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Glycan-deficient PrP stimulates VEGFR2 signaling via glycosaminoglycan

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ABSTRACT

Whether the two N-linked glycans are important in prion, PrP, biology is unresolved. In Chinese hamster ovary (CHO) cells, the two glycans are clearly not important in the cell surface expression of transfected human PrP. Compared to fully-glycosylated PrP, glycan-deficient PrP preferentially partitions to lipid raft. In CHO cells glycan-deficient PrP also interacts with glycosaminoglycan (GAG) and vascular endothelial growth factor receptor 2 (VEGFR2), resulting in VEGFR2 activation and enhanced Akt phosphorylation. Accordingly, CHO cells expressing glycan-deficient PrP lacking the GAG binding motif or cells treated with heparinase to remove GAG show diminished Akt signaling. Being in lipid raft is critical, chimeric glycan-deficient PrP with CD4 transmembrane and cytoplasmic domains is absent in lipid raft and does not activate Akt signaling. CHO cells bearing glycan-deficient PrP also exhibit enhanced cellular adhesion and migration. Based on these findings, we propose a model in which glycan-deficient PrP, GAG, and VEGFR2 interact, activating VEGFR2 and resulting in changes in cellular behavior.

1. Introduction

One of the most common protein post-translational modifications is N-linked glycosylation. N-linked glycosylation is important in proper protein folding, transit to the cell surface and secretion, in a protein, as well as cell context dependent manner [1–4]. The normal cellular prion protein, PrP, is a widely expressed, highly conserved, glycosylphosphatidylinositol (GPI)-anchored cell surface glycoprotein [5–7]. The two N-linked glycosylation sites on human PrP are located at the C-terminus at residues 181 and 197. A plethora of more than 40 proteins have been reported to bind PrP [8]. In addition, PrP also binds divalent cations, such as copper and zinc, lipids, nucleic acids and GAG [9–12]. However, other than being essential for the pathogenesis of prion diseases, the normal functions of PrP remain obscure; *Prnp*$^{-/-}$ mouse is normal without apparent phenotype [13,14]. Whether the two glycans are important in PrP biology is debated. In some cell models, glycan-deficient PrP is retained in the cytosol, unable to reach the cell surface [15,16]. On the contrary, other studies find them to be dispensable for cell surface expression [17,18]. Even though the two N-linked glycans are not essential for the pathogenesis of prion diseases, they do contribute to disease phenotypes as well as strain specificity of some infectious prion [19–22]. However, the underlying mechanisms by which N-linked glycans modulate disease phenotype or strain specificity are not known.

Because of these ambiguities, we investigated whether the two N-linked glycans were important in PrP biology using CHO cell, which lacked detectable endogenous hamster PrP [23]. We transfected a wild type (WT) human PrP; a single glycan site mutant (SM) PrP, in which one of the glycosylation sites, asparagine (N) 181, was altered to an alanine (A) (N181A); another SM PrP, in which the other site, N197, was mutated to an A (N197A); a double mutant (DM) PrP, in which both sites were replaced with A individually into CHO cells. These mutated PrP are collectively referred to as glycan-deficient PrP. We find that all glycan-deficient PrP are expressed on the cell surface in levels comparable to WT PrP. However, glycan-deficient PrP appears to partition more in lipid raft than wild-type PrP. Furthermore, glycan-deficient PrP forms complex with VEGFR2 and GAG resulting in the activation of VEGFR2 and the PI3K-Akt signaling pathway. These biochemical alterations also resulted in changes in cellular behavior; compared to cells with wild type PrP, cells with glycan-deficient PrP are more adhesive and more mobile. Collectively, these results suggest that in CHO cells, one of the functions of N-linked glycans on PrP is to negatively regulate PrP functions.

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2. Materials and methods

2.1. Cell lines

Chinese hamster ovary (CHO) cell line was kindly provided by Dr. Pamela Stanley of Albert Einstein College of Medicine, New York City, NY [24]. The cells were cultured with Minimum Essential Medium Alpha (11900024, Gibco), 1% antibiotic penicillin–streptomycin solution (PS) (03-031-1, Biological Industries) plus 10% fetal bovine serum (FBS) (04-001-1, Biological Industries). The pgsA-745 cell line was purchased from ATCC (CRL-2242), the cells were cultured with F-12K Medium (30-2004, ATCC) plus 10% FBS and 1% PS as suggested by the vendor. All cells were cultured in a 37 °C, 5% CO_2, 95% humidity incubator (Series 8000 WJ, Thermo Scientific).

2.2. Antibodies and other reagents

Anti-PrP monoclonal antibodies (Mabs) 4H2 and 7A12 were produced, purified, characterized and biotinylated as reported by us [25,26]. Antibodies against phospho (p)-Akt (Ser473) (4060), Akt (4691), p-SAPK/JNK (4668), SAPK/JNK (9252), HRP linked goat anti-mouse IgG (7076), HRP linked goat anti-rabbit IgG (7074), and LY294002 (9901) were purchased from Cell Signaling Technology. Antibodies against VEGFR2 (Flk-1) (sc-505), p-VEGFR2 (p-Flk-1) (sc-101820), flotillin-1 (sc-25506) and protein A/G PLUS-agarose (sc-2003) were purchased from Santa Cruz Biotechnology. Heparin antibody (MAB2040) was purchased from Merck Millipore. Receptor tyrosine kinase (RTK) inhibitors AG490 (S1143), Axitinib (S1005), BGJ398 (S2183), BMS754807 (S1124), Crizotinib (S1068), Ki8751 (S1363), Mubritinib (S2216), RGD peptides (S8008), SAR131675 (S2842), ZM306416 (S2897) were purchased from Selleck Chemicals (Texas, USA). Heparinase I and III (H3917) were purchased from Sigma. Proteinase inhibitor cocktail (11697498001) and 4′, 6-diamidino-2-phenylindole (DAPI) (10236276001) were purchased from Roche. Alexa Fluor 555 conjugated goat anti-rabbit IgG (A-21429), Alexa Fluor 555 conjugated goat anti-mouse IgG (A-21424), Alexa Fluor 647 conjugated goat anti-mouse IgG (A-21235), Alexa Fluor 647 conjugated streptavidin (S-21374), and Lipofectamine 2000 transfection reagent (11668-019) were purchased from Invitrogen. EZ-link Sulfo-NHS-Biotinylation kit (21425) was purchased from Thermo scientific. All the other chemicals were purchased from Amresco (OH, USA).

2.3. Construction of different human PrP mutants

Cloning vector pHAGE-CMV-MCS-IZsGreen and the packaging plasmids psPAX2, pMD2.G were kindly provided by Professor Zan Huang (Wuhan University). Wild type (WT) human *PRNP* (hPrP) DNA was created as described [27]. Constructions of hPrP-N181A, hPrP-N197A, hPrP-N181A-N197A (double mutation, hPrP-DM), and hPrP-DM-ΔKKRPK were made by site-directed gene mutagenesis kit (D0206, Beyotime, Shanghai, China). For the constructions of different PrPs-CD4 chimeric proteins, human CD4 transmembrane domain and cytosol tail were amplified with the indicated primer pairs (Table S1) containing restriction enzyme *Bsp119I* site before the GPI-anchor signal sequence in the forward primer, and restriction enzyme *BamHI* site in the reverse primer. The site of *Bsp119I* was deleted after CD4 transmembrane and cytosol domain was inserted into the PrP expressing constructs. All the constructs were sequence confirmed.

2.4. Expression of human PrP in CHO cells

Wild type and mutant PrP were inserted in the pHAGE-CMV-MCS-IZsGreen vector and transfected into 293 T cells as described [28,29]. Lentiviral particles were collected by centrifuging at 700 ×*g* for 10 min, and filtered with 0.45 nm filter. CHO or pgsA-745 cells were then infected with the virions for 6 h. PrP expression was confirmed by immunofluorescent staining followed by flow cytometric analysis.

2.5. Co-immunoprecipitation (Co-IP)

PrP expressing cells were seeded in culture medium at 37 °C, 5% CO_2, 95% humidity for 14 h. The cells were cultured for an additional 24 h in medium without FBS. After that cells were lysed in cell lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na_3VO_4, 1% Triton, 1 mM PMSF and 1 × proteinase inhibitor cocktail) and protein concentration was quantified using a Bio-Rad Protein Assay (500-0002, Bio-Rad). Before co-IP, cell lysates were pre-cleared with rabbit IgG and protein A/G agarose beads for 1 h at 4 °C. Pre-cleared lysates were incubated with 2 μg of indicated antibodies at 4 °C for 14 h. The protein A/G agarose beads were then added for an additional 3 h and incubated at 4 °C. After washing 6 times with cell lysis buffer, the beads were collected by centrifuging at 1000 ×*g* for 1 min. 30 μl 1.3 × reducing sample loading buffer (10% sucrose (W/V), 5% 2-mercaptoethanol (V/V), 2% SDS (W/V), 0.0625 M Tris–HCl pH 6.8, 0.0004% bromophenol blue (W/V)) were then added to the beads. Proteins co-purified with VEGFR2 were subjected to immunoblotting.

2.6. Immunoblotting

Cultured cells were harvested, lysed, and cell lysate were prepared as described by us [28]. The concentration of proteins was measured by Bio-Rad Protein Assay (500-0002, Bio-Rad). Comparable levels of proteins were loaded and separated on 10% SDS-PAGE gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane (HATF00010, Merck Millipore) and blocked in 3% BSA in TBST (137 mM NaCl, 20 mM Tris, 0.1% Tween-20, pH 7.6) overnight at 4 °C. To detect the proteins on nitrocellulose membrane, the blots were first probed with the primary antibody. Bound primary antibodies were then probed with a HRP-conjugated secondary antibody. The concentration of anti-PrP antibodies 4H2 was 1 μg/ml. All the other primary antibodies were used according to the manufacturer's instruction.

PNGase F (P0704S, NEB) treatment of PrP was performed according to manufacturer's instruction. Briefly, 20 μg proteins per sample were combined with 1 μl 10 × glycoprotein denaturing buffer and deionized water to make a 10 μl reaction volume on ice. The sample was then boiled for 10 min, and 2 μl 10 × G7 buffer, 2 μl 10% NP40, 5 μl deionized water and 1 μl PNGase F were added to make a total of 20 μl reaction. PNGase F treated samples were then subjected for immunoblotting as above.

2.7. Cell surface biotinylation

Cell surface biotinylation was performed according to manufacturer's protocol (21331, Thermo scientific). After protein biotinylation, the biotin conjugated proteins were pulled down with streptavidin beads (N-1000, Solulink). Proteins were further separated with 10% SDS-PAGE gel and immunoblotted with 4H2 accordingly. The amounts of unglycosylated, mono-glycosylated, and di-glycosylated PrP were quantified based on densitometry using the Image J software (NIH).

2.8. Immunofluorescence staining

CHO cells expressing WT-PrP, N181A-PrP, N197A-PrP, or DM-PrP were cultured as described in above. To detect PrP expression, cells were detected with Mab 4H2 (10 μg/ml) at 4 °C for 1 h. Bound antibodies were detected with Alexa Fluor 555 conjugated goat anti-mouse antibody (1 μg/ml) for 1 h at 4 °C.

For double labeling of PrP (GPI anchored and CD4 chimeric PrP) and GM1, the cells were seeded as above. GM1 was labeled with the Vybrant lipid raft labeling kits (V-34404, Molecular Probes) according to the