Normal cellular prion protein is a ligand of selectins: binding requires LeX but is inhibited by sLeX

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The normal PrP C (cellular prion protein) contains sLeX [sialyl-LeX (Lewis X)] and LeX. sLeX is a ligand of selectins. To examine whether PrPC is a ligand of selectins, we generated three human PrP C-Ig fusion proteins: one with LeX, one with sLeX, and the other with neither LeX nor sLeX. Only LeX-PrP C-Ig binds E- L- and P-selectins. Binding is Ca 2+-dependent and occurs with nanomolar affinity. Removal of sialic acid on sLeX-PrP C-Ig enables the fusion protein to bind all selectins. These findings were confirmed with brain-derived PrP C. The selectins precipitated PrP C in human brain in a Ca 2+-dependent manner. Treatment of brain homogenates with neuraminidase increased the amounts of PrP C precipitated. Therefore the presence of sialic acid prevents the binding of PrP C in human brain to selectins. Hence, human brain PrP C interacts with selectins in a manner that is distinct from interactions in peripheral tissues. Alternations in these interactions may have pathological consequences.

Key words: human brain, Lewis X (LeX) isotope, neural cell-adhesion molecule (NCAM), sialic acid, prion protein, selectin.

INTRODUCTION

The normal PrP C (cellular prion protein) is a highly conserved, GPI (glycosylphosphatidylinositol)-anchored, cell surface protein present on many cell types [1,2]. Currently, the normal physiological functions of PrP C remain unclear. PrP C binds metal and functions as a metal transporter [3,4]. PrP C has pro-apoptotic, as well as anti-apoptotic, activities [5,6]. Many different ligands for PrP C have been identified using bacteria-produced recombinant PrP C, yeast two-hybrid system, biochemical cross-linking and transfected cell lines, which overexpress PrP C [7]. Consequently, the significance of these interactions remains unclear.

The human PrP C has two highly conserved, N-linked glycosylation sites. The N-linked glycans on PrP are of the complex type, which contain terminal galactose, sialic acid and fucose attached to the innermost GlcNAc cores. Approximately 70 % of the terminal galactose on PrP C is modified with sialic acid. Another feature of PrP C is that its GPI anchor contains sialic acids [8]. Rodent PrP C also contains sulfate [9]. More detailed studies using MS with purified hamster and murine PrP C found that both of the N-linked glycosylation sites are glycosylated, resulting in the generation of more than 30 glycostructures, and the glycans contain a trisaccharide, LeX (Lewis X), and/or a tetrasaccharide, sLeX (sialyl-Lewis X) [10,11]. Currently, the biological significance of glycosylation in PrP C function and the pathogenesis of prion diseases are not completely understood. All prion diseases are believed to share the same pathogenic mechanism, which is based on the conversion of the PrP C into the infectious and pathogenic PrP 27-30 (scrapie prion protein) [2].

The selectins (CD62) are a family of cell surface molecules that are important in cell adhesion and migration [12–14]. There are three selectins: L-selectin (CD62L), which is found on most leucocytes; P-selectin (CD62P), which is present on activated platelets and endothelial cells; and E-selectin (CD62E), which is expressed exclusively on activated endothelial cells. All three selectins interact with carbohydrates in a Ca 2+-dependent manner, and sialic acid is an essential component of the carbohydrate recognition complex. Of all the selectin ligands, the most extensively studied is sLeX [15].

Most studies on selectins have been focused on the roles they play in migration of blood cells and interactions between blood cells and the vascular system in inflammation. Much less is known about the role selectins play in other systems, such as the CNS (central nervous system). Previously, Huang et al. [16] reported the presence of an L-selectin ligand on myelin in the mouse CNS [16]. Interestingly, binding of L-selectin to myelin is sialic acid-independent. The ligand was thought to be a surface protein with a lipid anchor [16]. The levels of L-selectin have been reported to be up-regulated during early stages of prion diseases in animals [17]. Because mouse and rat brain PrP C contain LeX and sLeX epitopes, PrP C in the CNS may interact with selectins. In the present paper, we investigate whether human brain-derived PrP C contains LeX and/or sLeX epitopes and whether human PrP C interacts with selectins.

MATERIALS AND METHODS

Cell lines

CHO (Chinese-hamster ovary)-K1, LEC11 and LEC12 cells lines were provided by Dr Pamela Stanley of Albert Einstein College of Medicine (New York, NY, U.S.A.). LEC11 and LEC12 cell lines are glycosylation mutants derived from the CHO-K1

Abbreviations used: CHO, Chinese-hamster ovary; CNS, central nervous system; GPI, glycosylphosphatidylinositol; HRP, horseradish peroxidase; LeX, Lewis X; mAb, monoclonal antibody; MoCD24, mouse CD24; NCAM, neural cell-adhesion molecule; PNGase F, peptide N-glycosidase F; PrP C, cellular prion protein; PrP 27-30, scrapie prion protein; PSGL-1, P-selectin glycoprotein ligand-1; sLeX, sialyl-LeX; SPR, surface plasmon resonance.

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cell line and are known to express two different GDP-fucose/N-acetylgalcosaminidase 3-α-L-fucosyltransferases, which are responsible for the differences in expression of Le^x and sLe^x epitopes on these mutant cell lines [48].

Antibodies and other reagents

Anti-PrP mAbs (monoclonal antibodies) 7A12, 8H4 and 8F9 were produced, affinity-purified and biotinylated as previously reported [49]. mAb 7A12, mAb 8H4 and mAb 8F9 recognize amino acid residues 135–145, 175–185 and 225–231 respectively. These mAbs are specific for PrP^C and have been characterized extensively. Antibodies against E-, L- and P-selectins, recombinant human E-, L-, P-selectin–IgG, Ig fusion proteins and human NCAM (neural cell-adhesion molecule–Ig) fusion protein were purchased from R&D Systems. All the fusion proteins were produced in NSO cells. Mouse IgM antibody, goat anti-mouse IgG–HRP (horseradish peroxidase) antibody and FITC–goat anti-mouse IgM were purchased from Chemicon. Mouse anti-human CD15 (clone HI98, IgM), rat anti-mouse CD24 and goat anti-rat IgG–HRP were purchased from BioLegend. Mouse anti-human CD15s (clone CSLEX1, IgM) was purchased from BD Pharmingen. Neuraminidase was purchased from ICN. Protein G–agarose beads were purchased from Roche. PNGase F (peptide N-glycosidase F) was purchased from New England Biolabs. All reagents for SPR (surface plasmon resonance) assays were supplied by the Biocore Core Facility of the Cleveland Clinic Foundation (Cleveland, OH, U.S.A.) through Biacore. Protein assay kits were purchased from Bio-Rad.

Generation of fusion proteins, transfection and purification of fusion proteins

Human full-length PrP^C DNA (nt 97–780) was PCR-amplified with primers. The forward primer was 5′-TACCGTTGGGACCGACTTCTA-3′ and the reverse primer was 5′-AGAGACATTATCTGGACTCTA-3′. The Ig fragment of human IgG was PCR-amplified with primers. The forward primer sequence was 5′-GCTCCCCGGGCGACTAGGA-3′ and the reverse primer was 5′-TCAATTACCCGCAGAGGGAG-3′. MoCD24 (mouse CD24) DNA was also PCR-amplified (nt 71–243) with forward primer (5′-TGTAACCGGTCTGCTACTCC-3′) and reverse primer (5′-CCTCCGGCGTGGGAGACCC-5′). All the PCR products were cloned into PGEM-T easy vector from Promega. To construct PrP–Ig and MoCD24–Ig, the PCR-amplified PrP and MoCD24 were cleaved with EcoRI and then inserted into the NotI site of mutated plBluescript II SK from Stratagene separately to create PrP–Ig or MoCD24–Ig. The fragment was cleaved with SacII and SacI and then inserted into PrP–Ig or MoCD24–Ig. The constructs were cleaved further with HindIII and NotI and then inserted into pcDNA3.1/mygro from Invitrogen to create PrP–Ig–pcDNA3.1 and MoCD24–Ig–pcDNA3.1.

Each construct was transfected individually into CHO, LEC11 and LEC12 cells with Lipofectamine™ 2000 from Invitrogen. Stable cell lines were selected and maintained with 50 µg/ml hygromycin (Invitrogen). Secreted PrP^C–Ig and MoCD24–Ig proteins were purified with Protein G–agarose beads. The PrP^C–Ig and MoCD24–Ig-positive fractions were identified by goat anti-human IgG–HRP. The pooled proteins were quantified by ELISA and Western blots with anti-PrP, anti-mouse CD24 mAbs or anti-human IgG–HRP. The concentration of each fusion protein preparation was determined using a Bio-Rad protein assay kit.

Preparation of brain homogenates and treatment of brain homogenates or fusion proteins with enzymes

Brain tissues from non-CJD (Creutzfeldt–Jakob disease) controls were homogenized in 10 vol. (10%, v/v) of ice-cold lysis buffer (10 mM Tris, 150 mM NaCl, 1% Nonidet P40 and 0.5% sodium deoxycholate) in the presence of 1 mM PMSF [22]. After spinning at 4100g for 10 min, the supernatants were stored in aliquots at −80°C [22]. Preparation of the anti-PrP mAb-coupled Protein G immunoadfinity column has been described in detail in [18]. An mAb 8H4-coupled column was used to enrich the total PrP population in total brain homogenate. Brain homogenates or affinity-purified fusion proteins were treated with neuraminidase to remove the sialic acid or with PNGase F to remove the N-linked glycan following the manufacturer’s protocol (New England Biolabs). The treated brain homogenates were subjected for ELISA or precipitation.

ELISA

To detect Le^x or sLe^x epitopes on PrP^C in human brain homogenate, the ELISA plate was coated with affinity-purified anti-PrP mAb 8H4 or 8F9 (2 µg/well) in duplicate and blocked with 4% (w/v) BSA in PBST (PBS with 0.05% Tween 20, pH 7.4, 40 µl of a 20% brain homogenate was added to each well, and incubated at room temperature (25°C) for 1 h with rocking. The bound PrP species were detected with anti-human CD15, CD15s (0.5 µg/µl, 1:300) or an irrelevant IgM for 1 h followed by a goat anti-mouse IgM–HRP antibody (1:500) and absorbance was read at 405 nm.

To detect Le^x or sLe^x epitopes on recombinant PrP^C–Ig and MoCD24–Ig proteins expressed in CHO, LEC11 or LEC12 cells, purified fusion proteins from the respective cell lines were coated on the plate. The subsequent steps for detecting Le^x and sLe^x epitopes were the same as described above.

To detect interactions between selectins and PrP^C–Ig or MoCD24–Ig fusion proteins, 2 µg/well human E-selectin–Ig, P-selectin–Ig, NCAM-1–Ig or 1.35 µg/well human L-selectin–Ig fusion proteins were coated on ELISA plates. PrP^C–Ig and MoCD24–Ig were then loaded in the presence of 10 mM Ca^2+. Bound PrP^C–Ig was detected with biotinylated anti-PrP mAb 8H4 (0.5 µg/well) and detected further with streptavidin–HRP. The bound MoCD24–Ig was first detected by rat anti-mouse CD24 and then goat anti-rat IgG–HRP (BioLegend).

SPR

SPR experiments were performed at 25°C with a Biacore 3000 at The Cleveland Clinic Foundation Microbiology Core Facility. To detect binding between PrP–Ig and selectins, E-, L- and P-selectins were immobilized via amine groups at pH 4.5, or PrP–Ig was immobilized via amine groups at pH 4.0 on a CMS chip. Binding between PrP–Ig and selectins in HBS-P solution (0.01 M Hepes, pH 7.4, 0.15 M NaCl and 0.005% Tween 20) with either Ca^2+ or Mg^2+ was measured at a flow rate of 20 µl/min. Regeneration was performed with 10 mM glycine (pH 3.0) at a flow rate of 20 µl/min. The data were simulated and the binding affinity of each interaction was obtained either through steady-state measurements if selectins were immobilized or through the Langmuir method if PrP–Ig was immobilized.

Precipitation of brain-derived PrP^C with selectin–Ig fusion proteins

Normal brain homogenate (40 µl) was incubated with 10 µg of E-selectin–Ig, L-selectin–Ig or P-selectin–Ig fusion proteins with 10 nM Ca^2+ or EDTA at room temperature for 1 h. The beads were then washed five times in PBST at 1000 g for 2 min.
RESULTS

Human brain PrP<sup>C</sup> contains Le<sup>x</sup> and sLe<sup>x</sup>

We first determined whether PrP<sup>C</sup> in human brain contains Le<sup>x</sup> and sLe<sup>x</sup> epitopes by ELISA. PrP<sup>C</sup> species in human brain homogenates were captured on ELISA plates with either anti-PrP<sup>C</sup> mAb 8H4 or 8F9. These two mAbs react with two distinct epitopes on PrP<sup>C</sup>. An anti-CD15 (Le<sup>x</sup>) or an anti-CD15s (sLe<sup>x</sup>) mAb was used individually to react with captured PrP<sup>C</sup>. Both mAbs reacted significantly with the brain PrP<sup>C</sup> species. An irrelevant, control IgM antibody did not react with the captured PrP<sup>C</sup> (Figure 1A). By RIA, it was found that the parental CHO cell line expresses neither Le<sup>x</sup> nor sLe<sup>x</sup> epitopes, the LEC11 and LEC12 cell lines are glycosylation mutants, which were derived from the parental CHO cell line. By RIA, it was found that the parental cell line, CHO, expresses neither Le<sup>x</sup> nor sLe<sup>x</sup> epitopes, the LEC11 cell line has more sLe<sup>x</sup> than Le<sup>x</sup> epitopes and the LEC12 cell line has Le<sup>x</sup> epitope [19].

We then purified the PrP<sup>F<sub>C</sub></sup>–Ig-CHO, PrP<sup>F<sub>C</sub></sup>–Ig-LEC11 and PrP<sup>F<sub>C</sub></sup>–Ig-LEC12 fusion proteins from the culture supernatants of the respective cell lines by Protein G-affinity chromatography, and determined whether these PrP<sup>F<sub>C</sub></sup>–Ig fusion proteins express either Le<sup>x</sup> or sLe<sup>x</sup> epitopes by ELISA. Plates were first precoated with an anti-PrP mAb, 8H4. Identical amounts of purified PrP<sup>F<sub>C</sub></sup>–Ig-CHO, PrP<sup>F<sub>C</sub></sup>–Ig-LEC11 or PrP<sup>F<sub>C</sub></sup>–Ig-LEC12 fusion proteins were then added on to the plates. Antibodies specific for CD15 (Le<sup>x</sup>) or CD15s (sLe<sup>x</sup>) or an irrelevant IgM were then added to react with the bound PrP<sup>F<sub>C</sub></sup>–Ig fusion proteins. We did not detect any binding with PrP<sup>F<sub>C</sub></sup>–Ig-CHO; thus this fusion protein lacks both Le<sup>x</sup> and sLe<sup>x</sup> epitopes (Figure 2A). Interestingly, PrP<sup>F<sub>C</sub></sup>–Ig-LEC11 reacted with anti-CD15s (sLe<sup>x</sup>) but not with anti-CD15 (Le<sup>x</sup>) antibodies. Therefore human brain-derived PrP<sup>C</sup> contains both Le<sup>x</sup> and sLe<sup>x</sup> epitopes.
Expression of PrP<sub>C</sub>–Ig fusion proteins in CHO, LEC11 and LEC12 cell lines

(A–C) Equal amounts of affinity-purified PrP<sub>C</sub>–Ig fusion proteins from CHO, LEC11 or LEC12 cells were added onto ELISA plates that had been coated with anti-PrP<sub>C</sub> mAb 8H4. Bound fusion proteins were then detected with either mouse IgM, anti-CD15 or anti-CD15s antibodies. The bound antibody was detected further with goat anti-mouse IgM–HRP. Neither Le<sup>X</sup> nor sLe<sup>X</sup> epitope was detected on PrP<sub>C</sub>–Ig CHO, only sLe<sup>X</sup> was detected on PrP<sub>C</sub>–Ig LEC11, and only Le<sup>X</sup> was detected on PrP<sub>C</sub>–Ig-LEC12. Results are the means ± S.D. for four experiments (n = 16 for each sample).

(D) Affinity-purified PrP<sub>C</sub>–Ig fusion proteins were applied to SDS/PAGE under non-reducing conditions and then immunoblotted with mAb 8H4. Under non-reducing conditions, the apparent molecular mass of the fusion protein is approx. 100 kDa. (E, F) Affinity-purified PrP<sub>C</sub>–Ig fusion proteins were treated with PBS or PNGase F, then applied to SDS/PAGE under reducing conditions and immunoblotted with mAb 8H4. PNGase F treatment reduced the molecular mass of the PrP<sub>C</sub>–Ig chimaera from 52 kDa to approx. 42 kDa. OD, absorbance.

mAb (Figure 2B), while PrP<sub>C</sub>–Ig-LEC12 reacted only with anti-CD15 (Le<sup>X</sup>) but not anti-CD15s (sLe<sup>X</sup>) mAb (Figure 2C). Similar amounts of PrP<sup>C</sup>–Ig proteins were captured with anti-PrP mAb because comparable levels of binding were detected when an anti-human Ig antibody instead of anti-CD15 or anti-CD15s was used to detect bound PrP<sup>C</sup>–Ig (results not shown). Therefore PrP<sup>C</sup>–Ig-CHO lacks both Le<sup>X</sup> and sLe<sup>X</sup>, PrP<sup>C</sup>–Ig-LEC11 has sLe<sup>X</sup> but lacks detectable Le<sup>X</sup>, and PrP<sup>C</sup>–Ig-LEC12 contains Le<sup>X</sup> epitope but is deficient in sLe<sup>X</sup> epitope.

Under reducing conditions in SDS/PAGE, the three fusion proteins migrated as proteins with a molecular mass of approx. 52 kDa (Figure 2D). Under non-reducing conditions in SDS/PAGE, the three proteins had an apparent molecular mass of 100 kDa, corresponding to the fusion protein dimer, which doubles the expected molecular mass of human PrP<sup>C</sup> combined with the Fc region of human IgG<sub>1</sub> (Figure 2E). When the fusion proteins were treated with PNGase to remove the N-linked glycans, they migrated as proteins with a molecular mass of approx. 42 kDa (Figure 2F). We did not detect any differences in the binding of multiple anti-PrP mAbs (n = 6) between PrP<sup>C</sup>–Ig and brain-derived PrP<sup>C</sup>; therefore it is unlikely that addition of the Ig portion has drastically altered the overall conformation of the PrP<sup>C</sup> (results not shown).

Binding of human PrP<sub>C</sub>–Ig fusion proteins to human selectin–Ig fusion proteins

We next determined whether the three PrP–Ig fusion proteins (PrP<sup>C</sup>–Ig-CHO, PrP<sup>C</sup>–Ig-LEC11 and PrP<sup>C</sup>–Ig-LEC12) interact with human selectin–Ig fusion proteins: P-selectin–Ig, E-selectin–Ig and L-selectin–Ig. All selectin fusion proteins were produced in NSO cells. An NCAM–Ig fusion protein produced in NSO cells was included as a control. None of the PrP<sup>C</sup>–Ig fusion proteins reacted with NCAM–Ig (Figure 3A). On the other hand, PrP<sup>C</sup>–Ig-LEC12, which contains Le<sup>X</sup>, is able to bind all three selectins. Neither PrP<sup>C</sup>–Ig-CHO, which lacks both Le<sup>X</sup> and sLe<sup>X</sup> epitopes, nor PrP<sup>C</sup>–Ig-LEC11, which carries the sLe<sup>X</sup> epitope, was able to bind selectins. Binding to selectins is PrP<sup>C</sup>–Ig fusion protein concentration-dependent (Figure 3B), and requires Ca<sup>2+</sup> (Figure 3C). Addition of EDTA, a Ca<sup>2+</sup> chelator, inhibits binding.

Treatment with neuraminidase enables PrP<sub>C</sub>–Ig-LEC11 to bind selectins

To determine whether the presence of sialic acid interferes with the binding of PrP<sup>C</sup>–Ig-LEC11 fusion protein to selectins, we treated this fusion protein with neuraminidase to remove the sialic acid residues. After neuraminidase treatment, PrP<sup>C</sup>–Ig-LEC11 is able to bind all three selectins (Figure 4). However, neuraminidase-treated PrP<sup>C</sup>–Ig-LEC11 remained unable to bind NCAM–Ig. These results provide strong evidence that the presence of sialic acid on PrP<sup>C</sup>–Ig-LEC11 prevents its binding to selectins.

Binding between PrP<sub>C</sub>–Ig-LEC12 and selectins is PrP<sub>C</sub>-specific

The Fc portion of the PrP–Ig fusion protein has one potential N-linked glycosylation site. Therefore the specificity of the interaction between PrP<sup>C</sup>–Ig-LEC12 and the selectins was validated further by using another fusion protein, mouse CD24–Ig fusion protein produced in LEC12 cells. We chose CD24 for the
Normal cellular prion protein is a ligand of selectins

Figure 3  PrP<sup>C</sup>–Ig from LEC12 cells binds E-, L- and P-selectins in a Ca<sup>2+</sup>-dependent manner

(A) ELISA plates were precoated with identical amounts of E-selectin–Ig, L-selectin–Ig, P-selectin–Ig or NCAM–Ig fusion protein. Identical amounts of affinity-purified PrP<sup>C</sup>–Ig fusion proteins from CHO, LEC11 and LEC12 cells were then added to the plates. The bound PrP<sup>C</sup>–Ig fusion proteins were then detected with a biotinylated anti-PrP<sup>C</sup> mAb, 8H4. The bound antibody was detected further with streptavidin–HRP. Only PrP<sup>C</sup>–Ig fusion protein from LEC12 cells binds all three selectins. None of the PrP<sup>C</sup>–Ig fusion proteins bind NCAM. Results are the means ± S.D. for four experiments (n = 12). (B) Binding between selectins and PrP<sup>C</sup>–Ig LEC12 is PrP<sup>C</sup>–Ig fusion protein concentration-dependent. (C) Binding between PrP<sup>C</sup>–Ig LEC12 and selectin–Ig fusion protein is eliminated in the presence of EDTA (10 mM). P-values between binding of PrP<sup>C</sup>–Ig LEC12 to selectin–Ig fusion proteins with or without EDTA were <0.005 for all three selectins. Results are the means ± S.D. for three experiments (n = 8 for each treatment). OD, absorbance.

Figure 4  Treatment with neuraminidase enables PrP<sup>C</sup>–Ig-LEC11 to bind all three selectins

ELISA plates were precoated with identical amounts of E-selectin–Ig, L-selectin–Ig, P-selectin–Ig or NCAM–Ig fusion proteins. Equal amounts of affinity-purified, PBS-treated or neuraminidase-treated PrP<sup>C</sup>–Ig LEC11 were then added on to the plates. The bound fusion proteins were then detected with a biotinylated anti-PrP<sup>C</sup> mAb, 8H4. Bound antibody was detected further with streptavidin–HRP. Results are the means ± S.D. for four experiments (n = 8 for each sample). Removal of sialic acid enables PrP<sup>C</sup>–Ig-LEC11 to bind all three selectin–Ig fusion proteins. Neuraminidase-treated PrP<sup>C</sup>–Ig-LEC11 remained unable to bind NCAM–Ig fusion protein. OD, absorbance.

Figure 5  Mouse CD24–Ig produced in LEC12 cells (MoCD24–Ig-LEC12) bound only P-selectin

(A) Mouse CD24–Ig expressed in LEC12 cells exists as dimer under non-reducing conditions with an apparent molecular mass of 98 kDa. Its apparent molecular mass under reducing conditions is 49 kDa. (B) ELISA plates were precoated with identical amounts of E-selectin–Ig, L-selectin–Ig, P-selectin–Ig or NCAM–Ig fusion protein. Identical amounts of affinity-purified MoCD24–Ig-LEC12 fusion proteins were then added onto the plates. The bound MoCD24–Ig-LEC12 proteins were then detected with rat anti-mouse CD24 antibody and the binding was detected further with goat anti-rat IgG–HRP. MoCD24–Ig-LEC12 fusion protein reacts only with P-selectin. Results are the means ± S.D. for three experiments (n = 6 for each treatment).
having an LeX epitope is insufficient for the fusion protein to bind all three selectins. From these experiments, we concluded that binding between PrP\(^{\text{C}}\)–Ig-LEC12 and the three selectins requires both the LeX epitope on PrP\(^{\text{C}}\) and the PrP\(^{\text{C}}\) protein backbone.

### Affinity of binding of PrP\(^{\text{C}}\)–Ig-LEC12 to selectin–Ig

We next determined the affinity of binding of PrP\(^{\text{C}}\)–Ig-LEC12 to the selectins by SPR. Affinity was determined through steady-state measurements or through the Langmuir method. We found that PrP\(^{\text{C}}\)–Ig-LEC12 binds to the three selectins with rather high affinity (\(K_d\)), ranging from approx. 66 nM for L-selectin, 220 nM for P-selectin and 340 nM for E-selectin (Table 1). The requirement for Ca\(^{2+}\) was also confirmed in these binding studies; replacement of Ca\(^{2+}\) with Mg\(^{2+}\) completely abolished the binding of PrP\(^{\text{C}}\)–Ig-LEC12 to the selectins (results not shown).

### Interactions between brain-derived PrP\(^{\text{C}}\) and selectins

When human brain homogenate was electrophoresed and immunoblotted with anti-PrP mAb 7A12, we observed the characteristic immunoreactive pattern with three bands (Figure 6A). The 36 kDa band is the full-length, fully glycosylated PrP\(^{\text{C}}\) species, while the two smaller PrP species are mainly N-terminally truncated PrP species [22]. We next sought to verify that selectins indeed bind human brain-derived, native PrP\(^{\text{C}}\). Human brain homogenates were first incubated with one of the four fusion proteins: E-selectin–Ig, P-selectin–Ig, L-selectin–Ig or NCAM–Ig. Precipitated proteins were then separated by SDS/PAGE and immunoblotted with anti-PrP C mAb, 7A12. All three selectins but not the NCAM–Ig fusion protein precipitated three proteins with apparent molecular masses of 29, 32 and 36 kDa, a pattern identical with the one seen in (Figure 6A). Proteins in normal human brain homogenates were precipitated with NCAM–Ig, and then immunoblotted with anti-CD15 mAb (Figure 7C); these differences were rather small, but significant. As expected, the amount of PrP\(^{\text{C}}\) precipitated with E-selectin–Ig was greater in the sample that had been previously treated with neuraminidase (Figure 7D). In samples that were immunoblotted with mAb 8H4, treatment of brain homogenate with neuraminidase slightly increased the mobility of the PrP species, reflecting the removal of sialic acid residues (Figure 7B).

Accordingly, treatment of brain homogenate with neuraminidase increased the binding of anti-CD15 mAb but decreased the binding of anti-CD15s (sLe\(^X\)) mAb in ELISA (Figure 7C), and remaining samples were precipitated with either E-selectin–Ig or NCAM–Ig, and then immunoblotted with anti-PrP\(^{\text{C}}\) mAb 8H4 (Figure 7D). In samples that were immunoblotted with mAb 8H4, treatment with neuraminidase slightly increased the mobility of the PrP species, reflecting the removal of sialic acid residues (Figure 7B).

**DISCUSSION**

Human PrP\(^{\text{C}}\)–Ig-LEC12 fusion protein with Le\(^X\) structure binds E-, L- and P-selectin–Ig fusion proteins in a Ca\(^{2+}\)-dependent manner. On the other hand, PrP\(^{\text{C}}\)–Ig-LEC11 fusion protein with sLe\(^X\) structure does not bind. However, removal of the sialic acid on PrP\(^{\text{C}}\)–Ig-LEC11 enables it to bind all three selectins. Most importantly, all three selectin–Ig proteins but not control proteins

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**Table 1 Binding affinity of PrP\(^{\text{C}}\)–Ig-LEC12 for selectin–Ig**

<table>
<thead>
<tr>
<th>Selectin</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
<th>Mean ± S.D.</th>
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<td>P-selectin</td>
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<tr>
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<td>60</td>
<td>67</td>
<td>57</td>
<td>66 ± 10.23</td>
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**Figure 6 Human brain PrP\(^{\text{C}}\) binds selectins and binding is Ca\(^{2+}\)-dependent**

(A) Proteins in normal human brain homogenate were separated by SDS/PAGE and then immunoblotted with anti-PrP\(^{\text{C}}\) mAb, 7A12. It showed the typical three bands at approx. 29, 32 and 36 kDa. (B) Proteins in normal human homogenates were precipitated with NCAM–Ig, E-selectin–Ig, L-selectin–Ig or P-selectin–Ig fusion protein. Precipitated proteins were then separated by SDS/PAGE and immunoblotted with anti-PrP\(^{\text{C}}\) mAb, 7A12. All three selectins but not the NCAM–Ig fusion protein precipitated three proteins with apparent molecular masses of 29, 32 and 36 kDa, a pattern identical with the one seen in (A). (C) When precipitation with selectin–Ig was carried out in the presence of 10 mM EDTA, no PrP can be precipitated by selectin–Ig fusion proteins. Therefore binding of all three selectin–Ig fusion proteins to brain-derived PrP\(^{\text{C}}\) is also calcium-dependent. I. P., immunoprecipitated.
The neuraminidase-treated brain homogenate than PBS-treated brain homogenate. The neuraminidase-treated, E-selectin–Ig-precipitated PrPC proteins also migrated slightly faster than the PBS-treated glycans on PrPC are critical for binding of selectin–Ig to PrPC. Controls. I. P., immunoprecipitated.

Equal amounts of PBS-treated control homogenates or neuraminidase-treated brain homogenates were added onto the ELISA plate. An anti-CD1 5 mAb or anti-CD15s precipitated with E-selectin–Ig or NCAM–Ig. Precipitated proteins were then separated by SDS/PAGE and immunoblotted with anti-PrPC mAb, 8H4. E-selectin–Ig precipitated more PrPC protein from immunoblotted with anti-PrPC mAb, 8H4. Neuraminidase treatment reduced the apparent molecular mass of PrPC as compared with the non-treated PrPC proteins. Brain-derived PrPC increases the amount of PrP C precipitated in a Ca 2+

also precipitated human brain-derived PrP C in a Ca 2+ and N-linked glycan-dependent manner. Removal of sialic acid from brain-derived PrP C increases the amount of PrP C precipitated with selectins. Both the Le X structure and the protein portion of PrP C are important in binding to selectins. An irrelevant protein, mouse CD24–Ig-LEC12, which bears Le X, failed to bind either E- or L-selectins, but did bind P-selectin, a known ligand of CD24 [20]. We hypothesize that interactions between PrP C and selectins may be important in normal cellular physiology and/or pathophysiology under some conditions.

PrP C–Ig-LEC12 protein binds the three selectin–Ig proteins with K d values in the nanomolar range: approx. 66 nM for L-selectin, 220 nM for P-selectin and 340 nM for E-selectin. These values are substantially higher than those reported earlier, with the exception of the binding of P-selectin to PSGL-1 (P-selectin glycoprotein ligand-1) on neutrophils [23]. The reported affinity between selectins and their respective ligands varies significantly, ranging from 70 nM for the binding of P-selectin to PSGL-1 to 7800 nM for the binding of the same selectin to sLe X [23,24]. Both selectin–Ig and PrP C–Ig proteins are dimeric, and thus bivalent. One may argue that their affinity may be overestimated. In fact, recombinant PrP does exist as a dimer [25]. Furthermore, PrP C is a GPI-anchored protein occupying microdomains on the cell membrane known as lipid rafts; each lipid raft is known to contain multiple GPI-anchored proteins [26]. Some selectins are also present on special domains, pseudopods, on the cell surface in multimeric forms [23]. Our conclusion that PrP C binds selectins with rather high affinity is also supported by our finding that all selectin–Ig proteins precipitated PrP C in human brain in a Ca 2+-dependent manner.

The ability of PrP C with Le X epitopes to bind selectins is reminiscent of the binding of P-selectins to PSGL-1. On the surface of lymphocytic cells, a significant percentage of the PSGL-1 present is non-functional, not binding selectins due to the lack of proper glycans. Therefore, in PSGL-1, binding to selectins requires the presence of sLe X epitope; in contrast, binding of PrP C to selectins necessitates the absence of the sLe X epitope. Addition of sLe X on PrP C may be a mechanism by which the interaction between PrP C and selectins is regulated.

Whether interactions between selectins and PrP C are important in cellular physiology is not known. PrP C is broadly expressed on the surface of human peripheral blood cells, including T-cells, B-cells, monocytes, neutrophils, dendritic cells as well as platelets [27,28]. Furthermore, memory T-cells, activated T-cells and macrophages express more PrP C than naive, resting T-cells or unstimulated macrophages. PrP C on leucocytes participates in signal transduction [29]. Human PrP C is physically present in the ‘immune synapses’, which are critical for the interactions between T-lymphocytes and antigen-presenting cells [30]. On the other hand, L-selectins are present on most leucocytes and P-selectins are present on platelets. Therefore it is highly likely PrP C and selectins will encounter each other, either on the same cell surface or on different cell types. However, the consequences of these interactions are not clear at this time. Conversely, PrP C is present on cultured human umbilical-vein endothelial cells [31]. Human brain endothelial cells also express PrP C [32]. Importantly,
expression of PrPc has been reported to be critical in the trans-endothelial migration of human monocytes [32]. Therefore selectins on blood cells can also interact with PrPc on endothelial cells, which may be important in cellular activation, adhesion or migration.

The nature of the N-linked glycans on PrPc is cell-type-dependent. In SDS/PAGE, PrPc species from human peripheral blood mononuclear cells migrate slower than brain-derived PrPc due to the difference of their N-linked glycans [33]. However, whether PrPc on human peripheral blood cells has LeX or sLeX epitopes is not known. Recently, we reported that the natures of N-linked glycans on PrPc change during normal aging in mouse [34]. Selective modification of the N-linked glycans on PrPc in different cell types or during normal aging will provide additional mechanisms for regulating the interactions between PrPc and selectins.

In contrast with human, most of the circulating lymphocytes in mice lack PrPc. PrPc is preferentially expressed on immature thymocytes, and in a subpopulation of bone marrow progenitor cells [35]. Since there is no obvious phenotypic alteration in leucocyte composition in peripheral or central lymphoid organs in mice lacking PrPc expression, interactions between PrPc on blood cells and selectins on vascular structures do not seem to be important in lymphocyte migration in mouse. However, a recent report found that PrPc−/− mice are deficient in recruitment of neutrophils after intraperitoneal injection of zymosan, suggesting that PrPc may be important in cell migration under certain conditions [36]. Another recent report found that bone marrow cells from PrPc−/− mice are less efficient in repopulating irradiated recipients, suggesting a possible defect in bone marrow stem cells in PrPc−/− mice [37]. Yet another report found that normal T-lymphocyte development is altered in mouse, which overexpresses PrPc on thymocytes [38]. Collectively, all these findings are also consistent with the interpretation that PrPc may be important in cell–cell interactions and cell migrations.

Leucocyte infiltration is rarely seen in prion diseases, but activation of microglial cells has been speculated to be important in prion diseases. PrPc is expressed on microglial cells, neurons and astrocytes. Activated astrocytes express increased levels of E- and P-selectins [39]. In prion disease, neurons and astrocytes have been shown to recruit microglia [40]. In PrPc−/− mouse brain, there were early up-regulation of L-selectin expression and down-regulation of P-selectin expression [17,41]. In vitro, direct cell-to-cell contact is required for PrPc spreading from one infected cell to another [42]. Migration and/or adhesion of microglia cells involving PrP and selectins may play a role in the spread of PrPsc.

Both cell-surface PrPc and selectins have signal transduction capability [43,44]. Binding of PrPc in the CNS by anti-PrPc antibody activates apoptosis and neurodegeneration in mice [45]. Both soluble selectins and PrPc are present in the CNS [46,47]. Potentially, binding of soluble selectins to PrPc may have pathological consequences similar to those of cross-linking with anti-PrPc antibody. Conversely, binding of soluble PrP on selectins may further activate selectin-bearing cells, such as glial cells, astrocytes or endothelial cells.

By expressing PrPc−Ig in cell lines with different capacity to add unique N-linked glycostructures, we have been able to identify novel binding partners of PrPc, the selectins. Three important issues remain to be addressed: (i) identifying the precise nature of the N-linked glycans that are important in these interactions, which will require much more detailed biochemical analysis; (ii) investigating whether interactions between PrPc and selectins are important in the normal physiology of the CNS; and (iii) determining whether these interactions are important in the pathogenesis of prion diseases. Experiments using sLeX−/− deficient mice may provide new insights into these questions.

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REFERENCES


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