Normal cellular prion protein is a ligand of selectins: binding requires Le^X but is inhibited by sLe^X

Chaoyang LI*, Poki WONG*, Tao PAN*, Fan XIAO*, Shaoman YIN*, Binggong CHANG*, Shin-Chung KANG*, James IRONSIDE† and Man-Sun SY*[1]

*Institute of Pathology, School of Medicine, Case Western Reserve University, Cleveland, OH 44107-1712, U.S.A., and †Division of Neuropathology, University of Edinburgh, Edinburgh, U.K.

The normal PrP^C (cellular prion protein) contains sLe^X [sialyl-Le^X (Lewis X)] and Le^X. sLe^X is a ligand of selectins. To examine whether PrP^C is a ligand of selectins, we generated three human PrP^C–Ig fusion proteins: one with Le^X, one with sLe^X, and the other with neither Le^X nor sLe^X. Only Le^X-PrP^C–Ig binds E-, L- and P-selectins. Binding is Ca^{2+}-dependent and occurs with nanomolar affinity. Removal of sialic acid on sLe^X-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 (Le^X) 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 brain 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, Le^X (Lewis X), and/or a tetrasaccharide, sLe^X (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^{Sc} (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 sLe^X [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 sLe^X and Le^X epitopes, PrP^C in the CNS may interact with selectins. In the present paper, we investigate whether human brain-derived PrP^C contains Le^X and/or sLe^X 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; Le^X, 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^{Sc}, scrapie prion protein; PSGL-1, P-selectin glycoprotein ligand-1; sLe^X, sialyl-Le^X; SPR, surface plasmon resonance.

[1] To whom correspondence should be addressed, at Room 5131, Wolstein Research Bldg, School of Medicine, Case Western Reserve University, 2103 Cornell Road, Cleveland, OH 44106-7288, U.S.A. (email mxs92@po.cwru.edu).

cell line and are known to express two different GDP-fucose:*N*-acetylgucosaminide 3-α-L-fusosyltransferases, 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 and have been characterized extensively. Antibodies against E-, L- and P-selectins, recombinant human E-, L-, P-selectin–IgG_1 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′-TACCGCTTGGAACC-GACGACCT-3′ and the reverse primer was 5′-AGAGACCATTA-TCCGGACTCTA-3′. The Ig fragment of human IgG_1 was PCR-amplified with primers. The forward primer sequence was 5′-GCTCCCGCGCGGCAGTCAGAA-3′ and the reverse primer was 5′-TCATTTACCCGGAGACAGGGAG-3′. MoCD24 (mouse CD24) DNA was also PCR-amplified (nt 71–243) with forward primer (5′-TGTACCCGTCTCGCTACCACC-3′) and reverse primer (5′-CCTCGACGGTGGGGGAGACCAC-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 pBluescript II SK from Stratagene separately to create PrP-pBlu or MoCD24-pBlu. The Ig fragment was cleaved with SacII and SacI and then inserted into PrP-pBlu or MoCD24-pBlu to generate PrP-Ig-pBlu and MoCD24-Ig-pBlu. The constructs were cleaved further with HindIII and NotI and then inserted into pcDNA3.1/Hygro 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/w) 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 4100 ***g*** for 10 min, the supernatants were stored in aliquots at −80 °C [22]. Preparation of the anti-PrP mAb-coupled Protein G immunoaffinity 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 CM5 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^C–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 mM 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.

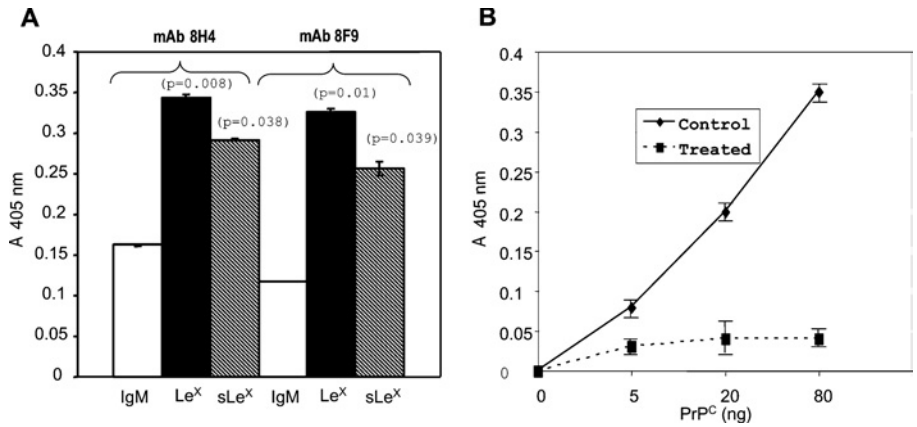

Figure 1 Human brain PrP^C had Le^X and sLe^X epitopes

(**A**) Human brain-derived PrP^C species were first captured with either anti-PrP^C mAb 8H4 (epitope between amino acid residues 175 and 185) or 8F9 (epitope between amino acid residues 225 and 231). Plates were then incubated with an irrelevant mouse IgM, anti-CD15 (Le^X) or anti-CD15s (sLe^X) mAbs. The bound antibody was detected further with HRP-conjugated anti-mouse IgM antibody (IgM–HRP). Both anti-CD15 and anti-CD15s but not IgM antibodies reacted significantly with the captured PrP^C. Therefore human brain-derived PrP^C contains both Le^X and sLe^X epitopes. Results are the means $\pm$ S.D. for three experiments ($n = 12$ for each treatment). *P*-values represent comparisons between binding of IgM control and binding of anti-CD15 or anti-CD15s antibodies. (**B**) Affinity-purified human brain PrP^C were first treated with PBS or neuraminidase. After treatment, identical amounts of proteins were added into ELISA plates that had been precoated with anti-human CD15s antibody. Captured PrPs were then detected with a biotinylated 8H4 antibody. The bound mAb 8H4 antibody was reacted further with a streptavidin-conjugated HRP. mAb 8H4 did not react with the neuraminidase-treated PrP^C, indicating that the anti-CD15s mAb failed to capture the neuraminidase-treated PrP^C. Results are the means $\pm$ S.D. for three experiments ($n = 12$ for each treatment).

Bound proteins were subjected to SDS/PAGE and Western blots. NCAM-1–Ig fusion protein was used as a control.

Western blotting

Proteins were separated by SDS/12 % PAGE and then electro-transferred on to PVDF membranes. After blocking in blocking solution from Roche at 4 °C overnight, detection was by biotinylated 7A12 or 8H4 mAb against PrP followed by streptavidin–HRP. Western blots were developed using SuperSignal® West Femto Maximum Sensitivity Substrate from Pierce.

Statistical methods

Two-tailed *P* values were based on two-sample Student's *t* test assuming unequal variance (Microsoft Excel 2003).

RESULTS

Human brain PrP^C contains Le^X and sLe^X

We first determined whether PrP^C in human brain contains Le^X and sLe^X epitopes by ELISA. PrP^C species in human brain homogenates were captured on ELISA plates with either anti-PrP^C mAb 8H4 or mAb 8F9. These two mAbs react with two distinct epitopes on PrP^C. An anti-CD15 (Le^X) or an anti-CD15s (sLe^X) mAb was used individually to react with captured PrP^C. Both mAbs reacted significantly with the brain PrP^C species. An irrelevant, control IgM antibody did not react with the captured PrP^C species (Figure 1A). The ELISA also did not react with bacterial-produced, recombinant full-length human PrP (rHu-PrP$^{23-231}$), which lacks the N-linked glycans, or with brain homogenates in which the PrP^C species were depleted with anti-PrP^C mAb (results not shown). We also confirmed the presence of the sLe^X epitope on human PrP^C by an alternative approach. Human PrP^C species were first affinity-purified by anti-PrP mAb chromatography [18]. Purified PrP^C species were treated either with PBS or with neuraminidase. After treatment, different amounts of treated PrP^C were added to an ELISA plate, which had been precoated with an anti-CD15s mAb. A biotinylated anti-PrP^C mAb, 8H4, was then used to detect bound PrP^C. In the control PBS-treated homogenates, binding of anti-PrP^C mAb 8H4 is detected in a PrP^C concentration-dependent manner (Figure 1B). In contrast, when the purified human brain PrP^C was first treated with neuraminidase to remove the sialic acid, binding of mAb 8H4 was greatly reduced (Figure 1B). No immunoreactivity was detected when rHu-PrP$^{23-231}$ was added in place of brain-derived PrP^C (results not shown). In summary, the ELISA is specific for both PrP^C and N-linked glycans and human brain-derived PrP^C contains both Le^X and sLe^X epitopes.

Chimaeric human PrP^C–Ig fusion proteins produced in CHO, LEC11 and LEC12 cell lines

We generated an expression plasmid, which encodes a chimaeric fusion protein containing the full-length human PrP^C (without the GPI anchor) and the Fc region of human IgG$_1$. We then stably transfected this construct into three well-characterized cell lines, CHO, LEC11 and LEC12. 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^X nor sLe^X epitopes, the LEC11 cell line has more sLe^X than Le^X epitopes and the LEC12 cell line has Le^X epitope [19].

We then purified the PrP^C–Ig-CHO, PrP^C–Ig-LEC11 and PrP^C–Ig-LEC12 fusion proteins from the culture supernatants of the respective cell lines by Protein G-affinity chromatography, and determined whether these PrP^C–Ig fusion proteins express either Le^X or sLe^X epitopes by ELISA. Plates were first precoated with an anti-PrP mAb, 8H4. Identical amounts of purified PrP^C–Ig-CHO, PrP^C–Ig-LEC11 or PrP^C–Ig-LEC12 fusion proteins were then added on to the plates. Antibodies specific for CD15 (Le^X) or CD15S (sLe^X) or an irrelevant IgM were then added to react with the bound PrP^C–Ig fusion proteins. We did not detect any binding with PrP^C–Ig-CHO; thus this fusion protein lacks both Le^X and sLe^X epitopes (Figure 2A). Interestingly, PrP^C–Ig-LEC11 reacted with anti-CD15s (sLe^X) but not with anti-CD15 (Le^X)

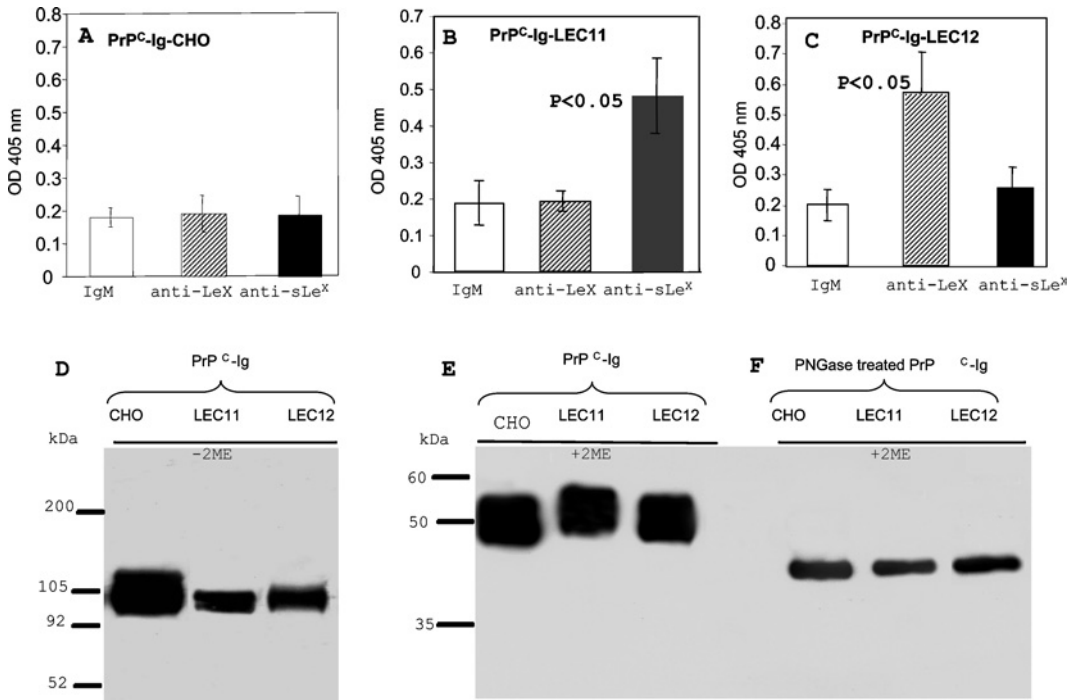

Figure 2 Expression of PrP^C–Ig fusion proteins in CHO, LEC11 and LEC12 cell lines

(**A**–**C**) Equal amounts of affinity-purified PrP^C–Ig fusion proteins from CHO, LEC11 or LEC12 cells were added on to ELISA plates that had been coated with anti-PrP^C 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^X nor sLe^X epitope was detected on PrP^C–Ig CHO, only sLe^X was detected on PrP^C–Ig LEC11, and only Le^X was detected on PrP^C–Ig-LEC12. Results are the means ± S.D. for four experiments ($n = 16$ for each sample). (**D**) Affinity-purified PrP^C–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^C–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^C–Ig chimaera from 52 kDa to approx. 42 kDa. OD, absorbance.

mAb (Figure 2B), while PrP^C–Ig-LEC12 reacted only with anti-CD15 (Le^X) but not anti-CD15s (sLe^X) mAb (Figure 2C). Similar amounts of PrP^C–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^C–Ig (results not shown). Therefore PrP^C–Ig-CHO lacks both Le^X and sLe^X, PrP^C–Ig-LEC11 has sLe^X but lacks detectable Le^X, and PrP^C–Ig-LEC12 contains Le^X epitope but is deficient in sLe^X 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^C combined with the Fc region of human IgG$_1$ (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^C–Ig and brain-derived PrP^C; therefore it is unlikely that addition of the Ig portion has drastically altered the overall conformation of the PrP^C (results not shown).

Binding of human PrP^C–Ig fusion proteins to human selectin–Ig fusion proteins

We next determined whether the three PrP–Ig fusion proteins (PrP^C–Ig-CHO, PrP^C–Ig-LEC11 and PrP^C–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^C–Ig fusion proteins reacted with NCAM–Ig (Figure 3A). On the other hand, PrP^C–Ig-LEC12, which contains Le^X, is able to bind all three selectins. Neither PrP^C–Ig-CHO, which lacks both Le^X and sLe^X epitopes, nor PrP^C–Ig-LEC11, which carries the sLe^X epitope, was able to bind selectins. Binding to selectins is PrP^C–Ig fusion protein concentration-dependent (Figure 3B), and requires Ca^{2+} (Figure 3C). Addition of EDTA, a Ca^{2+} chelator, inhibits binding.

Treatment with neuraminidase enables PrP^C–Ig-LEC11 to bind selectins

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

Binding between PrP^C–Ig-LEC12 and selectins is PrP^C-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^C–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

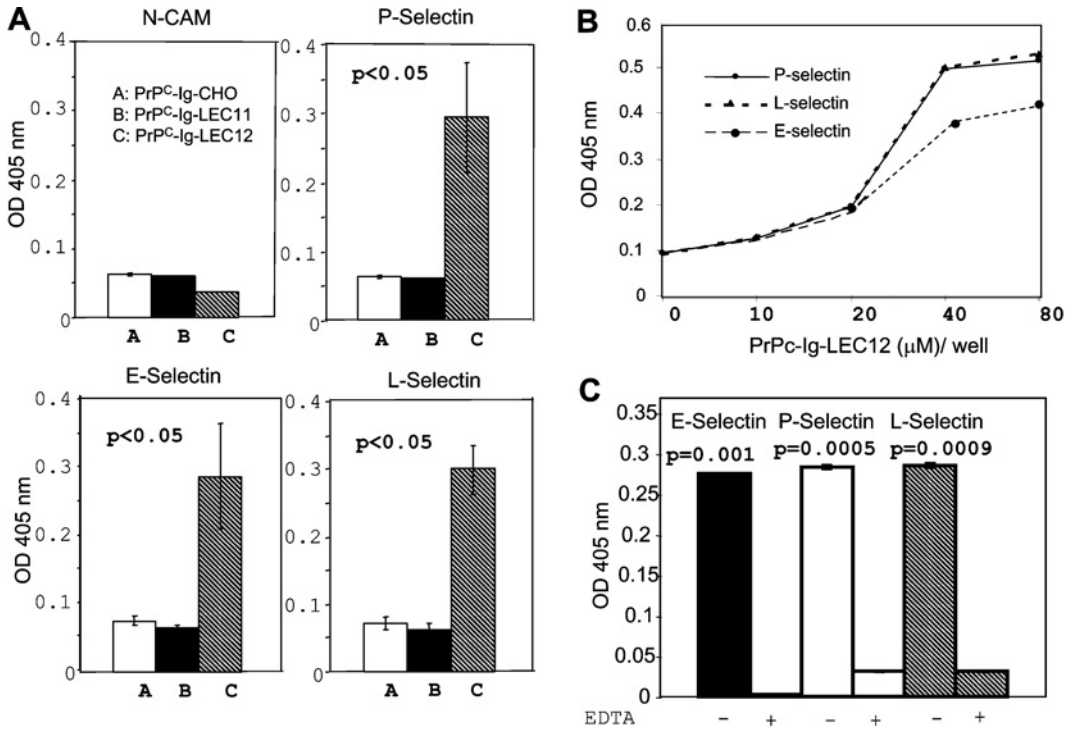

Figure 3 PrP^C–Ig from LEC12 cells binds E-, L- and P-selectins in a Ca^{2+}-dependent manner

(**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^C–Ig fusion proteins from CHO, LEC11 and LEC12 cells were then added to the plates. The bound PrP^C–Ig fusion proteins were then detected with a biotinylated anti-PrP^C mAb, 8H4. The bound antibody was detected further with streptavidin–HRP. Only PrP^C–Ig fusion protein from LEC12 cells binds all three selectins. None of the PrP^C–Ig fusion proteins bind NCAM. Results are the means ± S.D. for four experiments ($n = 12$). (**B**) Binding between selectins and PrP^C–Ig-LEC12 is PrP^C–Ig fusion protein concentration-dependent. (**C**) Binding between PrP^C–Ig LEC12 and selectin–Ig fusion protein is eliminated in the presence of EDTA (10 mM). *P*-values between binding of PrP^C–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.

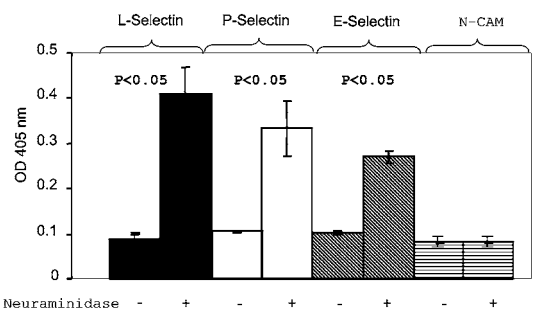

Figure 4 Treatment with neuraminidase enables PrP^C–Ig-LEC11 to bind all three selectins

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^C–Ig LEC11 were added on to the plates. The bound fusion proteins were then detected with a biotinylated anti-PrP^C 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^C–Ig-LEC11 to bind all three selectin–Ig fusion proteins. Neuraminidase-treated PrP^C–Ig-LEC11 remained unable to bind NCAM–Ig fusion protein. OD, absorbance.

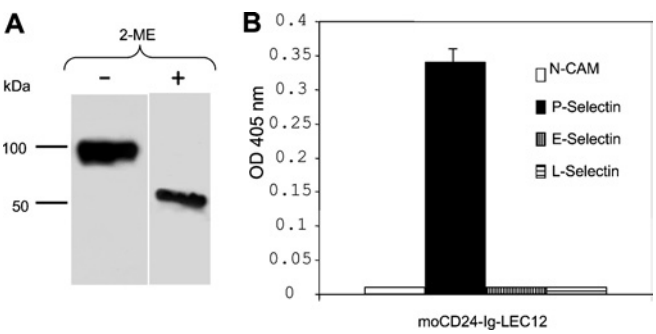

Figure 5 Mouse CD24–Ig produced in LEC12 cells (MoCD24–Ig-LEC12) bound only P-selectin

(**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).

following reasons: (i) similar to PrP^C, CD24 is a GPI-anchored protein; (ii) CD24 is present in the CNS as well as in peripheral tissues; and (iii) CD24 binds P-selectin [20]. Expressed CD24–Ig-LEC12 has detectable Le^X epitope (results not shown). Under non-reducing conditions, CD24–Ig-LEC12 also has a molecular mass of approx. 98 kDa; in the presence of reducing agent, 2-mercaptoethanol, it has a molecular mass of approx. 49 kDa (Figure 5A). As has been reported, CD24–Ig-LEC12 binds P-selectin but not E- or L-selectins [21]. CD24 is highly conserved between mouse and human. Our results also suggest that mouse CD24 binds human P-selectin (Figure 5B). Therefore simply

Table 1 Binding affinity of PrP^C–Ig-LEC12 for selectin–Ig

After selectin–Ig (experiments 1–3) or PrP–Ig (experiment 4) was immobilized on a CM5 chip through the N-termini, PrP-Ig (experiments 1–3) or selectin-Ig (experiment 4) was flowed through to detect the interactions. K_d values were calculated with either steady-state kinetics (experiments 1–3) or Langmuir method (experiment 4). ND, not determined.

	K_d (nM)				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Means ± S.D.
P-selectin	ND	234	226	211	224 ± 10.4
E-selectin	286	378	347	ND	337 ± 46.8
L-selectin	80	60	67	57	66 ± 10.23

having an Le^X epitope is insufficient for the fusion protein to bind all three selectins. From these experiments, we concluded that binding between PrP^C–Ig-LEC12 and the three selectins requires both the Le^X epitope on PrP^C and the PrP^C protein backbone.

Affinity of binding of PrP^C–Ig-LEC12 to selectin–Ig

We next determined the affinity of binding of PrP–Ig-LEC12 to the selectins by SPR. Affinity was determined through steady-state measurements or through the Langmuir method. We found that PrP^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^C–Ig-LEC12 to the selectins (results not shown).

Interactions between brain-derived PrP^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^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^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 mAb 7A12. All three selectins bring down three PrP^C species with molecular masses of 36, 32 and 29 kDa (Figure 6B). No immunoreactive band is detected in the sample incubated with NCAM–Ig. In another study, we also demonstrated that precipitation of native brain-derived PrP^C by selectin–Ig is Ca^{2+}-dependent (Figure 6C); in the presence of EDTA, no anti-PrP mAb immunoreactive band was detected in any of the samples. Collectively, these results provide conclusive evidence that human brain-derived PrP^C interacts with all three selectins.

We next confirmed that the N-linked glycans on PrP^C are important in the binding of PrP^C to selectins. Brain homogenates were first treated with PNGase F to remove the N-linked glycans prior to precipitation with selectin–Ig fusion proteins. Removal of the N-linked glycans on PrP^C drastically reduced the amounts of PrP^C precipitated by the three selectin–Ig proteins. Therefore the interaction between PrP^C and selectin is N-linked glycan-dependent (Figure 7A).

We also determined whether treatment of brain homogenates with neuraminidase increases the amount of PrP^C available for selectin–Ig binding. Brain homogenate was first treated either with

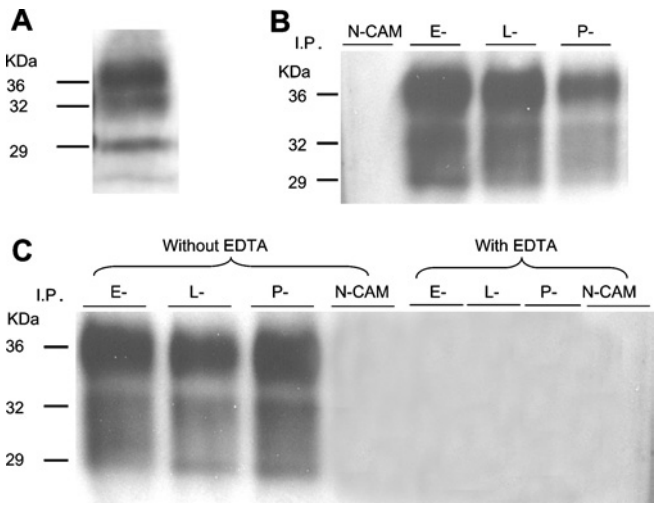

Figure 6 Human brain PrP^C binds selectins and binding is Ca^{2+}-dependent

(**A**) Proteins in normal human brain homogenate were separated by SDS/PAGE and then immunoblotted with anti-PrP^C mAb, 7A12. It showed the typical three bands at approx. 29, 32 and 36 kDa. (**B**) Proteins in normal human brain 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^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^C is also calcium-dependent. I. P., immunoprecipitated.

PBS or with neuraminidase. After treatment, each sample was then divided into multiple aliquots: one was immunoblotted with mAb 8H4 (Figure 7B), one was used to determine the binding of anti-CD15 (Le^X) or 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^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).

Accordingly, treatment of brain homogenate with neuraminidase increased the binding of anti-CD15 mAb but decreased the binding of anti-CD15s mAb (Figure 7C); these differences were rather small, but significant. As expected, the amount of PrP^C precipitated with E-selectin–Ig was greater in the sample that had been previously treated with neuraminidase (Figure 7D). Based on densitometry measurements, the amounts of PrP^C precipitated were approx. 20–40 % ($n = 3$) greater in neuraminidase-treated brain homogenates (results not shown). Again, neuraminidase-treated PrP^C has a faster mobility in SDS/PAGE. No PrP^C immunoreactivity was detected in the neuraminidase-treated sample first precipitated with NCAM–Ig. These results provide strong evidence that removal of sialic acid residues on human brain-derived PrP^C increases the interactions between PrP^C and selectins.

DISCUSSION

Human PrP^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^C–Ig-LEC11 fusion protein with sLe^X structure does not bind. However, removal of the sialic acid on PrP^C–Ig-LEC11 enables it to bind all three selectins. Most importantly, all three selectin–Ig proteins but not control proteins

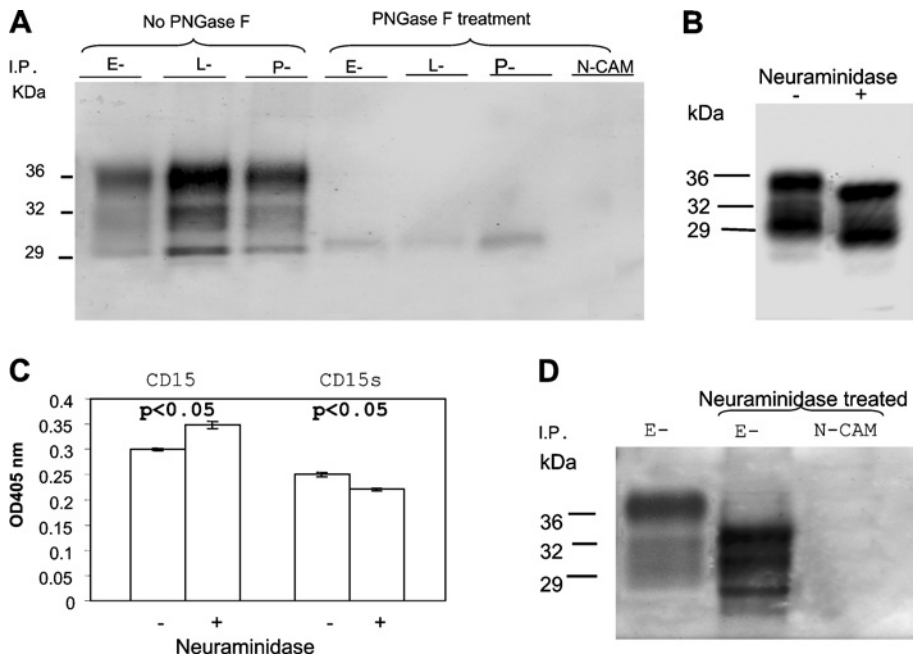

Figure 7 Selectin–Ig proteins do not react with brain-derived PrP^C lacking N-linked glycans

(**A**) Brain homogenate was treated with PBS or with PNGase F and then subjected to precipitation with either selectin–Ig or NCAM–Ig fusion proteins. The precipitated proteins were then separated by SDS/PAGE and immunoblotted with anti-PrP^C mAb, 8H4. Treatment with PNGase F greatly reduced the amounts of PrP^C precipitated by the three selectin–Ig fusion proteins. Therefore N-linked glycans on PrP^C are critical for binding of selectin–Ig to PrP^C. (**B**) Human brain homogenates were treated with PBS or neuraminidase. After treatment, proteins were separated by SDS/PAGE and then immunoblotted with anti-PrP^C mAb, 8H4. Neuraminidase treatment reduced the apparent molecular mass of PrP^C as compared with the non-treated PrP^C proteins. (**C**) ELISA plates were precoated with anti-PrP^C mAb, 8H4. Equal amounts of PBS-treated control homogenates or neuraminidase-treated brain homogenates were added onto the ELISA plate. An anti-CD15 mAb or anti-CD15s mAb were then added to react with the bound PrP^C species. The bound anti-CD15 or anti-CD15s antibodies were detected with a goat anti-mouse IgM–HRP. Anti-CD15 mAb reacted more strongly with neuraminidase-treated brain homogenate. On the other hand, binding of anti-CD15s antibody was reduced in neuraminidase-treated brain homogenates. Results are the means $\pm$ S.D. for two experiments ($n = 12$ for each treatment). *P*-values represent comparisons between samples treated with or without neuraminidase. (**D**) PBS-treated or neuraminidase-treated brain homogenates were precipitated with E-selectin–Ig or NCAM–Ig. Precipitated proteins were then separated by SDS/PAGE and immunoblotted with anti-PrP^C mAb, 8H4. E-selectin–Ig precipitated more PrP^C protein from neuraminidase-treated brain homogenate than PBS-treated brain homogenate. The neuraminidase-treated, E-selectin–Ig-precipitated PrP^C proteins also migrated slightly faster than the PBS-treated controls. I. P., immunoprecipitated.

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 PrP^C has been reported to be critical in the trans-endothelial migration of human monocytes [32]. Therefore selectins on blood cells can also interact with PrP^C on endothelial cells, which may be important in cellular activation, adhesion or migration.

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

In contrast with human, most of the circulating lymphocytes in mice lack PrP^C. PrP^C 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 PrP^C expression, interactions between PrP^C 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 $PrP^{C-/-}$ mice are deficient in recruitment of neutrophils after intraperitoneal injection of zymosan, suggesting that PrP^C may be important in cell migration under certain conditions [36]. Another recent report found that bone marrow cells from $PrP^{C-/-}$ mice are less efficient in repopulating irradiated recipients, suggesting a possible defect in bone marrow stem cells in $PrP^{C-/-}$ mice [37]. Yet another report found that normal T-lymphocyte development is altered in mouse, which overexpresses PrP^C on thymocytes [38]. Collectively, all these findings are also consistent with the interpretation that PrP^C 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. PrP^C 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 PrP^{SC}-infected 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 PrP^{Sc} 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 PrP^{Sc}.

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

By expressing PrP^C–Ig in cell lines with different capacity to add unique N-linked glycostructures, we have been able to identify novel binding partners of PrP^C, 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 PrP^C 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 sLe^X-deficient mice may provide new insights into these questions.

This work was supported in part by NIH (National Institutes of Health) grant NS-045981-02 and an award/contract from the U.S. Department of Army, DAMD17-03-1-0286. We thank Dr Satya P. Yadav of The Biocore™ Core Facility of The Cleveland Clinic Foundation, for performing the binding studies, and Dr Eric D. Roush of BioCore™ Co. for help in evaluating the binding results. We also thank Dr Ajit Varki of UCSD (University of California San Diego) for providing E- and L-selectin–Ig fusion proteins from HEK-293 cells (human embryonic kidney cells) and suggestions, and Dr Michael Lamm for a careful reading of this paper. This work is supported in part by NIH grant NS-045981-01 and an award/contract from the U.S. Department of Army, DAMD17-03-1-0286 (to M.S.S.).

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Received 13 December 2006/3 May 2007; accepted 11 May 2007
Published as BJ Immediate Publication 11 May 2007, doi:10.1042/BJ20061857