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Dynamic Regulation of alpha-Dystroglycan in Mouse Placenta

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Abstract

Alpha dystroglycan (α -DG) is a peripheral membrane protein important in cellular interaction with other cells and the extracellular matrix. Recent data suggests that the *Dag1* gene, which encodes α -DG, is important for implantation. In addition to its importance in cellular function, α -DG also serves as a cellular receptor for members of the *arenaviridae* family of viruses which can cause placental infection. Because of its apparent dual role in implantation and its role as a viral receptor, we sought to determine placental and uterine α -DG expression during mouse pregnancy. *Dag1* is expressed throughout gestation in the placenta and to a lesser extent in the uterus, with the highest levels in early gestation. By Western blot analysis, the glycosylated protein is also expressed and the pattern of glycosylation changes to favor the most highly glycosylated forms at mid gestation. These data support the idea that α -DG may be a target for evolutionary host-pathogen interactions at the maternal-fetal interface.

Keywords

placenta; dystroglycan

1. INTRODUCTION

The mammalian placenta is likely the result of evolutionary interaction between mother, developing fetus and infectious agents, especially viruses. Examples of tenacious viral infection (reviewed in [1], and for example [2–4]) and the expression of virus-derived proto oncogenes [5] support this assertion. Viral persistence in the placenta can be a source of maternal and or fetal morbidity, and this clinical problem continues to generate interest in the mechanisms by which the placenta is virally infected. For many viruses the cellular receptor is known, or highly evidenced. However, less is known about the expression of such receptors in placental cells.

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α -Dystroglycan (α -DG) is the cellular receptor for several members of the arenaviridae family [6, 7], which can cause maternal morbidity and mortality, persistent placental pregnancy loss, infection and congenital malformations [3, 8–11]. The alpha- and beta-subunits of Dystroglycan, a component of the dystrophin-glycoprotein complex, are encoded by a single gene and cleaved into two proteins by posttranslational processing. The molecular mass of α -DG is calculated as about 74kDa [12, 13], though it varies in size from 100–250kDa on a western gel, mainly due to glycosylation [14]. α -DG is known to undergo extensive glycosylation with both *N*- and *O*-linked glycans, and the *O*-linked structures are critical for its function [15, 16].

The level of glycosylation on α -DG varies according to the cell's tissue origin and developmental phase, [17–19]. Enzymes such as Protein O-mannosyl transferase 1 [20] are thought to participate in O-mannosylation of α -DG. This critical type of glycosylation is also thought to occur through the actions of enzymes such as LARGE, which is expressed in the placenta [21].

α -DG is commonly expressed in tissues that are strongly dependent on the existing extracellular matrix [22]. α -DG interacts with the transmembrane β -DG subunit and bridges the membrane with the extracellular matrix [23]. At extracellular sites in other tissues α -DG undergoes high affinity interactions with extracellular matrix proteins, i.e. laminin, and recognizes both pathogen and host derived ligands [24].

In humans, deficiency in α -DG is associated with muscular dystrophy [25]. Dystroglycan appears to be essential for the formation of Reichert's membrane, and disruption of the *Dag1* gene in mice results in embryonic lethality [26]. These data suggest that α -DG should be widely expressed in the placenta throughout gestation, and this was tested in mouse placentas from early and late gestation.

2. METHODS

2.1. ANIMAL HOUSING AND TIMED MATING

Adult (2–3 month old) C57BL/6 females were obtained from Jackson and housed under specific pathogen free Association for Assessment of Laboratory Animal Care approved conditions. The studies were approved by the Institutional Animal Care and Use Committee of the University of Vermont. Females were either never mated (control) or underwent timed mating and were euthanized on Day 8, 10, 14, 16, 17 or 18. Representative placentae (1–2), uterus and spleen were taken from each mouse for analysis. Uterus and spleen never-mated females served as controls. Tissues were snap frozen and stored at -80°C until used.

2.2. RNA ISOLATION

Total cellular RNA was isolated from frozen placenta and uterus, using the RNeasy mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions and was DNAase treated (Ambion-Applied Biosystems, Foster City, CA) and quantified by UV absorbance at 260 nm on a nanodrop spectrophotometer (NanoDrop, ThermoScientific, Willmington, DE).

2.3. REALTIME QUANTITATIVE RT-PCR

cDNA for each sample was generated from 1µg of RNA using the iScript cDNA Synthesis kit (Biorad, Hercules, California) with a mixture of random hexamers and Oligo dTs. cDNA reaction conditions were as follows: 25°C for 5min, 42°C for 30min, 85°C for 5min, followed by a 4°C hold. Quantitative PCR was performed using 1µl of the cDNA with Power SYBR green master mix (Applied Biosystems, Foster City, California) on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, California). These primers were used to detect α -DG and β -2 microglobulin (as a control): Alpha dystroglycan: 5' GGAGATCATCAAGGTGTCTGC; 3' AGCACTCACTGAGATGTA ATG C, (133 bp product) or β -2 microglobulin: 5' ATGCTATCCAGAAAACCCCTCAA; 3' CAGTTCAGTATGTTCCGGCTTCCC (79 bp product). The sequences of the primers were designed using the Primer Select program (DNASTAR Inc. Madison, WI). The reaction conditions were: 95°C for 10min, 95°C for 15sec and 60°C for 1min (40 Cycles) followed by dissociation melt curve analysis. The Relative Standard Curve method was used to calculate the amplification difference between α -DG and the control gene. Standard curves were generated for both the alpha dystroglycan and beta-2 microglobulin primer sets using a single sample which was serially diluted over the working range of the assay. The amount of target and endogenous control mRNA were determined by comparing the cycle threshold value to the standard curve. Relative target mRNA values were normalized by dividing the target quantity by the endogenous control quantity. Each sample was run in triplicate.

2.4. WESTERN BLOT ANALYSIS

Placenta, uterine tissue, and spleen were lysed in a 1X cell lysis buffer (Tris pH 7.5–50mM; NaCl-150mM; TritonX 100–1%; EDTA-1mM) containing protease inhibitors (Biotech, Rockford, IL) and phosphatase inhibitors (Sodium orthovanadate-1mM; Sodium pyrophosphate-2.5mM; NaF-1mM). Protein content in each sample was measured using the BCA kit (PIERCE Biotech). Twenty micrograms of protein from each sample was loaded and run on a 6% SDS PAGE gel with a broad range pre-stained marker (BIORAD, Hercules, CA). The proteins were then electrophoretically transferred onto a nitrocellulose membrane and probed with an antibody to α -DG (IIH6C4 Millipore, Billerica, MA) at a concentration of 1:500, followed by Horseradish Peroxidase (HRP)- conjugated goat anti-mouse IgM (Millipore). For normalization the blots were stripped and probed with an antibody to actin (Sigma, St Louis, MO) at a concentration of 1:2000, followed by HRP- conjugated goat anti-mouse IgG (BIORAD, concentration of 1:10000). Antibody binding was detected by chemiluminescence using Immobilon western HRP substrate (Millipore) and Bio-Rad's Chemidoc-XRS chemiluminescence detection system. Immunoreactive bands were imaged using the ChemiDoc XRS system (Bio-Rad). Densitometry of these bands was performed using Quantity One software (Bio-Rad). A tight box was drawn around each band and its densitometry was measured after subtracting the background. Densitometric values of α -DG bands were normalized to actin as an internal control. Boxes were drawn around bands at 100kda, 120kda, 150kda and 250kda for each sample and each band's densitometry was determined. The densitometric values of all these four bands were individually divided by the actin densitometry value.

2.5 STATISTICAL ANALYSIS

The results of quantitative RT-PCR for transcriptional expression of Dag1 in mouse placenta were analyzed by ANOVA followed by Student–Newman-Keuls as per the Sigmaplot 9.0 software (Point Richmond, CA). The uterine Dag1 expression was analyzed by ANOVA followed by Dunn’s method using the above software. Western blot studies for total glycosylation were analyzed by ANOVA followed by Tukey’s multiple comparison test, provided with the Graph pad prism 5 software (La Jolla, CA). For determination of relative glycosylation, the data was analyzed by the Kruskal-Wallis test. Statistical significance was set at $P < 0.05$

3. RESULTS AND DISCUSSION

3.1 DIFFERENTIAL EXPRESSION OF DYSTROGLYCAN (Dag1) DURING PLACENTAL DEVELOPMENT

We first examined transcriptional expression of the Dag1 gene by performing quantitative RT-PCR on tissues obtained from non-pregnant and pregnant mice in early (Day 8–12) and late (Day 14–18) gestation. Gene expression was detectable at all time points examined in both tissues (Fig. 1A). This is consistent with data that Dag1 is required for normal murine embryonic development [26] and further suggests that expression is important for normal pregnancy and development. Levels found in the uterus remained stable throughout gestation and similar to levels in the non -pregnant state (Fig. 1A). In contrast, we observed an increased level of mRNA expression in the placenta during early gestation (Day 8–12) and, interestingly, found a significant decrease in mRNA levels after Day 14 of gestation ($p < 0.050$). The rise in expression on Day 8 of gestation may be due to a role played by Dag1 in the implantation process [27]. However, there were no concomitant changes observed in the uterus during this time. With the caveats that our studies missed a very early surge or discreetly localized uterine expression, our data makes this assertion less likely. Another possibility is that the Dag1 gene products are important for early placental remodeling.

3.2. EXPRESSION OF α -DG DURING PLACENTAL DEVELOPMENT SUGGESTS REGULATED GLYCOSYLATION

To observe expression of the α -DG protein, we used Western blot analyses (Fig. 1B) to determine expression in the placenta at different days of gestation in comparison to peri-implantation or non- pregnant uteri. Previous work [19] suggests that differential glycosylation is responsible for variation in α -DG’s molecular mass, ranging from 100 –250 kDa. Consistent with this, our results indicate that mouse placental α -DG undergoes a change in expression pattern with time. For example (Fig. 1B, **middle panel**), on Day 8 of gestation, sharp bands were present at 100, 120 150 and 250 kDa. On Day 12 (Fig. 1B, **right panel**), of gestation, broader bands of approximately 150–250 kDa appeared. On day 14 and 16 the bands at higher, as opposed to lower, molecular weight were broader than at early gestation.

In late gestation (Fig 1B, **right panel**), around Day 18, a broad upper band is replaced by smaller sharper bands of approximately 150–200 kDa, and the band at 100–120 observed earlier faintly reappears. This suggests that α -DG undergoes temporal and differential

glycosylation in the placenta. The protein levels observed by Western Blot are consistent with the transcriptional expression studies in that the protein is expressed in the placenta throughout gestation, and at low levels in the uterus in the non- pregnant and peri-implantation state.

As a comparison to placenta, we chose to examine a tissue that does not undergo as extensive structural change during pregnancy. The spleen experiences an increase in size during pregnancy [28], but does not undergo the structural changes seen in the placenta. Thus, Western blotting was performed to compare the expression of alpha dystroglycan in spleen at different days of gestation. We found that the expression pattern of glycosylated alpha dystroglycan in the spleen remains uniform throughout gestation and there is no evident regulation observed during the course of gestation (Fig.1C). This is consistent with the idea that expression in the placenta is associated with, and possibly important for, structural modification.

To perform a semi-quantitative analysis of α -DG expression in the placenta, bands (Western Blot) representing the various forms of glycosylated α -DG at different gestational days were quantified by densitometry and compared to values obtained for actin. Fig. 2A **shows that** when the total densitometry units representing the glycosylated α -DG are normalized to units obtained for actin, there is no significant increase or decrease during the course of pregnancy. However, when we calculated the ratio of densitometry units representing the higher molecular weight bands (the sum of 150 and 250) to the units representing the lowest molecular weight band (100, Fig. 2.B), we revealed a relative increase in mid- gestation ($P=0.01$) that was consistent with our qualitative observations and supports the idea that α -DG in the placenta undergoes increased glycosylation at mid- gestation.

While we observed an increase in Dag1 expression by RT-PCR, studies of the protein by Western blot analysis showed variable but not significantly different levels of total glycosylated protein. The increase in relative glycosylation seen in mid- to late gestation, however, raises the issue that an early increase in mRNA indicates the increased presence of unmodified protein. Another explanation for our findings is that the β subunit and the α subunit are differentially regulated or detected, with the β subunit protein levels more closely paralleling transcription. Yet another, though less likely, explanation is the presence of alternative transcripts. These caveats raise the interesting possibility of more complex regulation of Dag1 and its gene products in the placenta during pregnancy. This could be addressed in future studies.

Our data is consistent with data showing dystroglycan expression in post-implantation mouse extraembryonic tissues (Day 5.5) and in early decidua [26],[27]. It is also consistent with data correlating deficiency with embryonic lethality. Our data also suggests that α -DG may be important in pregnancy maintenance, even late in gestation.

Moreover, our data suggests that α -DG undergoes differential glycosylation. The heterogeneity seen in pregnancy may represent differences in expression within distinct cell types or overall differences in the populations of cells expressing the molecule.

Our evidence suggests that α -DG, a cellular receptor for members of the arenaviridae, is expressed in the placenta and undergoes regulation during pregnancy. Thus, we speculate that the placenta is a potential target for viral-cellular interactions. These interactions could induce not only infection [3], but also the changes in growth, metabolism and expression of vital proteins that underlie viral persistence. Further studies on the regulation of this and similar molecules may shed light on the continuing evolutionary conflict involving mother, fetus, and infectious agents.

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Fig. 1. Expression of α -DG in placenta and uterus

Samples of placenta, uterus and spleen from non-pregnant and pregnant mice were used to determine α -DG expression.

1A. Transcriptional expression in placenta and uterus. Light grey bars: placenta. Dark grey bars: uterus. Y axis: Relative expression compared to expression of a housekeeping gene. X axis: pregnancy status or gestational age. NP: non pregnant and never mated. Inset numbers refer to the number of samples assayed.

1B. Representative western blots of uterus and placenta demonstrating the

glycosylation of α -DG. Left panel: Uterus. Homogenates from day 5 uterus were analyzed using the anti- α -DG antibody 11H6C4. Left panel: uterus. Middle panel: Tissues from day 8 gestation. Right panel: Tissues from mid-late gestation. The electrophoretic pattern shows the presence of four different bands (bracket) at 100 to 250 kD as determined by molecular weight markers (not shown). These bands represent different glycosylation levels. The thickness of these bands varies according to gestational age. Lower blots: bands obtained after stripping the blot and probing for β -Actin are shown for comparison.

1C. Representative western blots of spleen. Spleen was isolated from non-pregnant, never mated female mice and mice at various days of gestation. Homogenates of spleens were treated as in Figure 1B.

Fig. 2. Semi-quantitative analysis of glycosylated α -DG protein

Placenta and uterus were isolated from several pregnant and non pregnant mice. Western blotting was performed to determine expression of α -DG, using 11H6C4, which recognized only the glycosylated form of α -DG. **2A. Total normalized glycosylated α -DG.** The chemiluminescence of the individual α -DG specific bands at 100 KDa and above were quantified by densitometry, normalized to the band obtained for β -Actin in the same sample, and the resulting calculated normalized densitometry units were used to compare samples. Y

axis: The sum of the normalized densitometry units obtained for all the bands noted. X axis: day of gestation. Each symbol represents one sample from one mouse.

2B. Relative glycosylation in placental samples. The relative glycosylation was estimated by adding the β -Actin-normalized densitometry units of the 250 and 150 KDa band and dividing this sum by the units obtained for the 100 KDa band. Y axis: Ratio of densitometry units. X axis: day of gestation. Each symbol represents one sample from one mouse.