FEBS Letters 579 (2005) 4792–4796 FEBS 29857

Disruption of perlecan binding and matrix assembly by post-translational or genetic disruption of dystroglycan function

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Received 13 June 2005; revised 19 July 2005; accepted 25 July 2005

Available online 11 August 2005

Edited by Micheal R. Bubb

Abstract **Dystroglycan is a cell-surface matrix receptor that requires LARGE-dependent glycosylation for laminin binding. Although the interaction of dystroglycan with laminin has been well characterized, less is known about the role of dystroglycan glycosylation in the binding and assembly of perlecan. We report reduced perlecan-binding activity and mislocalization of perlecan in the LARGE-deficient Large^{myd} mouse. Cell-surface ligand clustering assays show that laminin polymerization promotes perlecan assembly. Solid-phase binding assays provide evidence for the first time of a trimolecular complex formation of dystroglycan, laminin and perlecan. These data suggest functional disruption of the trimolecular complex in glycosylation-deficient muscular dystrophy.**

Keywords: Dystroglycan; Laminin; Perlecan; Basement membrane; Congenital muscular dystrophy; Large^{myd} mouse

1. Introduction

Dystroglycan (DG) is a transmembrane protein that links the extracellular matrix (ECM) to the cellular cytoskeleton, and it has multiple roles in various tissues [1]. DG consists of the extracellular alpha subunit (α-DG), and the transmembrane beta subunit (β-DG), which are encoded by the same mRNA and cleaved in post-translational processing [2]. β-DG binds to intracellular dystrophin or utrophin, which then binds to actin filaments and extracellular α-DG. α-DG binds to several ECM proteins that contain laminin globular (LG) domains such as laminins, agrin, and perlecan [3]. Using recombinant LG domains, laminins and perlecan have shown to compete for binding to α-DG [4].

Recent studies demonstrate that the *O*-glycosylation essential for ligand-binding activity of α-DG takes place on the mucin-like domain [5]. Detailed analyses indicate that the N-terminal domain of α-DG is necessary for molecular recognition by a glycosyltransferase, LARGE, and that the DG-LARGE interaction is critical for the functional expression of DG [5]. Mutations in the *LARGE* gene have been found in human congenital muscular dystrophy type 1D, as well as in the Large^{myd} mouse [6,7]. Furthermore, recent studies suggest that the DG post-translational glycosylation pathway is a convergent target for several human muscular dystrophies, classified as ''dystroglycanopathies'' [3]. Hypoglycosylation of α-DG in dystroglycanopathies and Large^{myd} mice has been observed in conjunction with a reduction of laminin-binding activity [8].

Here, we investigate roles of DG in assembly of perlecan on the cell surface. Reduced perlecan-binding activity of DG and abnormal laminin/perlecan complexes were detected in Large^{myd} mice. By controlling ligand concentration and molecular interaction, we provide evidence for the first time of a trimolecular complex of DG, laminin, and perlecan. These data demonstrate the mechanism of the trimolecular complex formation and suggest its disruption in the pathogenesis of glycosylation-deficient muscular dystrophy.

2. Materials and methods

2.1. Animals, antibodies, and proteins

Wild type (C57BL/6) and Large^{myd} mice were bred at The University of Iowa from stock originally obtained from Jackson Laboratories (Bar Harbor, ME). All animal studies were authorized by the Animal Care Use and Review Committee at The University of Iowa.

Monoclonal antibody IIH6 against α-DG and rabbit polyclonal antibodies against perlecan (anti-PGI and anti-PGV) were described previously [8–10]. Anti-laminin and perlecan antibodies were obtained from Sigma and Chemicon, respectively.

Perlecan fragments domain I (PGI) and domain V (PGV) were prepared as previously described [9,10]. Laminin-1 and heparan sulfate proteoglycan (HSPG), derived from Engelbreth–Holm–Swarm (EHS) mouse sarcoma, were obtained from Biomedical Technologies Inc.

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0014-5793/$30.00
doi:10.1016/j.febslet.2005.07.059

and Sigma, respectively. Purification of α-DG and enrichment of DG with wheat germ agglutinin (WGA)-beads were also described previously [5,8]. *p*-Aminoethylbenzenesulfonyl fluoride (AEBSF)-treated laminin-1 was prepared using the method described by Colognato et al. [11].

2.2. Solid-phase binding assay

The solid-phase binding assay was described previously [8]. To measure IIH6-sensitive binding, IIH6 was used at 0.06 mg/ml. In cases where the DG-laminin complexes were tested, laminin was preincubated with immobilized DG in 3% BSA-LBB (10 mM triethanolamine, pH 7.6, 140 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) for 16 h prior to the addition of tested ligand. Ligand binding was detected by incubating with primary antibodies followed by HRP-conjugated secondary antibodies. All data were triplicate means.

2.3. Miscellaneous

Immunofluorescence analysis [8] and laminin-clustering assay [12] were described previously.

3. Results

3.1. Reduction of perlecan-binding activity of DG in Large^{myd} mice

To examine perlecan-binding activity of DG in Large^{myd} mice, DG preparations were enriched with WGA-chromatography from skeletal muscle. Western blotting with antibodies to the α-DG core protein confirmed that nearly all of the DG in the muscle sample bound to WGA-beads (data not shown). Solid-phase PGV binding assays showed a reduction of more than 80% of PGV binding in Large^{myd} mice (Fig. 1). This indicates that perlecan-binding activity requires the specific carbohydrate modification of α-DG. Therefore, we hypothesized that reduced ligand-binding activity of DG may cause displaced localization of ligand proteins.

3.2. Mislocalization of perlecan/laminin in Large^{myd} mice

We have reported previously that the Large^{myd} mouse has a disruption of glia limitans, the surface basement membrane in the brain, which leads to abnormal neuronal migration [8]. Here, we show that perlecan does not localize in regions where the glia limitans is disrupted (Fig. 2). In addition, the Large^{myd} mouse presents with an abnormal appearance of punctuate

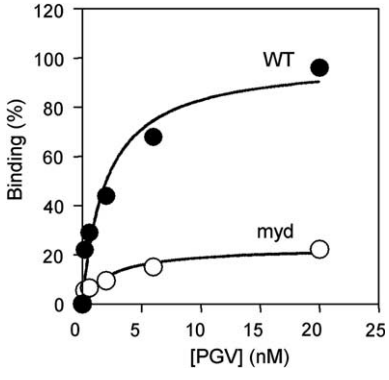

Fig. 1. *Reduction of perlecan-binding activity of DG in Large^{myd} mice.* WGA-bound materials from Large^{myd} (myd) or littermate control (WT) skeletal muscle extracts were immobilized and then incubated with various concentrations of PGV. Binding was detected with anti-PGV antibody. The maximal binding to the control preparations was set as 100%.

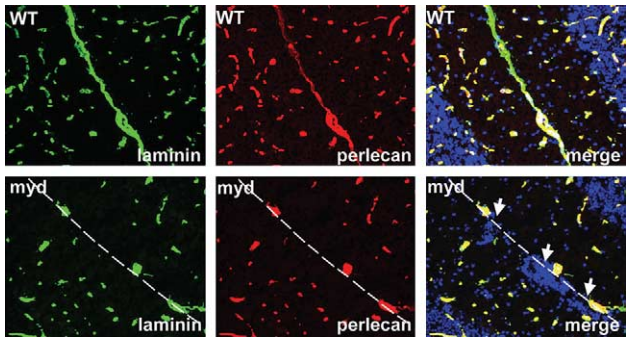

Fig. 2. *Immunolocalization of laminin and perlecan in Large^{myd} cerebellum.* Cryoselections from Large^{myd} (myd) and littermate control (WT) mice were stained with antibodies against laminin and perlecan. Staining in blue denotes DAPI. Dotted line indicates location of disrupted glia limitans between of cerebellar lobules. Arrows indicate clusters of abnormally migrated granule cells.

accumulations of laminin/perlecan throughout the cerebral cortex in locations where DG is not normally localized, whereas laminin/perlecan staining on microvessels appears normal (double arrows) (Fig. 3). We also observed similar punctuate accumulations of laminin and perlecan in brain-specific DG-null mice (data not shown). This suggests that perlecan interacts with laminin independently of DG, however DG may be required for proper location of perlecan.

3.3. Cell surface assembly of perlecan with DG-facilitated laminin-network

In order to understand the role of DG and laminin in membrane assembly of perlecan, cell-surface ligand clustering assays were performed. We observed defects of the cell surface laminin-clustering on Large^{myd} myoblast and fibroblast, however perlecan was not detected in these cell culture system (R.B, M.K, and K.P.C unpublished data). Since LARGE-deficiency results in a DG functional-null phenotype, we have used genetically engineered embryonic stem (ES) cells as a model system. We previously reported that that DG was required for the formation of laminin clusters on the surface of individual ES cells [12]. The clusters have been classified on the basis of three distinct morphologies: dots, lines, and plaques [13]. Fig. 4A shows colocalization of clusters of DG, laminin, and

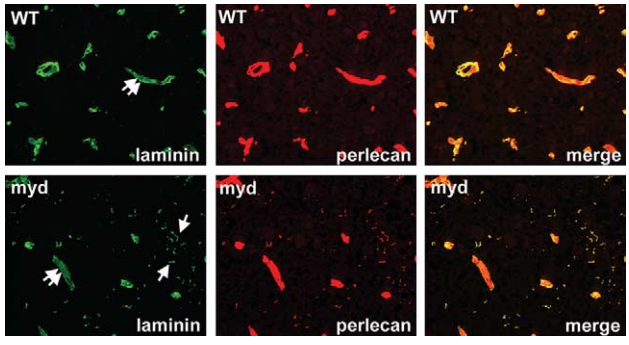

Fig. 3. *Immunolocalization of laminin and perlecan in Large^{myd} cerebral cortex.* Cryosections from Large^{myd} (myd) and littermate control (WT) mice were stained with antibodies against laminin and perlecan. The double arrow indicates normal staining of laminin and perlecan at microvessels within the cerebral cortex. The single arrow indicates areas of abnormal punctuate accumulations of laminin and perlecan in Large^{myd} cerebral cortex.

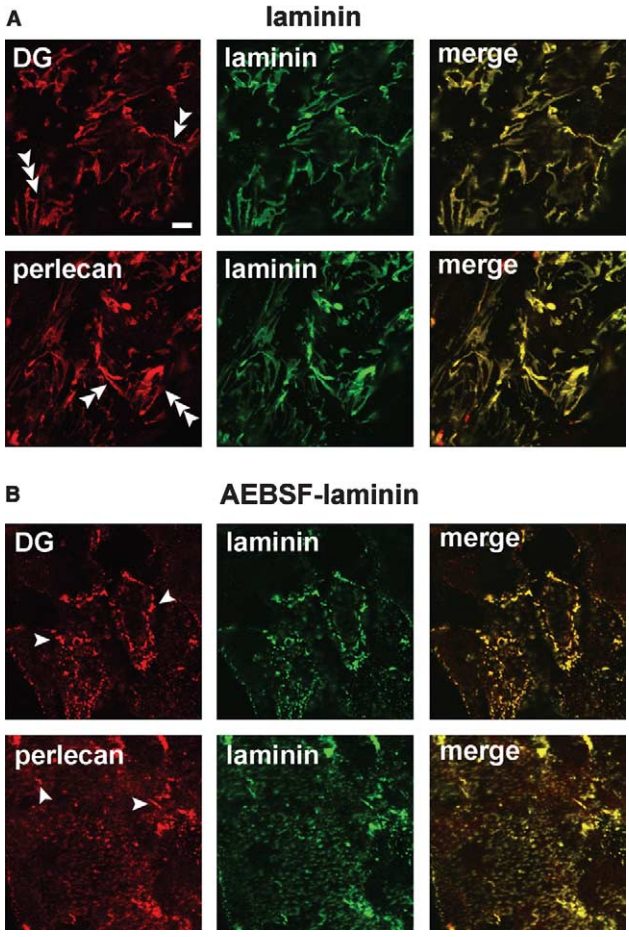

Fig. 4. *Cluster formation of laminin, DG, and perlecan on the surface of ES cells.* A laminin-clustering assay was performed on $DG^{+/-}$ ES cells. $DG^{+/-}$ ES cells were treated with 7.5 nM laminin-1 (A) or AEBSF-treated laminin-1 (B) and then incubated for 16 h. The cells were subsequently immunolabeled using anti-laminin, anti-α-DG (IIH6) or anti-perlecan antibodies, and examined by confocal microscopy. In all panels, the antibody used for staining is shown in the upper left corner of the panel. Each panel shows a representative small colony of ES cells. Single, double, and triple arrowheads represent dot, linear, and plaque clusters, respectively. Bar, 5 μm.

perlecan on the surface of $DG^{+/-}$ ES cells which were observed 16 h after the addition of exogenous laminin-1. Treatment of laminin-1 with AEBSF has been reported to decrease the self-association capability of laminin [11]. When AEBSF-treated laminin-1 was used, dot-like clusters of laminin-1 were most commonly observed, and such clusters were colocalized with DG and perlecan (Fig. 4B). These findings indicate that both DG and laminin self-association facilitates/is required for assembly of perlecan on the cell surface.

3.4. A trimolecular complex of DG/laminin/perlecan

Although perlecan and laminin have been reported to compete with each other for binding to α-DG, colocalization of laminin and perlecan with DG led us to hypothesize that perlecan assembles on a laminin oligomer whose terminal is anchored to α-DG. To examine this, we modified conventional solid-phase binding assays with monoclonal antibody IIH6, which recognizes functionally glycosylated α-DG. HSPG preparations were used as ligands for this assay. We confirmed the presence of perlecan in HSPG preparations by ELISA and Western blotting using antibodies to PGI and PGV (data not shown). When the HSPG preparations were incubated on the α-DG-immobilized wells, we observed perlecan-binding to α-DG (Fig. 5A, open squares). The presence of IIH6 inhibited the perlecan-binding to α-DG (closed squares), as is the

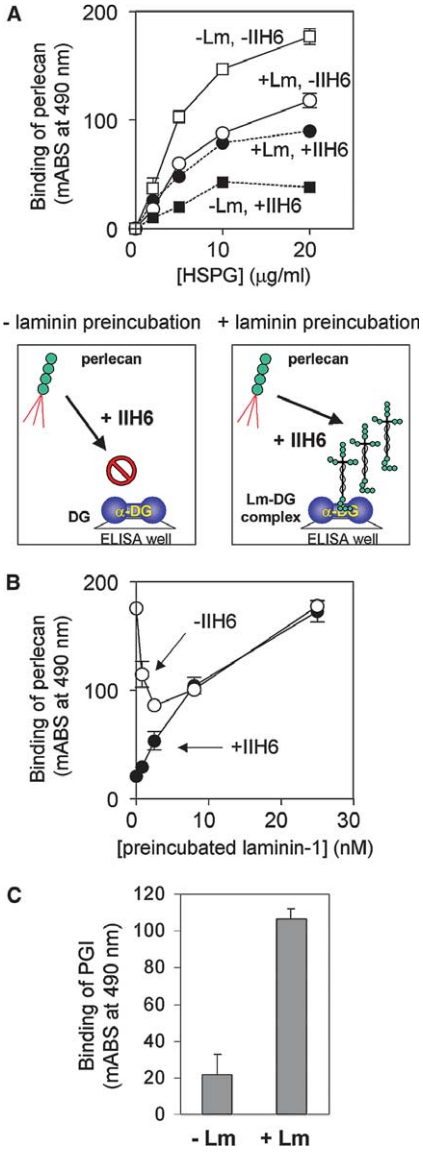

Fig. 5. *Trimolecular complex of α-DG, laminin, and perlecan.* (A) Secondary binding of perlecan to α-DG-laminin complex. After the preformation of α-DG-laminin complex, various concentrations of the HSPG preparations were added with (closed circles) or without (open circles) IIH6. The perlecan binding to immobilized α-DG without preincubation of laminin was also measured with (closed squares) or without (open squares) IIH6. Drawings illustrate that in the presence of IIH6, binding of perlecan to α-DG is inhibited, whereas perlecan binds to preformed α-DG-laminin complex through laminins on immobilized α-DG. (B) Laminin-1-concentration dependency of the secondary perlecan binding to α-DG-laminin complex. Various concentrations of laminin-1 were incubated on the α-DG-immobilized wells and then the HSPG preparations (4 μg/ml) were added with (closed circles) or without (open circles) IIH6. (C) Secondary binding of PGI to laminin-1-α-DG complex. α-DG-immobilized wells were preincubated with (right bar) or without (left bar) laminin-1 (7.5 nM), and then binding of recombinant PGI (9 μg/ml) was analyzed.

case for laminin-binding (data not shown). This IIH6-sensitive binding can be defined as a direct binding of perlecan or laminin to α-DG. For the formation of the trimolecular complex of DG/laminin/perlecan, laminin-1 was preincubated on the α-DG-immobilized wells, and then HSPG preparations were added with or without IIH6. IIH6-insensitive perlecan binding was detected after preincubation of laminin (open and closed circles). Because IIH6 blocks perlecan-binding on α-DG molecules that are unoccupied with laminin, the IIH6-insensitive binding represents perlecan-binding to preformed DG-laminin complex via laminin.

Next, we examined laminin concentrations during the preformation of the α-DG-laminin complex (Fig. 5B). The IIH6-insensitive binding of perlecan to the α-DG-laminin complex was dependent on laminin concentrations during preincubation (closed circles). In the absence of IIH6, binding of perlecan was inhibited by low concentrations of preincubated laminin (<2.5 nM). At a higher concentration range, the inhibitory effect was eliminated, and perlecan binding was increased. These data indicate that laminin and perlecan are competitive for binding to α-DG, but at higher concentrations of laminin, perlecan is capable of assembly on a laminin oligomer whose terminal is bound to α-DG. PGI is known to interact with laminins but not with α-DG [14,15]. Although we observed little binding of PGI after incubation with immobilized α-DG, clear increases of PGI-binding to the α-DG-laminin complex were detected (Fig. 5C). These data demonstrate that the laminin-perlecan interaction mediates perlecan assembly on the α-DG-laminin complex.

4. Discussion

A reduction in perlecan-binding activity in Large^{myd} and the inhibitory effect of IIH6 on the perlecan-DG interaction suggest that similar to laminin [8], perlecan binding to DG is regulated by glycosylation status of α-DG. Abnormal accumulations of perlecan in the cerebral cortex appear to contain laminin. Considering the antigen of laminin antibody used in this study, the abnormal punctuate structures most likely contain at least α1, β1, and/or γ1-chain. Although perlecan and laminin appeared to be normally localized in Large^{myd} skeletal muscle, electron microscopy studies showed microdisruptions of the basement membrane [16]. Thus, DG may be required for proper localization and assembly of its ligand proteins into an intact basement membrane with tissue-dependent manner.

We demonstrated that laminin self-association facilitates assembly of perlecan on the cell surface and show evidence for the first time of a binary interaction of perlecan and laminin on their receptor DG. Consistent with previous reports [4], a competition between perlecan and laminin for binding to α-DG was detected. Interestingly, the inhibitory effect of laminin on the perlecan–DG interaction was reduced at higher concentrations of laminin during preincubation due to the secondary binding of perlecan to a DG-anchored laminin polymer. That is, perlecan binding becomes indirect, rather than direct, in the presence of laminin. These data support a widely-accepted model, the ''receptor-facilitated laminin network model,'' where both initial laminin assembly on the cell surface and subsequent laminin self-association are necessary for the formation of the ECM network [11].

Laminin-1 used in the in vitro binding assay is reported to show high-affinity to DG [4,5] as well as laminin-2 which is expressed in adult skeletal muscle and brain [17,18]. Since domains responsible for binding to heparin and perlecan/nidogen complex are present in both laminin-1 and -2 [17], the mechanism of DG-dependent laminin-perlecan network formation is likely similar between laminin-1 and -2. Because of high-affinity to DG, perlecan could be a linker between DG and laminins that show low or no affinity to DG. This is supported by a recently proposed model that perlecan/nidogen complex links laminin-6 (α3/β1/γ1) on alveolar epithelial cells [19].

The present data indicate that the local concentrations of competing ligands in vivo, and relative binding affinities among these ligands and their receptors are likely critical to the ordered assembly of matrix, the nature of the molecular interaction between the matrix and membrane proteins, and the molecular proximity of the laminin/perlecan lattice to the cell surface. Clearly, it is important to elucidate an understanding of the temporal and spatial distribution of local concentrations of DG ligand proteins. In addition, non-DG type laminin acceptor/receptors, such as sulfatide, have been suggested to play important roles in ECM organization [20]. Overall, this study demonstrates a mechanism for the formation of a DG/laminin/perlecan complex on the cell surface, and suggests that its disruption might be associated with pathogenic events in congenital muscular dystrophies.

Acknowledgements: We thank Drs. Reinhard Fassler, Steven Moore, and Aaron Beedle, Ms. Carmen Nidey, and all members of the Campbell laboratory for their critical reading of the manuscript and fruitful discussion. We thank Jason Flanagan, Lindy McDonough, and Sarah Anderson for expert technical assistance. We also thank Chuck Lovig and The University of Iowa Hybridoma Facility. There are no potential financial conflicts of interest for any of the authors. This work was supported in part by the Muscular Dystrophy Association (K.P.C.). K.P.C. is an investigator of the Howard Hughes Medical Institute.

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