence and in the absence of laminin. Colocalization of DG with actin (red) can be revealed in all cases.

(B) Intensity correlation quotient (ICQ) of cells transfected with DG^{WT} and DG^{T190M}, both in the presence or in the absence of laminin. The ICQ varies from 0.5 (co-localisation) to -0.5 (exclusion) while random

staining and images impeded by noise will give a value close to zero. Data represent mean \pm SD of n=50 cells per sample. (C) Manders fractional index M₂ of cells transfected with DG^{WT} and DG^{T190M}, both in the presence or in the absence of laminin. M₂ gives the fraction of β -DG which colocalizes with actin. M₂ is significantly higher in cells transfected with DG^{WT} cultured on laminin, indicating a clustering of this protein in the actin domains. Data represent mean \pm SD of 3 independent experiments (n=50 cells per sample).

Fig.4 Fluorescence recovery after photobleaching.

A) Organization of DG at the plasma membrane: α -DG interacts with the ECM and binds non-covalently to β -DG (K_a , affinity constant of the α / β interaction), whose cytoplasmic domain interacts with the actin-cytoskeleton. The interaction with the cytoskeleton is responsible for the immobile fraction (R) of the β -subunit. B) Representative images of a typical FRAP experiment of cells transfected with DG^{WT} and DG^{T190M} are reported, in which the pre-bleach, the post bleach and the final recovery phases are shown. C and D) FRAP curves of the β -subunit of respectively DG^{WT} and (C) DG^{T190M} in absence (black squares) and in presence of laminin substrate (red squares).

E) The immobile fraction R for each construct in the presence and in the absence of laminin was calculated from the recovery curves in B and C. F) K_a for the association between α and β -DG. G) Linear relationship between R and M₂. The assumption that R represents the fraction of protein strongly associated with actin domains is enforced by its correlation with M₂, which is an indicator of β -DG clustering in the actin domains. Results represent the mean of 3 independent experiments (20 cells per sample).

Fig.5 Glycosylation affects cells migration

A) Representative displacements of cells are shown in a two dimensional spatial plot.

B) Recovered speed values from the maps are reported. The absence of laminin induced an overall decrease of cell speed, in particular the decrease in cell speed was -31% for DG^{WT} (p<0.0001) and -25% for DG^{T190M} transfected cells (p=0.024). On laminin substrate, compared to DG^{WT}, DG^{T190M} transfected cells displayed a marked reduction of cell migration (-55% in respect to DG^{WT} p<0.0001) indicating that the mutation associated with muscular dystrophy impairs the cell functionality and influences interaction with laminin.

C) Speed of spontaneous cell movement in function of K_a for the association between α and β -DG. Results represent the mean of 3 independent experiments (40 cells per sample).

Fig.6 Glycosylation affects cell migration by modulating filopodia dimensions and β -DG clustering.

Schematic representation of filopodia extension. The formation of filopodia is independent from α -DG glycosylation and in the absence of laminin, cells form membrane protrusions when transfected both with DG^{WT} and DG^{T190M}. However, in the presence of laminin cells transfected with DG^{WT} forms longer

filopodia compared to cells transfected with hypoglycosylated α -DG. The interaction between α -DG and laminin stabilizes the α/β -DG interaction and induces a further reorganization of the actin cytoskeleton. The β -DG that interacts with a hypoglycosylated α -DG is characterized by an increased rigidity that may act inhibiting some of the structural rearrangements induced by external stimuli.