Charles University in Prague

First Faculty of Medicine

PhD Thesis Autoreport

The characterization of blood platelet cellular prion protein

(Charakterizace buněčného prionového proteinu krevních destiček)

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Prague 2011

Doktorské studijní programy v biomedicíně

Univerzita Karlova v Praze a Akademie věd České republiky

Obor: Buněčná a molekulární biologie, genetika a virologie

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Školicí pracoviště: Ústav imunologie a mikrobiologie 1.LF UK

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Content

Summary

The conformational conversion of the cellular prion protein (PrPc) to the misfolded isoform (PrPsc) is the central pathogenic event in the transmissible neurodegenerative prion diseases. The recently shown transmissibility of variant Creutzfeldt-Jakob disease by blood transfusion emphasizes the need for better understanding of the PrPc in blood. In the current thesis, we focused on blood platelet PrPc, which has not been very well described so far.

In the first part of the thesis, platelet PrPc was characterized as glycosylphosphatidylinositol-anchored glycoprotein with dominant diglycosylated form. Platelet PrPc was shown to be sensitive to cleavage with proteinase K, which is a feature discriminating between cellular and pathological prion protein. We have confirmed that platelet PrPc binds copper ions by its N-terminal octapeptide repeat region. Regarding quantity of PrPc molecules expressed on blood elements we have proved that both platelets and red blood cells express considerable amount of PrPc and thus can not be neglected in the problematic of prions transmission by blood transfusion. The detailed study regarding PrPc localization in blood platelets is presented in the second part of the thesis. PrPc was shown to be expressed in α-granules as well as on the cytoplasmic membrane of platelets. Substantial amount of PrPc was found to localize in the lipid rafts. The majority of lipid raft associated PrPc was shown to be linked to platelet cytoskeleton. As for revealing the physiological role of PrPc in blood platelets further research needs to be done.

Taken together, blood platelets express indispensable amount of PrPc, which does not significantly differ from very well described neuronal PrPc. Thus, our results are support for next study of the role of platelet PrPc in the pathogenesis of prion diseases.

Souhrn

Centrálním dějem při vzniku transmisivních neurodegenerativních prionových chorob je konformační změna buněčného prionového proteinu (PrPc) na patologickou isoformu (PrPsc). V současnosti jsou už známé čtyři případy přenosu jedné z prionových chorob, variantní Creutzfeldt-Jakobovy choroby, krevní transfúzí. Porozumění vlastnostem a chování PrPc na krevních elementech je proto velmi důležité. Tato práce se zabývá buněčným prionovým proteinem na krevních destičkách.

V první části práce jsme charakterizovali PrPc krevních destiček jako protein ukotvený glykosylfosfatidylinositolovou kotvou, který je schopný vázat měďnaté ionty prostřednictvím N-koncové oktapeptidové repetice. Zjistili jsme, že ze tří možných glykoforem PrPc převládá na destičkách diglykosylovaná forma proteinu. Prokázali jsme, že PrPc krevních destiček není resistentní k proteinase K, čímž se odlišuje od patologické formy proteinu. Potvrdili jsme, že krevní destičky i erytrocyty nesou nezanedbatelné množství PrPc, a mohou se tak stát zdrojem infektivity při přenosu prionových chorob krevní transfúzí.

V druhé části práce jsme detailně popsali lokalizaci PrPc v krevních destičkách. Pomocí fluorescenčního značení jsme ukázali, že se PrPc nachází jak v α-granulích tak na cytoplasmatické membráně. Většina PrPc krevních destiček je součástí lipidových raftů a současně je napojena na destičkový cytoskeleton. Fyziologická funkce destičkového PrPc stále není známá, je potřeba další studium. Zjištěné vlastnosti destičkového PrPc jsou velmi podobné vlastnostem PrPc exprimovaného v mozkové tkáni popsaným v literatuře. Naše výsledky jsou základem pro další studium uplatnění destičkového PrPc v patogenezi prionových chorob a v jejich možném přenosu krví.

Introduction

The transmissible spongiform encephalopathies (TSE) or prion diseases constitute a unique group of fatal neurodegenerative disorders that occur in humans and mammals. One of the common hallmarks of all TSEs is the deposition of pathogenic prion protein (PrPsc) in the central nervous system in the form of amyloid fibrils (Carrell and Lomas 1997). The term PrPsc comes from the scrapie, which is a TSE affecting sheep and goats. The human TSEs are typically of late middle age and can be inherited, sporadic or acquired. The inherited forms include Familial Creutzfeldt-Jakob disease (fCJD), Fatal Familial Insomnia or Gerstmann-Straussler-Schienker syndrome, and can all be attributed to a mutations in gene coding prion protein PRNP (Ironside 1996). An example of sporadic TSE is sporadic CJD (sCJD), which has an unknown cause, and is the most prevalent among the other CJDs (85% of cases). Acquired forms can be iatrogenic (iCJD), as in the cases of transplantation of durra mater (Liscic, *et al* 1999) or the use of pituitary hormones from previously infected patients (Caboclo, *et al* 2002). A new variant form of CJD (vCJD) emerged in the United Kingdom in 1996 (Will, *et al* 1996), which has been associated with the consumption of meat infected with bovine spongiform encephalopathy (BSE) (Bruce 2000, Scott, *et al* 1999). vCJD affects young people and in contrast to sCJD has been shown transmissible by blood transfusion. Although the precise nature of the infectious TSE agent is uncertain, the abnormal form of PrPc - PrPsc co-purifies with infectivity in diseased tissues (Bolton, *et al* 1982) and it is supposed that the host protein – cellular prion protein (PrPc) serves as a template of PrPc conversion to PrPsc.

Table 1. The survey of human and animal transmissible spongiform encephalopathies.

TSE	Species
Sporadic Creutzfeldt-Jakob disease (sCJD)	human
Familial Creutzfeldt-Jakob disease (fCJD)	human
Variant Creutzfeldt-Jakob disease (vCJD)	human
Iatrogenic Creutzfeldt-Jakob disease (iCJD)	human
Gerstmann-Straussler-Schienker syndrome (GSS)	human
Fatal familial insomnia (FFI)	human
Kuru	human
Transmissible mink encephalopathy (TME)	mink
Bovine spongiform encephalopathy (BSE)	cattle
Chronic wasting disease (CWD)	cervids
Feline spongiform encephalopathy (FSE)	felines
Scrapie	sheep and goats

The infectivity is mainly accumulated in the brain tissue, however blood was also shown to be the source of infectivity. It was documented by four cases of transmission of vCJD by blood transfusion (Llewelyn, *et al* 2004, Peden, *et al* 2004). All patients were transfused with nonleukoreduced red blood cells. One case of likely transmission of vCJD infection by factor VIII concentrates has been reported in an elderly haemophilic patient in the UK, who had been treated with factor VIII produced from pooled plasma to which a donor, who subsequently died from vCJD, had contributed {Peden, 2010 #1352}. Very recent study proved that blood platelets can harbour infectivity – platelets from chronic wasting disease (CWD) affected deer were able to transmit the disease (Mathiason, *et al* 2010).

PrPc is glycosylphosphatidylinositol (GPI) anchored membrane glycoprotein with a molecular mass of 33 – 40 kDa encoded by PRNP gene located on chromosome 20. This highly conserved protein is expressed in nearly all tissues, being particularly abundant in neurons. PrPc is predominantly membrane protein, however it also cycles between the membrane and endocytic compartment (Shyng, *et al* 1993). PrPc co- and post-translational modifications include removal of a 22 amino acid N-terminal signal peptide, N-glycosylation at Asn181 and Asn197, formation of disulfide bond between Cys179 and Cys214 and replacement of the carboxy-terminus sequence at S231 by GPI-anchor. In the cells, PrPc is present as di-, mono- or unglycosylated molecules. PrPc is composed of about 40% alpha helix with less than 10% being in the beta sheet formation. The change of secondary conformation with increase of beta sheets to about 40% accompanies the formation of PrPsc (Lopez Garcia, *et al* 2000, Riek, *et al* 1996). The change in the protein conformation makes PrPsc more resistant to cleavage with proteinases e.g. proteinase K (PK).

Even though PrPc is expressed by many cell types and is highly conserved in mammals, its physiological role remains unclear. One possible explanation is that PrPc may serve multiple functions in diverse cell types. PrP knockout animals are viable and healthy; however, recent studies have demonstrated that PrP deletion may affect the organism under stress conditions. This was observed in PrP-deficient mice with induced anemia, which showed reduced erythroid cell and erythropoietin production (Zivny, *et al* 2007), and in irradiated mice, which had a decreased survival rate after transplantation of haematopoietic stem cells from PrP-null bone marrow (Zhang, *et al* 2006). PrPc has been shown to interact with a number of proteins such as Bcl-2, Hsp60, plasminogen, and laminin precursor receptors (Westergard, *et al* 2007). Other studies suggest that PrPc functions in cellular

resistance to oxidative stress (Milhavet and Lehmann 2002, Rachidi, *et al* 2003), in copper and zinc homeostasis (Watt and Hooper 2003), in synaptic transmission (Collinge, *et al* 1994), and in cell signaling (Mouillet-Richard, *et al* 2000). With regard to cell signaling, the association of PrPc with lipid rafts has been suggested to be critical. Lipid rafts are cholesterol-rich, detergent resistant microdomains of the cytoplasmic membrane. A number of studies strongly support a functional role for rafts in the onset of signaling events in different cell types, especially in immune (Horejsi, *et al* 1999, Montixi, *et al* 1998) and neuronal cells (Tsui-Pierchala, *et al* 2002). Like other GPI-anchored proteins, PrPc has been found in lipid rafts of diverse cells. The role of lipid rafts in the conversion of PrPc to the pathological prion protein in neuronal cells has been proposed (Kaneko, *et al* 1997, Taraboulos, *et al* 1995). However, the association of platelet PrPc with rafts has not been studied to date. This is important because raft associated PrPc may serve as a receptor or substrate for PrPsc and participate in blood transmission of prion diseases.

PrPc has been shown to be present on human blood elements as well as in plasma. However, there is a wide variation between the studies in the number of PrPc molecules found on each type of blood cell, which was attributed mainly to the method of detection and the choice of antibody (Table 2).

Table 2. Distribution of PrPc in blood (% from whole blood).

	(Barclay, *et al* 1999)	(MacGregor, *et al* 2000)	(Holada and Vostal 2000)
Platelets	96.3	84.2	44.9
RBCs	0	5.7	53.7
Mononuclear cells	2.8	7.6	1.3
Granulocytes	0.9	2.5	0.1

Previous studies have shown that PrPc is present on both internal and external platelet membranes of blood platelets. Moreover, upon platelet activation PrPc relocates to the surface along with other granule membrane protein CD62P (P-selectin) (Holada, *et al* 1998). Interestingly, in this study it was not possible to remove platelet PrPc from the cell membrane with phosphatidylinositol-specific phospholipase C (PIPLC). The up-regulation of PrPc on activated platelets together with CD62P suggested its localization in α-granules

of platelets. Platelet activation leads not only to PrPc up regulation on the surface, but also to the release of the protein on both microvesicles and exosomes (Robertson, *et al* 2006).

Aims of the study

The aim of this work was to examine the cellular prion protein (PrPc) in blood platelets from the view of its localization in blood platelets, its physiological function as well as possible involvement in the pathogenesis of prion diseases. The specific aims of this thesis were as follows:

1. To characterize biochemical properties of PrPc as compared with PrPc expressed in red blood cells and brain tissue.
2. To analyze PrPc localization in blood platelets with fluorescence microscopy.
3. To study the association of blood platelet PrPc with lipid rafts.
4. To follow possible PrPc role in blood platelet physiological function.

Materials and methods

Chemicals and antibodies: The chemicals used in the study were purchased from: Sigma, Amersham Biosciences, Pierce, New England BioLabs, Roche, Bio-Rad and Chemicon. The antibodies were purchased as follows: monoclonal anti-PrP antibodies AG4, AH6, DC2, FH11 and GE8 from TSE Resource Center, monoclonal 6H4 from Prionics, monoclonal 3F4 from Biodesign or Chemicon and monoclonal SAF32 from Cayman Chemical. Monoclonal anti-TSP antibody and rabbit polyclonal anti-actin were purchased from Santa Cruz Biotechnology, rabbit polyclonal anti-CD62P from BD Biosciences Pharmingen. Anti-mouse TRITC labeled and anti-rabbit FITC labeled secondary antibodies were from Jackson Immunoresearch Laboratories. Control mouse IgGs were purchased from Caltag. Anti-phosphotyrosine antibody was from Upstate. Alkaline phosphatase conjugated secondary antibodies were purchased from Biosource.

Blood platelets processing: Platelets were prepared either from platelet rich plasma (PRP) collected by apheresis or from citrated whole blood. PRP was subjected to gel filtration with subsequent resuspendation of platelets in Tyrode`s/HEPES buffer. Whole blood served as a source of both platelets and red blood cells. Organelles and membranes were prepared by platelets sonication and step centrifugation.

Fluorescence microscopy: Fluorescently labeled fibrinogen adhered or cytospined platelets were observed with a microscope Olympus AX70.

Lipid rafts isolation: Lipid rafts were isolated from detergent lyzed platelets by flotation assay on sucrose gradient according to Bodin et al (Bodin, *et al* 2005). To deplete cholesterol, saponin or methyl-β-cyclodextrin were used. In order to depolymerize actin before flotation assay, platelets were treated with potassium iodide.

Platelet cytoskeleton isolation: Platelet cytoskeleton was isolated by centrifugation of lyzed platelets as described earlier (Fox, *et al* 1988). In some experiments, the cytoskeleton was treated with potassium iodide and the lipid rafts were isolated as described by Bodin et al. (Bodin, *et al* 2005).

Platelet aggregation: Aggregation was measured using a BioData PAP-4 aggregometer with the collagen type I or ADP and epinephrine as agonists.

SDS PAGE and Western blot analysis: SDS-PAGE a Western blotting were performed in accordance with published protocols (Laemmli 1970). The densitometry was done using GS-800 Calibrated Densitometer with integrated Quantity One software (Bio-Rad).

Flow cytometry: Platelets were analyzed by a FACScan flow cytometer (Becton Dickinson) equipped with CELLQuestTM software.

Results

The biochemical characterization of platelet PrPc

In order to compare glycosylation pattern of platelet, red blood cells (RBC) and brain PrPc we performed western blotting analysis of platelet lysate, RBC ghosts and brain homogenate. As obvious from (Figure 1, A) the banding profile of RBC PrPc considerably differ to that of platelet and brain derived PrPc. In order to exclude resistance of platelet PrPc to cleavage with PK we performed the treatment of both platelet membranes and organelles with increasing concentration of the enzyme. As shown in (Figure 1, B), both platelet fractions were totally digested with 100 μg/ml of PK.

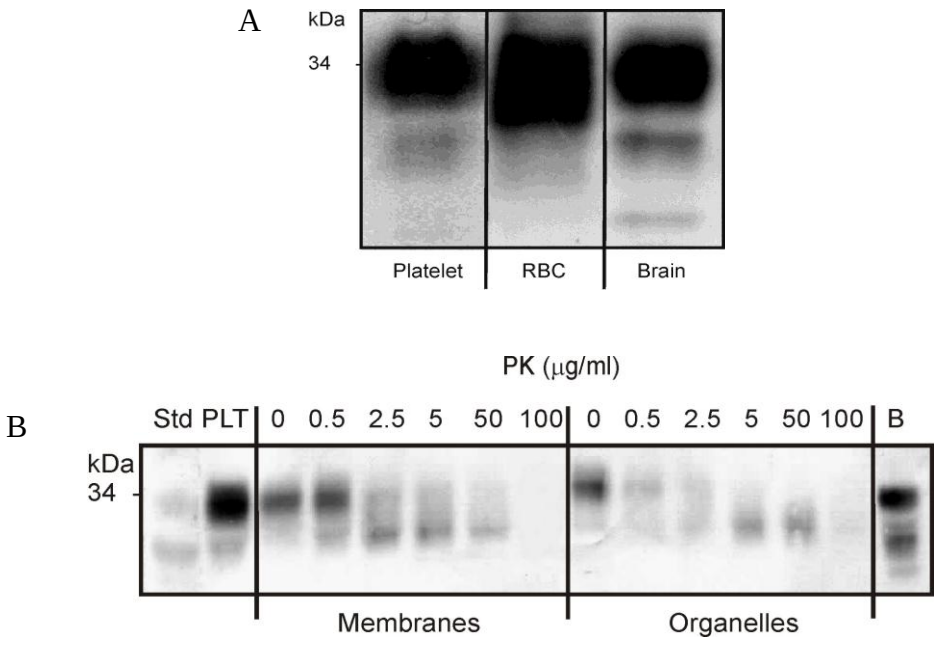

Figure 1. The biochemical characterization of platelet PrPc.
(A) The glycosylation pattern of platelet, RBC and brain PrPc was analyzed by SDS PAGE and developed with MAb AG4. (B) Both platelet membrane and organelle PrPc is sensitive to PK cleavage. Platelet fractions were treated for 30 minutes on ice with increasing

concentration of PK and the reaction was terminated with PMSF. The mebrane was developed with MAb 6H4. As a positive control for PrPc detection, the whole platelet lysate (PLT) and brain homogenate (B) were used.

The previous results demonstrated that platelet PrPc is resistant to cleavage by GPI-specific phospholipase C (PIPLC) (Holada, *et al* 1998). Using either PIPLC treatment of solubilized membranes with western blot detection or PIPLC treatment with subsequent phase separation we confirmed his results. We have proved the presence of GPI-anchor by treatment with hydrofluoric acid (HF), which cleaves the anchor alternatively, resulting in the decrease of the molecular weight of treated protein (Figure 2).

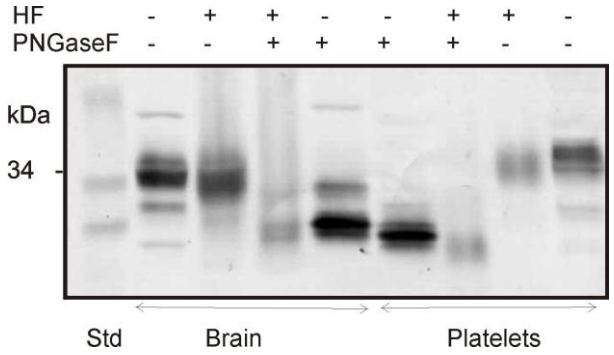

Figure 2. HF treatment proved that platelet PrPc is GPI-anchored protein.
Isolated platelets or 10% (w/w) brain homogenate were treated with 48% aqueous ice cold HF and part of the samples were subsequently deglycosylated with 10 U/ml of PNGase F. The membranes were developed with MAb 6H4.

PrPc is known as a copper binding protein and binding of copper was shown to induce rapid PrPc endocytosis. To test the effect of copper binding on platelet PrPc, we incubated platelets with increasing concentration of copper ions for 30 minutes at room temperature (Figure 3). PrPc expression was measured by flow cytometry using MAbs targeted against different PrPc epitopes. Adding of copper ions caused decrease only in FH11 binding. This MAb is targeted against N-terminal region of PrPc (PrP 46-59), which is in immediate vicinity of copper binding domain located between residues 60-91. The binding of MAbs 3F4 (PrP 109-114) and 6H4 (PrP 144-152) was not influenced.

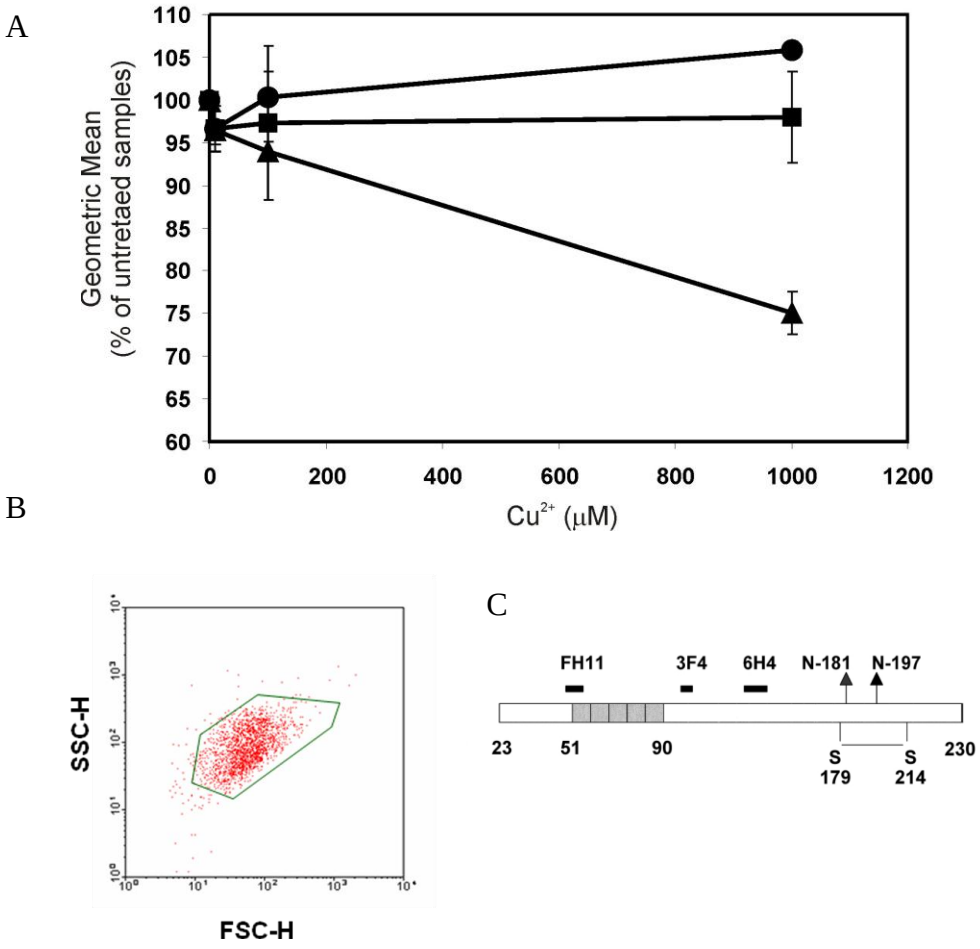

Figure 3. Copper ions decrease binding of anti-PrP MAb targeted against N-terminal region.
(A) Blood platelets were diluted to concentration 30×103/μl and incubated with increasing concentration of copper ions (CuSO4: 0, 10, 100 and 100 μM). Then platelets were labeled with PrP MAbs: FH11 (▲ PrP 46-59), 3F4 (■ PrP 109-114) and 6H4 (● PrP 144-152) for 20 minutes (all diluted 1:100). Labeled platelets were washed and analyzed by a FACScan flow cytometer. The graph represents the change in geometrical mean comparing to non treated samples. (B) The scattergram shows the population of isolated blood platelets. (C) The epitopes of antibodies used.

According to literature, blood platelets express much more PrPc then RBC (Barclay, *et al* 1999, MacGregor, *et al* 2000). To compare PrPc quantity in RBC and platelets we performed western blot analysis of RBC ghosts and several dilutions of platelets (Figure 4). Densitometry analysis of blot developed with 6H4 confirmed that one platelet (PLT) contains approximately 4 times more PrPc than one RBC ghost. This is an innovative insight into the distribution of PrPc in blood.

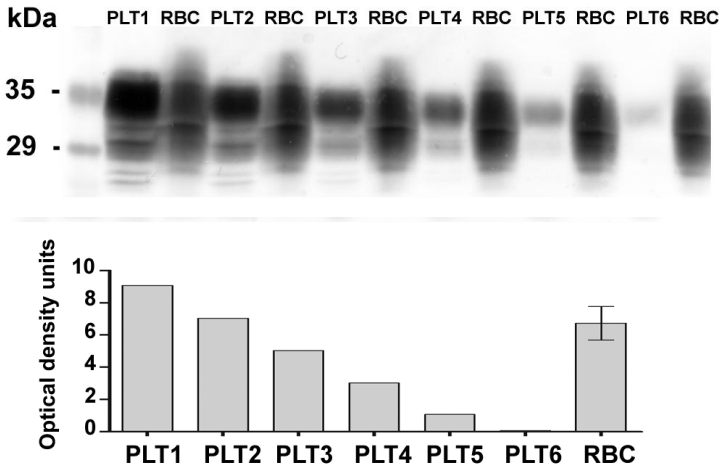

Figure 4. Semiquantitative analysis of platelet and RBC PrPc.
Western blot comparison of PrPc quantity in representative, standardized samples of RBC ghosts ($1x10^{10}$ RBC/ml) and platelets ($5x10^{9}$; $2.5x10^{9}$; $1.25x10^{9}$; $6.25x10^{8}$; $3.13x10^{8}$; $1.56x10^{8}$ PLT/ml). Samples were resolved by 12% SDS PAGE and developed with antibody 6H4. Densitometry of bands on blot demonstrates that RBC contain four times less PrPc then equal number of platelets.

Analysis of PrPc localization by fluorescence microscopy

According to published study about 70% of total PrPc in resting platelets resides inside the α-granules (Holada, *et al* 2006). Our aim was to confirm the intracellular localization of PrPc by fluorescence microscopy. We used two different approaches for preparing of samples. Firstly, platelets were adhered onto slides coated with fibrinogen, which leads to platelet activation with spreading and forming so called skirts. Secondly, platelets were centrifuged onto slides coated with poly-L-lysine, what leads to deposition of resting platelets. α-granular protein CD62P, which is expressed both on the cytoplasmic and granular membranes was used as a control. Its presence on the granules was confirmed by colocalization with α-granular marker thrombospondin. Labeling of resting permeabilized platelets with antibodies against PrPc and CD62P did not show any colocalization of these two proteins. However, activated platelets displayed different pattern. While the signal of PrPc and CD62P was still predominantly separated on the membrane, in the intracellular area both proteins were colocalized (Figure 5). The colocalization appears to occur in the cluster in the central area of the platelets, which belongs to the clustered organelles of activated platelets. The cytoplasmic localization of PrPc was excluded by co-labeling with cytosolic actin.

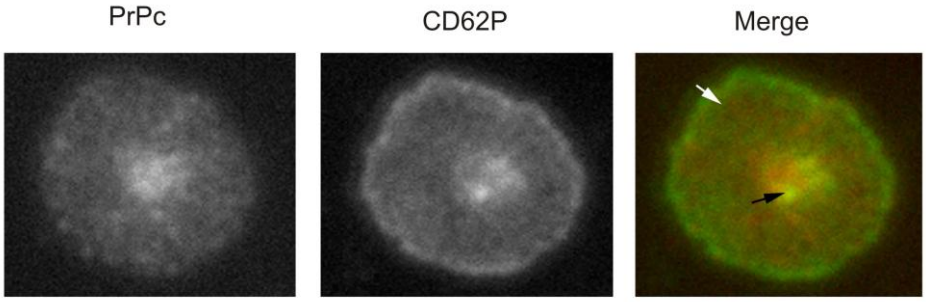

Figure 5. Labeling pattern of PrPc and CD62P in fibrinogen adhered platelets.
Platelets were adhered on fibrinogen coated slides, fixed with 1% paraformaldehyde and permeabilized with methanol. Slides were incubated with the mixture of anti-PrP MAbs 6H4 and 3F4 and anti-CD62P antibody followed by incubation with TRITC and FITC labeled secondary antibodies respectively. Note the separated signal of PrPc and CD62P on the membrane area (white arrows). The co-localized signals (yellow; black arrow) belong to fused organelles. The width of the image is 10 μm. The image is representative of four independent experiments.

PrPc association with lipid rafts

Isolation of lipid rafts was attempted with three different detergents: Triton X-100 and NP-40 at 4°C and Brij 98 at 37°C. All detergents led to flotation of PrPc and platelet raft markers. (GPI-anchored protein CD59 and transmembrane protein CD36) (Dorahy, *et al* 1996) to the interface between the 5% and 30% sucrose (Figure 6). The amount of PrPc recovered in rafts was relatively low, regardless of the detergent used for platelet solubilization: approximately 5–10% of total PrPc was recovered in the case of Triton X-100 solubilization. The amount of raft associated PrPc was not influenced by platelet activation.

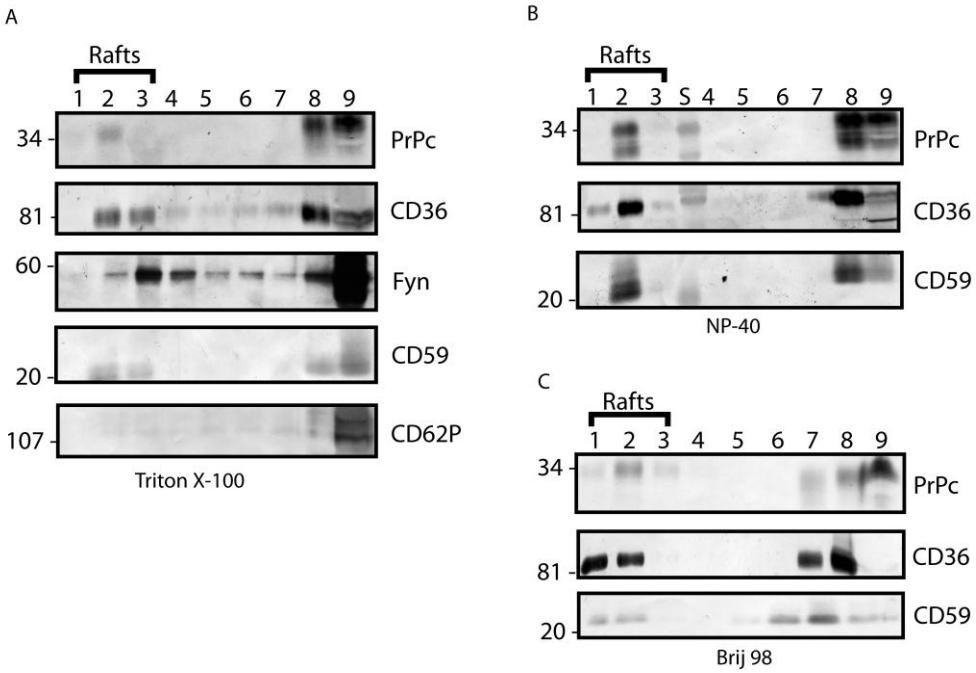

Figure 6. PrPc in blood platelets associates with lipid rafts.
Resting platelets were lysed in 1% Triton X-100 or 1% NP-40 at 4°C (A and B) or in 1% Brij 98 at 37°C (C) and then subjected to flotation assay in sucrose gradient. The proteins from nine fractions of sucrose gradient were analyzed by SDS PAGE, blotted and developed with MAb 6H4 against PrPc, anti-CD59, anti-CD36, anti-Fyn and anti-CD62P. 1 – top of the gradient, 9 – bottom of the gradient. The blots are representative of three to five independent experiments.

Lipid rafts strongly depend on the content of cholesterol, which was confirmed by saponin treatment as well as by methyl-β-cyclodextrin (MβCD) treatment. Here we present the treatment with the later (Figure 7). The identity of rafts was confirmed also by the presence of protein kinase Fyn (Ezumi, *et al* 2002). Dodecyl maltoside is detergent known to solubilize the rafts even at 4°C (Brdicka, *et al* 2000), so to further confirm the PrPc interaction with rafts, we lysed platelets with maltoside resulting in destroying of rafts and thus nor PrPc neither Fyn could be detected in rafts fractions.

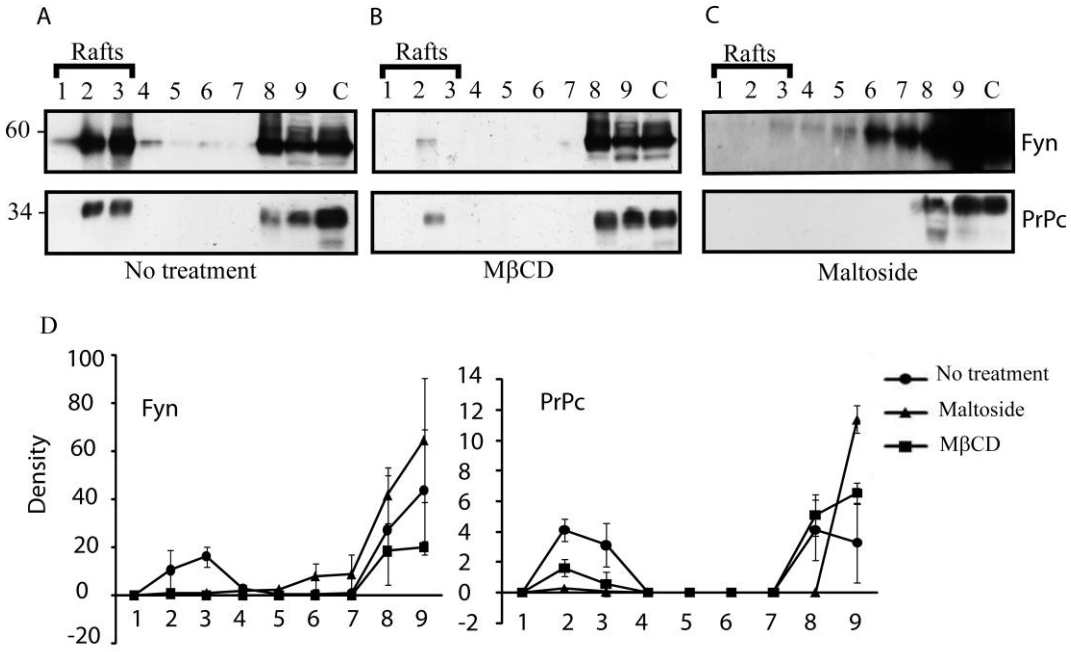

Figure 7. Platelet lipid rafts containing PrPc are sensitive to cholesterol depletion with MβCD, and can be solubilized with dodecyl maltoside.
(A) Lipid rafts were isolated from the platelet, lysed with 1% Triton X-100 at 4°C. (B) In order to deplete cholesterol, platelets were incubated with 20 mM MβCD at 37°C, extensively washed, and then subjected to lysis with Triton X-100 at 4°C. (C) Blood platelets were lysed in 1% n-dodecyl-ß-D-maltoside leading to the solubilization of rafts. In all three cases lysed platelets were subjected to flotation assay. The proteins from nine subsequent fractions of the sucrose gradient were analyzed by SDS PAGE, blotted, and developed with MAb 6H4 against PrPc and anti-Fyn. Insoluble pellets on the bottom of the tubes were not included in the preparation of electrophoretic samples. Lines C show the control platelet lysate. (D) Bands of 9 fractions were analyzed using densitometry. Data shown are mean optical density units ± SD, n=3.

As we have found that the majority of platelet PrPc is associated with platelet cytoskeleton, we performed the flotation assay with actin depolymerization of whole solubilized resting and activated platelets. Upon actin depolymerization, PrPc and control proteins CD36 and CD59 were markedly upregulated in raft fractions (Figure 8). We have also shown that cytoskeleton of activated platelets contains high amount of raft associated PrPc.

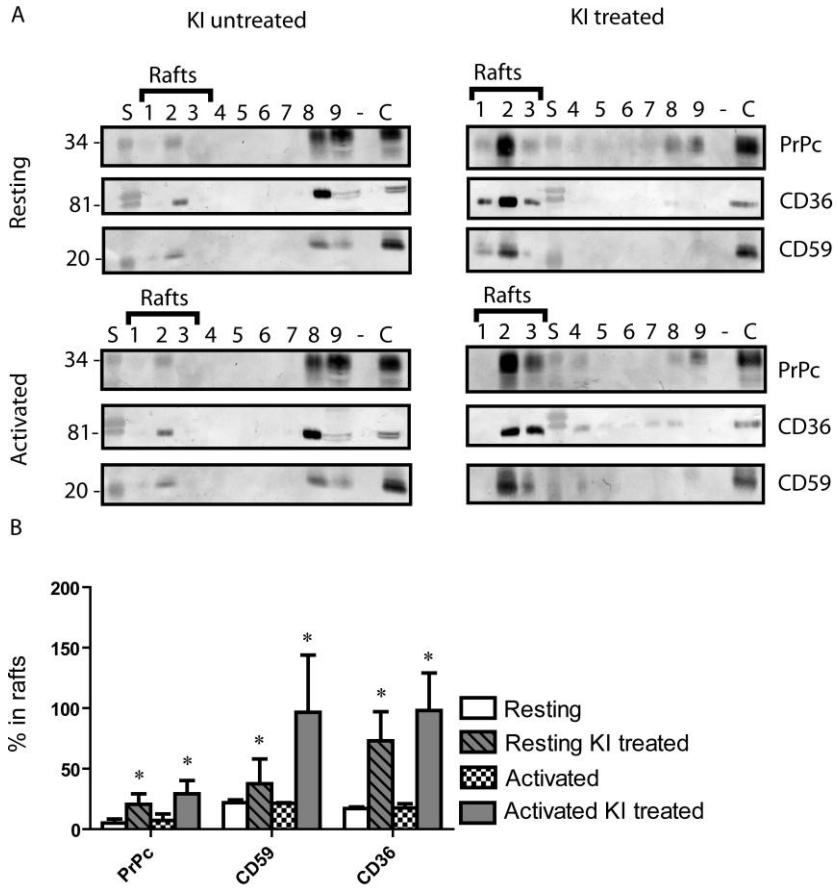

Figure 8. The majority of PrPc containing lipid rafts associates with platelet cytoskeleton.

Rafts were isolated by standard procedure using Triton X-100 at 4°C (A, left panels).) In order to depolymerize actin filaments resting or activated platelets were lysed with 1% Triton X-100 followed by treatment with 0.6 M KI for 30 min at 4°C (A, right panels). The increase of raft associated proteins upon actin depolymerization is shown in bar graph as densitometry analysis of the representative blots (B). The 100% here is the band density of appropriate protein in whole platelet lysate. Proteins from nine subsequent fractions were analyzed by SDS PAGE, blotted and developed with MAb 6H4 against PrPc, anti-CD59 and anti-CD36. 1 – top of the gradient, 9 – bottom of the gradient, Std – molecular weight standard, C – whole platelet lysate. The blots are representatives of four independent experiments.

Based on our observations, we propose model of platelet PrPc localization in different membrane domains (Figure 9).

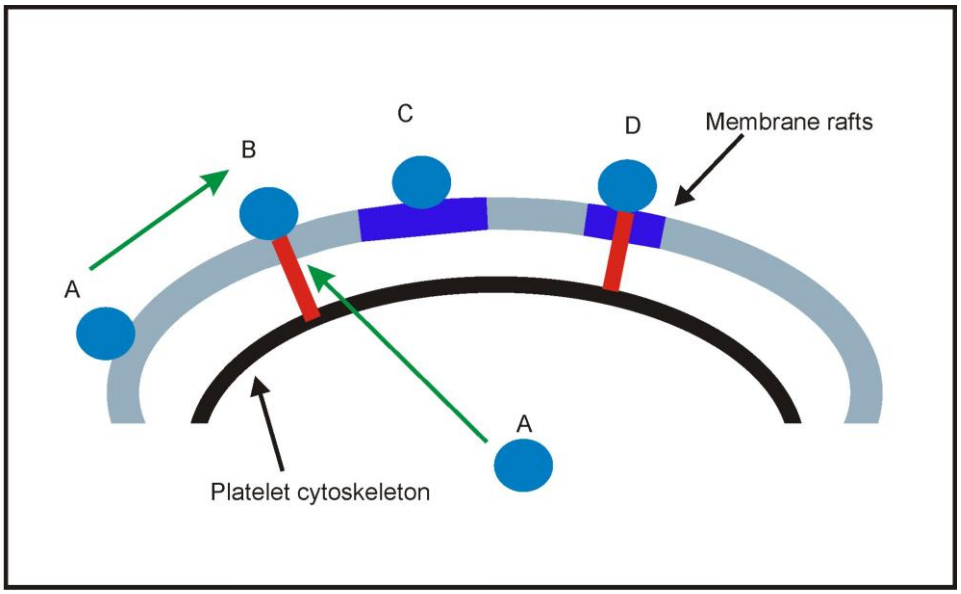

Figure 9. Model of different pools of PrPc.
We suggest that platelet PrPc consists of four pools (PrPc is shown as a blue circle). A – A pool which is either intracellular or membrane in resting platelets and upon their activation associates with cytoskeleton (platelet activation is illustrated by green arrow). B – PrPc associated with cytoskeleton. The association with cytoskeleton illustrated by red rectangle does not reflect the real character of the interaction, which is not known. C – PrPc associated with lipid rafts. D – PrPc associated with both lipid rafts and cytoskeleton. This pool is not possible to identify by flotation assay without actin depolymerization. Pools C and D are not influenced by platelet activation.

The study on PrPc involvement in platelet physiological function

In order to follow the effect of anti-PrP antibodies on platelet signaling events, we stimulated platelets with MAbs 3F4 and SAF32 and observed the tyrosine phosphorylation. However, no significant phosphorylation was observed comparing to control samples, not even when PrPc molecules were cross-linked. We have also tested the possible effect of anti-PrP antibodies on platelet aggregation triggered either by collagen or by ADP and epinephrine, but we did not achieve any effect.

Discussion

It is well known that PrPc serves as a template for pathogenic PrPsc alias prion which amplification and deposition in CNS is a hallmark of TSEs. As for vCJD the presence of infectivity in peripheral lymphoid tissues led to the fear that this disease might be transmitted by blood transfusion. Unfortunately, it was confirmed by four cases of vCJD transmission by non-leucodepleted red blood cells (Llewelyn, *et al* 2004, Peden, *et al* 2004). These reports have highlighted the importance of knowledge of infectivity distribution in different blood components. Since PrPc is crucial for infectivity accumulation, its expression on blood components is of importance. The role of platelet PrPc is not well understood yet, especially from the point of view of its physiological function and its involvement in TSE pathophysiology. Quite recently it was shown that platelets are able to carry the infectivity.

In the current thesis the glycoprotein pattern of platelet PrPc was very similar to that of brain PrPc, however one must be careful in glycoprofile evaluating with regard to complexity of antibodies as well as the sample source choice. PrPc glycosylation might influence its conformation and stability. It is not clear whether similarly glycosylated prions might preferentially interact with each other and undergo conformational change. As platelet PrPc was shown to be similarly glycosylated comparing to brain PrPc, we can hypothesize that it might be less resistant to conformational change induced by inoculum derived from brain tissue.

Interestingly, contrary to neuronal PrPc, platelet PrPc was previously shown to be resistant to cleavage with PIPLC (Holada, *et al* 1998). We have confirmed the presence of GPI-anchor, however, the cleavage site for enzyme PIPLC is likely to be modificated and thus inaccessible. The ability to bind copper ions, which is known for neuronal PrPc, was demonstrated for platelet PrPc as well.

We have followed the localization of PrPc using fluorescence microscopy. Interestingly, the signals of PrPc and CD62P on plasma membrane were always separated, with the PrPc signal spreading evenly on the platelet surface in the form of small spots. The pattern of PrPc fluorescence suggested that the protein might concentrate in membrane domains such as lipid rafts, similar to other GPI-linked proteins. Indeed, we demonstrated the association of PrPc with platelet lipid rafts using differing detergents and experimental conditions (Brouckova and Holada 2009). In contrast to that, CD62P was not found in rafts. Oddly, only a relatively small portion of PrPc was detected in the raft fractions of resting platelets. Interestingly, we have uncovered the big pool of raft PrPc linked to platelet cytoskeleton. The size of this pool

is relatively stable regardless of platelet activation status, although platelet activation leads to its increased association with the actin cytoskeleton. In addition, smaller pools of soluble PrPc and cytoskeleton-associated non-raft PrPc also seem to be present in platelets. Since raft associated PrPc might serve as a receptor for PrPsc or as a substrate for PrPsc conversion, our findings are interesting from the point of prion infectivity distribution in blood. Published studies of the infectivity distribution in rodents do not support platelets as a source of blood infectivity (Holada, *et al* 2002). However, we must bear in mind that rodent platelets in contrast to human platelets do not express significant level of PrPc (Holada and Vostal 2000). In addition, human platelets were recently shown to support in vitro PrPsc propagation by protein misfolding cyclic amplification (Jones, *et al* 2007). Blood platelets were also shown to have ability to transfer the infectivity when transfused from CWD deer to healthy deer (Mathiason, *et al* 2010). Protein association with either lipid rafts or cytoskeleton is supposed to be critical for platelet signaling, as it allows signaling molecules to interact. However, our attempt to find PrPc involvement in platelet signaling events was unsuccessful.

Based on the published data and on our observation, it is highly plausible that platelet PrPc might carry the infectivity in blood. As we have shown, red blood cells possess about 1,5-2 times more PrPc then platelets and thus are the possible source of infectivity as well (Panigaj, *et al* 2010).

Conclusion

The present work was focused on detailed study of blood platelet PrPc, its localization and physiological function. Our results are of importance for next study of platelet PrPc physiological function as well as for its role in the pathogenesis and transmission of prion diseases. The results might be summarized as follows:

1. Platelet PrPc was shown to be similarly glycosylated as PrPc expressed in brain tissue, however the glycoprofiles partially depended on antibody used.
2. The sensitivity of PrPc to digestion by proteinase K is similar for platelets, RBC and brain.
3. We have confirmed that platelet PrPc is GPI-anchored protein, however, contrary to brain PrPc it is PIPLC resistant.
4. Conformation of platelet PrPc was proved to be influenced by copper ions as shown by decreased binding of MAb FH11, which is targeted against octapeptide repeat region.
5. Comparing to one RBC, one platelet express more PrPc molecules, but when total cell number taken into account RBC possess considerable pool of blood cell associated PrPc.
6. Co-labeling of PrPc and α-granular marker CD62P proved the localization of platelet PrPc in α-granules. Although colocalized in intracellular compartment, on the cytoplasmic membrane these proteins formed isolated signals.
7. Our study is first to demonstrate the association of PrPc with lipid rafts in blood platelets and also first to demonstrate the association of PrPc with platelet cytoskeleton.
8. Our study demonstrates the existence of different forms of PrPc in platelet membranes (raft associated and non-raft PrPc; free, membrane skeleton or cytoskeleton associated).
9. Most of platelet PrPc is incorporated in rafts and simultaneously binds to platelet cytoskeleton.
10. Our study demonstrates that platelet activation changes the proportion of PrPc associated with cytoskeleton but not with membrane rafts.
11. In this study the function of PrPc in platelet signaling processes was not proved.

Based on our observations as well as previous studies, the model of PrPc localization in blood platelets is presented in Figure 10.

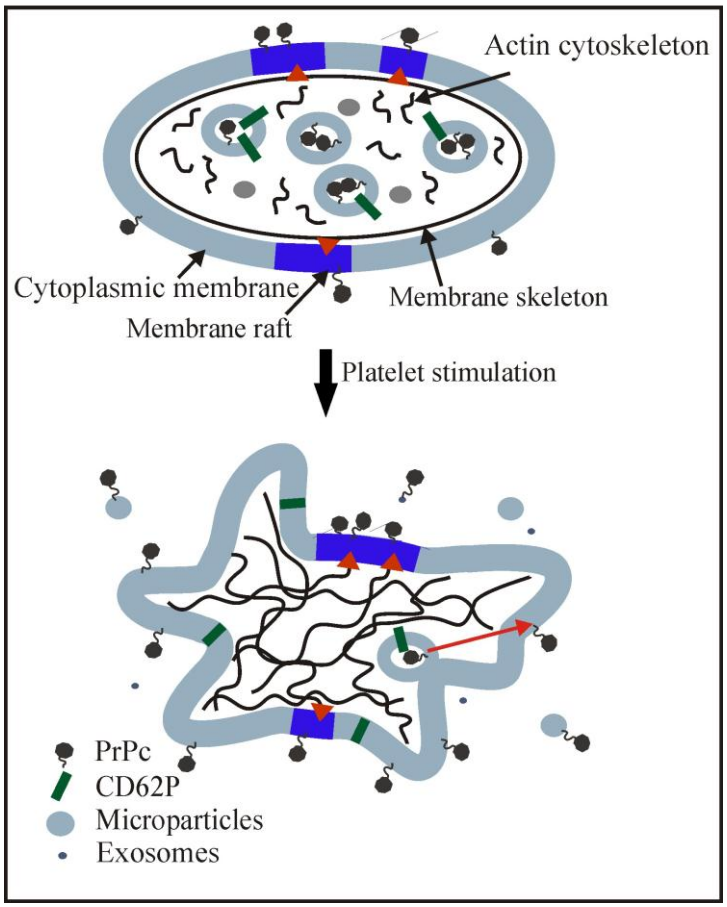

Figure 10. Model of PrPc localization in platelets.
The majority of PrPc is localized in the intracellular compartment of resting blood platelets, namely in α-granules. Upon platelet activation PrPc up regulates on the platelet surface due to organelles fusion with cytoplasmic membrane (illustrated by red arrow) and part of PrPc is released on both microvesicles and exosomes. Platelet activation is accompanied by assembly of actin cytoskeleton with membrane skeleton that coats the underside of the cytoplasmic membrane. This leads to change of platelet shape. Platelet PrPc is enriched within lipid rafts which in resting platelets associate with membrane skeleton. The association between lipid rafts and cytoskeleton is illustrated by red triangles. Platelet activation makes lipid rafts to cluster. It does not affect the amount of raft associated PrPc but due to cytoskeleton reorganization it leads to interaction of PrPc containing rafts with cytoskeleton assembled of membrane skeleton and actin cytoskeleton. α- granular marker CD62P is also up regulated on cytoplasmic membrane upon platelet activation with no protein localized in lipid rafts. The proportions of individual objects are just for illustration.

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List of publications

Publications related to the thesis:

Panigaj M., **Broučková A.**, Glierová H., Dvořáková E., Šimák J., Vostál J. G., Holada K.: Underestimation of the expression of cellular prion protein on human red blood cells. Transfusion **2010**, Nov 8. *IF 2,982*

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