PRIONS AND PLATELETS: A POSSIBLE ROLE FOR CELLULAR PRION PROTEIN

BY

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Winnipeg, Manitoba

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ABSTRACT

Cellular prion protein (PrPc) is a GPI-anchored protein, of unknown function, found in a various cell types throughout the body. It is now widely believed that a misfolded, protease resistant form of this protein is responsible for a group of fatal neurodegenerative diseases called transmissible spongiform encephalopathies (TSE), including Creutzfeldt-Jakob disease (CJD) and kuru in humans, scrapie in sheep, chronic wasting disease (CWD) in deer and elk and bovine spongiform encephalopathy (BSE) in cattle. Although the exact function of PrPc is unknown it binds copper and has been implicated in copper homeostasis, signal transduction and cell adhesion.

The pathogenesis of prion diseases is poorly understood, however the expression of PrPc in target cells is an absolute requirement for disease progression. Platelets have been shown to be the largest reservoir of PrPc in peripheral blood cells and studies in animal models have suggested platelets may also be involved in TSE infectivity.

In this study, we determine the exact location of PrPc within human platelets, examine the mobilization and release of PrPc from activated platelets on both microvesicles and exosomes and suggest a possible role for platelets in prion infectivity. In addition we examine the role of PrPc within normal platelet functions including aggregation, signal transduction and adhesion.
DEDICATION

It is the duty of the working class to educate themselves to their fullest potential.

- D.H. Lawrence 1885-1930

This work is dedicated to the memory of my Grandmother,

Catherine Quinn

A woman who recognized the value of a good education.
ACKNOWLEDGEMENTS

This project would never have been completed without the help and support of a large number of people.
First of all, I would like to express my sincere gratitude to my supervisor Dr. Archie McNicol for his patience and guidance throughout this project. You have not only been an excellent supervisor but a true friend.
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I wish to thank my Aunt Cathie and Uncle Archie not only for their support and encouragement during this time but for always being such a positive influence throughout my childhood.
To my good friends and “adopted family” the Tennents, I am so glad you decided to come to Canada.
Finally, a big thank you to “the Captain, for keeping my spirits up over the last few years.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACD</td>
<td>Acid citrate dextrose</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSE</td>
<td>Bovine spongiform encephalopathy</td>
</tr>
<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob Disease</td>
</tr>
<tr>
<td>CK</td>
<td>Casein kinase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PILPC</td>
<td>Phosphatidylinositol-specific phospholipase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl methyl sulphonyl fluoride</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion protein</td>
</tr>
<tr>
<td>PrPc</td>
<td>Cellular prion protein</td>
</tr>
<tr>
<td>PrPres</td>
<td>Protease resistant prion protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline with 0.05% tween20</td>
</tr>
<tr>
<td>TME</td>
<td>Transmissible mink encephalopathy</td>
</tr>
<tr>
<td>TSE</td>
<td>Transmissible spongiform encephalopathy</td>
</tr>
<tr>
<td>vCJD</td>
<td>variant Creutzfeldt-Jakob disease</td>
</tr>
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1. INTRODUCTION

In the course of studies to determine the causative agent of the group of diseases known as Transmissible Spongiform Encephalopathies (TSE), an insoluble protein, termed prion for “Proteinaceous Infectious Particle”, was partially purified from the brains of affected animals (Prusiner, S. B., 1998).

Two forms of the prion protein (PrP) were identified, the non-infectious cellular form (PrPc), which was expressed in a variety of normal neuronal and non-neuronal tissues including tonsils, spleen and blood cells, and a protease-resistant, form (PrPres) which is believed to be the agent responsible for TSEs (Martins, V. R. et al., 2002; Barclay, G. R. et al., 1999).

1.1 Cellular prion protein structure

PrPc is a glycoprophatidyinositol (GPI) anchored protein of 254 amino acids with a molecular mass of 33-40 kilodaltons (kDa) or, in its unglycosylated form, of 27kDa. Structurally, the protein has 5 glycine/proline rich octapeptide repeat regions, two N-linked glycosylation sites, a single disulfide bond and signaling peptides at both the amino and carboxyl terminii (Stahl, N. et al., 1987) (Figure 1). PrPc is composed of about 40% alpha helix formation with less than 10% being in the beta sheet formation. However the beta sheet content increases to about 50% in the infectious form (Martins, V. R., 1999) (Figure 2). The prnp gene is located on chromosome 20 and the protein is highly conserved among species, with a similarity of 85-97% among mammals (Gabriel, J. M. et al., 1992).
Figure 1. Structure of prnp gene (Vostal JG et al 2001)

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Figure 2. Structure of Normal cellular Prp and infectious isoform

The normal form of cellular prion protein on the left is composed of mainly alpha helical formation whereas the infectious misfolded isoform is predominantly beta sheet formation (http://www.cmpharm.ucsf.edu/cohen/media/pages/gallery.html)
1.2 Biosynthesis, cellular location and trafficking of PrPc

PrPc is synthesized in the rough endoplasmic reticulum, and passes through the golgi en route to the plasma membrane. During biosynthesis it undergoes a number of post-translational modifications including cleavage of the N-terminal signal peptide, addition of N-linked oligosaccharide chains, formation of a single disulphide bond, cleavage of C-terminal peptide and attachment of the GPI anchor (Turk, E. et al., 1988; Haraguchi, T. et al., 1989).

The majority of PrPc is found in detergent resistant domains, called lipid rafts, at the cell membrane of neuronal and non-neuronal cells, as well as of cultured cell lines (Vey, M. et al., 1996). However PrPc does not remain on the cell surface but cycles between the membrane and an endocytic compartment (Shyng, S. L. et al., 1993). Clathrin coated pits are the main structures responsible for endocytic uptake of PrPc (Shyng, S. L. et al., 1994).

PrPc has been demonstrated in the secretory granules of epithelial cells in the stomach (Fournier, J. G. et al., 1998), as well as on the surface of both quiet and activated platelets, (Barclay, G. R. et al., 1999; Holada, K. et al., 1998; MacGregor, I. et al., 2000).

1.3 Interaction of PrPc with other proteins

PrPc interacts with a large number of proteins (Table 1). The first interacting proteins identified were a pair of prion protein ligands Pli45 and Pli 110 (Oesch, B. et al., 1990); the former being glial fibrillary acidic protein, a marker for astrocytes which proliferate in response to TSE infections (DeArmond, S. J. et al., 1992).
Subsequently, a number of other PrPc-interacting proteins have been identified using a yeast two-hybrid system; these include the anti-apoptotic protein Bcl-2 (Kurschner, C. et al., 1996), the cellular chaperone heat shock protein 60 (Hsp60) (Edenhofer, F. et al., 1996), the 37kDa laminin receptor precursor (Rieger, R. et al., 1997), the synaptic vesicle marker synapsin1b, the adaptor protein Grb2 and a protein named prion interaction protein (Pint 1), for which no function has been determined (Spielhaupter, C. et al., 2001). Capellari et al co-immunoprecipitated PrPc from a M17 neuroblastoma cell line with antibodies to grp94, protein disulphide isomerase, calnexin, and calreticulin suggesting that PrPc interacts, with these proteins (Capellari, S. et al., 1999).

PrPc also binds to laminin in PC12 cells and rodent primary neurons, and this interaction promotes neurite outgrowth in these cells (Graner, E. et al., 2000). A number of additional cell surface proteins interact with PrPc including neuronal cell adhesion molecules (N-CAMs) (Schmitt-Ulms, G. et al., 2001), apolipoprotein 1 (APlp1) an amyloid precursor protein which has been implicated in Alzheimer's disease (Yehiely, F. et al., 1997), the 67 kDa laminin receptor (Gauczynski, S. et al., 2001) and glycosaminoglycans (GAGS) (Priola, S. A. et al., 1994; Pan, T. et al., 2002).

Finally, complementary hydropathy, a technique in which cDNA is used to generate a molecule which is a complementary mirror image of the target protein, identified a 66 kDa protein, the stress inducible protein STI-1, which bound to PrPc and which may be involved in neuroprotection (Zanata, S. M. et al., 2002).
Table 1. Protein molecules interacting with cellular prion protein

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Function</th>
<th>Possible binding site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synapsin 1b</td>
<td>Regulation of neurotransmitter</td>
<td>Intracellular vesicles</td>
<td>Spielhaupter (2001)</td>
</tr>
<tr>
<td>Grb2</td>
<td>Adapter protein</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Pint 1</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Caveolin 1</td>
<td>Signaling</td>
<td>Caveolae raft</td>
<td>Mouillet-Richard (2000)</td>
</tr>
<tr>
<td>CK2</td>
<td>Ser/Thr phosphorylation</td>
<td>Caveolae raft</td>
<td>Meggio (2000)</td>
</tr>
<tr>
<td>STII</td>
<td>HSP related</td>
<td>Cell surface</td>
<td>Zanata (2002)</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Apoptosis</td>
<td>Unknown</td>
<td>Kurschner (1996)</td>
</tr>
<tr>
<td>GAG</td>
<td>Biomolecular transport</td>
<td>Cell surface</td>
<td>Priola (1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pan (2002)</td>
</tr>
<tr>
<td>N-CAM</td>
<td>Adhesion</td>
<td>Caveolae-like domain</td>
<td>Schmitt-Ulms (2001)</td>
</tr>
<tr>
<td>GFAP</td>
<td>Cell repair</td>
<td>Unknown</td>
<td>Oesch (1990)</td>
</tr>
<tr>
<td>Pli 110</td>
<td>Unknown</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Hsp60</td>
<td>Chaperone</td>
<td>Unknown</td>
<td>Edendhofer (1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Stockel (1998)</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Apoptosis inhibitor</td>
<td>Unknown</td>
<td>Yehiely (1997)</td>
</tr>
<tr>
<td>Aplp1</td>
<td>Regulation of neurite outgrowth</td>
<td>Cell surface</td>
<td></td>
</tr>
<tr>
<td>Laminin receptor</td>
<td>laminin binding</td>
<td>Cell surface</td>
<td>Gauczynski (2001)</td>
</tr>
<tr>
<td>Gp94</td>
<td>protein disulphide isomerase</td>
<td>Unknown</td>
<td>Capellari (1999)</td>
</tr>
<tr>
<td>Calnexin</td>
<td>Chaperone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calreticulin</td>
<td>Chaperone</td>
<td></td>
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</table>
1.4 Putative functions of PrPc

As outlined above, PrPc binds to a large number of proteins, however its function(s) remains unclear. Early attempts to determine a function for PrPc using prnp knockout mice (PrP\(^{-/-}\)) were disappointing, since there were no apparent differences between the knockout and wild type mice (Bueler, H. et al., 1992). To date, four different strains of PrP\(^{-/-}\) mice have been developed (Manson, J. C. et al., 1994; Sakaguchi, S. et al., 1996). Two of the later strains developed ataxia which suggested a neuroprotective role for PrPc (Moore, R. C. et al., 1999).

1.4.1 Copper Binding

In 1992, Pan et al. demonstrated that PrPc from hamster brain bound copper (Pan, K. M. et al., 1992), and subsequent studies showed that PrPc can bind other metal ions, such as zinc, manganese and nickel, but with a much lower affinity than copper (Stockel, J. et al., 1998; Brown, D. R. et al., 2000). The main copper binding site in human PrPc is an N-terminal octapeptide repeat region encompassing residues 60-91 (Hornshaw, M. P. et al., 1995; Brown, D. R. et al., 1997a; Viles, J. H. et al., 1999). PrP\(^{+/-}\) mice have 50% lower copper concentrations in their synaptosomal fractions than wild type mice, suggesting that PrPc may play a role in copper regulation in neuronal synapses (Herms, J. et al., 1999). Similarly, there are higher levels of copper in mice over-expressing PrPc (Brown, D. R. et al., 1998). Interestingly the association with copper gives PrPc similar enzymatic activities to that of Cu\(^{2+}\)/Zn\(^{2+}\) superoxide dismutase (SOD-1) (Brown, D. R. et al., 1999) and SOD-1 activity in cultured neurons from PrP\(^{+/-}\) mice is 50% lower than that from normal mice, while activity from PrPc overexpressing mice is about 20%
higher than control mice (Brown, D. R. et al., 1997b). Taken together these studies suggested a role for PrPc in the protection against oxidative stress.

These studies, however, have not been substantiated. Waggoner and colleagues failed to detect any differences in copper levels between PrP\(^{-/-}\), wild type and PrPc over-expressing mice. In addition, no effect of PrPc on SOD-1 activity was seen, suggesting that PrPc may simply act as a carrier for copper ions (Waggoner, D. J. et al., 2000). In addition, recent in vivo studies using PrP\(^{-/-}\) and PrP over-expressing mice crossed with SOD-1 over-expressing mice failed to detect any effects of PrPc on SOD-1 activity (Hutter, G. et al., 2003).

1.4.2 Signal Transduction

The role of PrPc in signal transduction has also been investigated. As outlined above, two putative surface receptors for PrPc have been identified; the 37kDa/66 kDa laminin receptor (Gauczynski et al 2001) and STI-1 (Zanata et al 2001). A yeast two-hybrid system demonstrated that PrPc also interacts with the cytoplasmic adaptor protein Grb2, which acts as a link between extracellular receptors and intracellular signaling molecules (Koch, C. A. et al., 1991).

In cultured neuronal cells, PrPc indirectly increases the phosphorylation of the tyrosine kinase fyn and it has been suggested that caveolin–1 acts as the intermediate signaling molecule between PrPc and fyn (Mouillet-Richard, S. et al., 2000).

In addition, recombinant bovine PrPc has been shown to increase the phosphotransferase activity of the pleiotropic protein kinase, casein kinase 2 (CK-2), which is abundant in the brain and is involved in a number of signal transduction, as well as gene expression, pathways (Meggio, F. et al., 2000).
1.4.3 Cell Adhesion

Graner et al. (2000) demonstrated that, in addition to an interaction with the laminin receptor, PrPc is directly associated with laminin, and have suggested a role for PrPc in neuronal cell adhesion and neurite outgrowth.

Studies using the neuroblastoma cell line, N2A, demonstrated that cells over-expressing PrPc showed increased aggregation when compared to wild type N2A cells. Furthermore, when surface PrPc was removed by incubation with phosphatidylinositol-specific phospholipase C (PILPC), which cleaves GPI-linked proteins, aggregation levels returned to that of wild type cells (Mange, A. et al., 2002). Cross-linking PrPc with formaldehyde in N2A cell lines, revealed that PrPc was present in high molecular weight complexes and that these complexes were three splice variants of N-CAMs (Schmitt-Ulms, G. et al., 2001). These studies strengthened the argument for the involvement of PrPc in neuronal cell adhesion.

1.5 Transmissible Spongiform Encephalopathies

TSEs are a group of fatal neurodegerative diseases that affect both humans and animals. The animal TSEs include BSE in cattle, scrapie in sheep, CWD in mule deer and elk, transmissible mink encephalopathy in farmed mink and feline spongiform encephalopathy in zoological and domestic cats (Sigurdson, C. J. et al., 2003).

The human TSEs are typically diseases of late middle age and can be either inherited or acquired (Palmer, M. S. et al., 1992). The inherited forms include Familial CJD, Fatal Familial Insomnia or Gerstmann-Straussler-Schienker syndrome, and can all be attributed to a germline prnp gene mutation (Ironside, J. W., 1996). The acquired
forms can be iatrogenic, as in the cases of transplantation of dura matter (Liscic, R. M. et al., 1999), or the use of pituitary hormones from previously infected patients (Collins, S. et al., 1996), or can also be acquired through ingestion of PrPres. For example, in Papua, New Guinea, the prion disease Kuru was attributed to ritualistic cannibalism (Pedersen, N. S. et al., 2002), and the outbreak of variant CJD (vCJD) in the United Kingdom has been linked to ingestion of beef from cattle infected with BSE (Bruce, M. E. et al., 1997).

There have also been cases of sporadic CJD or atypical CJD, which have an unknown cause but have been thought to be due to a somatic mutation in the prnp gene (Poser, S. et al., 2000).

The physical symptoms of prion diseases may include visual disturbances, cerebellar ataxia (gait difficulties), myoclonus (muscle spasms) and progressive dementia. These symptoms are not present in all TSE cases. For example, vCJD differs from sporadic CJD in that it usually affects patients at a much younger age, and the duration of the disease is longer. However all of these forms of prion diseases are fatal and, at the present time, there is no treatment (Will, R. G., 2002).

At the tissue level, the family of diseases is characterized by the spongiform appearance of brains of affected individuals, due to vacuolation of neurons and surrounding neuropil. This vacuolation is accompanied by deposition of protease-resistant protein (Wells, G. A., 1993) (Figure 3).
Figure 3. Histology and Immunohistochemistry of TSEs

Example of TSE infected brain. A: Haemotoxylin and Eosin stained section showing vacuolation and degeneration of neurons (n) and surrounding neuropil (arrows). B: Ab to PrP demonstrates presence of protease resistant protein deposits in neuronal cell bodies (n) as well as along the neuronal processes (arrows). (Micrographs courtesy of Dr. Y Robinson, Canadian Food Inspection Agency)
1.6 The Role of PrPc in TSEs

The role of PrPc in TSEs has been widely studied and it is now widely accepted that PrPres is the agent responsible. This has led to the prion hypothesis which states that PrPc undergoes a conformational change to PrPres, which acts as a template for the transformation of additional PrPc to PrPres (Telling, G. C. et al., 1996). The mechanism of this conversion is poorly understood, although another protein, termed “Protein X”, has been implicated (Telling, G. C. et al., 1995; Kaneko, K. et al., 1997).

Studies using PrP^{−/−} mice suggest that the PrPc must be present in order for the disease to progress (Brandner, S. et al., 2000). PrP^{−/−} mice inoculated with murine scrapie were still healthy 500 days post inoculation compared to the wild-type mice which developed scrapie in less than 165 days post inoculation (Prusiner, S. B. et al., 1993). Furthermore, PrP^{+/−} mice grafted with PrPc expressing neuroectodermal tissue showed only neurodegeneration in the area of the graft after inoculation with scrapie (Brandner, S. et al., 1996)

1.7 Pathogenesis of TSE

The pathogenesis of prion diseases is also poorly understood. It has been proposed that ingested infectious prions are absorbed through Payers patches in the gut and accumulate in the spleen. The spleen is highly innervated and PrPres travels to the brain via peripheral nerves (Glatzel, M. et al., 2001).

Although this is the most likely route for neuroinvasion, it has been suggested that blood may also play a role in the pathogenesis of prion diseases (Ramasamy, I. et
al., 2003; Radebold, K. et al., 2001). For example, a number of studies in animals have produced prion infections from inoculation of buffy coats and other blood components (Bons, N. et al., 2002; Holada, K. et al., 2002; Cervenakova, L. et al., 2003). In 2000, Hunter et al used sheep, previously infected with BSE or scrapie, as blood donors to TSE-free sheep. Due to the long incubation period of BSE, these studies are expected to take 5 years to complete; however in 2002 the authors reported that 17% of the recipient animals were showing clinical signs of BSE and 19% were positive for scrapie, providing evidence for the transmission of BSE and scrapie via blood transfusion (Hunter, N. et al., 2002; Houston, F. et al., 2000).

Of importance there have been two recent reports of transmission of variant CJD by blood transfusion of blood from a donor later found to have been infected with vCJD (Llewelyn, C. A. et al., 2004; Peden, A. H. et al., 2004). The mechanism of transmission is currently not clear.

Recently the effectiveness of standard leucoreduction for removing TSE infectivity from whole blood was investigated (Gregori, L. et al., 2004). The removal of all white cells reduced infectivity by only 42%, suggesting that other blood components, cells or plasma, carry TSE infectivity. These data, when considered along with the animal studies of prion infectivity in blood, have highlighted both the significance of PrPc and the role of individual blood components in the pathogenesis of TSEs. Therefore in order to understand the transmission of the disease, it is important to determine the physiological behaviour and function of the normal cellular form of this protein in blood cells and plasma.
1.8 PrPc and Blood

Several flow cytometric studies on whole blood have shown PrPc to be present on the surface of a number of peripheral blood cells (Vostal, J. G. et al., 2001). There is a wide variation, however, between these studies in the number of PrPc molecules found on each type of blood cell (Table 2). For example, two studies reported that the majority of PrPc was associated with platelets with minimal levels in red blood cells (Barclay, G. R. et al., 1999; MacGregor, I. et al., 2000). In contrast, Holada et al reported only 44.9% associated with platelets and over 50 % to be associated with red blood cells (Holada, K. et al., 2000). These differences have been attributed to a number of factors including choice of antibody (Ab), method of detection and the type of reference beads used for calibration (Vostal, J. G. et al., 2001).

There are documented similarities between platelet secretion and neuronal exocytosis (Reed, G. L. et al., 2000). Neurons have both small and large dense core granules, which bear some resemblance to the alpha and dense granules of platelets. Indeed, dense granules have previously been used as a model to study serotonin uptake in neurons (Pletscher, A., 1986; Da Prada, M. et al., 1988). Since human platelets have been reported to contain high proportions of PrPc (Table 2), understanding PrPc function in platelets may also give insight into its function in neurons.

Previous studies have shown that PrPc is present on both internal and external platelet membranes, and that it relocates to the surface, along with other granule membrane proteins, when the platelet is activated (Holada, K. et al., 1998).
Although the exact location of internal PrPc has yet to be determined, the alpha granule membrane seem to be a good candidate as PrPc, like the alpha granule membrane protein P-selectin, is expressed on the surface of activated platelets.
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Platelets</td>
<td>96.3</td>
<td>84.2</td>
<td>44.9</td>
</tr>
<tr>
<td>RBCs</td>
<td>0</td>
<td>5.7</td>
<td>53.7</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>2.8</td>
<td>7.6</td>
<td>1.3</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>0.9</td>
<td>2.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>
1.9 Platelets

Platelets are anucleate blood cells produced by differentiation of bone marrow derived megakaryocytes (Italiano, J. E., Jr. et al., 2003b). Circulating platelets have a lifespan of around 10-12 days and are removed primarily by the spleen. Resting platelets are discoid in shape with an equatorial diameter of 2-3 microns (Figure 4).

1.9.1 Plasma membrane proteins

The platelet plasma membrane contains a number of membrane spanning proteins including members of the serpine, integrin, the leucine rich glygoprotein, the selectin, the quadraspan and the immunoglobulin supergene families. These proteins have a variety of functions including acting as receptors for various platelet agonists and adhesion molecules (Table 3) (Kunicki, T. J., 1989).

The open canallicular system (OCS) is continuous with the plasma membrane and is thought to be the channel through which internal platelet granules release their contents upon cell activation.

1.9.2 Platelet Granules

Alpha granules are the largest, and the most numerous, of the platelet granules. They contain a number of adhesive molecules, coagulation factors and growth factors. Fibrinogen, fibronectin and thrombospondin are also present in alpha granules, as are platelet factor 4, platelet derived growth factor and \( \beta \)-thromboglobulin. The alpha granule membrane contains a number of adhesive receptors which are expressed on the platelet surface after activation, consistent with a role for alpha granule membrane receptors in platelet adhesion (Wencel-Drake, J. D. et al., 1986).
### Table 3. Main glycoprotein receptors on platelet plasma membrane

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Receptor</th>
<th>Integrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td>GPIc/IIa</td>
<td>(\alpha_2\beta_5)</td>
</tr>
<tr>
<td>Collagen</td>
<td>GPIa/IIa</td>
<td>(\alpha_2\beta_1)</td>
</tr>
<tr>
<td>Thrombospondin</td>
<td>GpIV</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>GpV GPIb</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>GPIIb/IIIa</td>
<td>(\alpha_{III}\beta_3)</td>
</tr>
<tr>
<td>Laminin</td>
<td>GpIIa/Ic</td>
<td>(\alpha_6\beta_1)</td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>GPIb/IX</td>
<td></td>
</tr>
</tbody>
</table>
These receptors include CD62 (P-selectin, GMP-140.), which plays a role in the interaction of platelets with neutrophils (McEver, R. P. et al., 1984), GMP-33, which has been reported to be an N-terminal proteolytic fragment of thrombospondin (Damas, C. et al., 2001) and Gn24, a GTP binding protein thought to be involved in exocytosis (van der Meulen J. et al., 1991).

Dense granules are smaller, and less numerous (3-8 per platelet), than alpha granules and contain serotonin, ATP, calcium and ADP (Rendu, F. et al., 2001). The presence of calcium in these granules imparts an electron dense appearance when viewed with a transmission electron microscope. The release of serotonin and ADP following platelet activation promotes further platelet aggregation in a positive feedback manner. The membranes of dense granules have been shown to contain P-selectin (Israels, S. J. et al., 1992), the tyrosine kinase pp60src (Rendu, F. et al., 1989), as well as two almost identical proteins granulophysin (Gerrard, J. M. et al., 1991; Nishibori, M. et al., 1993) and CD63 (a lysosomal membrane protein) (Metzelaar, M. J. et al., 1991). Each of these proteins has been shown to relocate to the surface of activated platelets.

Lysosomal granules contain a number of lysosomal enzymes which have been suggested to play a role in clot lysis, and clearing of thrombi (Van Oost, B. A. et al., 1985). In addition to CD63 mentioned above, lysosomal granule membranes also contain the lysosomal-associated membrane proteins LAMP-1 and LAMP-2 (Silverstein, R. L. et al., 1992). Interestingly LAMP-2 has also been identified to be present in platelet dense granule membranes (Israels, S. J. et al., 1996).
The presence of peroxisomes in platelets remains contentious. Parmley et al suggest that they are in fact dense granules (Parmley, R. T. et al., 1974), whereas Breton-Gorius and Guichard demonstrated that they were distinct granules and unrelated to dense granules (Breton-Gorius, J. et al., 1975).

1.9.3 Tubular Systems

In addition to the open canalicular system, there are two other tubular systems within platelets. The first is a band of microtubules that encircles the platelet just below the plasma membrane. Formed from a single coiled microtubule from the megakaryocyte (Italiano, J. E., Jr. et al., 2003a), it is thought to be involved in platelet shape change in response to agonists.

The second system is the dense tubular system which is thought to be the main calcium sequestration and thromboxane synthesising organelle within platelets (Ebbeling, L. et al., 1992).

1.9.4 Platelet Function

The function of platelets is to form a primary hemostatic plug at the site of vessel injury (Gerrard, J. M. et al., 1976). This is a multi-step process involving platelet adhesion to the damaged vessel wall and subsequent aggregation to form the haemostatic plug. The initial stimulus is collagen which is exposed by the injured vessel wall and binds to receptors on the platelet membrane (Gerrard, J. M., 1988). This interaction of collagen with its receptor triggers a series of events including platelet shape change, associated with centralization of organelles and extension of pseudopods, the exposure of adhesive receptors, granule exocytosis, and the synthesis
and secretion of the pro-aggregatory eicosanoid thromboxane A$_2$ (Gerrard, J. M. et al., 1993).

Furthermore activated platelets release microvesicles (Figure 5). Microvesicles are derived from the plasma membrane, range in size from 100nm to 1 micron and are enriched in the anionic phospholipid phosphatidylserine which serves as the platform for both the VIIIa/IXa and the Va/Xa complexes in the coagulation cascade (Heijnen, H. F. et al., 1999). It is therefore believed that microvesicles play a procoagulant function (Siess, W., 1989; Fox, J. E. et al., 1990).

There is some evidence suggesting that, in addition to microvesicles, platelets also release exosomes (Heijnen, H. F. et al., 1999). Ranging in size between 40-100nm, exosomes are smaller than microvesicles and are thought to be derived from both alpha granules and multivesicular bodies; the latter of which are present in platelets and megakaryocytes and are believed to represent an intermediate stage in alpha granule formation (Heijnen, H. F. et al., 1998).

The precise function of platelet-derived exosomes remains to be determined although the low binding of factor X, prothrombin and annexin V to their surface suggests that they do not have the same pro-coagulant activity as microvesicles. The presence of the tetraspan protein CD63 on their surface however points to a possible role for exosomes in cell adhesion, since CD63 molecules are involved in adhesion of neutrophils to endothelial cells (Toothill, V. J. et al., 1990).
Figure 5. Electron Microscopy: Platelet Activation

Quiet platelet – discoid
alpha granule (a)
dense granule (d)
open canalicular system (ocs)

Activated platelet – shape change,
formation of pseudopods (p)

Granule centralisation, extrusion of
contents and formation of microvesicles
(mvs) (Bar=500nm)
1.10 Objectives

As outlined above, PrPc is present on both internal and external platelet membranes and relocates to the surface following platelet activation (Holada, K. et al., 1998). However the role PrPc, if any, in normal platelet function has not been addressed. The purpose of this work is therefore twofold:

- to determine the internal location of PrPc within platelets.
- to determine if PrPc is involved in normal platelet function mechanisms.
2. MATERIALS AND METHODS

2.1 Antibodies and reagents

2.1.1 Anti-PrP antibodies

The following anti-PrP Abs were used. The final concentrations or dilutions used are indicated in brackets.

- Monoclonal Ab 308 (3 µg/ml), raised against amino acids (aa)106-126 of human PrP, was purchased from Cayman Chemical Co. Ann Arbor, MI
- Monoclonal Ab 6H4 (1/1000 dilution), raised against aa 144-152 human PrP was purchased from Prionics, Switzerland
- Monoclonal Ab 3F4 (1/1000 dilution), raised against aa 108-111 human and hamster PrP, was purchased from DakoCytomation, Mississauga, Ontario
- Monoclonal Ab F89/160.1.5 (5µg/ml) raised against aa 142-145 ovine PrP was a generous gift from Dr. K. O’ Rourke, USDA, Pullman, WA
- Monoclonal Ab F99/97.6.1 (5µg/ml) raised against aa 220-225, was a generous gift from Dr. K. O’ Rourke USDA, Pullman, WA
- Polyclonal Ab 703 (1/2000 dilution), raised against full length bovine PrP, was purchased from Abcam Inc. Cambridge, MA

2.1.2 Other antibodies

- Anti-CD62P and anti CD63 Abs (1:100 dilution) were purchased from Abcam Inc. Cambridge, MA
- Anti calreticulin (1:100 dilution) was purchased from Stressgen Biotechnologies, Victoria B.C.
• Anti fyn (1:100 dilution) was purchased from BD Biosciences, Mississauga, Ont
• Anti-laminin receptor (1:100 dilution) was purchased from Neomarkers, Fremont, CA
• CD9-FITC mouse monoclonal (1:50 dilution) was purchased from DakoCytomation, Mississauga, Ontario

2.1.3 Reagents

Alexa fluor 488 (10μg/ml) was obtained from Molecular Probes, Eugene, OR., Texas Red conjugated secondary Ab (10μg/ml) was from Vector laboratories, Burlingame, CA, and horseradish peroxidase conjugated secondary Ab (1:1000 dilution) from DakoCytomation, Mississauga, Ontario

Thromboxane analogue U46619 was from Cayman Chemical Ann Arbor, MI, and equine tendon collagen was from Helena Laboratories, Beaumont, TX.

The “Seize” primary immunoprecipitation kit and the “Super Signal West Pico” chemiluminescence kit were obtained from Pierce Chemical Company, Rockford, IL. Hyperfilm was purchased from Amersham, Piscataway, NJ, and nitrocellulose membranes, and protein assay kits, were purchased from Bio-Rad, Mississauga, ON.

Secondary Abs conjugated to gold, bovine thrombin, metrizamide, protease inhibitors and all other reagents were from Sigma-Aldrich Canada Ltd, Oakville, ON, and were of the highest grade available.

2.1.4 Buffers

All buffers used are listed in appendix A.

2.2 Preparation of washed platelets

Blood from healthy donors, who had refrained from taking aspirin in the previous two weeks, was drawn into 20 ml syringes containing ACD anticoagulant in the ratio of
8.1 ml blood to 1.9 ml of ACD (Aster, R. H., 1972). The whole blood was transferred to 15 ml polypropylene tubes and centrifuged at 800 x g for 5 minutes at room temperature. The resultant platelet rich plasma was transferred to a fresh tube and centrifuged at 800 x g for a further 15 minutes to pellet the platelets. The supernatant was removed and the platelet pellet re-suspended in an appropriate buffer (McNicol, A. et al., 1997).

2.3 Preparation of whole platelet homogenates

Platelet rich plasma, prepared as outlined above, was transferred to a fresh polypropylene tube, 2mM EDTA was added and centrifuged at 800 x g for 15 minutes at 4°C to pellet the platelets. The pellet was re-suspended in Hepes/Tyrodes buffer and centrifuged at 800 x g for 15 minutes at 4°C. The washed platelet pellet was re-suspended in TBS to a volume of 1ml of buffer per 10ml of platelet rich plasma. After the addition of protease inhibitors (10µg/ml leupeptin, 1mM PMSF, 1mM benzamidine), the platelet suspension was dispensed into 200µl aliquots and stored at –80°C until use.

2.4 Fractionation of platelets

Platelet rich plasma prepared from 60ml blood, as outlined above, was transferred to a fresh tube, 2mM EDTA added, and centrifuged at 800 x g for 15 minutes at room temperature to pellet the platelets. The platelet pellet was re-suspended in wash buffer, and any remaining red blood cells were removed by a brief (10 second) centrifugation at 800 x g. The supernatant was transferred to a fresh tube and the red blood cell pellet was discarded. This step was repeated until the platelet preparation was free of red blood cell contamination. The platelets were centrifuged at 800 x g for 15 minutes and the resultant pellet re-suspended in homogenizing buffer containing protease inhibitors (1mM PMSF, 10µg/ml leupeptin, 1mM benzamidine). The platelets were sonicated for three 15-second
pulses at 4°C and the lysate layered on to a 40% metrizamide gradient (Rendu, F. et al., 1982). The gradient was centrifuged at 54,000 x g for 30 minutes at 4°C and the alpha granule enriched layer above the gradient was removed to a fresh tube. The metrizamide was removed and the dense granule-enriched pellet was washed with homogenizing buffer and finally re-suspended in Tris buffer pH 7.4. Both granule-enriched fractions were stored at –80°C until use.

2.5 Protein Determination

The protein concentrations of the samples for electrophoresis were determined by a Bio-Rad protein assay, following the manufacturer’s instructions. An aliquot (4µl) of the sample was mixed with 800µl of water and 200µl of Bio-Rad protein assay reagent added. The samples were mixed and absorbance at a wavelength of 595nM measured on a Gemini max spectrophotometer. Protein concentrations were determined from a standard curve made of varying concentrations of BSA.

2.6 Electrophoresis and Immunoblotting

Samples (10–15 µg of protein) were mixed with 3X SDS reducing sample buffer in the ratio 2:1 sample:buffer, denatured by incubation at 100°C for 5 minutes and the proteins separated on a 12% SDS polyacrylamide gel (175 volts for 1 hour.).

The proteins were transferred to 0.45µM nitrocellulose using a semi-dry transfer system (Invitrogen; 20 milliamps per gel for 1 hour). The transferred proteins were visualised by staining in 0.1% ponceau S for 30 seconds followed by rinsing in water.

The nitrocellulose was incubated in non-fat powdered milk (5% in TBS-T) for 30 minutes at room temperature, to block non-specific binding, and subsequently incubated in the appropriate Ab for 60 minutes at room temperature. Following 3 x 5 minute washes
in TBS-T, the nitrocellulose was incubated with an appropriate, horseradish peroxidase-conjugated secondary Ab for 30 minutes at room temperature. The nitrocellulose was washed (6 x 5 minutes in TBS-T) and incubated for 5 minutes in Super Signal West Pico chemiluminescence system. Following drying, the bands were visualised by exposure to Hyperfilm or by digital imaging on the Fluor–S Max multimager (Bio-Rad). Multiple exposures were made to optimise the intensity.

2.7 Immunofluorescence

Platelets were washed into Hepes/Tyrodes buffer, as described above, and aliquots (700µl) dispensed into eppendorf tubes. The platelets were stimulated with 1 unit/ml of thrombin (Sigma) over a time course of 0, 30, 60, and 120 seconds. The reaction was stopped, and fixed, by the addition 700µl of 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes. Some unstimulated platelet samples were permeabilised, after fixation, by incubation with 0.2% Triton-X 100 for 3 minutes prior to the addition of excess PBS.

In all case, samples were washed x 3 in PBS with 0.1% BSA, incubated in the appropriate primary Ab diluted in PBS/0.1% BSA overnight at 4°C, re-washed x 3 in PBS/0.1% BSA and incubated in the appropriate secondary Ab conjugated to either Alexa Fluor 488nm or Texas red for 30 minutes at room temperature in the dark.

Following a further washing x 3 in PBS the samples were re-suspended in 90% glycerol/ 10% PBS. A drop was placed on a slide and allowed to settle for 30 minutes before being visualised on an Olympus IX70 microscope with confocal attachment (Carsen Group, ON).
For double labeling experiments, the samples were washed in PBS with 0.1% BSA after labeling with the first fluorochrome then incubated in the second Ab, washed x3 in PBS with BSA then labeled with the second fluorochrome.

2.8 Mepacrine labeling of dense granules

Platelet dense granules were labeled with mepacrine according to a method by McNicol et al (McNicol, A. et al., 1994) Briefly, 1μl of 55mM mepacrine was added to every 1ml of platelet rich plasma and incubated at 37°C. ACD was added in the ratio of 1.9ml ACD: 8.1 ml platelet rich plasma and the platelets were pelleted by centrifugation as above. The platelet pellet was re-suspended in Hepes/Tyrodes buffer.

For double labeling experiments, aliquots of the mepacrine labeled platelets were fixed by addition of an equal amount of 4% paraformaldehyde in phosphate buffer and allowed to fix for 10 minutes. After fixation the platelets were washed in PBS /0.1% BSA then incubated in the appropriate primary Ab as described in section 2.7, re-washed in PBS/0.1% BSA then incubated in the appropriate secondary Ab conjugated to Texas Red. The samples were further washed in PBS/0.1% BSA and re-suspended in 90% glycerol/105 PBS and visualized as above.

2.9 Flow cytometry

Platelets were washed into Hepes/Tyrodes buffer as described above. The washed platelets were incubated with 1 unit/ml of thrombin, or saline control, for various times (0, 30, 60, 120 seconds) and the reaction terminated by addition of an equal volume of 4% paraformaldehyde in PBS. The platelets were allowed to fix at room temperature for 30 minutes, then centrifuged at 10,000 x g for 2 minutes and the paraformaldehyde removed. The pellet was washed x 3 in PBS with 0.1% BSA and centrifuged as above.
The platelets were re-suspended in an appropriate primary Ab and incubated for 60 minutes at room temperature (or overnight at 4°C), washed x 3 in PBS/0.1% BSA as above, and incubated in a fluorescein isothiocyanate (FITC)-conjugated secondary Ab (30 minutes; room temperature). All FITC incubations were performed in the dark. The samples were finally washed x 3 in PBS and re-suspended in PBS. Flow cytometry was carried out on a Becton Dickinson FacsCalibur flow cytometer with forward and side scatter set to a logarithmic scale.

2.10 Platelet microvesicle release

Following flow cytometric analysis an area was drawn around the unstimulated platelet sample according to the forward and side scatter properties, and labeled platelet region (P). A second region was drawn to gate on smaller, more granular particles and labeled microvesicle (MVS) region. The percentage of samples from each region was calculated and the number of fluorescent labeled events in each region determined (Heijnen, H. F. et al., 1999)

2.11 Microvesicle and Exosome preparation

Microvesicles and exosomes were prepared according to a method by Heijnen and colleagues (Heijnen, H. F. et al., 1999). Briefly, platelets were washed into Hepes/Tyrodes buffer and divided into 2ml aliquots. Pefabloc (4µM) was added to the buffer to prevent endogenous protease digestion, and 5µM RGDS peptide added to prevent platelet clumping. Platelets were incubated with either 1unit/ml thrombin or saline control, for 120 seconds and the reaction stopped by the addition of an equal amount of ice-cold ACD. Samples were centrifuged at 800 x g for 15 minutes to obtain the platelet pellet fraction (P). The supernatant was further centrifuged at 10,000 x g for
30 minutes to obtain the microvesicle fraction (MV). The supernatant from the MV fraction was centrifuged at 100,000 x g for 60 minutes to pellet the exosomes (EX). All pellets were re-suspended in PBS. Samples were used for either immunoblotting or electron microscopy.

To ensure that exosomes were being produced, the supernatant from the 10,000 x g spin was mixed with 2M sucrose, and a 0.8-0.25M sucrose gradient was layered on top. This gradient was centrifuged at 65,000 x g for 16 hours and 500µl fractions were collected. These fractions were diluted in 4.5ml PBS and centrifuged at 100,000 x g for 60 minutes at 4°C. The pellets were resuspended in PBS for slot blots or electron microscopy.

### 2.12 Slot Blots

Pellets from the sucrose gradient fractions were mixed with 30 µl of PBS and 10µl was bound to a nitrocellulose membrane using a Schleicher and Schuell slot blot apparatus. The membrane was incubated in 5% non-fat powdered milk in TBS-T to block non-specific binding then incubated in the appropriate Ab diluted in TBS for 60 minutes at room temperature or overnight at 4°C. Following washing in TBS-T (3x5 minutes), the membrane was incubated in the appropriate secondary Ab conjugated to horseradish peroxidase for 30 minutes at room temperature. The membranes were washed in TBS-T (6 x 5 minutes), incubated in Super Signal West Pico chemiluminescence substrate and visualised on a Bio-Rad Fluoro S Max imaging system. Densitometry was performed using Quantity One software (Bio-Rad). The presence of exosomes in these fractions was confirmed by staining with a monoclonal Ab to CD63.
2.13 Electron microscopy and immunocytochemistry

2.13.1 Resin sections of whole platelets

Platelet pellets were re-suspended in Hepes/Tyrodes buffer as described above. The washed platelets were incubated with 1 unit/ml of thrombin, or saline control, for various times (0, 30, 60, 120 seconds) and the reaction terminated at by the addition of an equal amount of 0.1% glutaraldehyde in White’s Saline. The samples allowed to fix for 10 minutes at room temperature then processed for electron microscopy according to the protocol in Appendix B.

Sections (80 nm) were prepared on a Leica ultramicrotome and picked up on 300 mesh copper grids. Grids were stained in lead citrate for 10 minutes, washed in distilled water and dried before being examined in a Tecnai electron microscope.

2.13.2 Whole mounts of sucrose gradient fractions

Pellets from the sucrose gradient fractions were re-suspended in 30µl of PBS as described above and adsorbed onto 300 mesh formvar coated nickel grids for 10 minutes. Excess PBS was blotted with filter paper and the grids were fixed with 2% paraformaldehyde in PBS for 10 minutes. After fixation, the grids were washed in PBS with 1% BSA and stained for immunocytochemistry.

2.14 Antibody staining

Grids for immunocytochemistry were incubated in PBS with 1% BSA for 15 minutes then incubated in the appropriate Ab for 30 minutes. The grids were washed 3 x 5 minutes in PBS with 0.1% BSA and incubated in the secondary Ab conjugated to 10nm gold for 30 minutes. After gold labeling, the grids were washed 3 x 5 minutes in PBS.
followed by washing x 3 in deionised water. The grids were then stained in 3% uranyl acetate, dried and examined in a Tecnai transmission electron microscope.

2.15 Platelet Aggregation Studies

Platelet pellets were re-suspended in Hepes/Tyrodes buffer as described above. Aggregation studies were carried out on a Peyton Aggregometer (McNicol, A. et al., 1993). Briefly, platelet aliquots (400µl) were stimulated with the thromboxane A<sub>2</sub> analogue U46619 or collagen. In some studies anti-PrP Abs were added to the platelet samples and incubated at 37°C for 5 minutes before addition of agonist.

2.16 Platelet Adhesion Assays

Flat bottomed microtitre plates (96 well) were coated with fibrinogen, laminin, collagen or fibronectin (each at 10mg/ml). Coated wells were incubated with 1%BSA in calcium free Hepes/Tyrodes buffer for 90 minutes at room temperature before being used for adhesion assays. Platelets, washed into Hepes/Tyrodes buffer as described above, were incubated with various concentrations of anti-PrP Abs for 5 minutes. Platelets (200µl) were added to the wells and allowed to settle for 15 minutes. The wells were washed with PBS, incubated with anti-CD9, a platelet plasma membrane marker, conjugated to FITC for 30 minutes. The wells were washed twice in PBS and fluorescence determined on a Gemini Max fluorospectrophotometer with excitation and emission set to 495nm and 530nm respectively. BSA was used as a negative control and the background counts were subtracted from the samples. Platelets incubated with anti-PrP Abs were compared controls that had no Ab added.
2.17 Immunoprecipitations

Immunoprecipitations with anti-PrP monoclonal Ab 6H4 were carried out using a “Seize” primary immunoprecipitation kit following the manufacturer’s instructions. Platelets, in Hepes /Tyrodes buffer, were lysed using the mammalian cell lysis buffer provided. The lysates were then added to a column containing 6H4 Ab bound to sepharose beads, rocked overnight at 4°C and the proteins in the eluate separated on a 10% SDS PAGE gel, transferred to nitrocellulose and immunoblotted with Abs raised against to laminin receptor, fyn and calreticulin.
3 RESULTS

3.1 Cellular prion protein is present on platelet alpha granule membranes

Immunofluorescence staining of quiescent, non-permeabilised platelets with anti-PrP Abs demonstrated the presence of PrPc on the plasma membrane (Figure 6A, 6B). Following platelet stimulation with 1 unit/ml of thrombin there was an apparent increase in the surface levels of PrPc (Figure 6C, 6D).

Similar studies in permeabilised platelets which allowed access of detecting Abs to internal organelles showed the presence of PrPc on internal membranes (Figure 7A, 7D). Double immunolabeling, confocal microscopic studies of permeabilised platelets co-localised PrPc with CD62 (Figure 7A, 7B, 7C), an acknowledged alpha granule membrane marker (Furie, B. et al., 2001) but not CD63 (Figure 7D, 7E, 7F), which is present on the membranes of dense granules and lysosomes (Nishibori, M. et al., 1993).

Platelets were incubated with mepacrine which is actively and selectively accumulated by dense granules, and subsequently double stained with anti-PrP or anti CD62 Abs. Visualization, using a secondary Ab conjugated to Texas Red, confirmed the absence of PrPc on the membranes of dense granules (Figure 8).

Immunoblotting of platelet sub-cellular preparations with an anti-PrP Ab, was consistent with the presence of PrPc in whole platelet homogenates and in the alpha granule-enriched fraction, but not in the dense granule-enriched fraction (Figure 9). Taken together these data are consistent with the presence of PrP in alpha granule, but not dense granule, membranes.
Figure 6. Immunofluorescent staining of PrPc in quiet and activated intact platelets

Immunofluorescent staining of quiet (panels A and B) and activated (panels C and D) platelets with Ab 308 showed presence of PrPc on the plasma membrane (large arrow). Following activation with 1 unit/ml of thrombin, the internal stores of PrPc relocated to the outside of the cell (small arrow). Panels A and C – light microscopy, panels B and D Ab 308 followed by secondary antibody conjugated to FITC.
Figure 7. Double immunofluorescent staining of PrPc, CD62 and CD63 in resting permeabilised platelets
Figure 7. Double immunofluorescent staining of PrPc, CD62 and CD63 in resting permeabilised platelets

Top row: Permeabilised platelets stained with anti-PrP Ab 308 and visualized using a secondary Ab conjugated to AF488 (A), anti-CD62 Ab, visualizing a secondary Ab conjugated to Texas Red (B), overlay of A and B (C). Bottom row: Stained with the anti-PrPc Ab 308 and visualized using a secondary Ab conjugated to AF488 (D), stained with anti-CD63 Ab and visualized using a secondary Ab conjugated to Texas Red (E), overlay of D and E (F) (n=3).
Figure 8. Double immunofluorescent staining of dense granules, PrPc and CD62 positive granules in resting permeabilised platelets

A: Platelets labeled with mepacrine were subsequently stained with anti-PrP Ab3F4 and visualized using a secondary Ab conjugated to Texas Red. The dense granules are stained bright green (arrows), and the PrPc labeled granules are stained red. No co-localization was seen with dense granules and PrPc.

B: Platelets labeled with mepacrine were subsequently stained with anti-CD62 Ab and visualized using a secondary Ab conjugated to Texas Red. The dense granules are stained bright green (arrows), and the CD62 positive alpha granules are stained red. No co-localization was seen with dense granules and CD62
Figure 9. Western blot of platelet fractions

(A) Western blotting with anti-PrP Ab 308 revealed a 35-40 kDa band in the whole homogenate (H) and alpha granule enriched fraction (A) but not the dense granule fraction (D).

(B) Blotting with anti-CD63 Ab showed enrichment of this protein in both the alpha granule enriched fraction and the dense granule fraction. The presence of CD 63 in the alpha granule enriched fraction could be due to the presence of lysosomal granules in this fraction or the retention of some dense granules above the metrizamide gradient. (n=2)
3.2 Cellular prion protein translocates to the surface, and is released, on platelet activation.

Immunoblotting of lysates from thrombin stimulated platelets with an anti-PrP Ab showed a decrease in PrPc levels over time (Figure 10), although there was significant donor variability in the time course of the loss of PrPc. Densitometric analysis of the representative blot showed that, prior to thrombin stimulation, the density of the pellet sample was 1.75 ODunits/mm². This fell to 0.8 ODu/mm² at 120 secs following activation. This decrease in platelet-associated PrPc from these activated platelets was accompanied by a corresponding accumulation of PrPc in the releasate (Figure 11).

3.3 Thrombin causes the release of microvesicles from platelets

Thrombin-activated platelets caused the release of microvesicles, determined by flow cytometry, as has been previously reported (Heijnen, H. F. et al., 1999). Under resting conditions most of the events fall within the platelet region, however the number of events in the microvesicle region increased over time (Figure 12). There was significant donor variability in the time course with maximum release observed between 60 and 300 sec.

Analysis of the flow cytometry event numbers in the representative experiment (Figure 12) showed that in resting platelets, 0.8% of the total events corresponded to microvesicles. Following stimulation by thrombin the percentage of microvesicles increased to 12% of total events at 60 seconds, and to 43.6% of total events at 120 seconds. There was a corresponding decrease in the number of events in the platelet region falling from 98% at 0 seconds thrombin to 80% at 60 seconds and finally 45.5% at 120 seconds of thrombin stimulation.
Platelet pellets and supernatants from a timecourse of thrombin stimulation. Blotting with anti-PrP Ab 308 confirmed that PrPc was being released from activated platelets in a time-dependent manner. The supernatants from the activated platelet samples were spun at 200,000 x g and the pellets blotted with Ab308. By 120 seconds stimulation, the intensity of the PrPc band from the platelet pellet had diminished to about 50% of the other samples. PrPc was detected in the corresponding supernatant at 120 seconds. (n=3)
Densitometry was performed on immunoblots of pellets and supernatants from the timecourse of thrombin stimulated platelets in figure 9.

There was a slight decrease in density from the 60-second pellet sample, but by 120 seconds the density of the pellet had fallen from 1.75 ODu/mm² in the unstimulated sample to 0.8 ODu/mm². There was a corresponding rise in the density of the 120-second supernatant sample, showing that PrPc was being released to the supernatant.
A timecourse of thrombin stimulation was analysed using a forward scatter vs. side scatter dot plot. Gates were drawn around the platelet (P) and microvesicle (MVS) fractions. At 0 seconds thrombin the MVS region constituted 0.8% of the total events counted. By 60 seconds this percentage has risen to 12%, and by 120 seconds had increased to 43.6% of total events. (representative of 8 experiments)
3.4 Cellular prion protein is present in microvesicles and exosomes

Flow cytometry using an anti-PrP Ab demonstrated that the fluorescence intensity of PrPc was 37.7% in unstimulated platelets, consistent with the presence of PrPc on the platelet surface. Following thrombin stimulation this increased to 67.8% at 30 seconds, then fell to 42.9% at 60 seconds (Figure 13A). As outlined above, thrombin stimulated the release of microvesicles although the level of PrPc on the surface of the microvesicles remained relatively constant (Figure 13A). This suggests that the PrPc remains on the surface of the microvesicles.

A similar pattern of results were seen in platelet samples labeled with Abs directed against CD62 and CD63 (Figure 13B,C). As expected the there was considerably lower surface levels of CD62 and CD63, than of PrPc, in the unstimulated platelets.

Immunogold labeling of a whole mount of thrombin-stimulated platelets demonstrated the presence of PrPc around the periphery of the cell and at the tips of pseudopods. In addition there is some evidence for the presence of PrPc on small vesicles released from platelets (Figure14).

Previous studies have shown that, in addition to microvesicles, thrombin stimulates the release of exosomes from platelets (Heijnen, H. F. et al., 1999). Given the relatively low levels of PrPc on the surface of released microvesicles (Figure 13A), the possible association of PrPc with exosomes was therefore examined.
At 0 seconds of thrombin, 98.6% of total events fell within the platelet gate. This number decreased over time until at 60 seconds thrombin only 56.8% of events fell within this gate. There was a corresponding increase in events in the microvesicle gate, rising from 2% at 0 seconds thrombin to 37.5% of total events at 60 seconds thrombin.

Fluorescence intensity of PrPc was at 37.7% in unstimulated platelets. This amount increased to just below 67.8% at 30 seconds, but fell to 42.9% at 60 seconds of thrombin stimulation. Surface PrPc on microvesicles remained relatively constant between 3.9-4%. (Representative of 3 experiments)
97.5% of total events fell into the platelet fraction in the unstimulated sample. By 120 seconds stimulation this had fallen to 51.7%. Events in the MVS fraction rose from 1.6% in the unstimulated sample to 27.2% after 120 seconds of thrombin stimulation.

Surface CD62 intensity on the unstimulated platelet fraction was 13.9%, rising to 62.9% by 30 seconds then falling to 51.7% by 120 seconds. CD62 on microvesicles remained relatively constant at between 2.4 and 3.6% (Representative of 3 experiments)
98.3% of total events fell into the platelet fraction in the unstimulated sample. By 120 seconds stimulation this had fallen to 50.6%. Events in the MVS fraction rose from 1.7% in the unstimulated sample to 49.4% after 120 seconds of thrombin stimulation.

Surface CD63 intensity on the unstimulated platelet fraction was 25.5%, rising to 87.7% by 30 seconds then falling to 58% by 120 seconds. CD63 on microvesicles rose to only 0.38%. (Representative of 3 experiments)
Figure 14. Immunogold labeling of activated platelets

A: Immunogold labelling with anti-PrP MoAb 6H4 demonstrated the presence of PrPc at the periphery of the activated platelet and at the end of a pseudopod (P)

B: Release of membrane vesicles from an activated platelet. Gold- labelled PrPc is seen on these vesicles as well as on a smaller vesicle in the top right area of the picture (arrows).

C: Another example of PrPc seen in smaller vesicles released from activated platelets. These vesicles ranged from 40-100nm suggesting that they are exosomes. (Bar =200nm)
Exosomes were prepared by differential centrifugation, and separation through a sucrose gradient, of the releasate of thrombin-stimulated platelets. Slot blot analysis, and associated densitometry, with an anti-CD63 Ab confirmed the presence of exosomes in fractions 3 and 4, as previously reported (Heijnen, H. F. et al., 1999).

Immunoblotting using an anti-PrP Ab was consistent with the presence of PrPc in the exosome fraction (Figure 15 A and B). There was also significant PrPc present in fraction 1, likely representing soluble PrPc.

Transmission electron immunomicroscopy identified exosomes, which were considerably smaller than released microvesicles, stained with CD63 (Figure 16A). Subsequent staining with an ant-PrP Ab was consistent with PrPc on exosomes (Figure 16B).

Fraction 10 was also examined for the presence of PrPc by transmission electron immunomicroscopy. This fraction contained PrPc on both various membrane fragments and larger granules which, by size, are possibly microvesicles. PrPc seemed to be polarized to one end of these larger granules suggesting a possible lipid raft association (Figure 17).
Fractions from the sucrose gradient in figure 13A were bound to nitrocellulose and blotted with antip PrP and anti-CD63 MoAbs. Fractions 3 and 4, which contained large amounts of both PrPc and CD63 corresponded to the density of exosomes and were subsequently collected and used for immunoelectron microscopy.

Fraction 1 is most likely a soluble form of PrPc. Most of the remaining PrPc was found in fraction 10 (pellet). Although there was a substantial amount of CD63 in the exosome fraction, most of the CD63 was found in the membranes in fraction 10.
Figure 16A. CD63 Immuno electron microscopy of isolated exosomes

Presence of exosomes in fractions 3 and 4 was confirmed by staining with anti-CD63 MoAb. (A and B). Structures between 40-100nm are exosomes labelled with 10nm gold (Larger structure in panel B is most likely a microvesicle).
Figure 16B. PrPc Immuno electron microscopy of isolated exosomes

Immunolabelling of whole mounts of exosomes from fraction 3 and 4 with anti-PrP MoAb 3F4. PrPc was seen on released exosomes (A). Higher magnification of single exosome (B)
Fraction 10 from the sucrose gradient was seen to contain various membrane fragments and larger granules which ranged from 150-350nm in size consistent with microvesicles. PrPc staining seemed to be polarized to one end of these granules (arrows).
3.5 Cellular prion protein is not involved in platelet aggregation

Platelets were incubated with several antibodies to PrP prior to stimulation by either U46619 or collagen, each at the minimal concentration required to produce aggregation. Initial studies suggested that anti-PrP Abs 6H4 and 3F4, but not 308, inhibited aggregation in response to both agonists (Figure 18). However, both 6H4 and 3F4 contained sodium azide which is a powerful inhibitor of platelet aggregation. Thus it is unlikely that blocking PrPc had any effect on platelet aggregation. Furthermore neither 6H4 nor 3F4 were used in subsequent platelet function studies.

3.6 Cellular prion protein may be involved in platelet adhesion

Platelet adhesion to collagen, fibrinogen, fibronectin or laminin was measured using an anti-CD42 Ab directly conjugated to FITC. Pre-incubation of platelets with anti-PrP antibodies 308, 8G8, F89 and F99 each inhibited platelet adhesion to all four matrices to some degree, with inhibition ranging between 15-23% (n=3) (Table 4).

3.7 Cellular prion protein co-precipitates with calreticulin

Platelet eluates from an anti-PrP Ab (6H4) column were separated by SDS-PAGE and immunoblotted with Abs to calreticulin, the laminin receptor and fyn. Calreticulin, but neither the laminin receptor nor fyn, was present in the eluate of both unstimulated and thrombin-stimulated platelets (Figure 19A). Furthermore, immunoblotting of a platelet eluate from a corresponding anti-calreticulin column demonstrated the presence of both calreticulin and PrP (Figure 19B). Taken together these data are consistent with PrPc associating with calreticulin in platelets.

Immunofluorescence, using anti-PrP and anti-calreticulin Abs, of permeabilised unstimulated platelets showed the significant co-localization of PrPc and calreticulin
(Figure 20). However there was additional, non-PrPc-associated calreticulin present in the platelet cytoplasm.
Figure 18. Platelet Aggregation

1μM U46619 caused complete aggregation of platelets. Monoclonal antibodies to prion protein were added at a 1/400 dilution 5 minutes before aggregation. 3F4 and 6H4 completely blocked U46619 aggregation while 308 had no effect. (n=3)
Table 4. Platelet Adhesion Assays

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Antibody</th>
<th>Control</th>
<th>308</th>
<th>8G8</th>
<th>F89</th>
<th>F99</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>100%</td>
<td>81.3±5.4</td>
<td>80.8±6.3</td>
<td>81.0±4.6</td>
<td>85.9±6.5</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>100%</td>
<td>82.0±2.5</td>
<td>77.9±1.7</td>
<td>78.8±4.3</td>
<td>80.5±4.4</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>100%</td>
<td>87.4±5.6</td>
<td>86.1±6.1</td>
<td>83.0±3.0</td>
<td>85.4±2.9</td>
<td></td>
</tr>
<tr>
<td>Laminin</td>
<td>100%</td>
<td>81.±9.5</td>
<td>82.7±9.8</td>
<td>81.8±8.0</td>
<td>79.8±6.5</td>
<td></td>
</tr>
</tbody>
</table>

Platelet adhesion to collagen, fibrinogen, fibronectin or laminin was measured +/- preincubation with various antibodies to PrP. All antibodies inhibited adhesion to some degree. Inhibition to the various matrices ranged from 15-23% with Ab 8G8 the most effective in inhibiting adhesion to fibronectin at 23%. (n=3)
**Figure 19. Immunoprecipitation with antibody to PrPc and Calreticulin**

**A.** Eluates, of quiet (Q) and thrombin stimulated platelets (A), from an anti-PrP 6H4 Ab column were blotted with anti-calreticulin (CR), -Laminin receptor (LRc) and -Fyn Abs. Only calreticulin gave a positive signal in both quiet and activated platelets.

**B.** Platelets immunoprecipitated with anti-calreticulin Ab and blotted with anti-calreticulin Ab and anti-PrP Ab 308. Both gave a positive signal. (n=3)
Figure 20. Double labelling with antibodies to calreticulin and prion protein

Permeabilised platelets were labelled with anti-PrP Ab 308 with the secondary antibody conjugated to FITC (A), and anti-calreticulin Ab with the secondary antibody conjugated to Texas Red (B). The yellow areas in the overlay of both images showed some co-localization of PrPc and calreticulin (C) (n=2)
4. DISCUSSION

Historically TSEs were relatively rare and obscure fatal neurodegenerative diseases of humans and animals. However they were highlighted in the mid 1980s with the outbreak of BSE in the United Kingdom, the cause of which was suggested to be due to a combination of feeding meat and bone meal to cattle coupled with a change in rendering practices. (Taylor, D. M. et al., 2003; Taylor, D. M. et al., 1997). Between 1988 and 2004 a total of 179,085 cattle were diagnosed as positive for BSE http://www.defra.gov.uk/animalh/bse/statistics/weeklystats.html#pass, with 214,080 being slaughtered to date. This disease is not unique to the U.K.; a total of 25 countries worldwide have reported at least 1 or more positive cases of BSE since 1987 http://home.hetnet.nl/~mad.cow/. The finding of BSE in cattle causes an immediate and great impact on the economy of affected countries. For example, the single Canadian case in May of 2003 caused the immediate slaughter of 2000 herd-mates and offspring, and the closure of various borders to the import of Canadian beef had a devastating effect on the Canadian beef industry with an estimated loss of $5billion. (Canadian Cattlemens Association http://www.cattle.ca/) The recent discovery of two more cases in Western Canada may hamper negotiations with the United States to reopen the border to importation of live cattle.

Another result of the BSE outbreak in the U.K. was the emergence of a variant form of CJD which was first identified in 1996. It was linked to the digestion of beef contaminated with BSE (Lasmezas, C. I. et al., 2001). The CJD Surveillance Unit in the U.K. was set up in 1990 to monitor all new cases of CJD (Ironside, J. W., 1996), and to
date there have been a total of 147 human deaths from vCJD (http://www.cjd.ed.ac.uk/figures.htm).

Although the pathogenesis of CJD is still uncertain, the presence of PrPres in peripheral lymphoid tissue led to the fear that TSEs may be transmitted though blood transfusion (Lee, C. A. et al., 1998). In 1998, in an effort to prevent blood-borne transmission, the U.K. adopted the practice of universal leucodepletion. The decision arose from findings that described B lymphocytes as playing a major role in TSE pathogenesis (Klein, M. A. et al., 1997). However the effectiveness of leucodepletion to completely remove TSE infectivity has still to be established. Indeed, Klein et al. later downplayed the role of B lymphocytes in TSE pathogenesis and suggested a stronger role for follicular dendritic cells of the spleen (Klein, M. A. et al., 1998).

Although there have been no reports of classical CJD being transmitted by blood transfusion, there have recently been two reports linking the acquisition of vCJD to blood transfusions (Peden, A. H. et al., 2004). These reports have brought into question the effectiveness of leucodepletion and the possibility that PrPres may be carried on blood components other than leucocytes (Gregori L. et al., 2004).

Pathogenesis studies using PrP<sup>−/−</sup> mice have demonstrated that propagation of PrPres cannot occur in the absence of PrPc (Brandner S. et al., 2000) and, since a number of studies have shown PrPc to be present on platelets as well as leucocytes (Holada, K. et al., 2000), the objective of the current study was to further investigate the role of PrPc within platelets.

Previous studies have shown PrPc to be present on both external and internal platelet membranes; furthermore the internal store relocates to the surface on platelet
activation. In addition flow cytometric studies suggested an alpha granule location for PrPc since it relocated to the surface in a similar fashion to P-selectin, however the exact internal location had not been determined (Holada, K. et al., 1998). In the current study, by using a combination of imuno-blotting of platelet granule fractions and immunofluorescent co-localization studies, we have unequivocally shown that PrPc is located on the alpha granule membrane, but not on either dense granule or lysosomal granule membranes. Furthermore, we have shown that, following thrombin stimulation, PrPc relocates from these granule membranes to the plasma membranes, and is eventually released from the platelet surface. This is, in part, agreement with an earlier study which described a soluble form of PrPc being released from stored platelet packs (Bessos, H. et al., 2001).

It has been shown that on activation, platelets release two types of membranous packages; microvesicles, which are plasma membrane derived and range from 100nm to 1 µm, and smaller exosomes, which range between 40-100nm and are derived from alpha granules and multivesicular bodies (Heijnen, H. F. et al., 1999). We therefore investigated the possibility that PrPc was being released on exosomes and/or microvesicles.

We initially used flow cytometry to determine the amount of PrPc that was released on the microvesicles from thrombin-activated platelets, and although the number of microvesicles being released from activated platelets increased, the amount of PrPc in the microvesicle fraction did not rise above 4%. This suggested that the released PrPc was either in a soluble form or attached to membranes or vesicles that were much smaller than microvesicles. Since exosomes are smaller than 100nm they are beyond the limits
of detection by flow cytometry and it was therefore possible that some PrPc may be released on these vesicles.

Using a sucrose gradient, we isolated exosomes from thrombin-activated platelets and found increased amounts of PrPc present in both soluble and exosome containing fractions. Immunoelectron microscopy confirmed the presence of PrPc in the exosome fraction. Larger granules with smaller internal vesicles were also observed in the releasate from activated platelets. PrPc was located on the both the external and internal membranes of these structures. The size of these structures (approximately 500μm) and the presence of many internal vesicles are consistent with them being multivesicular bodies.

Therefore the current study has shown, for the first time that PrPc is released from activated platelets on both exosomes and microvesicles.

Recent bioassays using mouse models, demonstrated infectivity in both platelets and platelets poor plasma (Cervenakova et al., 2003). Although this study did not isolate exosomes from plasma it is possible that exosomes were present in the platelet poor plasma fraction and may be contributing to infectivity. Of interest, Fevrier and colleagues have recently demonstrated that cultured neuronal cells can release PrPres on exosomes (Fevrier, B. et al., 2004), suggesting that exosomes may be a likely source of prion infectivity. While the presence of PrPc on exosomes released from platelets does not necessarily indicate the presence of PrPres, the involvement of plasma exosomes in the transmission of TSE infection from blood cannot be ruled out and is an area which obviously merits further investigation.
It is widely acknowledged that the spleen is the major site for accumulation of PrPres (Jeffrey, M. et al., 2000). The spleen is also the main sequestration organ for senescent platelets (Berger, G. et al., 1998). It is therefore possible that any PrPres that may be carried on platelets or released granules could contribute to the accumulated PrPres in the spleen.

The role of PrPc in normal platelet function was also investigated. Initially the role of PrPc in platelet aggregation was examined. Blocking PrPc with a number of antibodies initially showed promise as two of the antibodies used completely blocked aggregation in response to the thromboxane analogue U46619. However, further investigation revealed that these antibodies contained sodium azide, which is itself, a potent inhibitor of platelet aggregation. Subsequent experiments using sodium azide-free antibodies had no effect on aggregation suggesting that PrPc was not involved in this function.

PrPc had been previously shown to be involved in cell adhesion in neuronal cells. We therefore investigated the possibility that it could also be involved in platelet adhesion. Blocking PrPc with a number of antibodies inhibited platelet adhesion to fibrinogen, collagen, fibronectin and laminin matrices by up to 25%. Although apparently a low level of inhibition, it should be noted that platelets contain a number of other receptors on their surface that are involved in adhesion imparting a degree of high adhesive capacity and a degree of receptor redundancy. These studies suggest a possible role for PrPc in platelet adhesion. However further studies are required to ensure that the inhibition of adhesion is a result of the specific inhibition of PrPc rather than a non-specific steric interference by the antibodies. In addition the effects of anti-bodies against
individual regions of PrPc should be used to determine the specific domain responsible for adhesion.

A number of studies in neuronal cells have suggested that PrPc is associated with a number of protein-protein interactions important for signaling pathways. These studies have implicated interactions between PrPc and calreticulin, laminin receptor and fyn. In our hands, calreticulin, but neither the laminin receptor nor fyn, co-precipitated with PrPc. Furthermore immunofluorescence double labeling studies suggested that PrPc may also co-localise with calreticulin. This is the first time that an association between PrPc and calreticulin has been described in platelets.

Interestingly calreticulin has been shown to be present on the surface of platelets (Elton, C. M. et al., 2002), and earlier studies have suggested the dense tubular system as a possible location for calreticulin (Arber, S. et al., 1992), although the ring like structure in activated platelets suggests a granule membrane location. In the current study PrPc was found to be mainly present in alpha granule membrane. To date there are no reports of a granular location for calreticulin although this could be addressed by applying the same fractionation and double labeling techniques that we have used in this study to characterise the location of calreticulin within platelets.

It may be also possible to elucidate a role for PrPc within platelets through its association with calreticulin. Calreticulin is known to be a multi functional calcium binding protein and an endoplasmic reticulum chaperone protein (Michalak, M. et al., 2002). It has been implicated in a number of processes including glycoprotein folding (Ellgaard, L. et al., 2003), cell adhesion and integrin dependent calcium signaling (Coppolino, M. G. et al., 1997). In platelets, it has been associated with collagen
receptors (Elton, C. M. et al., 2002), and thrombospondin signaling (Goicoechea, S. et al., 2002).

The tyrosine kinase fyn is also involved in the collagen activation pathway in platelets (Briddon, S. J. et al., 1999), and PrPc has been shown to phosphorylate fyn through caveolin-1 signaling in a neuronal cell line (Mouillet-Richard, S. et al., 2000). It may also be possible that both PrPc and calreticulin are associated during collagen signaling in platelets although investigation of this possibility warrants a more intensive approach than this project allows.

As this work progressed it became increasingly clear that further investigation into the various roles for PrPc within platelet function were necessary. The role that this protein plays in cell adhesion or signal transduction alone, warrants much further research than could be carried out here and we can only hope that this work may be done in future projects.

5. LIMITATIONS

The initial objective of these studies was to pinpoint the exact location of PrPc within platelets and, if possible, determine a role for the protein within normal platelet function. The localization of PrPc to platelet-derived exosomes raises the possibility that there may be a role for platelet derived exosomes in TSE pathogenesis. Investigating this role however is outside the scope of this project for a number of reasons:
• One approach would be to isolate exosomes from the plasma of previously infected animals and inoculate a new set of animals with these isolates. However the long incubation times of TSE infection put this type of experiment outside the time frame for this project. For example ME7 strain of scrapie has an incubation time of 171 days in the C57/BL strain of mice (Bruce, M. E. et al., 1991).

• There is also the possibility that the exosomes, which carry infectivity in plasma may be derived from other blood cells or endothelial cells. Like platelets, endothelial cells have also been shown to release microvesicles which contain PrPc (Simak, J. et al., 2002), although there have been no reports of endothelial cells releasing exosomes.

The antibodies used for the functional studies were all raised against different areas of the PrP peptide and therefore may have had varying degrees of effectiveness. Since PrPc is a GPI anchored protein, an alternative approach would be to remove the protein by incubation in phosphatidylinositol-specific phospholipase C (PILPC), which breaks the GPI anchor. However Holada et al found that the PrPc expressed on activated platelets was resistant to PILPC (Holada K. et al., 1998), so an alternative method of blocking PrPc would have to be found. A possibility is to use a peptide that would bind to PrPc thus rendering it inactive.
6. CONCLUSIONS

The experiments carried out in this study have enabled us to reach a number of conclusions about the location and possible function of PrPc in human platelets.

In addition to being located on the plasma membrane, we have shown PrPc to be located internally on the alpha granule membrane but not on dense granule or lysosomal granule membranes. Furthermore, this internal store of PrPc relocates to and is subsequently released from the plasma membrane when the platelet is activated with thrombin.

The PrPc from activated platelets is released in a soluble form as well as on microvesicles and exosomes.

As to a role for PrPc in normal platelet function, blocking PrPc with antibodies has shown that PrPc is not involved in platelet aggregation however it may be involved in platelet adhesion since blocking PrPc with antibodies inhibited adhesion to laminin, collagen, fibronectin and fibrinogen.

PrPc may also play a role in signal transduction since it co-precipitates calreticulin. However it did not co-precipitate fyn or the laminin receptor as had been reported in other cell lines.
7. APPENDICES

Appendix A. Buffer solutions

ACID CITRATE DEXTROSE ANTICOAGULANT (ACD)

Trisodium citrate 86mM  
Citric Acid 70mM  
Dextrose 200mM

HEPES /TYRODES BUFFER

Sodium Chloride 134mM  
Sodium hydrogen carbonate 12mM  
Potassium chloride 2.9mM  
Di-sodium hydrogen phosphate 0.6mM  
Magnesium chloride 1mM  
Hepes 8.4mM

Adjust pH to 7.4. Add 0.09% dextrose and 0.3% BSA before use.

TRIS BUFFERED SALINE (for whole platelet homogenate preparation)

Tris 20mM  
NaCl 130mM

Adjust pH to 7.0. Add 1mM PMSF, 10µg/ml leupeptin and 1mM benzamidine before use.

WASH BUFFER

Sodium chloride 130mM  
Potassium chloride 5mM  
Sodium dihydrogen orthophosphate 1mM
Sodium hydrogen carbonate  24mM  
Dextrose  10mM  
Sucrose  2.5mM  
EDTA  2mM  
BSA  0.7%  
Adjust pH to 7.4

HOMOGENIZING BUFFER

Hepes  25mM  
Potassium chloride  500mM  
Magnesium sulphate  2mM  
Dextrose  10mM  
Sodium citrate  20mM,  
Adenosine triphosphate  5mM  
Sucrose  100mM  
Adjust to pH 7.0. Add 1mM PMSF, 10µg/ml leupeptin, and 1mM benzamidine before use.

METRIZAMIDE GRADIENT

Metrizamide  40%  
KCl  0.35M  

150mM TRIS/HCl BUFFER pH 8.8

Tris  3.3g  
Deionised water  80ml  

Adjust pH to 8.8 with hydrochloric acid and bring volume to 200ml with deionised water
50mM TRIS/HCl BUFFER pH 6.8

Tris 6g
Deionised water 80ml

Adjust pH to 6.8 with hydrochloric acid and bring volume to 100ml with deionised water

12% POLYACRYLAMIDE RESOLVING GEL (Laemmli, U. K., 1970)

Deionised water 3.3 ml
30% Acrylamide/bis-acrylamide 29:1 4.0 ml
150mM Tris/HCl buffer pH 8.8 2.5 ml
10% SDS 0.1 ml
1% Ammonium persulfate 0.1 ml
Temed 0.004 ml

4% POLYACRYLAMIDE STACKING GEL

Deionised water 6.8ml
30% Acrylamide/bis-acrylamide 29:1 1.7ml
50mM Tris/HCl buffer pH 6.8 1.25ml
10% SDS 0.1ml
Ammonium persulfate 0.1ml
Temed 0.01ml

4X SDS SAMPLE BUFFER

1M Tris /HCL 2.4ml
SDS 8g
80% Glycerol 2.4ml
Bromophenol blue 4mg
Deionised water 2.1 ml

**TRIS/GLYCINE/SDS RUNNING BUFFER**

Tris 25mM
Glycine 192mM
SDS 0.5.%

**TRIS/GLYCINE TRANSFER BUFFER**

Tris 25mM
Glycine 192mM
Methanol 20%

**TRIS BUFFERED SALINE (TBS)**

Tris 50mM
Sodium Chloride 138mM
Adjust pH to 8.0

**TBS-T**

As above but with the addition of 0.05% Tween-20

**PHOSPHATE BUFFERED SALINE (PBS)**

Sodium Chloride 145mM
Sodium dihydrogen orthophosphate 1.45mM
di-sodium hydrogen phosphate 17mM
Adjust pH to 7.0
**WHITE’S SALINE SOLUTION A**

Sodium Chloride  240mM  
Potassium Chloride  100mM  
Magnesium sulphate  45mM  
Calcium nitrate  60mM  

Adjust pH to 7.4

**WHITE’S SALINE SOLUTION B**

Sodium hydrogen carbonate  12.5mM  
Potassium phosphate  3mM  
Phenol Red  0.002mM  

Dissolve in 100ml deionised water and adjust pH to 7.4

**0.1% GLUTARALDEHYDE IN WHITES SALINE**

Deionised water  8.875ml  
White’s saline solution A  0.5ml  
White’s saline solution B  0.5ml  
8% Glutaraldehyde  0.125ml  

**3% GLUTARALDEHYDE IN WHITES SALINE**

Deionised water  5.24ml  
White’s saline solution A  0.5ml  
White’s saline solution B  0.5ml  
8% Glutaraldehyde  3.74ml
**1% OSMIUM TETROXIDE**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
<th>ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionised water</td>
<td></td>
<td>7.0</td>
</tr>
<tr>
<td>0.1M Calcium Chloride</td>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>Potassium ferricyanide</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>4% osmium tetroxide</td>
<td></td>
<td>2.5</td>
</tr>
</tbody>
</table>
Appendix B. Processing schedule for transmission electron microscopy

1. Fix platelet samples by addition of an equal volume of 0.1% glutaraldehyde in Whites saline; 10 minutes.
2. Centrifuge at 2000 x g; 5 minutes.
3. Remove supernatant and incubate pellet in 3% glutaraldehyde in Whites saline; 60 minutes.
4. Centrifuge at 2000 x g; 5 minutes.
5. Discard supernatant and incubate pellet in 1% osmium tetroxide solution; 90 minutes.
6. Remove osmium tetroxide and wash pellet in deionised water; 2 x 5 minutes.
7. Incubate pellet in 3% uranyl acetate overnight at 4°C.
8. Wash pellet in deionised water; 2 x 5 minutes.
9. Incubate in 70% ethanol; 30 minutes.
10. Incubate in 90% ethanol; 30 minutes.
11. Incubate in 100% ethanol; 2 x 30 minutes.
12. Incubate in propylene oxide; 2 x 10 minutes.
13. Incubate in a 1:1 mixture of propylene oxide and epon/araldite resin; 60 minutes.
14. Incubate in epon/araldite resin; 2 x 60 minutes.
15. Embed pellet in fresh epon/araldite resin and cure at 60°C; 24 hours.
8. REFERENCES


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