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Ing. Adéla Broučková

The characterization of blood platelet cellular prion protein

(Charakterizace buněčného prionového proteinu krevních destiček)

PhD. Thesis
Supervisor: Ing. Karel Holada, PhD.

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Klíčová slova: *buněčný prionový protein (cellular prion protein), krevní destičky (blood platelets), lipidové rafty (lipid rafts), krevní transfúze (blood transfusion)*

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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.

Contents

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
CD	cluster of differentiation
CD62P	P-selectin
CJD	Creutzfelt-Jakob disease
CNS	central nervous system
CWD	Chronic wasting disease
DELFIA	dissociation-enhanced fluoroimmunoassay
DMSO	dimethyl sulfoxide
DRMs	detergent resistant membranes
EDRF	erythroid differentiation-related factor
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPI	epinephrine
FDC	follicular dendritic cells
FITC	fluorescein isothiocyanate
Fyn	Fyn proto-oncogene
GALT	gut associated lymphoid tissue
GAM	goat anti-mouse
GP	glycoprotein
GPI	glycosylphosphatidylinisotol
GSS	Gerstmann-Straussler-Schienker syndrome
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IgG	immunoglobulin G
LB	lysis buffer
Lck	lymphocyte-specific protein tyrosine kinase
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MβCD	methyl-β-cyclodextrin

MAb	monoclonal antibody
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFA	paraformaldehyde
PFA	paraformaldehyde
PGE1	prostaglandin E1
PIPLC	phosphatidylinositol-specific phospholipase C
PK	proteinase K
PLCγ2	phospholipase C, gamma 2
PLT	platelets
PM	promethazine
PMCA	protein misfolding cyclic amplification
PMSF	phenylmethylsulfonyl fluoride
PNGase F	N-glycosidase F
PRNP	prion protein gene
PRP	platelet rich plasma
PrP	prion protein
PrPc	cellular prion protein
PrPsc	scrapie prion protein
RBC	red blood cells
sCJD	sporadic Creutzfelt-Jakob disease
SD	standard deviation
SDS	sodium dodecyl sulfate
Src	v-src sarcoma viral oncogene
THB	tyrode`s/HEPES buffer
TRAP	thrombin receptor activating peptide
TRIS	tris(hydroxymethyl)aminomethane
TRITC	tetramethyl rhodamine
TSE	transmissible spongiform encephalopathy
TSP	thrombospondin
vCJD	variant Creutzfelt-Jakob disease
WBC	white blood cell

Introduction

Transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies (TSE) are a group of fatal neurodegenerative diseases which affect both human and animals (Table 1.). 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 fibrils. 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 Prnp mutation (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). In 1996 a new variant form of CJD (vCJD) emerged in the United Kingdom (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).

Table 1. The survey of transmissible spongiform encephalopathies which affect both human and animals.

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 first clinical case of BSE was reported in 1985 and the disease peaked in 1992. Epidemiological studies suggested that the source of infection involved feed contaminated with the meat-and-bone meal as a source of protein intake. The demographic and clinical features of vCJD and the temporal relation with BSE epidemic point to a cause-effect relationship between those two prion diseases (Figure I.). Also several experimental observations point out correlation between BSE and vCJD. For example, transgenic mice expressing the bovine prion protein were able to propagate BSE prions and there was no species barrier for transmission from cattle to mice. Moreover, these mice also propagated prions taken from patients who had the vCJD. Mice propagating either bovine or human prions had identical symptoms and incubation period, whereas mice propagating scrapie prion differed.

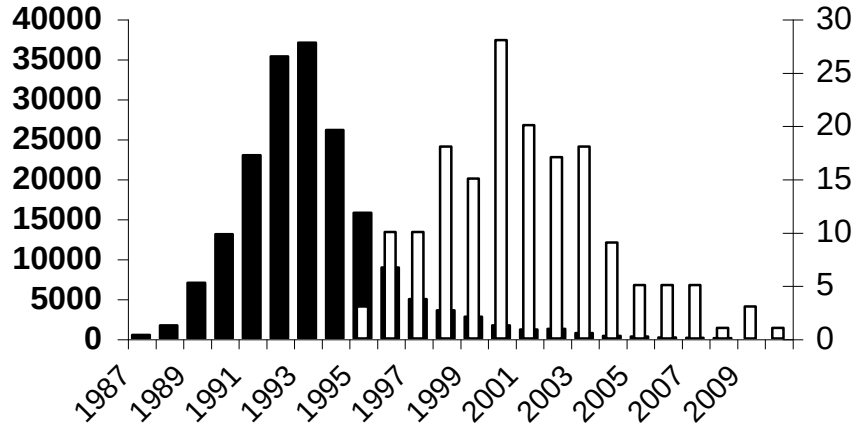


Figure I. The number of BSE (■) and vCJD (□) in Great Britain.
The number of BSE are reported in thousands, number of vCJD in units. The data are adapted from World Organization for Animal Health (OIE) and Department of Health UK. The total number of vCJD cases in Great Britain as reffered in May 2011 was 171 comparing to 1228 of sCJD cases recorded during the same time period. The number of vCJD deaths, which were neuropathologicaly confirmed, is 119. Remaining 52 were not confirmed.

In contrast with typical cases of sCJD, vCJD affects young patients (with the average age of 27 years compared with the average age of sCJD which is 60) and has longer incubation time (14 months compared with 4.5 months for sCJD). All TSEs are

characterized by the spongiform appearance of brain, due to vacuolation of neurons and surrounding neuropil (Figure II.). However, microscopic examination of the brain tissue of vCJD patients revealed a different pattern from that seen in the sporadic form of the disease - so called florid plaques. Florid plaques are deposits of abnormal proteins surrounded by vacuoles and they are present in large numbers in vCJD. vCJD also differs from sCJD in the distribution of prions in the tissues, notably in the spleen, appendix, tonsils and lymph nodes. This was confirmed in the study which used primate model to compare prion distribution of BSE, vCJD, sCJD and iCJD prion strains (Herzog, *et al* 2005). Further, BSE and vCJD had very similar distribution in peripheral tissues, while sCJD and iCJD prions were predominantly found in the CNS.

It is interesting that there is obviously no overwhelming species barrier between human and cattle regarding transmission of prions. Interestingly, prions that are isolated from one species are often less infectious to other species, as evident by the longer incubation times and reduced attack rates in these other species. For example, a prion species barrier occurs between mice and hamsters. On the other hand, transmission of prions from cattle to human is an example of jumping this barrier (Bruce, Will et al. 1997; Hill, Desbruslais et al. 1997). This phenomenon is connected to the existence of prion strains, which are TSE isolates that, after inoculation into distinct hosts, cause disease with consistent characteristics, such as incubation period, distinct patterns of prions distribution and spongiosis and relative severity of the spongiform changes in the brain. Strains may be also associated to biochemical hallmarks such as electrophoretic mobility and glycoform pattern of PrPsc on western blots. Thus it is assumed that BSE prion strain is highly identical with vCJD prions.

In CJD patients polymorphism in the human PRNP gene at codon 129, encoding either valin or methionin, was proved to correlate with age at disease onset or the duration of illness. Thus it was proposed, that methionin homozygotes are more susceptible to disease. So far, all clinical cases of vCJD, thought to result from the BSE prion agent, have shown methionine-methionine homozygosity at the M129V polymorphism of the PRNP gene. However, recent study reported a case of a patient, who was a heterozygote at codon 129 of PRNP, yet succumb to vCJD (Peden, *et al* 2004).

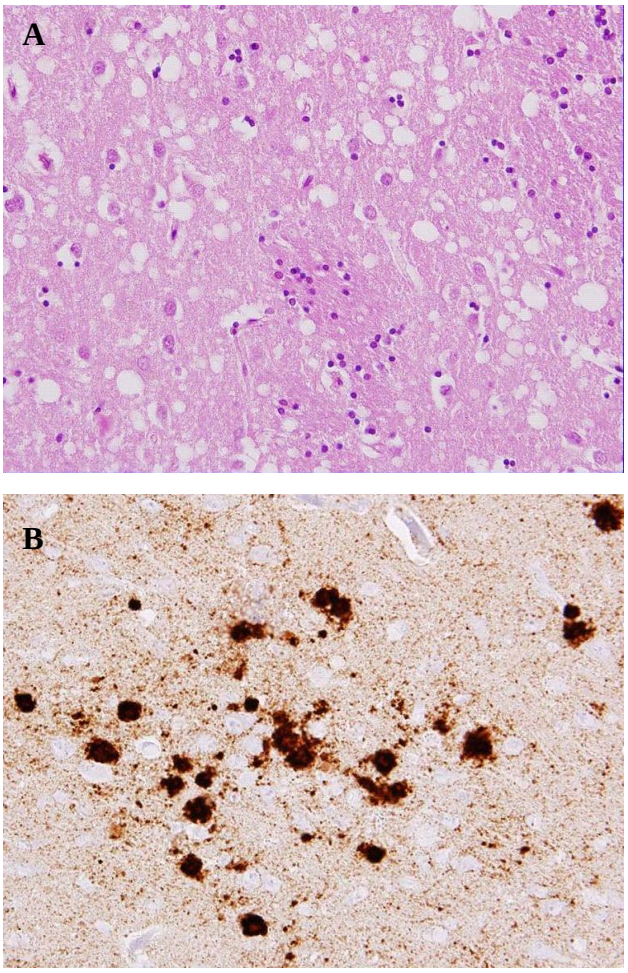


Figure II. Neuropathological changes in the brain of GSS patient. (A) Histological analysis of the brain tissue (striatum) stained with hematoxylin and eosin revealed the spongiform dystrophy characterized by numerous vacuoles. (B) Immunostaining of the cortex shows typical PrPsc deposition pattern. Original magnification of both images - 400×. Published with the kind agreement of MUDr. Radoslav Matěj, PhD., Department of Pathology and National Reference Laboratory TSE-CJN, Thomayer University Hospital, Prague.

Prions and pathogenesis of TSEs

To date, the clearest phenotype of PrP knockout mice is their resistance to infection with prions (Bueler, Aguzzi et al. 1993; Sailer, Bueler et al. 1994). This solidifies the central tenet of prion theory: the requirement of host protein for prion replication. The host protein is referred to as cellular prion protein (PrPc). According to the protein only hypothesis (Prusiner 1998), an interaction between the pathogenic prion and endogenous

PrPc is sufficient to cause the template-driven formation of more prions, where very the PrPc serves as a template.

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). In the previous text the term "prion" (proteinaceous infectious particle) is used instead of PrPsc. Prion is more general and includes both PrPsc and its possible partner in crime.

Initially, the infectious agent was thought to be a slow virus (Cho 1976), which was suggested to consist of an agent-specific nucleic acid enveloped in a host specific protein. This theory might explain the lack of an immune response by the host as well as strain variation (Kimberlin 1982). However, nowadays the modified protein PrPsc is considered to be a major component of infectivity. Prions have unconventional properties, such as resistance to UV irradiation, exposure to high pressures or temperatures and formaldehyde treatment. Further, the secondary structure of PrPsc will be discussed and as for posttranslational modifications, any are known.

As is mentioned above, PrPc serves as a template for PrPsc. However, the exact mechanism of conversion of PrPc to PrPsc is not known and it is unclear whether some third interaction partner (above mentioned as a partner in crime) is needed. Study focused on the role of different chaperones (e.g. GroEL and Hsp104) proved their ability to promote the conversion of PrPc to PrPsc but only in the presence of PrPsc (DebBurman, *et al* 1997). Some evidence suggests that nucleic acids might play role in the conversion as the presence of either RNA or DNA promoted the conversion of PrPc to PrPsc *in vitro* (Cordeiro, Machado et al. 2001; Gomes, Millen et al. 2008). Interestingly, PrPsc might be amplificated *in vitro* by so called protein misfolding cyclic amplification (PMCA, the method is explained on page 17). The most common source of PrPc in this assay is normal brain homogenate, which might also carry the interaction partners. The place of conversion in the cell also remains undiscovered; it may be either inside the cell, in the course of PrP recycling or on the cytoplasmic membrane. The conversion of PrPc to PrPsc probably occurs in lipid rafts as is discussed further (page 29).

In the case of acquired forms of TSEs such as BSE or vCJD the infectious agent must first enter the body. The exact mechanism is not known. It has been proposed that prions after crossing intestinal epithelium are adsorbed through Payers patches and enteric nervous system serves as an entry into the central nervous system (CNS) (Chiocchetti, *et al* 2008). Alternative route to CNS may include cells of immune system. The significance of

B lymphocytes in TSE pathogenesis was shown based on the transgenic mice studies. However it is plausible that B lymphocytes do not deliver prions themselves, but provide signals for the maturation and maintenance of other cell types needed for prion transmission (Kosco-Vilbois, *et al* 1997). Follicular dendritic cells (FDC) were shown to be the plausible source of infectivity since prions were shown to accumulate in FDC in the gut associated lymphoid tissue (GALT). This was confirmed in experiments using transgenic mice deficient in GALT components, in which prion transmission was blocked (Mabbott, *et al* 2001). The connection between complement receptors on FDC and the spread of prions was suggested, as mice genetically engineered to lack complement factors (Klein, *et al* 2001), as well as mice depleted for the C3 complement component (Mabbott and Bruce 2001), exhibited enhanced resistance to peripheral prion inoculation. An alternative route of prions to CNS may involve blood constituents, however it remains unclear how and whether prions cross the blood-brain barrier.

Another mystery in TSE pathogenesis is how PrPsc or other abnormal forms of PrP cause CNS pathology. One of the theories is that PrPsc possess novel toxic properties, for example may block axonal transport, interfere with synaptic function, or trigger apoptotic pathways. An alternative hypothesis postulates that the cause of neurodegeneration in TSEs is the lost of PrPc function upon its conversion to PrPsc. A third possible hypothesis involves a subversion of the normal neuroprotective function of PrPc, where upon interaction with PrPsc is PrPc converted to transducer of neurotoxic signals. Recently, it was suggested that PrPc is a high-affinity cell-surface receptor for soluble synthetic Aβ oligomers on neurons and thus may play a role in the pathophysiological process of Alzheimer`s disease as well (Lauren, *et al* 2009)

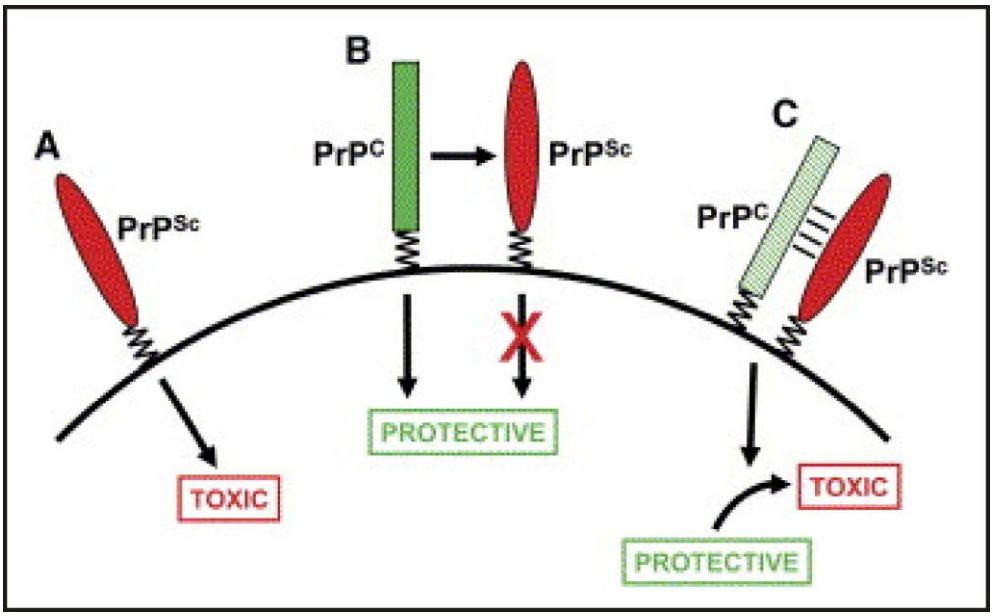


Figure III. Models for the cellular toxicity of PrPsc.
(A) Toxic gain-of-function mechanism. PrPsc possesses a novel neurotoxic activity that is independent of PrPc. (B) Loss-of-function mechanism. Normal PrPc possesses a neuroprotective function that is lost upon conversion to PrPsc. (C) Subversion-of-function mechanism. The normal, neuroprotective activity of PrPc is subverted by binding to PrPsc. Cross-hatching of the rectangle representing PrPC indicates a change in its signaling properties such that a neurotoxic rather than a neuroprotective signal is delivered. Adopted from (Harris and True 2006).

Diagnosis and treatment of TSEs

TSEs are fatal diseases and neither treatment nor pre-mortem screening diagnostics tests are available. Current diagnosis of human TSEs is based mainly on clinical examination, i.e. neurological symptoms, electroencephalography, magnetic resonance imaging of the brain and spinal fluid tests. Definitive diagnosis can be only made post-mortem by brain histological analysis (Ingrosso, *et al* 2002, Kordek 2000). Due to BSE epidemic, the system of evaluated and validated biochemical tests for cattle brain samples was adopted. An accurate method is histology and immunohistochemistry post-mortem analysis of the brain tissue, however it is time consuming, labor intensive and cannot be carried out on a large scale (Heim and Wilesmith 2000). Other approved tests for post-mortem diagnosis of PrPsc in cattle brain include western blotting, different settings of ELISA and conformational dependent immunoassay based on different binding of antibody

to native and denatured PrP (Safar, *et al* 1998). However, as for the brain tissue all these methods are available only for post mortem diagnosis.

Concerning the emergence of vCJD which may be transmitted by blood, the need for a pre-symptomatic diagnosis was emphasized. Routine diagnosis is limited to the examination of external tissues or fluids. The presence of PrPsc in tonsils and appendix indicates that a lymphoid tissue biopsy might be useful for diagnosing pre-symptomatic individuals (Hilton, *et al* 1998, Schreuder, *et al* 1996). Presence of PrPsc in periphery tissue was also reported for patients suffering from sCJD, for example in skeletal muscle, spleen samples and olfactory epithelium (Glatzel, *et al* 2003, Zanusso, *et al* 2003). However, these kinds of tissues still would need an invasive type of examination. Potentialities for non-invasive examination are urine or blood.

Detection of PrPsc in urine was reported in humans as well as in infected animals with TSE (Shaked, *et al* 2001). Shiga et al. reported the detection of PrPsc in the urine from patients with vCJD, however others have concluded, that this finding was related to bacterial contamination (Furukawa, *et al* 2004, Shiga, *et al* 2003). Dabaghian et al. detected in the urine proteinase K resistant proteins and suggested that they are formed by interaction between PrP and immunoglobulin proteins (Dabaghian, *et al* 2008).

However blood remains the most suitable material for screening tests. Many methods have been tested to detect prions in blood; however none of them have sufficient sensitivity and specificity. Almost all of these methods rely on the detection of PrPsc with monoclonal antibodies using a variety of formats, including western blotting, enzyme-linked immunoassays and capillary electrophoresis (Schmerr, *et al* 1994). The enthusiasm about the latter was suppressed by two studies showing the inaptitude of this method for detection of prions both in brain tissue and in blood of vCJD patients (Cervenakova, *et al* 2003, Lourenco, *et al* 2006). The theoretical limit of detection of immunoassays is set by the affinity of antibodies for their respective antigen. Moreover, one of the steps of immunoassays is degradation of the sample with proteinase K (PK) which leads to complete digestion of PrPc. As PrPsc is partially resistant, the cleavage with PK generates a large PK-resistant C-terminal core fragment termed PrP27–30 that in human prion diseases has a gel mobility of 19–21 kDa for the unglycosylated form, which might be detected on western blots.

In case of partial sensitivity of PrPsc to this treatment the amount of the protein to be measured would be yet more limited. The fact that PrPsc levels in blood are diminutive significantly diminishes applicability of these immunoassays.

To avoid this bottle neck, PrPsc conformational specific antibodies are being developed. Korth and colleagues produced antibody 15B3, which specifically recognizes bovine, murine and human PrPsc but not PrPc, however its utilization failed due to its low affinity (Korth, *et al* 1997). By immunization of animals with peptides containing tyrosine-tyrosine-arginine repeat motif several PrPsc specific antibodies were produced (Paramithiotis, *et al* 2003). Another reported PrPsc specific antibody is V5B2 recognizing PrPsc from patients affected by CJD (Curin Serbec, *et al* 2004). Recently, a monoclonal antibody 6H10 was produced, which was also shown to neutralize prion infectivity by binding to its C-terminus (Horiuchi, *et al* 2009). A quite controversial phenomenon is an anti-DNA antibody OCD4 and DNA-binding protein g5p both recognizing PrPsc, since according the authors this binding suggest that PrPsc forms a complex with nucleic acid (Zou, *et al* 2004).

There are some methods which are promising to overcome the problem of very low quantity of PrPsc in the sample, one of them being protein misfolding cyclic amplification (PMCA) (Saa, *et al* 2005). PMCA is *in vitro* technique that mimics the replication of prions *in vivo* (Saborio, *et al* 2001). Samples are subjected to cycles of incubation and sonication in the presence of large amount of normal PrPc. The procedure specifically amplifies the prions, because in the absence of abnormal protein, PrPc is not converted. Then, PrPsc might be efficiently detected by western blot. As a source of PrPc homogenized normal brain was commonly used. Recently, sulfated dextran was used to enhance the amplification of PrPsc by PMCA (Murayama, *et al* 2010). Furthermore, blood platelets were shown to serve as an usable substrate for amplifying of vCJD prions showing that platelet PrPc can be transformed to pathological prion (Jones, *et al* 2007). Using PMCA, prions were detected in blood of scrapie infected sheep (Thorne and Terry 2008), in hamster blood (Castilla, *et al* 2005), in mice blood (Tattum, *et al* 2010) and also allows pre-symptomatic detection of PrPsc which was performed on brain tissues (Soto, *et al* 2005). However, this method has not been optimized for PrPsc detection in human blood and present protocols are not sensitive and specific enough for clinical use. Moreover, Deleault`s study showed that PrPsc can be generated de novo and stochastically by PMCA

in the absence of pre-existing prions which raises the concerns about the specificity of this method (Deleault, *et al* 2007).

Another strategy to amplify PrPsc is the cell infectivity assay (Klohn, *et al* 2003). In this assay PrPsc susceptible N2A cells are exposed to infectious material, grown and subsequently tested for the presence of PK resistant form of PrP. However the limitation of this assay is that no cell lines are available to amplify cattle prions and there is only one cell line available to amplify CJD prions - neuroblastoma SHSY-5Y, but with non-reproducible results (Nuvolone, *et al* 2009).

The promising approach in TSE pre-clinical diagnosis might be some surrogate markers in blood. One of the first adept reported was an erythroid differentiation-related factor (EDRF) (other name is alpha-Hemoglobin stabilizing protein, AHSP), which was down regulated in blood samples from clinically and subclinically prion-infected animals (Miele, *et al* 2001). However, when EDRF mRNA and protein levels were quantified in blood from healthy individuals and from patients with CJD, no consistent differences were found (Appleford, *et al* 2008).

Prion diseases do not elicit any immunological response what complicates generating of prion vaccines. It was shown that host organisms are tolerant to both PrPc and PrPsc derived from that species and, furthermore, that this state of tolerance is not broken either by scrapie infection or by immunization with scrapie prions injected in adjuvant (Kascsak, *et al* 1987). However highly potent monoclonal antibodies have been raised in mice in which the prion protein gene has been deleted by gene targeting, some of them have been shown to treat prion infected neuroblastoma N2A cells (Enari, *et al* 2001). The in vivo complication is impermeability of blood-brain barrier for such antibodies. Different study proved prolonged survival times in scrapie infected mice, which were actively immunized with prion protein derived peptide (PrP 105-125) (Schwarz, *et al* 2003).

To date, several compounds have been described which decrease the PrPsc concentration in different scrapie-infected cell lines or prolong the incubation period in animal models. They belong to different classes, including sulphated polyanions such as dextran sulphate (Farquhar and Dickinson 1986, Kimberlin and Walker 1986), amphotericin B derivatives (Pocchiari, *et al* 1987), tetracyclic compounds (Forloni, *et al* 2002), Congo red (Caughey, *et al* 1993), tetrapyrroles (Caughey, *et al* 1998), branched polyamines (Supattapone, *et al* 1999), and ß-sheet breakers derived from PrP peptides (Soto, *et al* 2000). Nevertheless, none of them was effective when given around the time of

clinical phase. The promising compounds are quinacrine and chlorpromazine partly because of their ability to cross blood-brain barrier (Dohgu, *et al* 2004). Most of these compounds were tested in cell culture model and results differed upon type of cells, but testing *in vivo* did not bring any detectable effects (Barret, *et al* 2003).

Prions in blood

The blood infectivity of humans with vCJD was documented by four cases of transmission of the disease by blood transfusion (Llewelyn, et al 2004, Peden, et al 2004). All patients were transfused with nonleukoreduced red blood cells. Recently, 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, *et al* 2010). There have been no cases of transfusion transmitted sCJD or of fCJD. The fact that vCJD patients have got significant deposition of PrPsc in lymphoreticular tissues and leukocytes continuously recirculate between blood and lymphoreticular tissues suggests, that lymphocytes might be main carriers of prions. The distribution of infectivity in blood was so far studied on animal models including sheep, rodents and primates. Thus in BSE infected primates buffy coat (which consists mainly of leukocytes) turned out to be an efficacious way of transmitting prions (Bons, *et al* 2002). However in this study the inoculation was done intracerebrally, which is the most effective way of prion infection, and they inoculated only one animal. By intracerebral inoculation of the blood specimens from spiked blood into healthy animals, infectivity was also detected in plasma, cryoprecipitate and Cohn fractions I-III, whereas no appreciable levels of infectivity were detected in Cohn fractions IV and V (Brown, *et al* 1998). Another study tested the infectivity of blood platelets and mononuclear cells, which were isolated from hamsters in terminal stage of scrapie and subsequently intracerebrally inoculated into hamsters (Holada, *et al* 2002b). The authors concluded, that platelets are nor significant source of infectivity whereas mononuclear cells contain higher infectivity dose – 1.75 ID/ml for platelets versus 22.6 ID/ml for mononuclear cells, which is consistent with studies with buffycoat infectivity. However, these studies were performed either with human blood spiked with scrapie prions or in rodents which have different PrPc expression on blood elements then humans (Holada and Vostal 2000). Since prion distribution may depend on

PrPc expression which differs between species these results may not be so easily extrapolated to humans. Very recent study proved that blood platelets can harbor infectivity – platelets from chronic wasting disease (CWD) suffered deer were able to transmit the disease to healthy deer and healthy mice (Mathiason, *et al* 2010).

Cellular prion protein

Cellular prion protein (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 co- and post-translational modifications include removal of a 22 amino acid N-terminal signal peptide, N-glycosylation at N181 and N197, formation of disulfide bond and replacement of the carboxy-terminus sequence at S231 by GPI-anchor. Contrary to brain PrPc, which might be easily cleaved of with GPI-specific phospholipase C (PIPLC), platelet PrPc is resistant to this treatment (Holada, *et al* 1998). This might be explained by different type of anchorage, by some modification of cleavage site for example by glycosylation or by inaccessibility of the anchor for the enzyme due to contiguous protein. In the cells, PrPc is present as di-, mono- or unglycosylated molecules. Thus on the SDS PAGE gel it appears as three bands as shown for example in Fig. 7. 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. PK as mentioned earlier. PrPc is predominantly membrane protein, however it also cycles between the membrane and endocytic compartment (Shyng, *et al* 1993) with clathrin coated pits responsible for the endocytic uptake (Shyng, *et al* 1994). In the cells depleted of clathrin by RNA interference the clathrin independent endocytosis of PrPc was shown and interestingly it did not depend neither on caveolin-pathway suggesting that PrPc might be internalized via multiple pathways (Kang, *et al* 2009).

Physiological function of PrPc

Although many approaches have been utilized to understand the physiological function of PrPc, it still remains unknown. There was a great expectation that ablation of PRNP in the mouse would reveal the normal function for this enigmatic gene. However PrP knockout mice did not have any overt phenotype (Bueler, *et al* 1992, Manson, *et al* 1994) except some minor defects in circadian rhythm and sleeping (Collinge, *et al* 1994, Tobler, *et al* 1996). Later it became plausible that PrP deficiency might manifests only upon physiological challenge. This was observed in prion deficient mice which upon induction of anemia turned to have reduced erythroid cell and erythropoietin production (Zivny, *et al* 2007) or in irradiated mice which had decreased survival after transplantation of hematopoietic stem cells from PrP-null bone marrow (Zhang, *et al* 2006).

One of the strategy to find PrPc function focuses on its interactors. A large number of candidates have been identified as potential PrP-binding partners using different approaches such as yeast two-hybrid screens, co-immunoprecipitation, cross-linking and others (Tab. 2).

The physiological function of such interactors (adhesion, apoptosis etc.) may help in revealing PrPc function. Thus interaction with laminin and vitronectin led to hypothesis that PrPc is involved in cell adhesion (Graner, *et al* 2000, Hajj, *et al* 2007). However the results are not consistent and due to used methods there is no clear physiological relevance.

Several studies suggested PrPc function in the cellular resistance to oxidative stress. Since PrPc displays a copper-dependent superoxide dismutase (SOD) activity, it may detoxify reactive oxygen species by itself (Brown, *et al* 1999). However, these studies were done with recombinant PrP and data are not consistent with other authors (Jones, *et al* 2005). Other hypothesis is that PrPc just regulates activities of SODs (Waggoner, *et al* 2000) but on the opposite, *in vivo* studies in mice failed to detect any effects of PrPc on SOD activity (Hutter, *et al* 2003).

PrPc possess a copper binding site in N-terminal octapeptide repeat region, which led to suggestion that PrPc is copper binding protein (Brown, *et al* 1997, Hornshaw, *et al* 1995). The role of PrPc in copper uptake or efflux was suggested based on correlation between PrPc expression levels and the copper content of cells or tissues as was shown in mice (Brown 2003). It was also proposed that PrPc may play a role in regulating copper release at the synapse (Herms, *et al* 1999). However these results were negotiated by

Harris et al who assessed the effect of PrPc expression level on the amount of brain copper in mice and did not find any correlation (Waggoner, *et al* 2000).

Several lines of evidence have emerged recently suggesting that PrPc may have an anti-apoptotic activity, which has been demonstrated in variety of experimental systems, including cultured mammalian cells, yeast and mice. Thus in human neurons PrP inhibited Bax-induced cell death depending upon the presence of octapeptide repeat region of PrP (Bounhar, *et al* 2001). In recent study the anti-apoptotic activity of PrPc was demonstrated *in vivo* upon virus infection (Nasu-Nishimura, *et al* 2008). Different study revealed, that the toxicity of cytosolic misfolded PrP is mediated by its binding to Bcl-2, which could be prevented by the chaperones Hsp70 and Hsp40 (Rambold, *et al* 2006). On the other hand PrP was shown to interact in the yeast two-hybrid system with Bcl-2 suggesting its pro-apoptotic activity (Kurschner and Morgan 1995). In consequence to anti-apoptotic function, a role of PrPc in the development of tumors was also suggested. For example in a human breast carcinoma MCF7 cell line, PrPc overexpression increased cell resistance to tumor necrosis factor α (Diarra-Mehrpour, *et al* 2004). PrPc was also shown to negatively regulate the formation of metastasis of mesenchymal embryonic cells expressing ras and myc proteins (Muras, *et al* 2009).

Indispensable amount of publication suggest PrPc to function in cell signaling. This may be related to PrPc localization in microdomains of the cytoplasmic membrane called lipid rafts which are known to serve as a molecular scaffold for signal transduction.

Krebs et all suggested PrPc role in monocytes signaling based on study where PrP stimulation led to tyrosine phosphorylation of $ERK_{1,2}$ and Akt kinase (Krebs, *et al* 2006). A broadly used method to determine PrP involvement in signaling is its cross-linking with antibodies, which in neuroectodermal cell line 1C11 triggered a caveolin-1-dependent coupling of PrPc to the tyrosine kinase Fyn (Mouillet-Richard, *et al* 2000). More recently the crosslinking of PrPc in the identical cell line led to activation of tyrosine kinase Fyn, NADPH oxidase and extracellular-regulated kinases (Schneider, *et al* 2003) as well as to change in activities of G protein-coupled serotonin receptors (Mouillet-Richard, *et al* 2005). However, the cross-linking with antibodies just simulates possible physiological extracellular ligand(s), which real existence has not been proved yet. Among candidates for such ligands in PrPc coupled signaling is stress-inducible protein 1, which has been shown to bind to PrPc in HEK 293T cells and induce neuroprotective signals via a cAMP/protein kinase A-dependent pathway that rescues cells from apoptosis (Zanata, *et al* 2002).

Another candidate is neural cell adhesion molecule (N-CAM), which was shown to be recruited to rafts upon binding of PrPc with subsequent activation of Fyn kinase and enhancing neurite outgrowth (Santuccione, *et al* 2005).

Table 2. Putative PrP interactors

Candidate interactor	Candidate function	Reference
Grb2	Signal transduction	(Loertscher and Lavery 2002)
Pint1	Unknown	(Loertscher and Lavery 2002)
Bcl-2	Apoptosis	(Kurschner and Morgan 1995)
Hsp60	Chaperone	(Edenhofer, *et al* 1996)
Caveolin-1	Signaling	(Mouillet-Richard, *et al* 2000)
Laminin receptor precursor (LRP)	Extracellular matrix interaction	(Gauczynski, *et al* 2001)
Tubulin	Microtubule subunit	(Nieznanski, *et al* 2005)
N-CAM	Cell adhesion	(Schmitt-Ulms, *et al* 2001)
STI-1	Heat shock protein	(Zanata, *et al* 2002)
NRAGE	Activator of apoptosis	(Bragason and Palsdottir 2005)
Pli 45 and Pli 110	Unknown	(Oesch, *et al* 1990)
Nrf2	Transcriptional activator	(Yehiely, *et al* 1997)
Aplp1 (amyloid precursor-like protein 1)	Transcriptional activator	(Yehiely, *et al* 1997)

PrPc in human blood

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 3). Concerning red blood cells (RBC), Barclay et al. found that they express little or no PrPc (Barclay, *et al* 1999) contrary to Holada et al who reported over 50% of blood PrPc to be expressed on RBC (Holada and Vostal 2000). Using a new time-resolved dissociation-enhanced fluoroimmunoassay (DELFIA), MacGregor et al showed that majority of PrPc is present in plasma and platelet fraction (MacGregor, *et al* 1999). PrPc was also detected on $CD34^+$ bone marrow stem cells and on mature peripheral lymphocytes and monocytes, whereas PrPc on granulocytes was negligible (Dodelet and Cashman 1998). As for plasma PrPc, endothelial cells were suggested as its source, since

they were shown to release PrPc positive particles upon apoptosis (Simak, *et al* 2002, Starke, *et al* 2002).

PrPc expression on non-human blood cells is considerably different from human (Holada, *et al* 2007), given the doubts about TSE transmission studies performed on animal models. For example mouse, which is the most common model, do not express any detectable platelet PrPc (Holada and Vostal 2000).

Table 3. 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

Blood platelets

Platelets are anucleated blood cells derived from megakaryocytes. Circulating platelets have life span of around 10-12 days and are removed from circulation primarily in the spleen. Resting platelets are discoid in shape with an equatorial diameter of 2-3 μm. The cytoskeleton system plays a crucial role in platelet morphology. It consists from three layers: a spectrin based skeleton that is adherent to the cytoplasmic side of the plasma membrane; a single microtubule coil and a rigid actin filament-based network that fills the cytoplasmic space of the cell. Actin is a major platelets protein and about 40% of it is in the form of filaments (F-actin), the rest in the form of monomeric globular G-actin that fills the cytoplasm. During platelet activation the level of F-actin markedly increases. A variety of secretory granules is synthesized and packaged during megakaryocytes development. The most abundant are α-granules, which contain proteins essential for platelet adhesion during vascular repair. Next are dense granules, which contain small molecules that play important roles in cell activation. Dense granules are smaller and less numerous then α-granules (3-8 per platelet). Upon platelet activation the content of granules is released and

promotes further platelet activation and aggregation in a positive feedback manner. Lysosomal granules contain a number of lysosomal enzymes, which are supposed to play a role in clot lysis and clearing of thrombi. The content of platelet granules is present in Table 4.

Table 4. Platelet granules content. Adopted from Michelson 2002 (only the most important content is referred).

α-granules	**Dense granules**	**Lysosomes**
CD62P (P-selectin) Factor V Platelet-derived growth factor Platelet factor 4 Thrombospondin Fibrinogen $\alpha_{IIb}\beta_3$ IgG fibronectin	ATP, ADP GTP, GDP P, Pyrophosphate Ca Mg P Serotonin LAMP - 2 CD63 (LAMP - 3)	LAMP – 1 LAMP - 2 CD63 Acid hydrolases

Main platelets function is to form a primary haemostatic plug at the site of vessel injury (Gerrard and White 1976). This is a multistep process involving platelet adhesion to the damaged vessel wall, activation and subsequent aggregation to form the haemostatic plug. Upon activation platelets change shape, the organelles are centralized and pseudopods are formed, subsequently the adhesive receptors are exposed and the content of granules is spilled. Activated platelets release microvesicles ranging in size from 100 nm to 1 μm, which are supposed to promote coagulation cascade. Furthermore platelets release exosomes ranging in size between 40-100 nm, which are thought to be derived from α-granules and multivesicular bodies (Heijnen, *et al* 1999).

Senescent platelets are removed primarily by macrophages in the spleen, exceptionally severely damaged platelets are removed more quickly by hepatic macrophages.

PrPc in blood platelets

Previous studies have shown that PrPc is present on both internal and external platelet membranes. 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 platelet PrPc was not possible to remove from the cell membrane with phosphatidylinositol-specific phospholipase C (PIPLC), which is an enzyme able to cleave GPI-anchored proteins. Similar attribute was reported for PrPsc (Stahl, *et al* 1990), on the other hand PrPc is efficiently cleaved out of the membrane of different cell lines. Several reasons for this resistance of PrPc were suggested, among them alternative membrane anchoring or inaccessibility of GPI-anchor to PIPLC. Conversely another study showed release of PrPc from platelets by PIPLC treatment (Perini, *et al* 1996). However, in this study long term deposited blood was used. The up-regulation of PrPc on activated platelet together with CD62P suggested its localization in α-granules of platelets. Starke et al detected PrPc in α-granules using confocal immunofluorescence and immunoelectron microscopy (Starke, *et al* 2005). They also proposed that PrPc is present in the cell cytoplasm, however this was not referred anywhere else. The α-granular location was confirmed by recent study using platelets of patients suffering from Grey platelet syndrome, platelets which lack α-granules (Holada, *et al* 2006). In resting platelets the majority of PrPc was found inside the cells and was up regulated to the surface of normal as well as Hermansky-Pudlak syndrome platelets lacking dense granules, whereas in Grey platelet syndrome platelets PrPc failed to up regulate. 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). In such way another source of blood PrPc is generated, as exosomes are known to persist in blood plasma (Caby, *et al* 2005). This source of PrPc is similar to that of endothelial cells which was described earlier. Interestingly, based on the cell lines study exosomes were proposed to play a role as a vehicle for transport of PrPsc from cell-to-cell (Fevrier, *et al* 2004). It raises the question whether also exosomes present in plasma and expressing PrPc might serve similarly.

Lipid rafts

The recent Keystone Symposium on Membrane Rafts and Cell Function has adopted the definition for the rafts as follows: Membrane rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize the cellular processes (Pike 2006). Previously used term lipid rafts was replaced by the term membrane rafts. However, the term lipid rafts is still more used so it

is used in this thesis as well. Lipid rafts contain 3 to 5-fold the amount of cholesterol found in the surrounding membrane and they are enriched in sphingolipids such as sphingomyelin, which might be even 50% elevated compared to plasma membrane. Inner-leaflet lipids, such as phosphatidylethanolamine and anionic phospholipids, are particularly depleted. The majority of phospholipids within the rafts are fully saturated and together with elevated cholesterol, which is inserted between the lipids in the rafts and fills the voids, it makes the rafts to be tightly packed.

Rafts are not readily solubilized in non-ionic detergents, a property that is a result of tight packing. This has given rise to the acronym detergent resistant membranes (DRMs). Similar type of domain are caveolae, however they can be distinguished by the presence of the cholesterol-binding protein caveolin-1 (Murata, *et al* 1995).

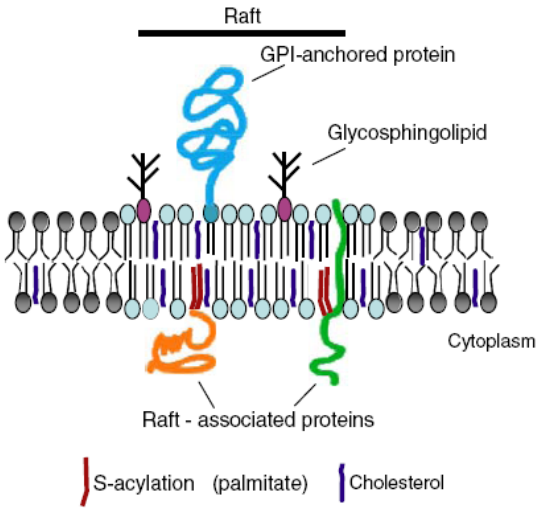


Figure IV. The model of lipid raft.
Lipid rafts have got special lipid composition which is represented here by different color of circles which represent lipids. Proteins interacting with rafts possess some type of raft-targeting signal, for example S-acylation or GPI-anchor. (Taken from (Chichili and Rodgers 2009)).

Traditional method of rafts preparation involves lysis in cold 1% Triton X-100 and subsequent flotation of lysate in a 5% to 30% sucrose density gradient where they distribute in the top fractions of the gradient. To date a wide variety of detergents have been used to isolate rafts including NP-40, octylglucoside, CHAPS, Brij 96, Brij 98 and others (Chamberlain 2004). Several detergent-free methods have also been reported, but

only for isolation of caveolae microdomains (Smart, *et al* 1995, Song, *et al* 1996). Method introduced by Smart *et al.* is based on cell membrane isolation by cell lysis in isotonic sucrose buffer, membranes are then sonicated in order to release the rafts which are isolated by flotation in a continuous gradient of Opti-Prep in isotonic solution. Song's *et al.* preparation involves the lysis of whole cells in a sodium carbonate buffer (pH 11). This buffer is used because the elevated pH helps in the removal of peripheral membrane proteins. Following sonication of the lysate, rafts are centrifuged on a discontinuous sucrose gradient and accumulate at the 5% and 35% sucrose interface. Direct visualization of lipid rafts is problematic due to their small size. However some attempts were used, for example immunostaining of ganglioside GM1, which is a marker of rafts, or immunofluorescence of GPI-anchored proteins (another type of raft markers) (Mayor, *et al* 1994). By cross-linking of these markers, Harder et al achieved better rafts visualization (Harder, *et al* 1998). Immunofluorescence examination revealed patches appearance suggesting specific clustering of raft-associated markers. Also other high resolution immunofluorescence studies using fluorescence energy resonance transfer (Varma and Mayor 1998), laser trap single particle tracking (Pralle, *et al* 2000) and single molecule microscopy (Schutz, *et al* 2000) provided a clear evidence that lipid rafts exist in vivo.

As cholesterol is the critical structural component of the rafts its depletion leads to disorganization of lipid raft microdomains and to dissociation of proteins bound to rafts. One of the possible attempts is inhibition of cholesterol synthesis by use of drugs like lovastatin and squalestatin, however it might affect other events in the cell. Among the most widely used cholesterol depleting agents belong membrane pores forming agents such as saponin, digitonin, streptolysin-O; drugs which sequester cholesterol: filipin, nystatin and amphotericin; and cholesterol depleting methyl-β-cyclodextrin (MβCD) (Fig. V.). Most of the agents become incorporated into membranes, whereas MβCD is a strictly surface-acting agent and can selectively and rapidly remove cholesterol from the plasma membrane in preference to other membrane lipids.

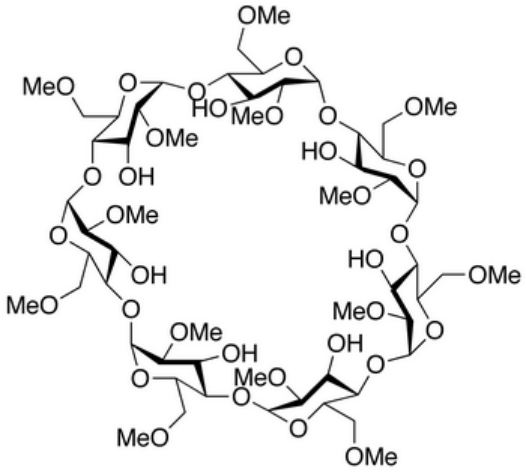


Figure V. The structure of methyl-β-cyclodextrin.
Methyl-β-cyclodextrin is cyclic oligosaccharide, which is commonly used for cholesterol extraction in order to disrupt lipid rafts.

A number of studies strongly support a functional role for rafts in the onset of signaling events in different cell types especially in immune cells (Horejsi, *et al* 1999, Montixi, *et al* 1998). Rafts might modulate signaling events by localizing signaling molecules within one membrane compartment thus enabling their interaction. Alternatively the translocation of signaling molecules in and out of rafts could regulate their activity, as was suggested for T cells tyrosine kinase Lck (Filipp, *et al* 2003). To date different signaling pathways were localized within rafts including G protein coupled receptors (Haasemann, *et al* 1998), the epidermal growth factor receptor (Waugh, *et al* 1999), the platelet-derived growth factor receptor (Liu, *et al* 1996) and various GPI-linked proteins, for example CD59 which provides inhibition of complement mediated lysis.

Prion and lipid rafts

Like other GPI-anchored proteins, PrPc was found in lipid rafts of diverse cells (Brown and Rose 1992, Harmey, *et al* 1995, Taraboulos, *et al* 1995). The possible relation between PrPc association with rafts and its physiological function was described in the

section about physiological function of PrPc (page 21). A variety of evidence indicates that lipid rafts play some role in the conversion of PrPc to PrPsc. However, there are two different theories. Baron et al. in cell free conversion system found, that GPI-anchored PrPc present in rafts is resistant to conversion to PrPsc, unless its GPI-anchor is cleaved by phospholipase C to release soluble PrPc (Baron, *et al* 2002). However when PrPsc was inserted into the rafts of recipient cells by membrane fusing agent polyethylene glycol the transformation of PrPc occurred. It may be that GPI-anchor stabilizes the conformation of PrPc within rafts unless the PrPsc inserts to the same domains. On the other hand cholesterol depletion leading to perturbation of PrPc-raft association diminished the formation of PrPsc (Taraboulos, *et al* 1995). Similarly, the replacement of GPI-anchor of PrPc with transmembrane polypeptide leading to shift of PrPc to non-raft region also diminished the formation of PrPsc (Kaneko, *et al* 1997). Moreover, Naslavsky et al. found both PrPc and PrPsc attached to lipid rafts, suggesting that the transformation occurs here (Naslavsky, *et al* 1997).

Interestingly, Walmsley et al shown that GPI-anchor is not necessary for targeting of PrPc to rafts giving the doubt about Kaneko`s and Taraboulos`s study. They demonstrated that amino acid residues 23 – 90 of the flexible N-terminal domain are sufficient for association of transmembrane PrPc with rafts (Walmsley, *et al* 2003). However the transmembrane domain of their PrPc was different comparing to that in previously mentioned studies. Another study disputed even about relevance of PrPc membrane attachment for its transformation to PrPsc (Nishina, *et al* 2004). On the other hand incubation of N2A cells with polyene macrolide antibiotic filipin, which induces the shedding of PrPc, inhibited the formation of PrPsc (Marella, *et al* 2002). To increase the confusion about this task, Bate et al. published the study where GPI-anchor analogue glucosamine-PI caused the replacement of PrPc from lipid rafts and subsequently inhibited PrPsc formation (Bate, *et al* 2010). To conclude, it is not clear yet, whether PrPc needs to be anchored to the membrane to be transformed to PrPsc.

Association with rafts was also reported to be critical for progression of prion disease. Transgenic mice infected with scrapie expressing PrPc lacking the GPI-anchor had minimal clinical manifestation, although an abnormal protease-resistant PrP was deposited as amyloid plaques in brain tissue (Chesebro, *et al* 2005). It was suggested that PrPsc formation within lipid rafts affects signaling events involving PrPc, leading to removal of neuroprotective signals. The lack of membrane anchorage would then prevent disease

progression possibly by disrupting the signal transduction processes (as explained in Figure III).

Lipid rafts in platelets

Evidence, shown by a number of studies, indicates that lipid rafts act in platelets as foci that integrate adhesion and signaling molecules. It was demonstrated that in resting blood platelets lipid rafts are uniformly distributed over the cytoplasmic membrane and platelet activation leads to their clustering (Bodin, *et al* 2003a, Gousset, *et al* 2002). Dorahy et al isolated lipid rafts from platelets by lysis with 1% Triton X-100 and examined them with an electron microscopy (Dorahy, *et al* 1996). He revealed a heterogeneous population of vesicles ranging in size from 20 to 1000 nm, which was enriched in CD36 and kinases Src and Lyn. Platelet rafts were found to be caveolin negative.

Transmembrane protein CD36, which is in platelets also referred to glycoprotein IV (GPIV), is considered to be a platelet raft marker. And as such is used also in this thesis. In platelet membrane CD36 associates with different tyrosine kinases (Huang, *et al* 1991) and this association was recently shown to be lipid mediated (Thorne, *et al* 2006) pointing out the importance of membrane raft environment.

A deal of studies proved the importance of rafts in platelet function. Platelet adhesion to the exposed subendothelium at sites of tissue damage is one of the steps required for the prevention of bleeding. At high shear forces adhesion of platelets through the surface glycoprotein GpIb-IX-V is an important initial mechanism of the repair process. At low flow rates, collagen and fibrinogen can support adhesion of platelets. The integrin $\alpha 2\beta 1$ is a collagen receptor involved in platelet adhesion. Another collagen receptor, GPVI, restricted to megakaryocytes and platelets, is also strongly involved in platelet activation process (Watson, *et al* 2001). GPVI is a multisubunit receptor in which the ligand binding subunit is noncovalently associated with Fc receptor γ-chain (FcRγ) that contains an immunoreceptor tyrosine activation motif (ITAM). Locke et al demonstrated that GPVI is not present in rafts of resting platelets but is recruited to these domains upon platelet activation (Locke, *et al* 2002). The complex GPVI-FcRγ was shown to interact with the immunoreceptor tyrosine-based inhibitory motif-containing receptor, PECAM-1, and this interaction occurs in rafts. Depletion of cholesterol with methyl-β-cyclodextrin (MβCD),

leading to raft disruption, causes abortion of this interaction (Lee, *et al* 2006). Thus, strong arguments are in favor for a role of lipid rafts in GPVI-mediated platelet activation.

Another platelet signaling molecule, Fc receptor FcγRIIa, was shown to be a part of rafts (Bodin, *et al* 2003b). FcγRIIa is a component of both the GPVI and the GP Ib-IX-V that mediates activation of platelets by von Willebrand Factor. The tyrosine phosphorylation initiated by FcγRIIa is less sensitive to cholesterol depletion then collagen-mediated tyrosine phosphorylation suggesting different significance of rafts for these two activation pathways.

The significance of lipid rafts in platelet activation processes was proved in several studies showing the influence of raft disruption on platelet aggregation. Cholesterol depletion with MβCD markedly diminished aggregation response to stimulation with estradiol (Reineri, *et al* 2007), with low doses of thrombin or TRAP (Bodin, *et al* 2005), or stimulation of GPVI receptor (Quinter, *et al* 2007). However it was found that cholesterol depletion did not affect maximal platelet aggregation of platelets stimulated with high doses of thrombin indicating that rafts are not mandatory for αIIβIII-mediated platelet aggregation.

Cholesterol depletion also affects platelet morphology, which became spherical with eversion of the open canalicular system. However, their dense and α-granules remain intact. Moreover treatment with MβCD leads to shedding of microvesicles (Gousset, *et al* 2002).

Taken together, the membrane compartmentalization appears to be required for maintenance of platelet haemostatic function particularly platelet activation via receptors.

The relation between lipid rafts and cytoskeleton

Eukaryotic cell membranes are heterogeneous environment, dynamically organized, which is crucial for cell viability as well as for their physiological functions. Thus, the changes in membrane structure and composition may lead to different pathologies. Eukaryotic cytoskeleton contains three kinds of cytoskeletal filaments – microfilaments which composed from actin, intermediate filaments and microtubules which are formed from tubulin. Microtubules and actin filaments have nucleotide binding and hydrolyzing activity, whereas intermediate filaments, which serve as cytoskeletal "identity cards" that distinguish different cell types, have no known enzymatic activity. Disassembly of the

actin filaments, which is involved in cell motility, adhesion, and endocytosis, leads to fusion of intracellular vesicular membranes with the plasma membrane. Microtubules are involved in cell locomotion, movement of organelles, and mitosis. Both actin filaments and microtubules are implicated in membrane trafficking in mitotic cells, what relates to their interaction with lipid rafts.

Studies on immune cells provided evidence that lipid rafts regulate membrane-cytoskeletal communication by regulating actin dynamics and recruiting cytoskeletal associated proteins (reviewed in (Meiri 2005)). In T cells, actin was isolated within lipid rafts and was also found to associate with known lipid raft markers (Jordan and Rodgers 2003). Recent study using fluorescence resonance energy transfer revealed that cytoskeleton promotes clustering of lipid rafts proteins (Chichili and Rodgers 2007). In platelets Bodin et al revealed that lipid rafts specifically associate with cytoskeleton upon platelet activation (Bodin, *et al* 2005). They found the similar lipid composition of cytoskeleton isolated from activated platelets and from lipid rafts. Moreover, they extracted rafts from actin cytoskeleton and found that most of rafts were recovered in cytoskeleton of activated platelets. As the raft-cytoskeleton association was inhibited by agonists of fibrinogen-GPIIb-IIIa and did not occur in type Glanzman`s thrombastenic platelets, they proposed that it depends on integrin GPIIb-IIIa.

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

Platelet preparation

Platelet rich plasma (PRP), obtained as an aliquot from plateletpheresis, and citrate anticoagulated whole blood were provided by department of transfusion medicine of Institute of Hematology and Blood Transfusion in Prague with informed consent of all donors in accordance with the Ethical Committee regulations of the Institute. The donors had not taken aspirin or other platelet-active drugs for at least seven days before blood donation. PRP was supplemented with 1 μM Prostaglandin E1 (PGE_1, Sigma) to prevent platelet activation and platelets were separated from plasma proteins by gel filtration on Sepharose CL-2B (Amersham Biosciences) into a Tyrode`s/HEPES buffer (THB: 4 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, 3 mM NaH_2PO_4, 0.35% BSA; pH 7.4). Sepharose column was immediately washed with 400 ml H_2O and stored in 0,05% NaN_3 for next platelet isolation. If not used immediately, platelets supplemented with the mixture of proteases inhibitors (0.5 mM PMSF, 1 μM leupeptin, 1 μM pepstatin and 100 μM EDTA) (all from Sigma) were stored frozen at -80°C. Whole blood was processed by centrifugation: PRP was prepared by centrifugation at 250×g for 15 minutes at room temperature. Such prepared PRP was used for fluorescence microscopy. Platelet count was measured in whole blood as well as after isolation procedures using cell counter ADVIA 60 (Bayer).

Platelet membrane and platelet organelles preparation.

Blood platelets were isolated by gel filtration as described above and they were resuspended at a concentration $1x10^9$/ml in THB. Platelets were then disrupted by sonication (3 x 3 sec., amplitude 60, small tip; Ultrasonic homogenizer 4710, Cole-Parmer Instrument Co.) in the presence of the cocktail of protease inhibitors Complete (Roche). Samples were chilled on ice for 1 min between each sonication. Non-disrupted platelets were pelleted at 1500 g for 5 minutes and discarded. The organelle fraction was sediment by centrifugation at 14 000 g for 15 minutes at 4°C and subsequently washed with THB. The membrane fragments were collected in the supernatant. We also tested membrane preparation by ultracentrifugation – in this approach the supernatant from previous step

was centrifuged 200 000 x g for 2 hours at 4°C, however for our purposes the membranes in the solution were sufficient.

Platelet activation

Platelet count was set to 0.7-1×10^9/ml and platelets were left to rest for 60 minutes. Aliquots of platelets were activated with 0.1 or 1 U/ml Thrombin (Sigma) for 10 minutes at 37°C or with 20 μM TRAP (SFLLRNP, thrombin receptor activating peptide) for 10 minutes at 37°C. In some cases the visible aggregation of the platelet might be observed. Aliquots of resting platelets were further stabilized with 1 μM PGE1.

Isolation of red blood cell membranes

RBC membranes were isolated as described recently (Panigaj, *et al* 2010). Whole citrated blood was diluted 1:1 with platelet wash buffer (PWB: 12.9 mM citrate, 30 mM glucose, 120 mM NaCl, 5 mM EDTA; pH 6.5) and centrifuged (300g, 15 min., RT). Platelet rich plasma (PRP) was transferred into new tube and sedimented cells were resuspended in PWB. This step was repeated three times to remove majority of platelets and plasma. RBC, which in this step contain contaminating leukocytes (WBC), were resuspended in cold PBS (pH 7.4) and centrifuged (1500g, 10 minutes, 4°C). Supernatant and buffy coat containing majority of WBC was discarded together with upper quarter of RBC phase. Middle half of RBC phase was transferred to a new tube, diluted with the same volume of PBS and checked for cell contamination on cell counter. Subsequently RBC were lysed with 14 volumes of ice cold lysis buffer (LB: 5 mM sodium phosphate, 1 mM EDTA; pH 7.4). Remaining contaminating platelets and WBC were removed by centrifugation (2000g, 5 minutes, 4°C). Subsequently RBC ghosts were sediment at 20,000×g (40 minutes, 4°C), washed three times with LB, and finally resuspended at a concentration 1x10^{10}/ml in LB supplemented with 5 mM EDTA and 0.5 mM PMSF and stored frozen at -80°C.

Preparation of brain homogenate

Human brain tissue (lobus frontalis) was homogenized (1:9) in ice cold TBS (20 mM Tris, 145 mM NaCl; pH 7.4) with 1 mM EDTA and 1 mM protease inhibitor PMSF using

ground glass pestle homogenizer followed by sonication (3 x 1 sec., amplitude 60, small tip). Sonicated sample was continuously chilled on ice. Coarse fragments were removed by centrifugation (4000× g, 10 minutes, 4°C) and supernatant – 10% (w/v) brain homogenate - was stored frozen in aliquots at -80°C.

Comparison of quantity of PrPc on platelets and RBC using western blot.

The cells were isolated as described above with avoiding the contamination of RBC ghosts and platelets with other cells. The analysis was done by comparing the intensity of PrPc bands on blots among samples prepared from 1×10^{10} RBC/ml and serial dilutions of platelets: 5×10^{9}; 2.5×10^{9}; 1.25×10^{9}; 6.25×10^{8}; 3.13×10^{8}; 1.56×10^{8} PLT/ml. PrPc was detected by MAb 6H4 or by a mixture of MAbs GE8, AG4 and AH6 (1 μg/ml of each; see the list of antibodies below). The comparison was done between platelets and RBC from the same individuals and repeated 3-times with cells isolated from three donors.

Proteinase K (PK) treatment

Platelet membranes and organelles in THB were prepared as described above and treated 30 minutes on ice with increasing concentration (0, 0.5, 2.5, 5, 50, 100 μg/ml) of PK. RBC ghosts and platelets isolated from the same donor and normal brain homogenate were set to the same protein content (1500 μg/ml) using BCA Protein Assay (Pierce), lyzed with 1% Triton X-100 and treated for 30 minutes on ice with increasing concentration (0, 2.5, 5, 50 μg/ml) of PK. The reaction was terminated by 5 mM phenylmethylsulfonyl fluoride (PMSF) and samples were analyzed by Western blotting.

Treatment with phosphatidylinositol-specific phospholipase C (PIPLC) and PNGase F

Platelet membranes prepared as described above were solubilized with 1% Triton X-100 for 10 minutes and subsequently treated with 1 U/ml PIPLC (Sigma) for 30, 60, 90 and 120 minutes at 37°C.

In order to deglycosylate PrPc we used an amidase N-glycosidase F (also known as PNGase F; New England BioLabs). Samples were denatured with Glycoprotein denaturing

buffer (0.5% SDS, 40 mM dithiotreitol) at 100°C for 10 minutes, cooled and supplemented with cocktail of protease inhibitors (described above). The reaction took place in G7 buffer (50 mM sodium phosphate; pH 7.5) supplemented with 1% NP-40 with 10 U/ml of PNGase F overnight at 37°C.

Phase separation with Triton X-114

50 µl of PIPLC treated platelets were mixed with 150 µl 2% Triton X-114 in 10 mM Tris-HCl (150 mM NaCl; pH 7.4), incubated on ice for 30 minutes and subsequently at 37°C for 5 minutes. The mixture was centrifuged for 2 minutes at 13,000×g at room temperature and the aqueous and detergent phase were separated. Another 50 µl of Triton X-114 was added to an aqueous phase and 50 µl of Tris-HCl buffer to detergent phase and the phase separation was repeated. The aqueous and detergent phases were pooled and analyzed by Western blotting. As a marker of aqueous phase cytosolic protein p42 developed with antibody anti-CD45RA (Holada, *et al* 1995) was used.

GPI-anchor cleavage with hydrofluoric acid (HF)

Washed platelets, which have been frozen and stocked at -80°C, were precipitated with four volumes of pre-chilled methanol at -20°C overnight. After centrifugation at 20 000×g for 20 minutes the protein pellets were dissolved in 100 µl of 48% aqueous ice cold HF and incubated on ice for 48 hours. Subsequently the samples were methanol precipitated, centrifuged and pellets were washed with methanol, dried out and resuspended in Laemmli sample buffer.

Electron microscopy

Washed blood platelets or isolated platelet organelles were fixed with 2% glutaraldehyde in PBS pH 7.2 for two hours at 22°C, washed three times in the same buffer and embedded in an Araldite–Epon mixture. The samples were observed with a Tesla 500 electron microscope at the Institute of Inherited Metabolic disorders (1st Faculty of Medicine, Charles University in Prague).

Fluorescence microscopy of platelets

Fibrinogen adhered platelets. The coverslips were treated with chromic acid for 1 hour and rinsed with distilled water. The fibrinogen (Sigma) solution 200 μg/ml in Tris buffer (50 mM Tris, 0.1 M NaCl; pH 7.4) was applied for 1 hour. After washing with Tris buffer, coverslips were blocked with 1% BSA for 1 hour and stored at 4°C. Before platelet adhesion coverslips were incubated again in 1% BSA in phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4; pH 7.4). PRP prepared as described above was applied on coverslips for 1 hour at room temperature. Non adhered platelets were washed out with PBS.

Cytospin sedimented platelets. PRP was prepared by centrifugation of whole blood as described above. Subsequently PRP was tenfold diluted so that approximate platelet count was 30,000/μl. Platelets were then sediment onto poly-L-lysine coated slides (Sigma) by centrifugation at 1000×g for 5 minutes. In some experiments diluted platelets were activated in suspension with 20 μM TRAP at 37°C for 10 minutes prior to centrifugation. Such diluted platelets do not aggregate, which would be inconvenient for further observation.

Platelets prepared as described above were fixed with 1% PFA for 10 minutes and treated with pre-chilled methanol for 10 minutes. To control their morphology platelets were stained according to May-Grunwald-Giemsa method. Coverslips were blocked with 1% BSA in PBS and then incubated with a mixture of mouse monoclonal antibodies against PrPc 6H4 and 3F4, mouse monoclonal anti-thrombospondin (Santa Cruz Biotechnology) rabbit polyclonal anti-CD62P (BD Biosciences Pharmingen) or rabbit polyclonal anti-actin (Santa Cruz Biotechnology) for 1 hour. All primary antibodies were diluted 1:100 in PBS supplemented with 0.1% BSA. Coverslips were washed three times for 5 min with PBS and incubated for 30 minutes with anti-mouse TRITC labeled or anti-rabbit FITC labeled secondary antibodies (both Jackson Immunoresearch Laboratories) diluted 1:100 in PBS supplemented with 0.1% BSA. After washing the slides were mounted with Mowiol 4-88 (Sigma) and observed with a microscope Olympus AX70.

Lipid rafts isolation – flotation assay

Platelet lipid rafts were isolated by flotation assay as was described previously (Baglia, *et al* 2003). Washed resting or activated platelets ($0.7\text{-}1\times10^9$/ml) in THB were lysed in presence of protease inhibitor cocktail Complete (Roche) by adding of appropriate detergent: Triton X-100, NP-40, n-dodecyl-β-D-maltoside or Brij 98 (all from Sigma) at a final concentration 1% (w/v). To achieve appropriate solubilization, samples were briefly vortexed. All subsequent steps were performed at 4°C or alternatively at 37°C in case of control experiments and those with Brij 98 (Schuck, *et al* 2003). Lysed platelets were held on ice for 20 min and then adjusted to 40% (w/v) sucrose by addition of an equal volume of 80% (w/v) sucrose in Citrate buffer (12.9 mM citrate, 30 mM glucose, 120 mM NaCl, 5 mM EDTA; pH 6.5). For all flotation assays sucrose gradient was generated by layering 6 ml of 30% (w/v) and 2 ml of 5% (w/v) sucrose over the samples in ultracentrifuge polyallomer tubes 14×89 mm. Alternatively, the step sucrose gradient was made by addition of 35-30-25-20-15-10-5% sucrose solution on the top of the sample. The samples were then ultracentrifuged in an Optima LE-80K at 200,000 × g at 4°C for 20 h in SW40 Rotor (Beckman Instruments). Nine 1 ml aliquots were collected from the top of each ultracentrifuge tube and precipitated with four volumes of pre-chilled methanol overnight at -20°C. Air dried pellets were resuspended in 100 μl Laemmli sample buffer. In most cases, aliquot taken from the bottom of the ultracentrifuge tube containing the sediment (in Figures shown as aliquot 9) was hard to dissolve, hence it can not be quantitatively compared to the other aliquots. Lipid rafts were also isolated from RBC ghosts using the same protocol.

Cholesterol depletion

Platelets were pre-treated with 0.05% saponin (Sigma) for 30 minutes at 4°C prior to lysis with detergent (e.g. Triton X-100) in order to deplete cholesterol from cytoplasmic membranes as described in literature (Cerneus, *et al* 1993, Naslavsky, *et al* 1997). Alternatively, platelets were incubated with 20 mM methyl-β-cyclodextrin (MβCD) for 30 minutes at 37°C. Subsequently platelets were washed three times by centrifugation at 800×g in a fivefold excess of THB and lysed in appropriate detergent.

Isolation of lipid rafts from actin depolymerized platelets.

500 µl of resting or Thrombin (1 U/ml) activated platelets were sediment at 1000×g and lysed with 50 µl 1% Triton X-100 (w/v) supplemented with mixture of proteases inhibitors Complete. Lysed platelets were then treated with 500 µl of 0.6 M KI for 30 minutes at 4°C in order to depolymerize actin. Simultaneously 550 µl of resting or Thrombin activated platelets were lyzed with Triton X-100 according to standard lipid rafts isolation protocol. An equal volume of 80% (w/v) sucrose was added and samples were processed by flotation assay. Before sucrose addition 50 µl of the lyzate was collected as a control and frozen till the electrophoresis. In some cases this control sample was collected after sucrose addition, so the control is a half of that described above. In Figure 24, B we presented the bar graph, were appropriate proteins are compared to the control lyzate which band density was set to 100%. If the control lyzate was collected after sucrose addition as described above, we had to multiply twice the control band density.

Isolation of lipid rafts from isolated platelet cytoskeleton.

To isolate rafts from cytoskeleton fraction, the cytoskeleton pellet isolated as described below, either from resting or activated platelets was treated with 0.6 M KI for 30 minutes at 4°C according to method introduced by Bodin et al. (Bodin, *et al* 2005). Subsequently samples were processed by flotation assay.

Platelet cytoskeleton isolation

Platelet cytoskeleton was isolated as described in (Fox, *et al* 1988) with minor modification. 500 µl of washed resting or activated platelets ($0.7\text{-}1\times10^9$/ml) were lysed by addition of an equal volume of cold Lysis buffer (100 mM Tris-HCl, 10 mM EGTA, 2% Triton X-100 (w/v); pH 7.4) with protease inhibitor cocktail Complete and vortexed. Platelets were not chilled before lysis as this might influence the actin assembly (Hoffmeister, *et al* 2001). Cytoskeleton was immediately sediment at 15,600×g for 4 min. The pellet was washed once with THB and resuspended in 50 µl Laemmli sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.004% bromphenol blue; pH 6.8). To isolate membrane skeleton, supernatant from cytoskeleton isolation was centrifuged at 100,000×g for 20 hours with subsequent dissolving of the pellet in 50 µl of Laemmli sample buffer.

Dissolution of the thrombin-aggregated cytoskeleton required vigorous vortexing and brief sonication.

Platelet aggregation

Aggregation was measured using a BioData PAP-4 aggregometer (Horsham, PA, USA) with PRP diluted to 250×10^3/μl using platelet-poor plasma, which was obtained by centrifugation of PRP at 2500×g for 20 minutes. Aliquots of PRP were placed in cuvettes containing magnetic stirrer bars and incubated for 5 minutes at 37°C whilst stirring (1000 rpm). Platelet aggregation was recorded as optical density by platelet aggregation system. Platelets aggregated with 10 μM ADP and 2 μM epinephrine (EPI) or with 100 μg/ml collagen type I (all agonist were purchased as a PAR/Pak II kit from BioData Corporation) were used as a positive control. After adding the agonists the change in light transmittance was recorded for about 20 min. In order to follow the effect of anti-PrP antibodies binding on PrPc molecule, platelets were stimulated with anti-PrP MAbs GE8, AH6, 3F4 or SAF32 (all diluted 1:100) for 10 minutes before induction of aggregation with ADP/EPI. In order to crosslink PrPc, goat F(ab`)2 anti-mouse IgG`s (GAM) were added (dilution 1:100) for another 10 minutes. As a negative control GAM was used by itself, alternatively the possible effect of mouse IgG`s was excluded by incubation of the samples with control mouse IgG`s (Caltag). In some experiments we followed the effect of anti-PrP antibodies on platelet disaggregation induced by promethazine (PM) (Sigma), which is an α_2-adrenergic receptor antagonist (Mondoro and Vostal 2002). Here we added promethazine in final concentration 500 μM after 4 minutes of aggregation, which was induced by 10 μM ADP. When anti-PrP MAb was included in the experiment, it was added after platelet aggregation induced by ADP before promethazine treatment. In other settings, platelets were first incubated with anti-PrP MAb, then aggregated with ADP and then disaggregated with promethazine. The dilution of MAbs was 1:100 in all experiments.

The stimulation of platelets with anti-PrP antibodies and its influence on platelet phosphoproteom

Blood platelets were isolated by gel filtration as described above and supplemented with 2 mM $CaCl_2$. All samples were incubated simultaneously with or without appropriate stimuli for 5 minutes at 37°C. As a negative control we used: non treated platelets, platelets

incubated only with GAM (1:100) or with mouse IgGs (1:100) (Abcam). Positive control were platelets stimulated with 10 U/ml thrombin (Sigma), with 100 μg/ml collagen type I (BioData Corporation) and samples stimulated with MAbs with subsequent activation with thrombin or collagen. Platelets stimulated with antibodies were incubated with MAbs 3F4 and SAF32 (all diluted 1:100). If cross-linking followed, GAM (1:100) was added for another 5 minutes. In order to confirm or exclude the specificity of 3F4 binding, platelets were incubated with both antibody and 110 μM peptide consisting of the PrPc residues 102–114 (SSKPKTNMKHMAG) containing the epitope of the antibody. Stimulation was terminated by platelet lysis with pre-heated Laemmli sample buffer and samples were analyzed by Western blotting using Anti-Phosphotyrosin antibody (Upstate) diluted 1:2500.

SDS PAGE and Western blot analysis

Samples were heated to 100°C for 5 minutes in non reducing Laemmli sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.004% bromphenol blue; pH 6.8), resolved in 10% polyacrylamide gels and electrotransferred by wet blotting to a 0.2 μm nitrocellulose membrane (Bio-Rad). Membrane was blocked with 5% milk solution (Bio-Rad) in TBS and subsequently, without washing, incubated in 1% milk with primary antibodies overnight at 4°C. Then membranes were three times washed in TBS with 0,1% Tween and once in TBS with 5% milk. After that the membranes were incubated in 5% milk solution with appropriate alkaline phosphatase conjugated secondary antibody (Biosource). After incubation the membranes were three times washed with TBS. Antibodies were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (BCIP/NBT; Chemicon). The anti-PrP antibodies are listed in Table 5.

Table 5. List of anti-prion antibodies

Antibody	Epitope	Provider
AG4	PrP 37-50 (?PrP 147-163)	TSE resource center
AH6	PrP 159-174	TSE resource center
GE8	PrP 183-191	TSE resource center
FH11	PrP 46-59	TSE resource center

DC2	PrP 39-46	TSE resource center
3F4 (1562)	PrP 106-112	Biodesign, Chemicon
SAF32	PrP 79-89	Cayman Chemical
6H4	PrP 144-152	Prionics

Densitometry

The densitometry was done using GS-800 Calibrated Densitometer with integrated Quantity One software (Bio-Rad). Membranes were scanned and the PrPc signal was measured as the summary of the intensities of all three PrPc glycoforms.

Flow cytometry analysis of copper treated platelets

Blood platelets were isolated by gel filtration as described above and diluted to concentration $30\times10^3/\mu l$ in THB. They were incubated with increasing concentration of copper ions ($CuSO_4$: 0, 10, 100 and 1000 μM) for 30 minutes at room temperature and then labeled with PrP MAbs linked to a FITC: FH11 (PrP 46-59) (TSE Resource Center), 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 (Becton Dickinson) equipped with CELLQuestTM software. Platelets were identified by their characteristic forward and side light scatter. Gates were set around single platelets, and 5000 events were analyzed. As a negative control we used platelets without any antibody or with unrelated FITC-labeled antibody.

Results

The biochemical characterization of platelet PrPc

The glycoforms of platelet PrPc

In order to compare glycosylation pattern of platelet, RBC and brain PrPc we performed western blotting analysis of platelet lysates, RBC ghosts and brain homogenate. As obvious from Figure 1. the banding profile detected with anti-PrP MAb AG4 (PrP epitope 37-50) considerably differs. The brain PrPc has got all three glycoforms apparent.

RBC PrPc appears as one smeared band, so that di- and monoglycosylated PrPc can not be discriminated. Platelet PrPc has got dominant diglycosylated form, weaker monoglycosylated and hardly detectable unglycosylated form.

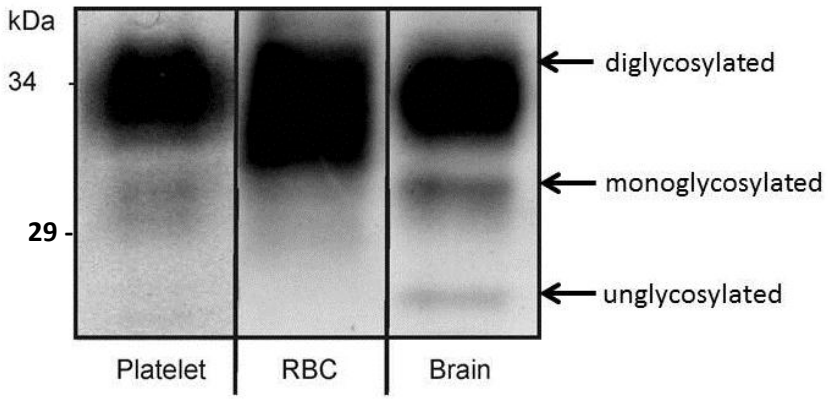


Figure 1. The glycosylation pattern of platelet, RBC and brain PrPc.
Platelet lysate ($2{,}5\times10^{9}$/ml), RBC ghosts (1×10^{10}/ml) and 10% (w/w) brain homogenate were analyzed by SDS PAGE and developed with MAb AG4.

When MAb DC2 (PrP epitope 39-46) directed against similarly localized epitope to that of AG4 was used for analysis, the banding profile of PrPc derived from platelets was comparable to PrPc from brain homogenates with the most dominant diglycosylated form (Fig. 2). Similar tendency was observed when using MAbs 3F4 (PrP epitope 106-112) directed against intermediary region of the protein and 6H4 (PrP epitope 144-152) directed against core protein region. Interestingly, MAb AH6 (PrP epitope 159-174), which has slightly shifted epitope comparing to 6H4, revealed different distribution of PrPc glycoforms. As for platelets, monoglycosylated form of PrPc predominated, whereas brain PrPc gave more intensive band od unglycosylated PrPc. Distribution of PrPc glycoforms developed with MAb GE8 (PrP epitope 183-191), which binds to C-terminal epitope, is comparable to that of developed with AH6 for platelet PrPc. Regarding brain PrPc, using MAb GE8 resulted in hardly detectable diglycosylated protein and higher intensities of mono- and unglycosylated forms.

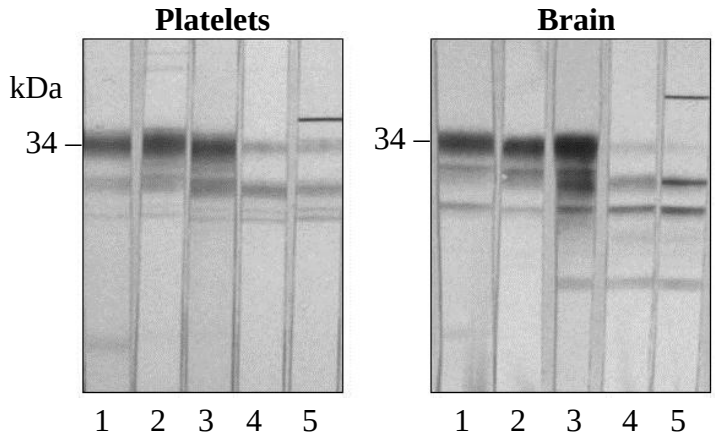


Figure 2. The glycosylation pattern of PrPc depends on the choice of anti-PrP antibody.
Platelet lysate ($2{,}5\times10^9$/ml) and 10% (w/w) brain homogenate were analyzed by SDS PAGE, the membranes were cut and the strips were incubated in the tray with different anti-PrP MAbs: 1 - DC2, 2 – 3F4, 3 – 6H4, 4 – AH6, 5 – GE8.

Sensitivity of platelet PrPc to proteinase K (PK)

In order to exclude resistance of platelet PrPc to cleavage with PK we performed the treatment of both platelet membranes and organelles isolated as described in the Methods section with increasing concentration of the enzyme (0, 0.5, 2.5, 5, 50 and 100 μg/ml).

The purity of organelle fraction was checked by electron microscopy (Fig. 3).

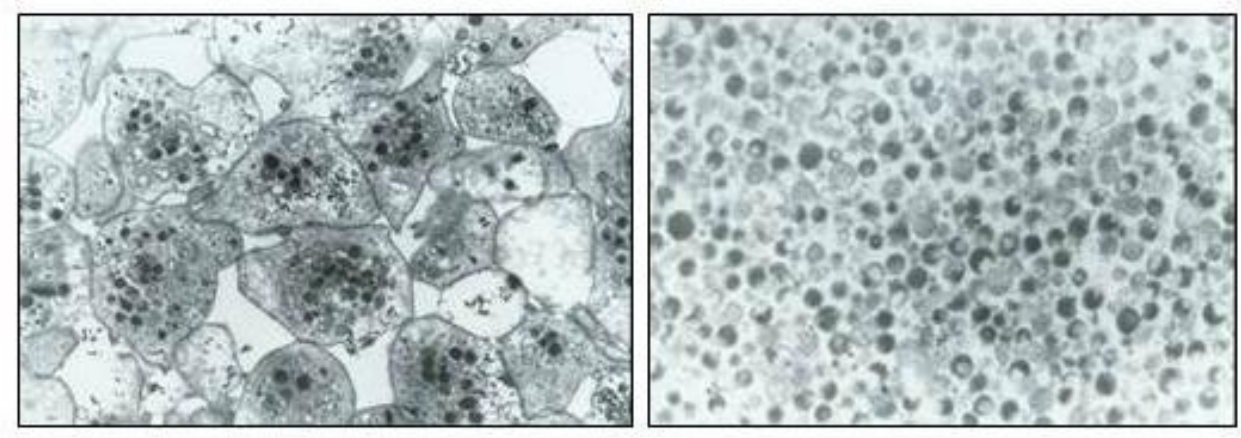


Figure 3. Control of blood platelet organelle purity.
Blood platelets as well as their organelles isolated by platelet disruption and centrifugation were fixed in buffered glutaraldehyde and processed for observing with electron microscopy. Left panel – whole blood platelets. Right panel – isolated organelles. Original magnification ×21,000.

As shown in Figure 4., both platelet fractions were totally digested with 100 μg/ml of PK. The sensitivity of platelet membrane PrPc to PK was also compared to that of brain and RBC (Fig. 5). Membranes of all samples were isolated and treated with the enzyme. The figure demonstrates the gradual cleavage with similar sensitivity of samples tested. 100 μg/ml of PK led to complete PrPc digestion (data not shown). In case of brain PrPc the thin band of molecular weight around 20 kDa appears, which was described by Jimenez-Huete et al. as amino-truncated peptide of PrPc (Jimenez-Huete, *et al* 1998).

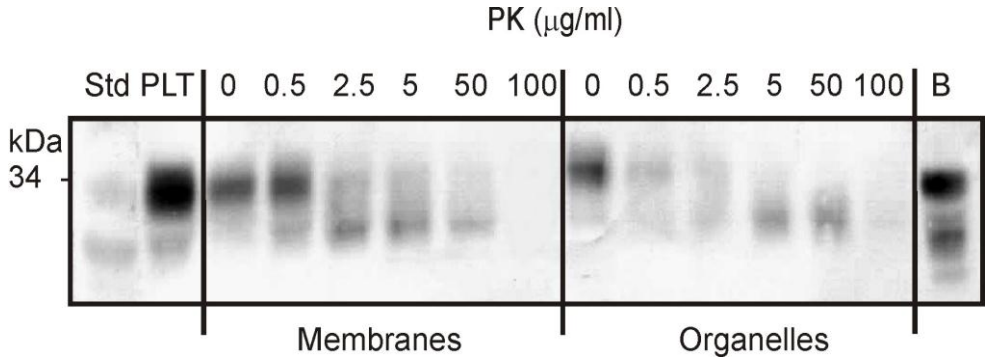


Figure 4. Both platelet membrane and organelle PrPc is sensitive to PK cleavage. Platelets isolated by gel filtration were sonicated and organelles were isolated by centrifugation whereas membranes left in the supernatant. Both fractions were treated for 30 minutes on ice with increasing concentration of PK and the reaction was terminated with PMSF. Samples were then resolved by 12% PAGE-SDS and developed with MAb 6H4. As a positive control for PrPc detection, the whole platelet lysate (PLT) and brain homogenate (B) were used.

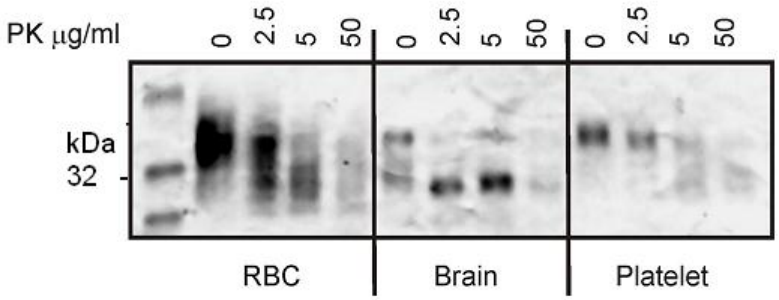


Figure 5. Platelet PrPc is similarly sensitive to PK as PrPc in RBC and brain homogenate.
RBC ghosts, 10% (w/w) brain homogenate and lysed platelets were set to the same protein content, lysed with 1% Triton X-100 and treated for 30 minutes on ice with increasing concentration of PK. The reaction was terminated with 5 mM PMSF and samples were resolved by 12% PAGE-SDS and developed with MAb 6H4.

Is platelet PrPc anchored by GPI-anchor?

Previous results demonstrated that platelet PrPc is resistant to cleavage by GPI-specific phospholipase C (PIPLC) (Holada, *et al* 1998).

The possible inaccessibility of the anchor for the enzyme due to contiguous protein was excluded by PIPLC treatment of the platelet membranes solubilized with Triton X-100 (Fig. 6). In order to achieve more visible band of PrPc, samples were deglycosylated so only one – unglycosylated band is detected. It is obvious then even when solubilized platelet membranes were treated with PIPLC for 120 minutes, there is no change in the molecular weight implying the GPI-anchor cleavage.

The cleavage of the anchor would have caused the decrease of the electrophoretic mobility on the SDS PAGE gel as illustrated on Fig. 7, since the cleavage causes lower binding of SDS and so decreased mobility. In our case no decrease was detected.

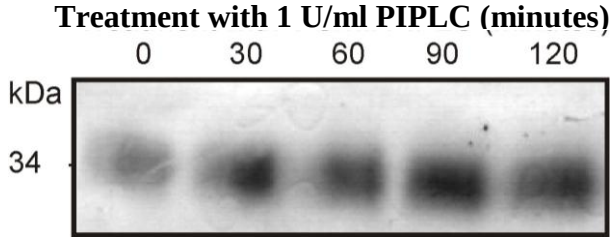


Figure 6. Platelet PrPc is resistant to shedding by PIPLC.
Platelet membranes were isolated by platelet homogenization as a supernatant after centrifugation of organelle fraction. They were solubilized with 1% Triton X-100 and subsequently treated with 1 U/ml PIPLC for different times. Samples were deglycosylated using 10 U/ml of PNGase F and analyzed by SDS-PAGE on 12% gel.

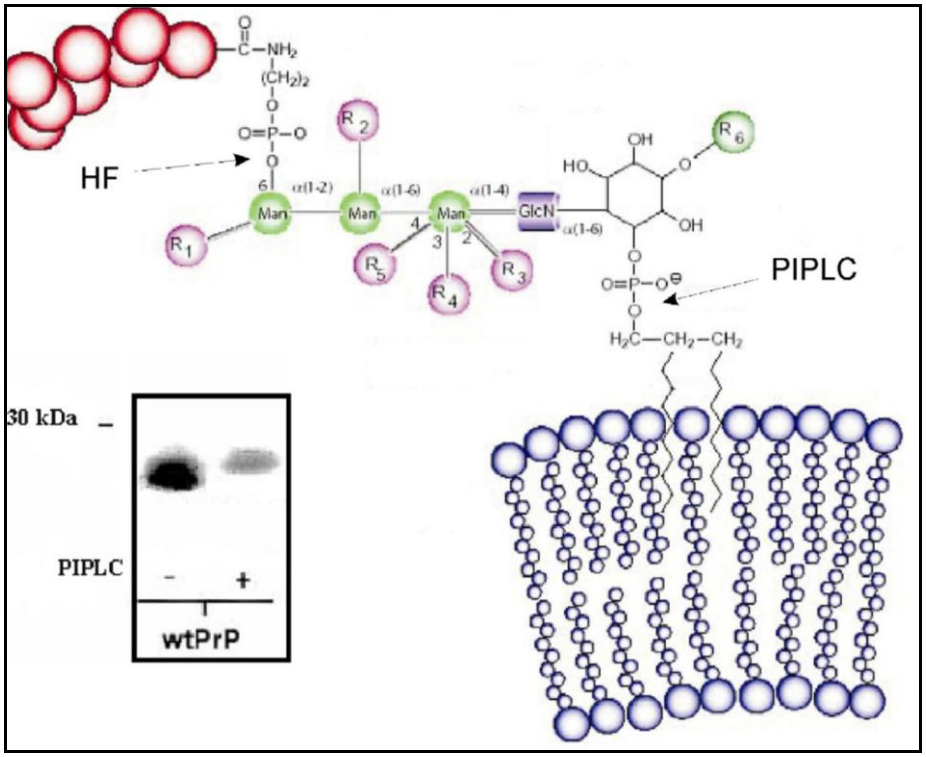


Figure 7. The cleavage -of GPI-anchor of PrPc by PIPLC or HF.
GPI-anchored proteins are linked at their carboxy-terminus through a phosphodiester linkage of phosphoethanolamine to a trimannosyl-non-acetylated glucosamine (Man3-GlcN) core. The reducing end of GlcN is linked to phosphatidylinositol (PI). PI is then anchored through another phosphodiester linkage to the cell membrane through its hydrophobic region. The Man3-GlcN oligosaccharide core may undergo various modifications. Release of GPI-anchored protein can be accomplished by treatment with PIPLC, which specifically hydrolyzes the phosphodiester bond of phosphatidylinositol. This is accompanied by decrease of electrophoretic mobility on SDS-PAGE electrophoresis as shown on inserted Western blot. Alternatively, hydrofluoric acid (HF) cleaves the phosphodiester bond of phosphoethanolamine leading to decrease of molecular weight as shown in Figure 9. Adapted from www.Sigmaaldrich.com and (Hooper 2001).

We have also tested the method of phase separation. This method allows to discriminate between membrane bound proteins (which would recruit into detergent phase) and cytosolic or cleaved proteins (which would recruit into aqueous phase). The platelets were treated with PIPLC and subsequently subjected to the phase separation. Thus, if PrPc was cleaved, it would recruit to aqueous phase. However, no PrPc was detected there confirming the resistance of the protein to PIPLC (Fig. 8). The weak PrPc bands in aqueous phase are attributed to contamination by detergent phase as they have the same density in both PIPLC treated and un-treated samples. The staining of cytoplasmic protein p42 proves that the contamination of detergent phase by aqueous phase was negligible.

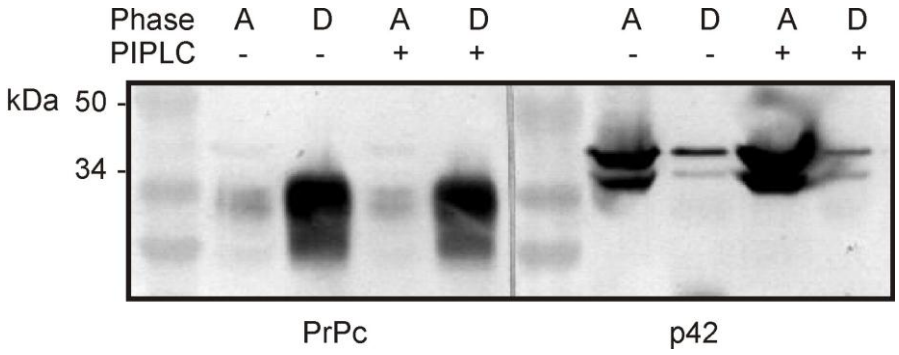


Figure 8. Phase separation with Triton X-114 confirms PrPc resistance to PIPLC. Lysed platelets were treated with 1 U/ml PIPLC for 1 hour. Then, samples were extracted with Triton X-114 and divided by step centrifugation to aqueous (A) and detergent (D) pools. Detergent phase thus recruit hydrophobic transmembrane and membrane anchored proteins whereas aqueous phase recruits water soluble cytosolic and cleaved proteins. Samples were resolved by 12% SDS-PAGE and developed with MAb 6H4 and MAb anti-CD45RA detecting platelet cytoplasmic protein p42.

GPI-anchor is also possible to cleave from the membrane with hydrofluoric acid (HF), which cleaves the anchor in different position then PIPLC with subsequent decreasing in molecular weight of the protein because of loosing of the sugar moiety as illustrated in Figure 7. In order to better discriminate the changes in molecular weight, the protein was deglycosylated by treatment with PNGaseF. HF treatment led to obvious decrease in PrPc molecular weight of control brain PrPc as well as of platelet PrPc (Fig. 9), confirming the presence of GPI-anchorage of PrPc.

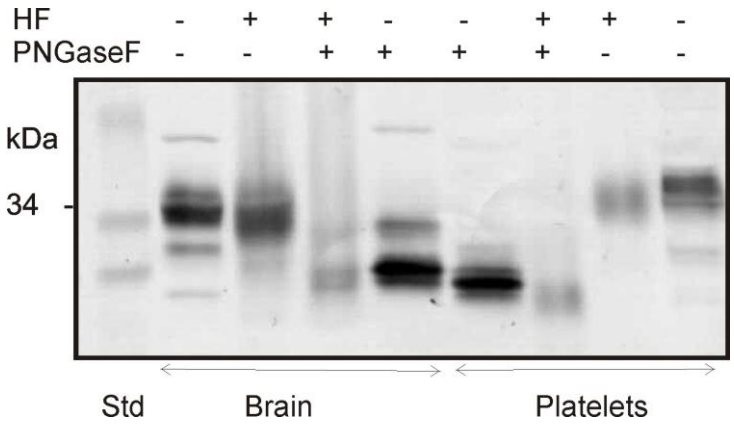
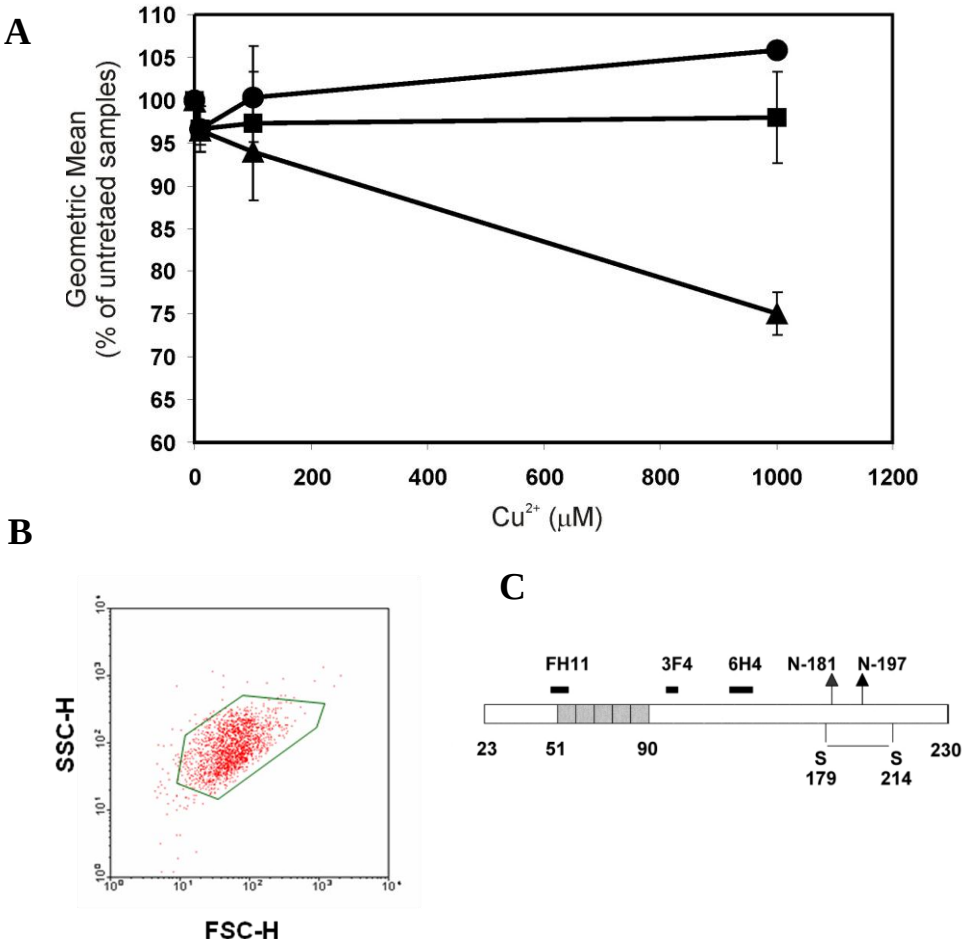


Figure 9. HF treatment proved that platelet PrPc is GPI-anchored protein.
Isolated platelets or 10% (w/w) brain homogenate were treated with 48% aqueous ice cold HF and part of the samples were subsequently deglycosylated with 10 U/ml of PNGase F. Samples were resolved by 12% SDS-PAGE and developed with MAb 6H4. Cleavage of GIP-anchor leads to decrease of protein molecular weight.

Influence of copper ions on platelet PrPc conformation

PrPc is known as a copper binding protein. To test the effect of copper binding to platelet PrPc, we incubated platelets with increasing concentration of copper ions for 30 minutes at room temperature. PrPc expression was measured by flow cytometry using MAbs targeted against different PrPc epitopes. The population of blood platelets for



analysis was gated as shown in Figure 10 B. 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. PrPc expression on the cytoplasmic membrane of copper treated platelets did not change in time as was shown by time course experiment (data not shown).

Figure 10. Copper decreases binding of anti-PrP MAb targeted against N-terminal region.
(A) Blood platelets were diluted to concentration 30×10^3/µl and incubated with increasing concentration of copper ions (CuSO4: 0, 10, 100 and 100 µM). Then platelets were labeled with PrP MAbs: FH11 (▲ epitope PrP 46-59), 3F4 (■ epitope PrP 109-114) and 6H4 (● epitope PrP 144-152) for 20 minutes (all diluted 1:100). Labeled platelets were washed and analyzed by a FACScan flow cytometer. The geometric mean of untreated platelets was set to 100%. 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 location of the epitopes of antibodies used.

Semiquantitative analysis of platelet and RBC PrPc

To compare PrPc quantity in RBC and platelets we performed western blot analysis of RBC ghosts and several dilutions of platelets (Fig. 11). In order to exclude the contamination with other cell types we counted cell numbers in each step of cell isolation. In final platelet lysate there was one contaminating WBC per 680 platelets and in final RBC ghosts suspension there was one contaminating WBC per 1400 RBCs. In a first set of experiments on 3 donors, one PLT contained 4 to 8 times more PrPc than one RBC ghost when measured with MAb 6H4 or 8 to 16 times more when measured with a mixture of PrP MAbs GE8, AG4 and AH6 (not shown). In the second set of experiments, PLT and RBC ghosts were prepared from blood of 5 separate donors and then pooled to obtain representative mixed PLT and RBC ghost samples. Densitometry analysis of blot developed with 6H4 confirmed that one PLT contains approximately 4 times more PrP^c than one RBC ghost.

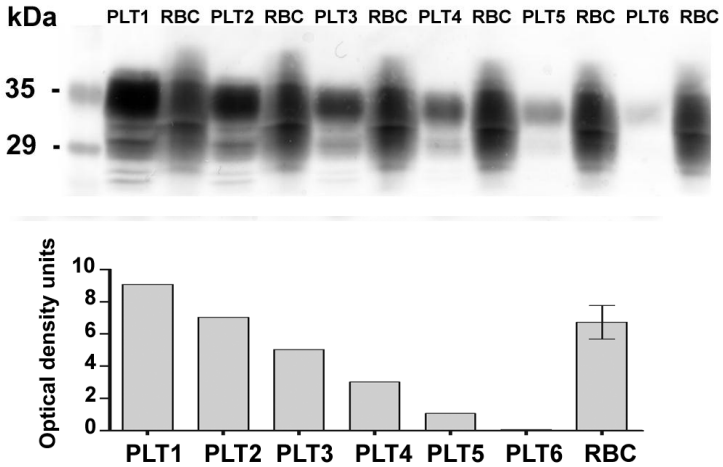
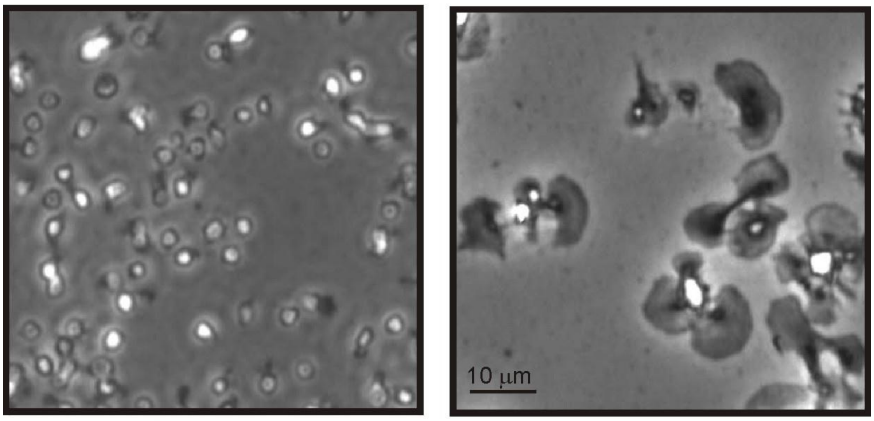


Figure 11. Semiquantitative analysis of platelet and RBC PrPc.
Western blot comparison of PrPc quantity in representative, standardized samples of RBC ghosts ($1x10^{10}$ RBC/ml) and platelets ($5x10^{9}$; $2.5x10^{9}$; $1.25x10^{9}$; $6.25x10^{8}$; $3.13x10^{8}$; $1.56x10^{8}$ PLT/ml). Samples were resolved by 12% SDS PAGE and developed with antibody 6H4. Densitometry of bands on blot demonstrate that RBC contain four time less PrPc then equal number of platelets.

Analysis of PrPc localization by fluorescence microscopy – method optimization

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. First, platelets were adhered onto slides coated with fibrinogen, what 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. Alternatively, platelets were activated in the suspension before centrifugation onto poly-L-lysine coated slides leading to sample with activated platelets but with slightly different appearance then adhered platelets. The differences in the morphology of resting or activated platelets are shown on Figure 12.



Resting **Activated**

Figure 12. Morphology of resting cytospined and activated adhered platelets.
Blood platelets were either centrifuged onto poly-L-lysine coated slides resulting in resting platelets or adhered onto fibrinogen coated slides resulting in activated platelets. Both were fixed with 1% PFA and observed using fluorescence microscope in phase contrast. Activated platelets are significantly bigger with distinct skirts.

The most critical step in sample preparation was the permeabilization and fixation of the platelets. This is the list of approaches that we have tested:

Table 6. Fixation and permeabilization agents tested for fluorescence observation of blood platelets.

Fixation	**Permeabilization**
1% PFA	Triton X-100 (0.05; 0.1; 0.2%)
1% PFA	Digitonin 0.1%
1% PFA	Triton X-100 0.1% → Methanol
Methanol → Acetone	
1% PFA	Methanol

The methodology using 1% PFA with subsequent permeabilization either with 0.1% Triton X-100 or 0.1% digitonin led again to almost undetectable signal of PrPc (data not shown). This might be because of masking of the epitope or dissolving of the membrane enfolding PrPc leading to washing out the protein. Neither decreasing (0.05%) nor increasing (0.2%) of Triton X-100 concentration led to better results. We have also modificated the protocol so that after fixation with PFA and permeabilization with Triton X-100 samples were stabilized with -20°C methanol either for 1 or 5 minutes. Unfortunately the loss of signal was still present. We have tested the widely used

methodology including 10 minutes treatment with methanol and subsequent 1 minute treatment with acetone. However, the signal of proteins after this protocol was unsatisfactory probably due to washing out of some pool of the proteins.

Finally, the most suitable methodology for our purpose was the platelet fixation with 1% PFA with subsequent methanol permeabilization as described in Methods section.

The immunolabeling protocol appeared to be no less complicated bottleneck. When only monoclonal antibody 3F4 was used for PrPc staining, the signal was very weak. It happens probably due to epitope (lysine) modification with PFA. Finally the mixture of 3F4 and 6H4 were found to provide satisfactory signal. Regarding secondary antibodies, we had to exclude Cy3-labeled antibodies, since they gave high level of background fluorescence. FITC-labeled anti-rabbit and TRITC-labeled anti-mouse immunoglobulin polyclonal antibodies proved as the best choice for our applications.

Immunolabeling of CD62P

CD62P (P-selectin) is a transmembrane protein which is present on the cytoplasmic membrane and in α-granules. It was previously shown that upon platelet activation it up regulates to cytoplasmic membrane along with PrPc (Holada, *et al* 1998). Therefore CD62P was selected as a control protein for our fluorescence study. The fluorescence pattern of CD62P in resting non permeabilized platelets is shown in Figure 13. The pattern of permeabilized platelets is very similar to that of non permeabilized, only some of the platelets have more intensive labeling in the central area. However, upon platelet activation is the fluorescence pattern of CD62P markedly changed with apparent labeling of formed "skirts". Moreover, in permeabilized platelets, there is intensive fluorescence in the central area of the platelet belonging most likely to the clustered organelles. These results demonstrated that the intracellular pool of CD62P was successfully labeled in activated platelets.

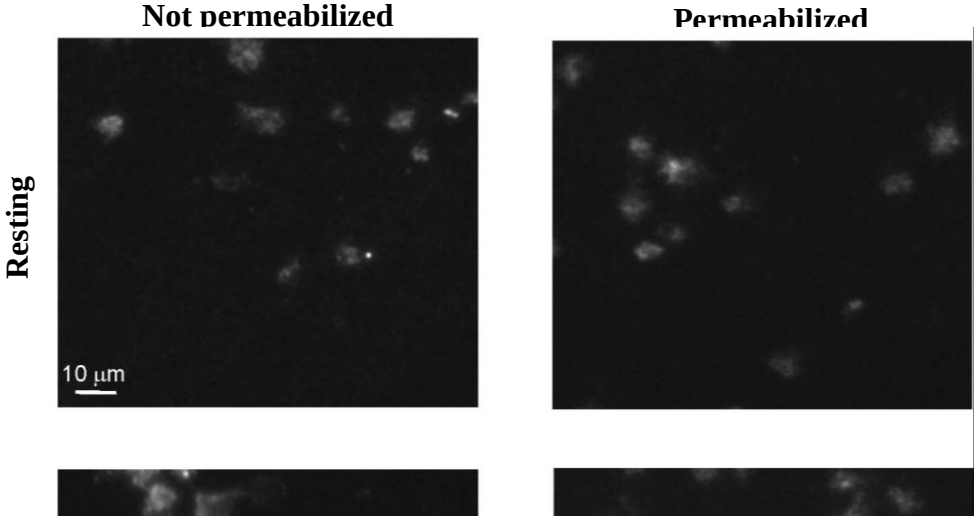


Figure 13. The influence of platelet status and permeabilization on CD62P labeling. Blood platelets were centrifuged onto poly-L-lysine coated slides either resting or activated with 20 μM TRAP. Platelets were fixed with 1% PFA and alternatively permeabilized with methanol (right panels). Samples were labeled with anti-CD62P antibody and FITC-labeled secondary antibody and observed with fluorescence microscope. The image is representative of two independent experiments.

Immunolabeling of CD62P and thrombospondin (TSP)

TSP is an α-granular 450 kDa glycoprotein which was chosen to further study our methodology of immunolabeling of organelles. Contrary to previously presented CD62P, TSP is not expressed on the membranes. To follow the fluorescence pattern of two α-granular protein, we performed the co-immunolabeling of CD62P and TSP. As shown in Figure 14 the colocalization was detected in the cluster in the central area of the resting cytospined platelets supporting our protocol. The same pattern was gained even for activated platelets (data not shown).

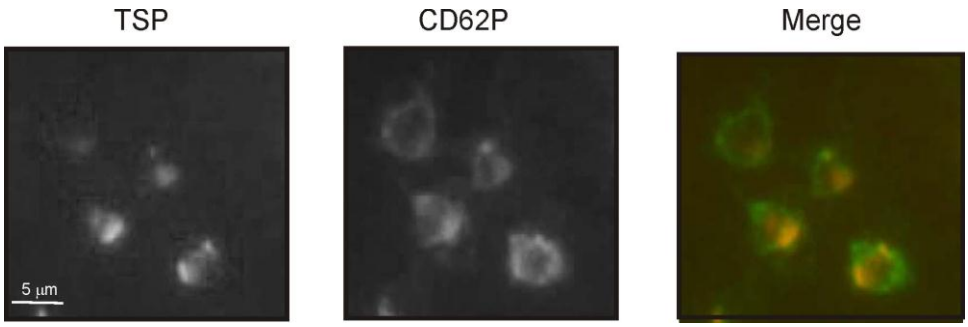


Figure 14. CD62P and thrombospondin (TSP) colocalize in blood platelets.
Blood platelets were centrifuged onto poly-L-lysine coated slides. Platelets were fixed with 1% PFA and permeabilized with methanol. Samples were labeled with anti-CD62P and anti-TSP antibodies and in next step with FITC- and TRITC-labeled secondary antibodies respectively. The image is representative of three independent experiments.

Immunolabeling of PrPc and CD62P

Labeling of resting permeabilized platelets with antibodies against PrPc and CD62P did not show any colocalization of these two proteins (Fig. 15, A).

Activation of platelets with 20 μM TRAP without subsequent permeabilization led to the same pattern of PrPc and CD62P as for resting platelets (Fig. 15, B). However, when activated platelets were permeabilized with methanol they displayed different pattern. While on the membrane area the signal of PrPc and CD62P was still predominantly separated, in the intracellular area both proteins were colocalized (Fig. 15, C). The colocalization appears to occur in the cluster in the central area of the platelets, which probably belongs to the clustered organelles of activated platelets.

The colocalization of PrPc and CD62P was observed also in the central area of platelets adhered onto fibrinogen (Fig. 15, D). Adhering of platelets onto fibrinogen allows achieving better resolution of membrane signals. So contrary to quite blurred signal of poly-L-lysine adhered platelets, here it is possible to observe separated signals of PrPc and CD62P (Fig. 16).

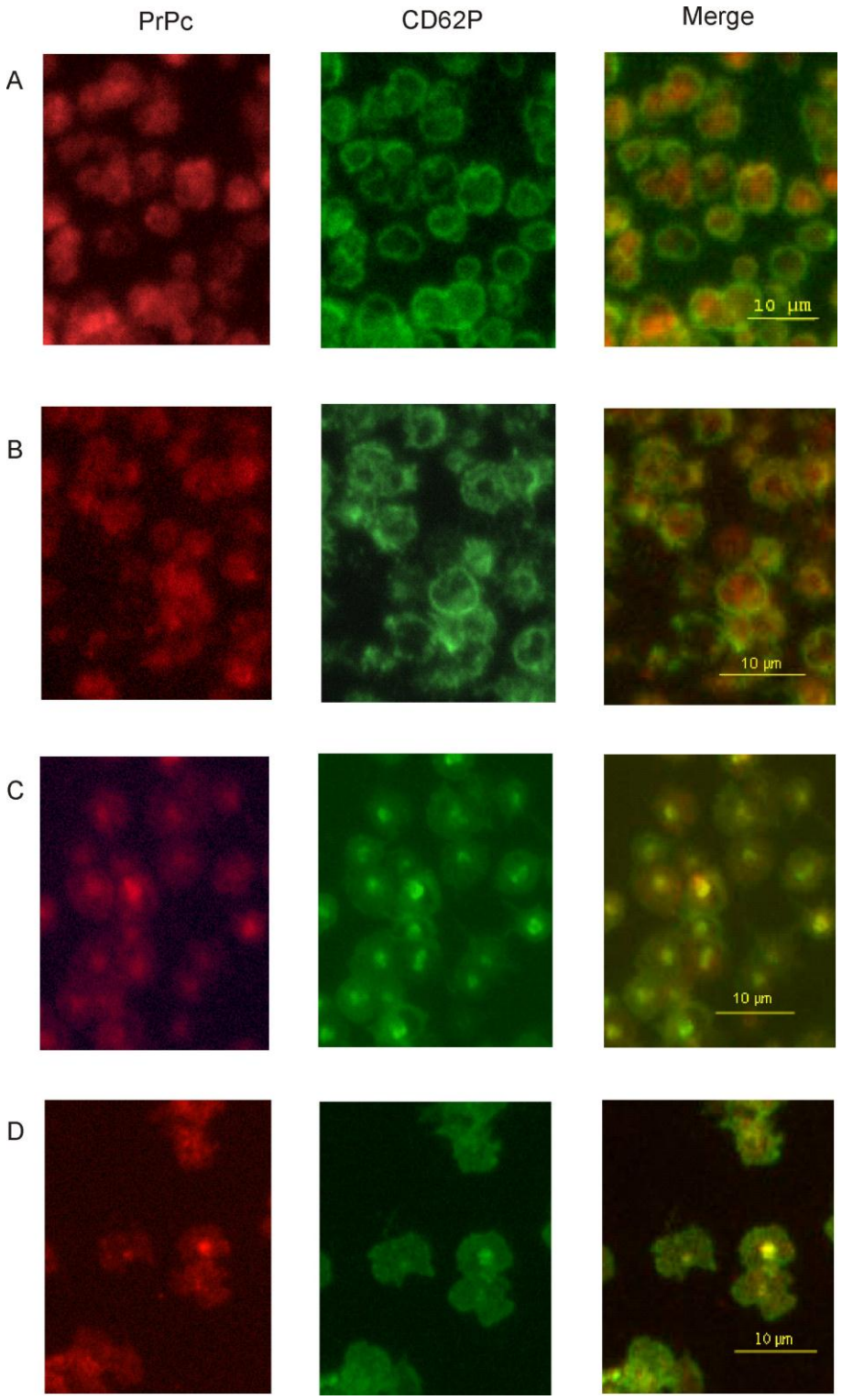


Figure 15. PrPc and CD62P colocalize in activated platelets.
Resting platelets (A) or platelets activated with TRAP (B, C) were deposited on poly-L-lysine coated slides. Alternatively platelets were adhered on fibrinogen coated slides that causes their activation (D). Platelets were fixed with 1% PFA (all panels) and permeabilized with methanol (A, C, D). In panel B are not-permeabilized platelets. 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.

The colocalization of PrPc and CD62P (yellow color) is obvious in organelles of activated permeabilized platelets but not on their membranes (C, D). The images are representative of two to four independent experiments.

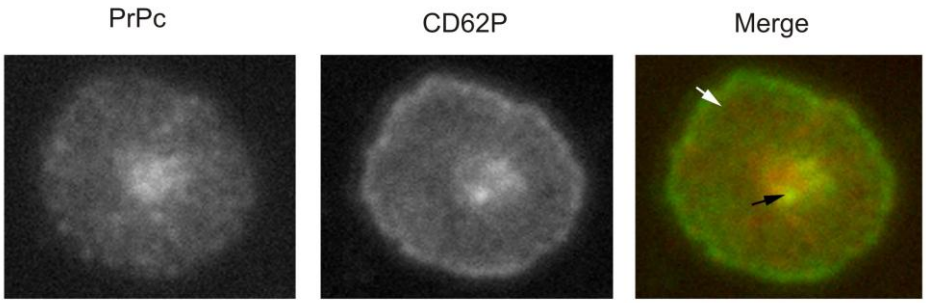


Figure 16. Demonstration of different labeling pattern of PrPc and CD62P on membrane area in fibrinogen adhered platelets.
Platelets were adhered on fibrinogen coated slides, fixed with 1% PFA and permeabilized with methanol. Slides were incubated with the mixture of anti-PrP MAbs 6H4 and 3F4 and anti-CD62P 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.

Immunolabeling of PrPc and actin

In the case of resting permeabilized platelets the signal of PrPc does not colocalize with signal of CD62P as shown above, although they are supposed to share the same platelet compartments. Our aim was to exclude the cytosolic localization of PrPc. For this purpose we performed co-labeling of PrPc and cytosolic actin. The proteins were not colocalized neither in resting nor in fibrinogen adhered platelets (Fig. 17) suggesting that PrPc detected in permeabilized resting platelets is either of cytosolic membrane or of organelle origin.

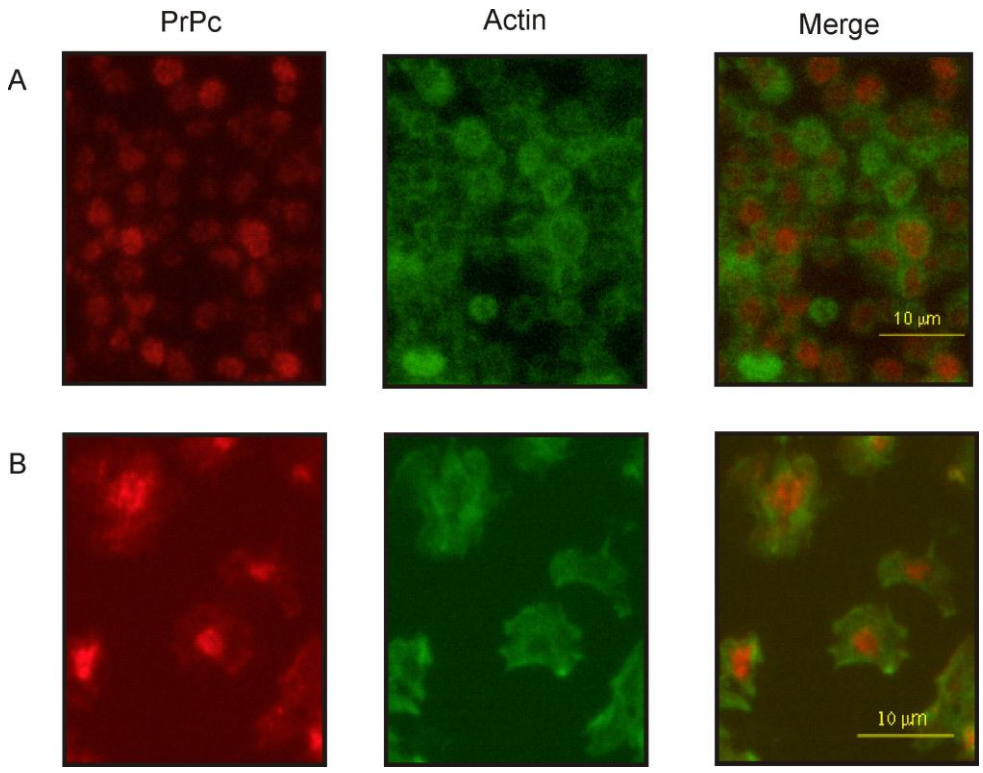


Figure 17. The exclusion of cytoplasmic localization of PrPc by co-labeling with actin. Platelets were deposited on poly-L-lysine coated slides (A) or adhered onto fibrinogen coated slides (B), fixed with 1% PFA and permeabilized with methanol. PrPc was visualized after incubation with the mixture of anti-PrP MAbs 6H4 and 3F4 and TRITC-coupled secondary antibody. Actin was visualized with anti-actin antibody and FITC-coupled secondary antibody. This figure demonstrates that PrPc and actin do not colocalize either in resting (A) or in activated (B) platelets and thus excludes the cytosolic localization of PrPc.

PrPc association with lipid rafts

NP-40 and Triton X-100 were used for rafts isolation at 4°C (Figure 18). Extraction was also performed with Brij 98 which has ability to preserve lipid rafts even under physiological temperature (Fig. 18, C). The lay out in panel D shows the sucrose gradient used for most of the experiments with except of those with step gradient. All detergents used led to flotation of PrPc as well as GPI-anchored protein CD59 and transmembrane protein CD36 to the interface between the 5 and 30% sucrose. As expected CD62P was not recruited to raft fractions under any conditions used. The identity of rafts was confirmed also by the presence of protein kinase Fyn (Ezumi, *et al* 2002). Inherent imprecision in the collection of the aliquots can occasionally lead to contamination of the lower, non-raft fractions (e.g. fraction 4) with traces of raft proteins. The amount of PrPc recovered in rafts

was relatively low regardless of the detergent used for platelet solubilization, approximately 5-10% of total PrPc in the case of Triton X-100 solubilization which was used in all subsequent experiments of this study. Platelet raft markers were found to float in higher proportions: 25% for CD59, 20% for CD36.

To compare membrane raft distribution of platelet PrPc to RBC PrPc we performed also flotation assay of RBC ghost (Fig. 19). Interestingly, more PrPc was recruited to lipid rafts when ghosts were lysed with Triton X-100 contrary to platelet PrPc, which was more enriched in rafts when platelets were lysed with NP-40. It suggests that platelet PrPc containing lipid rafts differ from RBC PrPc containing lipid rafts.

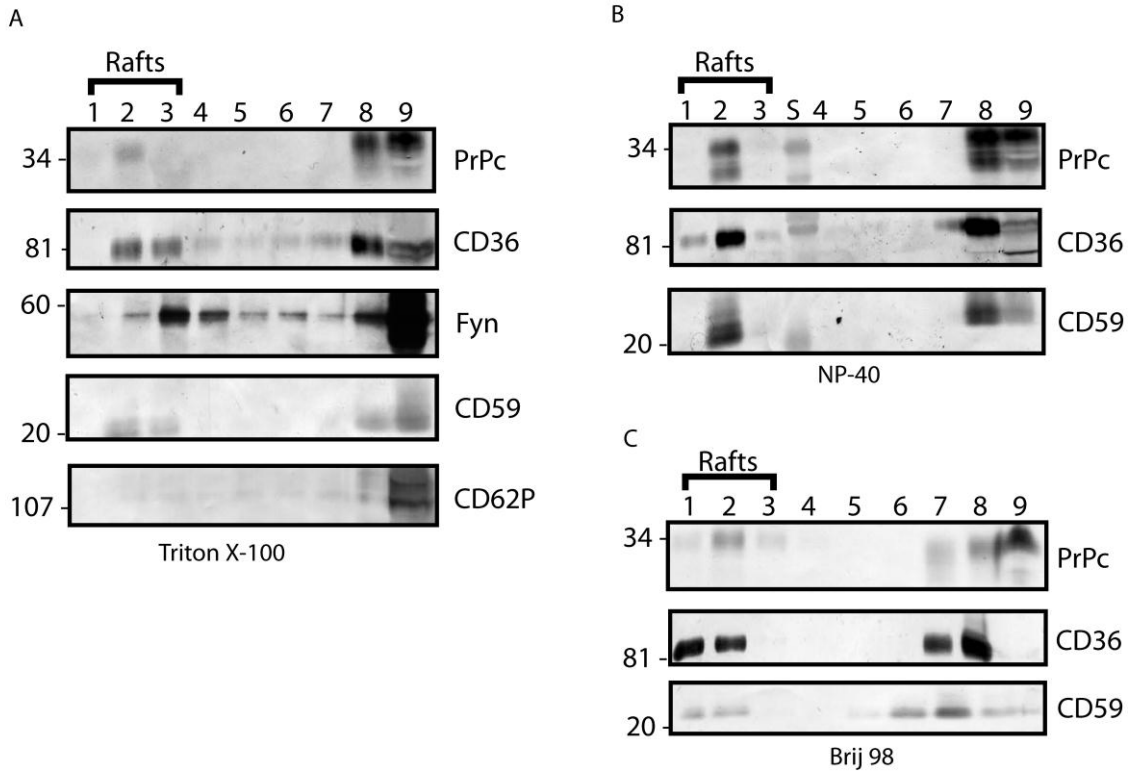


Figure 18. PrPc in blood platelets associates with lipid rafts.
Resting platelets were lysed in 1% Triton X-100 or 1% NP-40 at 4°C (A and B) or in 1% Brij 98 at 37°C (C) and then subjected to flotation assay in sucrose gradient. The proteins from nine fractions of sucrose gradient were analyzed by SDS 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.

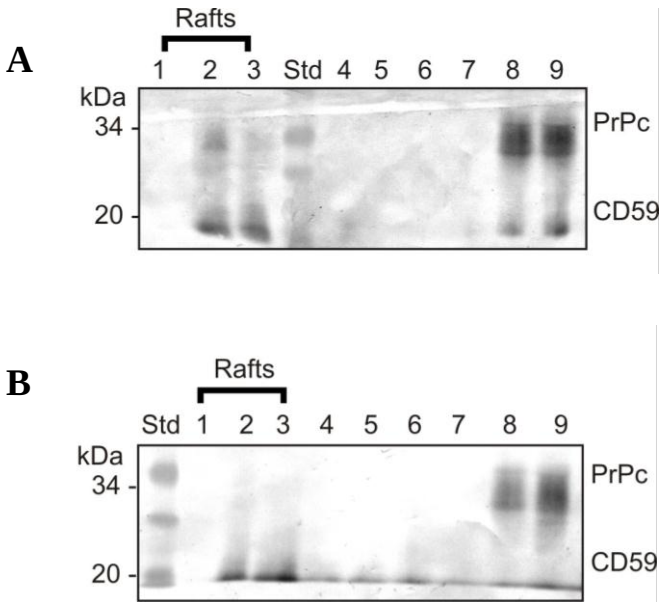


Figure 19. RBC PrPc resides in different lipid rafts then platelet PrPc.
RBC ghosts were isolated to final concentration 1×10^{10}/ml, lysed in 1% Triton X-100 (A) or 1% NP-40 (B) at 4°C and then subjected to flotation assay in sucrose gradient. The proteins from nine fractions of sucrose gradient were analyzed by SDS PAGE, blotted and developed with MAb 6H4 and anti-CD59. 1 – top of the gradient, 9 – bottom of the gradient, Std – molecular weight standard. The blots are representative of two independent experiments.

Flotation assay on sucrose step gradient

In several studies the floatation assay was performed on sucrose step gradient in order to more precisely separate lipid rafts with different densities as well as to reveal cytoskeleton bound rafts. We have tested this approach, however we have not found any difference from standard protocol (Fig. 20). Here, only samples from resting platelets are shown, since flotation assay of activated platelets gave comparable results. To follow the actin distribution in step gradient, blots were also developed with anti-actin antibody, showing that all actin remains in the bottom fractions (referred to 8 and 9). The result was the same even for samples treated with detergent NP-40 (data not shown).

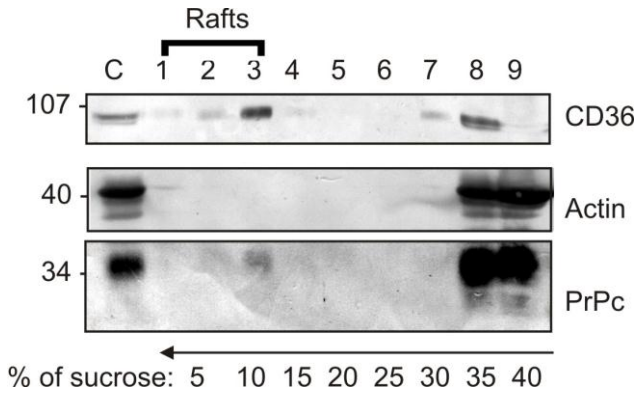


Figure 20. Flotation assay on step gradient does not reveal the presence of actin in raft fractions.
Lipid rafts were isolated using 1% Triton X-100 by flotation assay on step sucrose gradient. The proteins from nine subsequent fractions were analyzed by SDS PAGE, blotted and developed with MAb 6H4 against PrPc, anti-actin and anti-CD36. 1 – top of the gradient, 9 – bottom of the gradient, Std – molecular weight standard, C – whole platelet lysate. The blot is representative of two independent experiments.

PrPc containing lipid rafts disintegrate at 37°C and are cholesterol dependent

In standard *in vitro* protocol, lipid rafts are insoluble in Triton X-100 and NP-40 at low temperature (4°C), but an increase of the temperature to 37°C leads to rafts solubilization. No PrPc or platelet raft markers were found to float when raft extraction was accomplished at 37°C (Fig. 21).

To investigate the importance of cholesterol in the maintenance of proteins association with lipid rafts we performed its depletion with both saponin and methyl-β-cyclodextrin (MβCD). The buoyancies of CD36, CD59 as well as PrPc in flotation assay were totally abolished by saponin treatment (Fig. 21, C). The same results were obtained even with lower (0.01%) or higher (0.2%) concentration of saponin (data not shown).

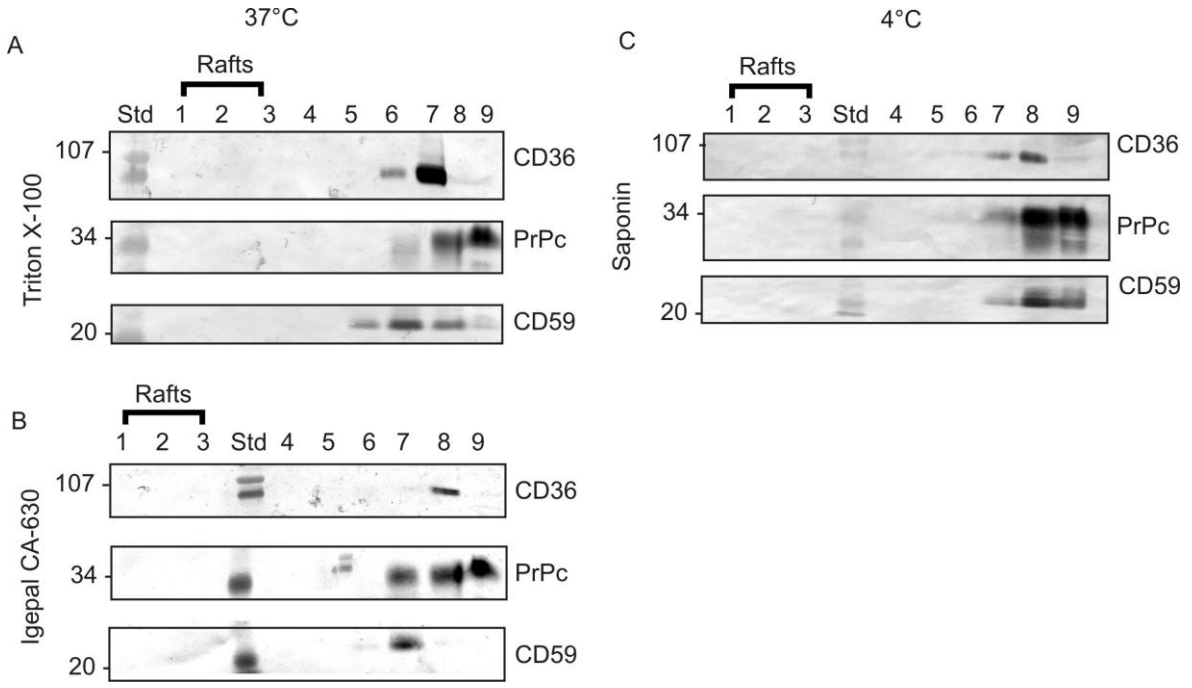


Figure 21. PrPc association with lipid rafts is temperature and cholesterol dependent. Resting platelets were lysed in 1% Triton X-100 or 1% NP-40 at 37°C (A and B) and then subjected to flotation assay in sucrose gradient. Increased temperature or depletion of cholesterol by treatment with 0.05% saponin (C) prior to lysis with Triton X-100 totally abolished flotation of the proteins. The proteins from nine subsequent fractions of sucrose gradient 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. The blots are representative of three independent experiments.

Similarly to saponin treatment, the use of 20 mM MβCD decreased the buoyancy of PrPc. As a control protein we used in this experiment raft associated protein Fyn, which was depleted from rafts as well (Fig. 22). A relatively high content of PrPc in rafts in comparison to non-raft fractions is caused by refraining from including insoluble pellets on the bottom of the tubes into fraction 9 in this set of experiments. Dodecyl maltoside is detergent known to solubilize the rafts even at 4°C (Brdicka, *et al* 2000). 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 (Fig. 22).

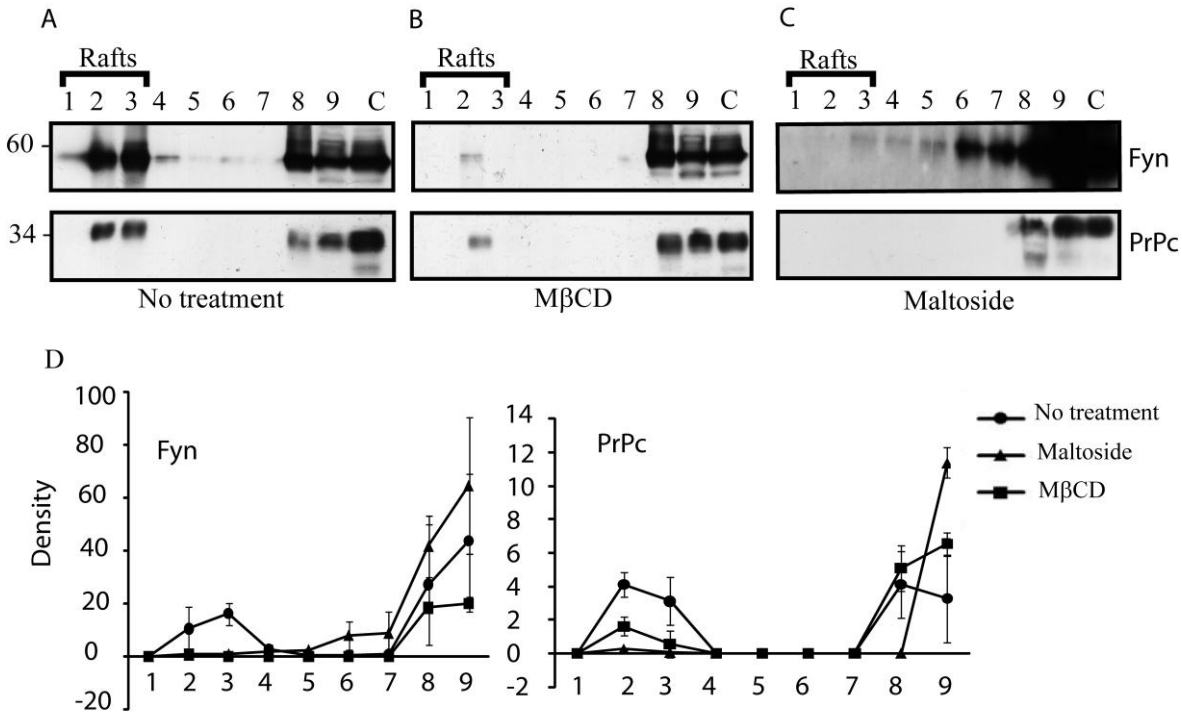


Figure 22. Platelet lipid rafts containing PrPc are sensitive to cholesterol depletion with MβCD, and can be solubilized with dodecyl maltoside.
(A) Lipid rafts were isolated from the platelet, lysed with 1% Triton X-100 at 4°C. (B) In order to deplete cholesterol, platelets were incubated with 20 mM 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.

Platelet activation does not influence PrPc incorporation into lipid rafts.

The distribution of PrPc, CD59 and CD36 into rafts was followed both in resting and activated platelets. However, no significant effect of platelet activation on the distribution into rafts was observed for PrPc as well as for the other proteins (Fig. 23).

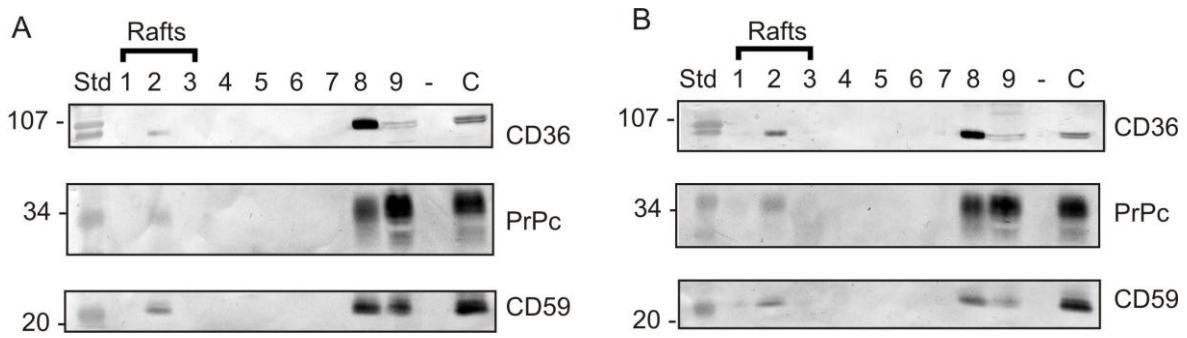


Figure 23. Platelet activation does not change the amount of raft associated PrPc. Lipid rafts were isolated using 1% Triton X-100 both from resting (A) and thrombin activated (B) platelets. The proteins from nine subsequent fractions were analyzed by SDS PAGE, blotted and developed with MAb 6H4 against 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 representative of three independent experiments.

Majority of PrPc in platelets is associated with platelet cytoskeleton

In order to elucidate the possible interaction of PrPc with platelet cytoskeleton we aimed to isolate both actin cytoskeleton and membrane skeleton and follow the distribution of PrPc by western blotting.

Actin cytoskeleton might be isolated as a low speed pellet (here designated as P1) after cell lysis with ice cold 1% Triton X-100. Platelet activation is known to lead to enrichment in actin in P1 as shown on Coomassie-stained polyacrylamide gel and western blot in Figure 24. As mentioned in the Methods section, platelets were not chilled before lysis as this influence the actin assembly as was confirmed by inhibition of actin increase in P1 (data not shown). Membrane skeleton was isolated as a high speed pellet (here designated as P2) by ultracentrifugation of the supernatant after P1 isolation.

Western blots revealed that the relative quantity of PrPc in the cytoskeleton pellet P1 increased from approximately 40% to 60% after platelet activation. This increase was accompanied by a significant decrease of PrPc in the membrane skeleton pellet (P2). The effect of platelet activation on protein distribution was similar either with 20 μM TRAP or 0.1 U/ml thrombin (data not shown). A portion of PrPc representing the soluble protein not associated with the cytoskeleton was detected in the supernatant (S2) after centrifugation of the membrane skeleton. The amount of PrPc in this fraction showed a tendency to decrease from approximately 21% to 14% after platelet activation, but this decrease was not statistically significant. In a control experiment, the lack of staining of protein p42

confirmed that the cytoskeleton was not contaminated with cytoplasm, as the protein remained in the supernatant fraction (S) (Fig. 24, C).

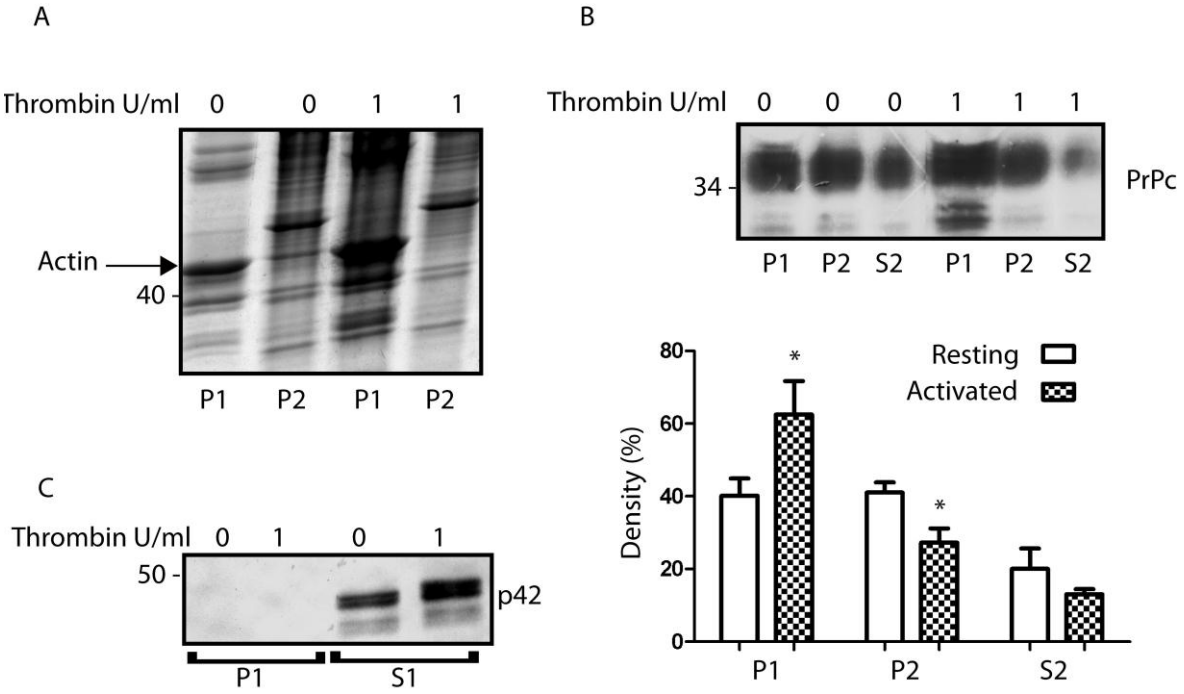


Figure 24. PrPc associates with the platelet cytoskeleton.
The actin cytoskeleton (P1) was isolated from resting or thrombin (1 U/ml) stimulated platelets as a detergent resistant pool after solubilization with 1% Triton X-100. The membrane skeleton (P2) was further isolated by high speed centrifugation of the supernatant (S1) after sedimentation of the actin cytoskeleton. (A) Coomassie brilliant blue stained gel of the actin cytoskeleton and membrane skeleton shows an increase of the actin band in P1 upon platelet activation. (B) Western blot of cytoskeleton fractions P1, P2, and supernatant S2 after sedimentation of P2, from either resting or activated platelets, was developed with 6H4 against PrPc. Data in the bar graph represent relative mean optical density of bands in percentages ± SD, when the cumulative density of P1, P2, and S2 was assigned 100% (* $p<0.05$, $n = 3$). (C) The actin cytoskeleton (P1) and supernatant (S1) were analyzed for the presence of platelet cytosolic marker p42 to exclude P1 contamination by cytoplasm. The blots are representative of three independent experiments. A major part of PrPc containing rafts is associated with cytoskeleton.

To determine whether some part of raft associated PrPc is bound to platelet cytoskeleton we used a protocol shown on the schema (Fig. 25). The first arm of the scheme (A) represents the "classic" flotation assay as described in Figure 18. Next, the flotation assay was performed upon actin depolymerization in whole solubilized resting or

activated platelets (B). Finally, the cytoskeleton isolated from platelets was depolymerized and used as a source material for the flotation assay (C).

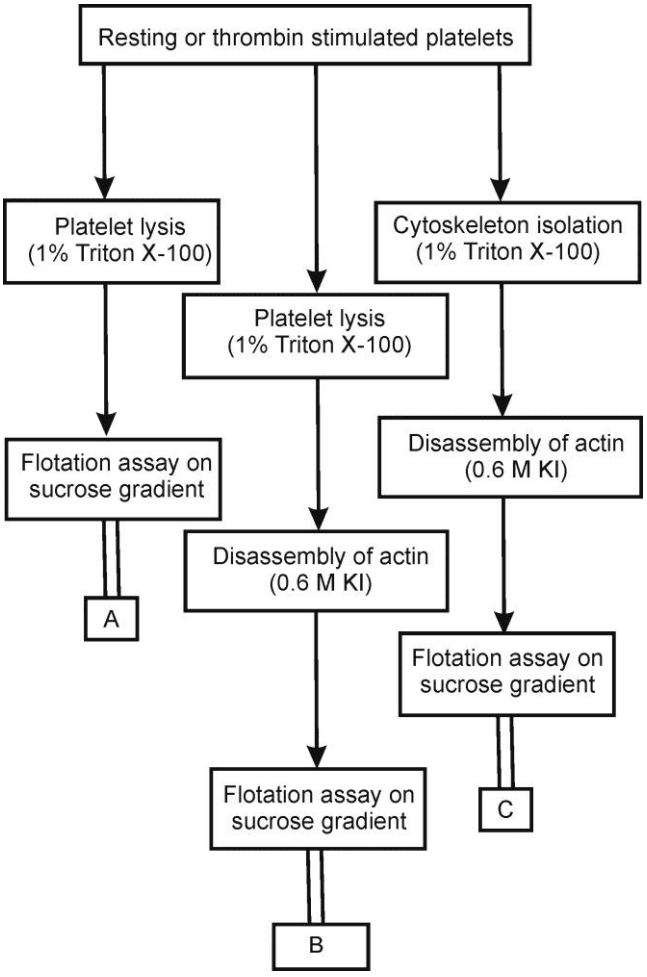


Figure 25. The schema of different approach in lipid rafts isolation.
A – Flotation assay of whole platelet lysate. B – Flotation assay of whole platelet lysate upon actin disassembly with 0.6 M KI. C - Flotation assay performed with isolated platelet cytoskeleton.

Cytoskeleton depolymerization led to significant up regulation of both PrPc and control proteins CD36 and CD59 in raft fractions (Fig. 26, right panel). Interestingly, a portion of PrPc remained in non-raft fractions 8 and 9 even after actin depolymerization, while the amount of CD36 and CD59 in those fractions was negligible. It suggests that this pool of PrPc is not raft associated. Actin depolymerization led to a similar increase of the monitored proteins in rafts isolated from both resting and activated platelets (right panels). A bar graph (Fig. 26, B) shows densitometry analysis of Western blot results. The data are

presented as a percentage of the density of bands in the control (C) whole-platelet lysate (the details are described in the Methods section). The amount of CD59 in rafts isolated from activated depolymerized platelets seemed to be higher than in depolymerized resting platelets; however, Student`s t-test confirmed that the difference between resting and activated platelets (KI treated or untreated) did not reach significance for any studied protein. On the other hand, significant differences were found between KI untreated and treated samples ($p<0.05$, $n=3$).

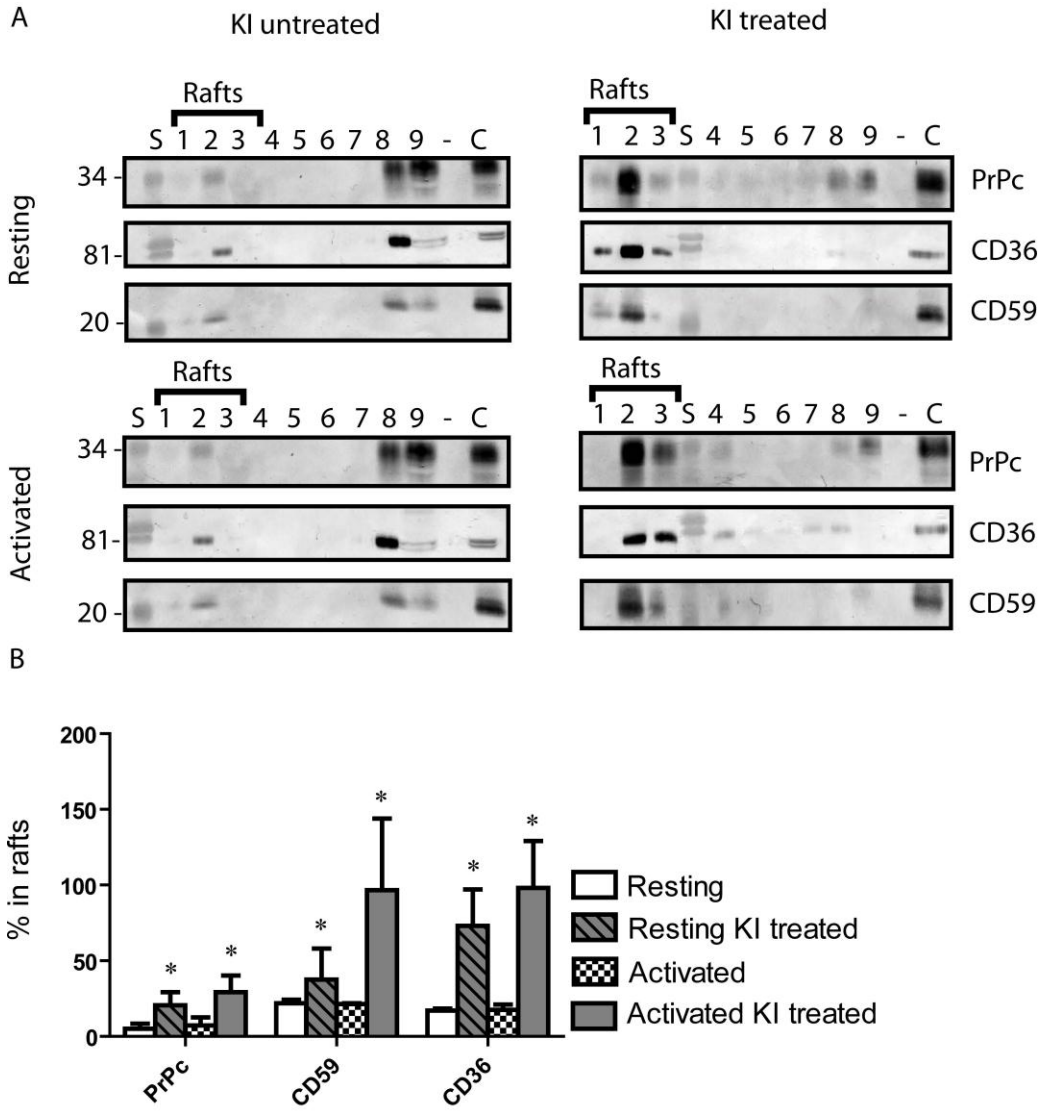


Figure 26. The majority of PrPc containing lipid rafts associate with platelet cytoskeleton.

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 three independent experiments.

To further confirm that part of raft PrPc is linked to the platelet cytoskeleton, we isolated lipid rafts from the depolymerized P1 pellet (described above), both from resting and activated platelets. Almost no PrPc or CD59 was detected in rafts isolated from the actin cytoskeleton of resting platelets (Fig. 27, A); however, silver stained gels confirmed no loss of the proteins during cytoskeleton isolation (data not shown). This suggests that rafts containing PrPc and CD59 in resting platelets might have been "lost" as membrane skeleton bound, since membrane skeleton is not recruited to the cytoskeleton pellet of resting platelets. In opposition to PrPc and CD59, transmembrane protein CD36 was visibly present in rafts isolated from the P1 pellet of resting platelets, albeit at a relatively low quantity. The situation dramatically changed after platelet activation. Rafts containing PrPc as well as CD59 and CD36 were clearly present in the cytoskeleton pellet P1 isolated from activated platelets (Fig. 27, B). Isolation of lipid rafts from the cytoskeleton also revealed a cytoskeleton associated pool of PrPc, which is not associated with rafts (fraction 9 in Fig. 27, B).

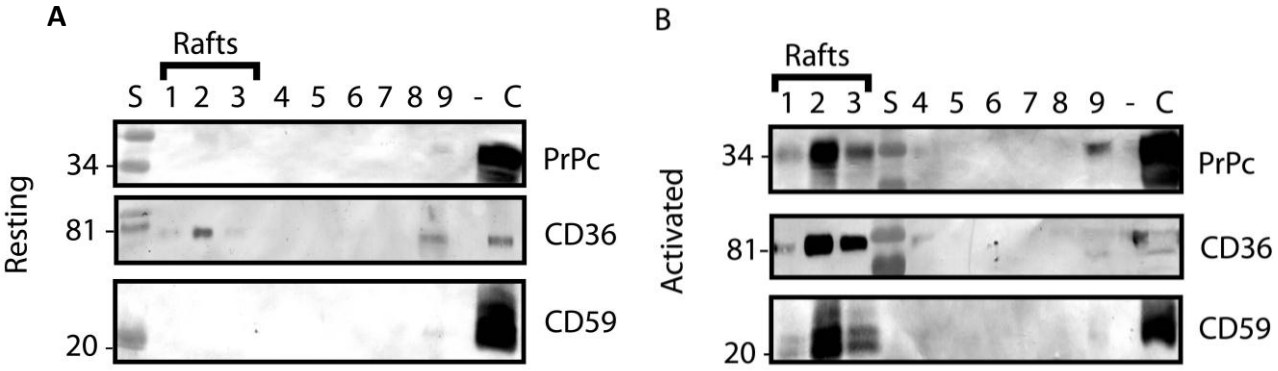


Figure 27. Isolation of lipid rafts from the platelet actin cytoskeleton.
Lipid rafts were isolated from the actin cytoskeleton P1 of resting (A) or thrombin (1 U / ml) activated platelets (B). Cytoskeleton pellets were treated with 0.6 M KI for 30 min at 4°C to depolymerize actin filaments and then subjected to flotation assay. Proteins from nine collected 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, S - molecular weight standard, C - whole platelet lysate. Platelet activation dramatically increased the amount of PrPc and raft markers CD36 and CD59 in rafts isolated from the platelet actin cytoskeleton pellet P1. The blots are representative of three independent experiments.

The study on PrPc involvement in platelet physiological function

The influence of PrPc engagement with anti-PrP antibodies on proteins phosphorylation

In order to follow the effect of anti-PrP antibodies on platelet signaling events, we stimulated platelets with MAbs 3F4 and SAF32 directed against epitopes 106-112 and 79-89 respectively. Subsequently the tyrosine phosphorylation was detected on western blots. As shown in Figure 28, thrombin (10 U/ml) activated platelets, which served as a positive control, exhibited increase in tyrosine phosphorylation comparing to non stimulated platelets (line 1 and 2). Binding of MAb 3F4 led to the phosphorylation of "double band" protein (shown by arrow), which might be also found upon stimulation with thrombin. When platelets were stimulated with 3F4 with subsequent thrombin treatment, the same band has appeared. Contrary, MAb SAF32 neither mouse IgGs did not cause phosphorylation of this protein. However no additional and specific tyrosine phosphorylation was detected. In the lines of 3F4 stimulated platelets there is also stronger phosphorylation in the molecular weights higher than 70 kDa, however this can be seen also in platelets incubated with control mouse IgGs. Comparable results were obtained using MAb 6H4 (not shown).

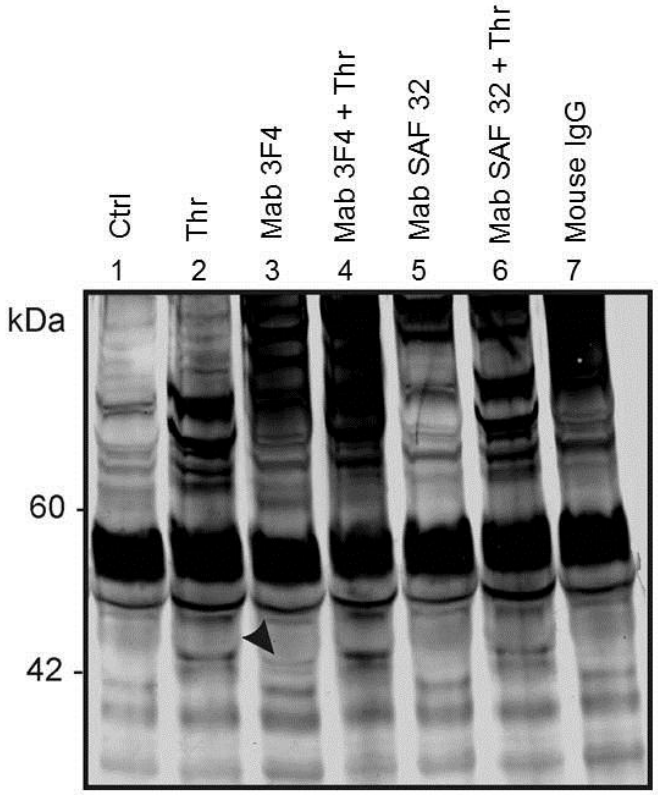


Figure 28. The influence of PrPc engagement on tyrosine phosphorylation in platelets. Platelets were stimulated with thrombin (Thr) as a positive control, with MAbs 3F4 or SAF32, with the same MAbs and subsequently with thrombin and with mouse IgGs as a negative control. Non stimulated platelets are referred to Ctrl in the figure. Stimulation was terminated with pre-heated sample buffer and samples were resolved by 12% SDS-PAGE and developed with anti-phosphotyrosin antibody. The arrow points to band of phosphorylated protein, which is also present upon thrombin stimulation but not upon SAF32 stimulation. The blot is representative of three independent experiments.

In order to enhance the effect of platelet stimulation via PrPc molecules, PrPc was cross-linked by stepwise binding of primary and secondary antibody. As a secondary antibody we used goat F(ab`)2 anti-mouse IgG`s (GAM). Negative controls could be found in line 1 (the unstimulated platelets) and in line 9 (platelets with DMSO). In lines 2 and 3 there are positive controls, i.e. platelets stimulated either with thrombin (10 U/ml) or with collagen (190 μg/ml). It is obvious that comparing to unstimulated platelet the phosphorylation of platelet proteins is higher in stimulated platelets and slightly higher in collagen activated platelets. As a primary anti-PrP antibody we used 3F4 or SAF-32; the results for the latter are not shown, however they were comparable to that of 3F4 stimulated platelets. As could be seen from Figure 29., the extent of phosphorylation is

almost comparable between platelets stimulated only with anti-PrP antibody (line 4) and platelets where PrPc was cross-linked (line 6). The huge band bellow 150 kDa in case of cross-linked sample (red asterisk) could be seen also in contiguous line, where only GAM was added as well as in line 11 (cross-linked mouse IgGs), so it has got no relevance and probably comes from non-specific detection of GAM. The intensive smeared band (yellow asterisk) above 150 kDa in line were 3F4 stimulated platelet were loaded (line 4) is not specific as well, since it can be observed also when binding of anti-PrP antibody was blocked with peptide PrP 102-114 as well as in mouse IgGs stimulated platelets (line 10). Two last lines in the figure show, that the phosphorylation is in some extent increased also when platelets were incubated with mouse IgGs and when mouse IgGs were cross-linked with the GAM.

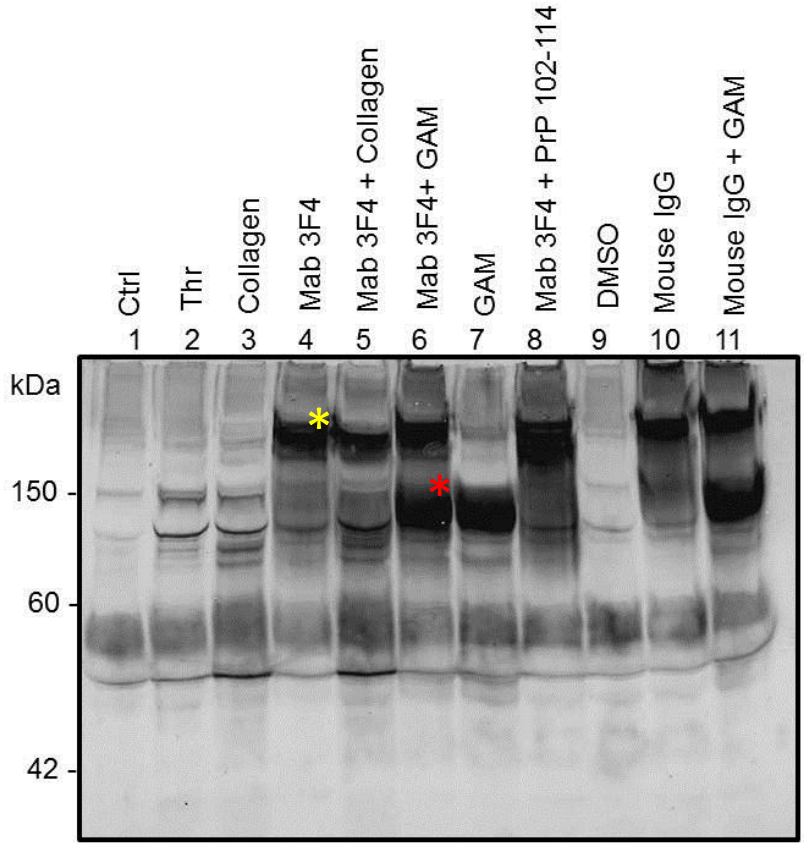


Figure 29. PrPc cross-linking does not have any specific effect on protein phosphorylation in blood platelets.
Platelets were stimulated with thrombin (Thr) and collagen as positive controls. Non stimulated platelets are referred to Ctrl (line 1) and to DMSO treated platelets (line 9). In line 4 there are platelets incubated with anti-PrP antibody 3F4, in next line incubation with MAb was followed with collagen stimulation. The effect of PrPc cross-linking is presented in line 6, as a negative control serves line 7 and 11. The specific effect of 3F4 antibody was

checked by its blocking with peptide PrP 102-114 (line 8). Stimulation was terminated with pre-heated sample buffer and samples were resolved by 12% SDS-PAGE and developed with anti-phosphotyrosin antibody. The meanings of asterisks are explained in the text. The blot is representative of two independent experiments.

PrPc engagement with anti-PrP antibodies does not influence platelet aggregation

The physiological function of platelets called the aggregation and the effects of different agonists on this function is possible to study *in vitro*. The Figure 30. presents the results from platelets incubation with MAb GE8 (PrP epitope 183-191). As obvious, upon addition of collagen to PRP, a lag phase occurs followed with a large, single wave of aggregation. To follow the effect of PrPc engagement, the platelets were incubated with anti-PrP antibody prior to stimulation with collagen. The green line shows the aggregation response when platelets were incubated with 1:100 diluted anti-PrP MAb GE8 prior to stimulation with collagen and it is obvious that aggregation was not influenced by such treatment. The blue line shows the aggregation response of platelets incubated with GE8, which was subsequently cross-linked with GAM and then stimulated with collagen, and again, no difference comparing to collagen treated platelets was observed.

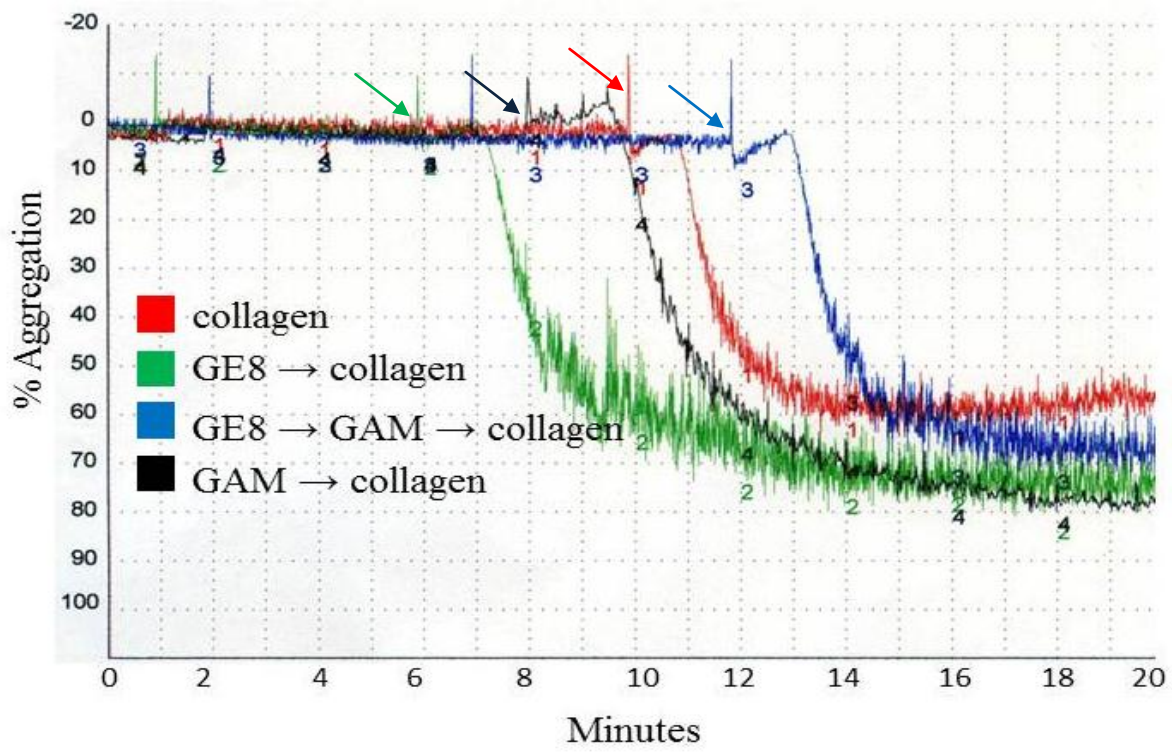


Figure 30. Anti-PrP MAb GE8 does not influence collagen induced platelet aggregation.
Platelets were aggregated with 100 µg/ml collagen (red line). In tested sample, platelets were incubated with anti-PrP MAb GE8 before stimulation with collagen (green line). In order to test the effect of PrPc crosslinking, goat $F(ab`)_2$ anti-mouse IgG`s (GAM) was added after the incubation with GE8 (blue line). As a negative control GAM was used by itself (black line). In all samples, the incubation with appropriate combinations of antibodies was followed with induction of aggregation with collagen. The arrows denote the addition of collagen. This is the representative tracing from three separate experiments.

Platelet aggregation was also performed with combination of agonists – 10 µM ADP and 2 µM EPI. This combination leads to slightly different aggregation wave comparing to that of induced by collagen. It can be seen in Figure 31. B (red line) – a large single wave without lag phase and with approximately 70% aggregation. In Figure 31. A, three days old blood platelets were used, therefore the aggregation response is lower. The green lines show the aggregation response when platelets were incubated with 1:100 diluted anti-PrP MAbs GE8 (A) or with SAF 32 (PrP epitope 79-89) (B) prior to stimulation with ADP/EPI. No effect of anti-PrP MAbs was observed. Neither cross-linking of PrPc molecules changed aggregation response (black lines). The results were comparable for other MAbs tested – 3F4 (Fig. 32) and AH6 (not shown).

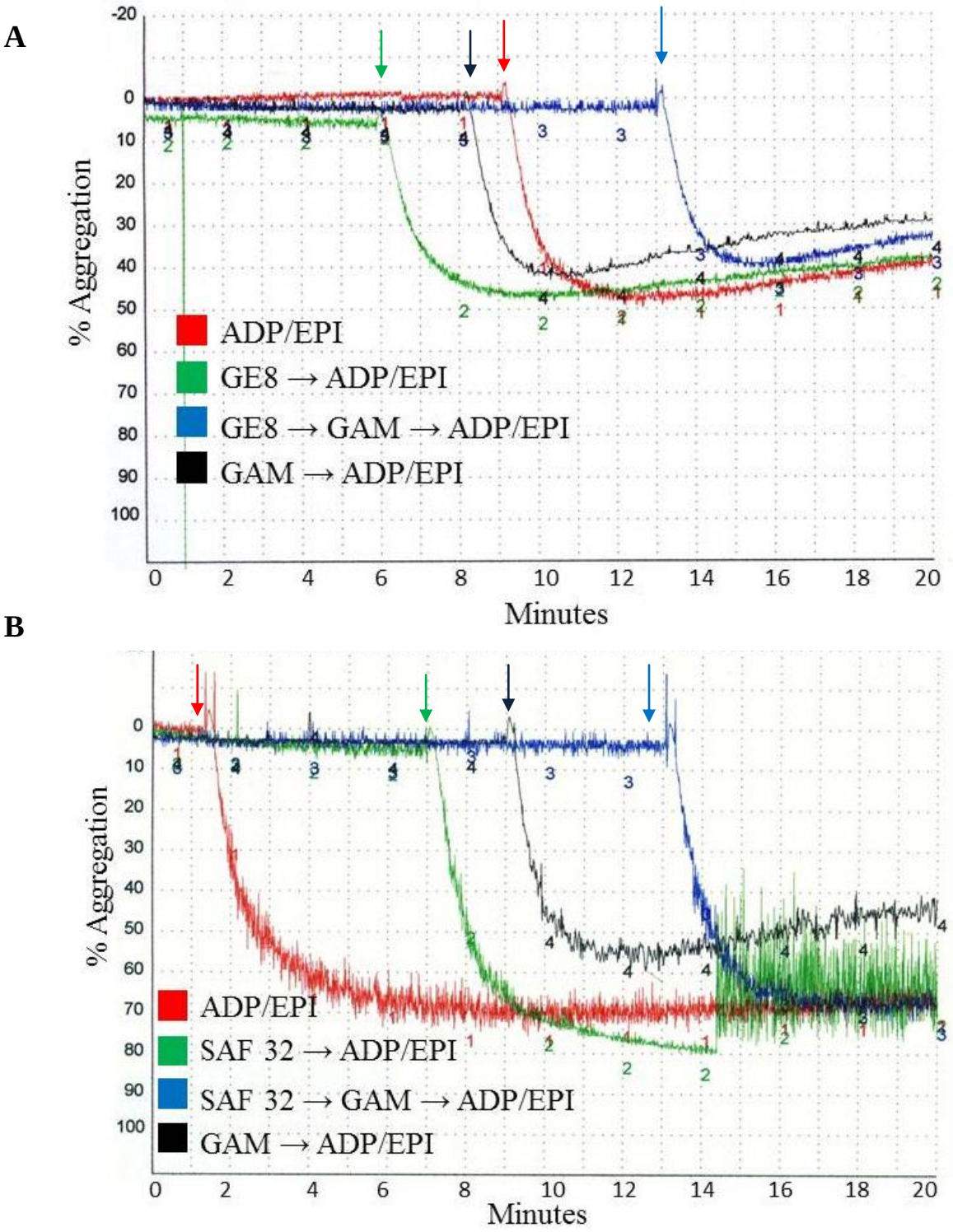


Figure 31. Anti-PrP MAbs GE8 and SAF 32 do not influence ADP/EPI induced platelet aggregation.

Platelets were aggregated with 10 µM ADP and 2 µM epinephrine (EPI) (red lines). In samples illustrated by green lines platelets were incubated with either anti-PrP MAb GE8 (A) or with anti-PrP MAb SAF 32 (B) and subsequently stimulated with ADP/EPI. In order to test the effect of PrPc crosslinking, goat F(ab`)$_2$ anti-mouse IgG`s (GAM) was added following the incubation with GE8 (blue lines). As a negative control platelets were incubated with GAM and subsequently aggregated (black lines). The arrows denote the

addition of ADP/EPI. Representative tracings from three separate experiments are shown in each panel.

In the Figure 32., the protocol was slightly modificated. Red line still represent platelets aggregated with 10 µM ADP and 2 µM EPI. Green line illustrates that neither incubation of platelets with anti-PrP MAb 3F4 (PrP epitope 106-112) caused any change in aggregation response. Blue line represents platelets, which were firstly stimulated with 10 µM ADP, which led to 20% aggregation, and 2 µM EPI was added after another 7 minutes (illustrated by arrows). Next, platelets were supplemented with MAb 3F4 simultaneously with ADP, they were left for 7 minutes to follow possible disaggregation effect and then EPI was added to check the ability of platelets to response (black line). Neither in such circumstances was any effect of anti-PrP MAb revealed. The same experiment with the same result was performed with GE8 (not shown).

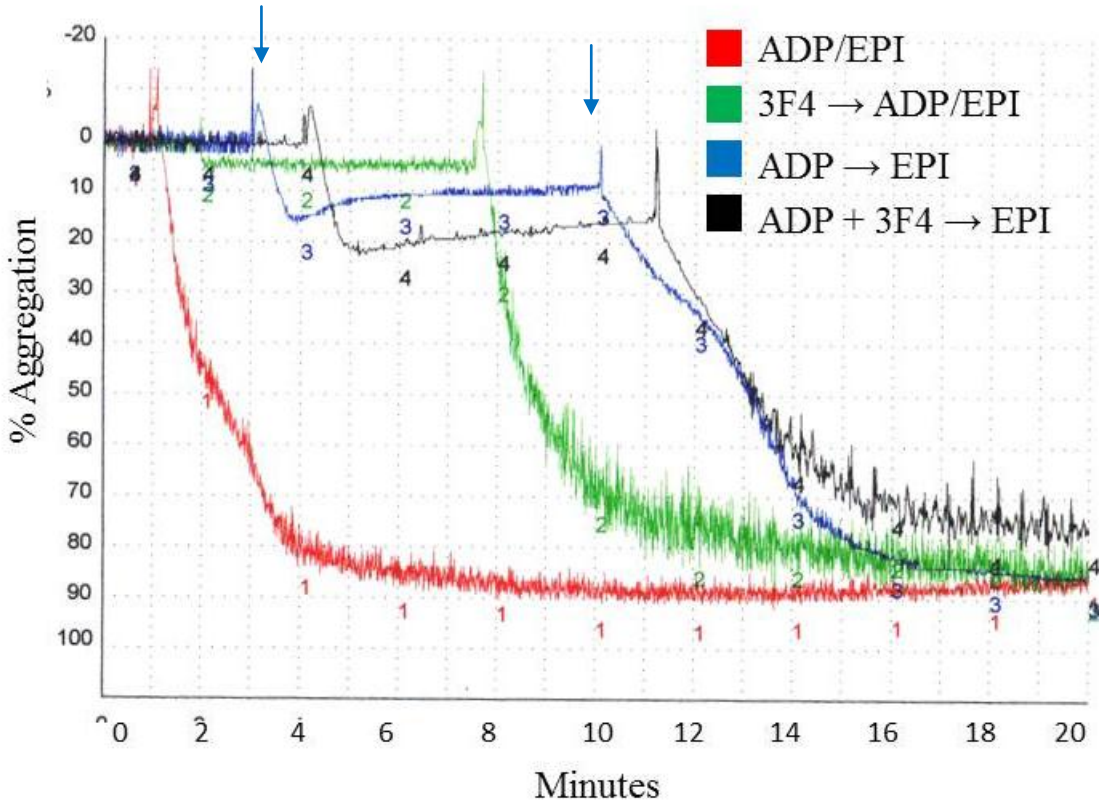


Figure 32. Anti-PrP MAb 3F4 do not influence ADP/EPI induced platelet aggregation and do not influence ADP aggregated platelets.
Platelets were aggregated with 10 µM ADP and 2 µM epinephrine (EPI) simultaneously (red line) or in successive steps (blue line). As for the latter approach, the time points when agonists were added are depicted with blue arrows. In the sample illustrated by green line platelets were incubated with anti-PrP MAb 3F4 and subsequently stimulated with the combination of ADP/EPI. Black line illustrates the sample were platelets were supplemented with both ADP and 3F4 in one point and EPI was added 7 minutes later. This is the representative tracing from two separate experiments.

In order to study possible effect of anti-PrP MAbs on platelet disaggregation, we used promethazine (PM) in concentration 500 µM. In Figure 33., the red line shows platelet aggregation induced with 10 µM ADP which reached about 20%. When PM was added, the aggregation was unequivocally reversed (green line). To follow the hypothetical role of anti-PrP MAbs we contrived two protocols:

In the first, which is shown by blue line, platelets were aggregated with ADP, the aggregation was allowed to proceed for 5 minutes, then MAb was added and after another five minutes aggregation was inhibited with PM. In the second approach (black line), platelets were firstly incubated with MAb and after that aggregated with ADP, the aggregation ran for 5 minutes and then PM was added. As shown in Figure 33., no effect of 3F4 antibody was observed.

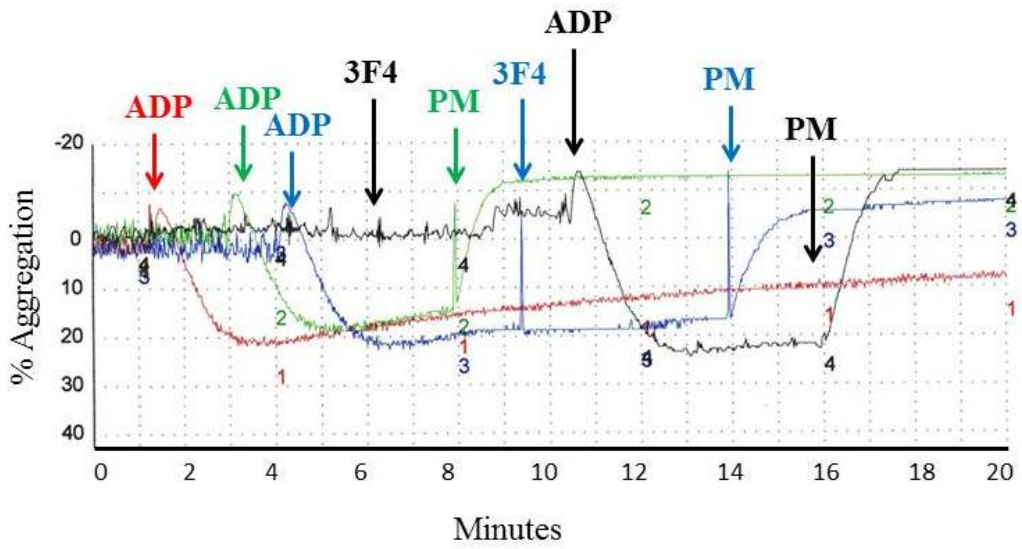


Figure 33. Anti-PrP MAb 3F4 does not influence blood platelet disaggregation induced with promethazine (PM).
Red line illustrates platelet aggregation with 10 μM ADP and green line disaggregation induced with 500 μM PM. Blue and black lines represent samples incubated with MAb 3F4. The arrow, whose colour corresponds to the colour of the lines, shows the time points where the agonist, antibody and PM were added. This is the representative tracing from two separate experiments.

Discussion

It is well known that PrPc serves as a substrate 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. As PrPc is crucial for infectivity accumulation, its expression on blood components is of importance. As for platelets, the presence of PrP mRNA was reported by Perini et al. (Perini, *et al* 1996) and subsequently PrPc was observed by flow cytometry on the plasma membrane of platelets (Holada, *et al* 1998).

The 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. The knowledge of PrPc physiological function is crucial for the development of diagnostic approaches as well as therapeutic interventions.

The biochemical characterization of platelet PrPc

The glycosylation of platelet PrPc

The role of PrPc glycosylation for its physiological function as well as prion pathogenesis was previously suggested. In *in vitro* studies, the addition of N-glycan structures favored proper folding of proteins *in vitro* (Live, *et al* 1996). As for PrPc, N-glycosylation might stabilize its conformation and reduce the efficiency of conversion to PrPsc (Ermonval, *et al* 2003). Contrary to this, recent study showed that glycosylation favors the disease development (Cancellotti, *et al* 2010). In this study they used transgenic mice expressing different glycosylated forms of PrP and they have shown, that mice without diglycosylated PrP had slowed-down or even none onset of the disease and mice expressing only unglycosylated PrPc did not develop clinical disease. In this study we used a panel of monoclonal antibodies directed against different PrPc epitopes. We have shown that glycosylation pattern of platelet PrPc is very similar to that of brain PrPc and differs from RBC PrPc.

The choice of antibodies used for protein detection influence the proportion of detected isoforms and the trend of isoforms proportion was very similar between brain and platelet PrPc. Thus antibodies which have got epitopes localized in the N-terminus of the protein (AG4, DC2) provided high intensity of diglycosylated isoform. On the other hand, antibodies with C-terminal localized epitopes (GE8) exhibited high intensity of unglycosylated isoform. This difference between N- and C-terminus directed antibodies is in agreement with the recent study of Kuczius et al. (Kuczius, *et al* 2007), who followed the brain PrPc. Interestingly, MAb AH6, which has got epitope in the core protein region, did not bind to diglycosylated forms of PrPc similarly to GE8. When using 6H4 we found the highest intensity for diglycosylated isoform both in platelets and in the brain homogenate. As for the latter Kuczius et al. reported different pattern of labeling with 6H4 – they found unglycosylated isoform of PrPc to be the most dominant. This might be explained by the recent finding, that the PrPc glycoform patterns vary considerably in the same individual depending on the kind of tissue and even between different brain regions (Beringue, *et al* 2003). Thus Kuczius et al. used samples which comprised mostly of cortex and cerebellum, whereas in our case the source of brain tissue was frontal lobe.

In another study Kuczius et al. used a broad panel of monoclonal antibodies in order to characterize glycoband profiles of brain and platelet PrPc (Kuczius, *et al* 2010).

Contrary to our study they concluded that glycoprofiles of platelet and brain PrPc differ. When antibodies directed against N-terminus of the protein were used (SAF34, 8G8, 3F4 and Pri308) the diglycosylated form of platelet PrPc predominated whereas unglycosylated form was almost invisible. The brain PrPc displayed predominated diglycosylated isoform as well, but the unglycosylated isoform was easily detectable. Interestingly, they also used antibodies targeted against C-terminus of PrP (Pri917), which led to high signal of unglycosylated platelet PrPc contrary to brain PrPc in which predominated the diglycosylated isoform.

To sum up, in our hands 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 more sensitive to conformational change induced by inoculum derived from brain tissue.

Sensitivity of platelet PrPc to PK

One of the features of PrPsc is partial resistance to cleavage by PK. PrPsc is also known to be resistant to PIPLC treatment as is platelet PrPc. Our aim was to exclude the possibility that the resistance of platelet PrPc to cleavage by PIPLC is caused by conformational similarity to PrPsc which should demonstrate as increased resistance to PK. As platelets are known to express PrPc both on the cytoplasmic membrane and intracellularly in α-granules, we performed PK treatment of platelet membranes as well as of platelet organelles. Isolated organelles simulate naturally present exosomes, which are released into blood stream for example during platelet activation. We confirmed that both pools of platelet PrPc are sensitive to PK treatment, so it cannot mimic PrPsc under appropriately chosen conditions. We also demonstrated that neither RBC PrPc is resistant to PK cleavage. This finding is relevant to the development of prion screening tests utilizing blood as a starting material, where the platelet and red blood cell PrPc may complicate detection of PrPsc by providing unwanted background.

Is platelet PrPc anchored by GPI-anchor?

Previously, Holada et al. reported that platelet PrPc can not be removed from platelet membrane by PIPLC treatment (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. The resistance to PIPLC treatment might be explained by different type of anchorage, by some modification of cleavage site for example by glycosylation, by inaccessibility of the anchor for the enzyme due to contiguous protein or by the existence of transmembrane form of PrPc. The latter was reported on platelets of patients with paroxysmal nocturnal hemoglobinuria which are unable to synthetize GPI - anchor. However, in the same study they did not find transmembrane PrPc on normal healthy platelets (Holada, *et al* 2002a). We also excluded the inaccessibility of the anchor for PIPLC cleavage, as the PrPc in lysed membranes was resistant as well. Finally, another method for GPI-anchor cleavage was applied. HF treatment confirmed that platelet PrPc is really anchored by GPI since the molecular weight of treated protein became lower reflecting the cleavage of GPI- anchor at the phosphodiester bond of phosphoethanolamine (Fig. 7). To conclude, platelet PrPc is GPI-anchored protein, but the cleavage site for enzyme PIPLC is likely to be modificated and thus inaccessible. Such an example of PIPLC resistant GPI-anchor is anchor of acetylcholinesterase on human erythrocytes, which is resistant due to inositol acylation (Roberts, *et al* 1988). We have confirmed that platelet PrPc is GPI-anchored which is important for the study of physiological function of PrPc, as GPI-anchored proteins are known to assembly in lipid rafts. Such localization is tightly connected to their signaling functions giving to platelet PrPc the potency to play such role (Horejsi, *et al* 1999).

Influence of copper ions on platelet PrPc conformation

PrPc possess a copper binding site in N-terminal octapeptide repeat region and it binds copper with low micromolar affinity. To test if the binding of copper has influence on its conformation and if it promotes PrPc endocytosis we incubated platelets with different concentrations of copper ions and followed its effect on the binding of different prion antibodies. We have detected the inhibition effect of copper ions on binding of anti-PrP MAb FH11 which is directed against epitope in N-region 46-59 close to copper binding region of PrPc. On the other hand, binding of MAbs directed against far-distant epitopes, 3F4 and 6H4, was not influenced. This result suggests that the decrease of FH11

binding was likely caused by copper induced PrPc conformation change of its N-terminal part which diminished binding of the antibody. The induction of PrPc endocytosis, which was described in another study (Pauly and Harris 1998), might be excluded, since that would cause decrease of binding of all MAbs tested. We have measured the expression of PrPc on platelets incubated with copper ions and it was not influenced. The importance of copper induced conformation change was proposed by Wong et al. who suggested that copper converts PrPc to a proteinase resistant conformation (Wong, *et al* 2000). However, later it was observed that this resistance was caused by inhibition of PK by copper ions instead by actual change of PrPc attributes (Stone, *et al* 2007). In our study we used about one hundred times higher copper concentration then that in plasma, therefore, the physiological relevance of our finding is speculative. However, copper ions might be specifically transported in the cell hypothetically leading to increase in local copper concentration. Such a carrier is ceruloplasmin, which is a plasma protein and moreover its GPI-anchored form was recently identified (Fortna, *et al* 1999). This GPI-anchored ceruloplasmin was localized in lipid rafts, which are also the site of PrPc enrichment and so might be the site of copper concentration for PrPc usage. Thus, the conformation change caused by copper ions might be a part of physiological process leading to fulfillment of PrPc role in the cell.

Comparing of PrPc expression of blood platelets and Red blood cells

According to published studies, most of PrPc associated with blood cells reside in platelets and RBC express only low amount of PrPc, however the results were inconsistent depending on detecting methods and antibodies used (Barclay, *et al* 1999, MacGregor, *et al* 1999). On the contrary Holada et al. assessed that RBC possess 53.7% of blood cell associated PrPc whereas platelets 44.9% (Holada and Vostal 2000). Using quantitative flow cytometry with MAb 6H4 the amount of PrPc on one RBC was set to approximately 270 molecules and on one platelet approximately 600 molecules (Holada, *et al* 2007). It was previously estimated that 70% of platelet PrPc is localized inside the cells (Holada, *et al* 2006). It means that the content of PrPc per one platelet including its intracellular pool is roughly seven times higher than in one RBC. We have confirmed this estimation by western blot analysis, which showed that one platelet express on average four times more PrPc than one RBC. However when total number of platelets and RBC is taken into account, RBC possess about 1,5-2 times more PrPc then platelets what is in agreement

with previous Holada`s study and make RBC PrPc pool largest among cellular fractions of blood. We have also demonstrated that different anti-PrP MAbs bind to PrPc with different sensitivities, which effect was especially obvious for RBC. Very the differences between MAbs biding explain inconsistency in PrPc RBC expression reported by different authors as mentioned above. MAb 3F4, which was used in Barclay et al. study, binds RBC PrPc very poorly. On the other hand both platelet and brain PrPc might be satisfactorily detected with this antibody. We suggested it might be caused by RBC PrPc post-translational modification of 3F4 epitope (KTNMKHM) (Panigaj, *et al* 2010). Interestingly, when analyzing PrPc on transferrin receptor positive (CD71+) erythroid precursors from cord blood, the equal binding of 3F4 and 6H4 was measured suggesting, that the modification of RBC PrPc proceeds after maturation and release of RBC to circulation. On the basis of decreased binding of 3F4 to brain PrPc treated *in vitro* with glyoxilic acid, glycation of RBC PrPc was proposed as the plausible mechanism of RBC PrPc modification.

To conclude, both platelets and RBC express considerable amount of PrPc, which can not be neglected in the problematic of prions transmission by blood transfusion. This was recently alerted by four known cases of vCJD transmission by blood, where the disease was transmitted by non leucodepleted red blood cells. Very recent study proved that also blood platelets can harbor infectivity – platelets from chronic wasting disease (CWD) suffered deer, which similarly as human platelets express PrPc, were able to transmit the disease to both healthy deer and mice (Mathiason, *et al* 2010).

Study of PrPc localization in blood platelets by fluorescence microscopy

The expression of PrPc on blood platelets has been shown by several studies and the existence of intracellular pool of PrPc was previously suggested according to increased expression on cytosolic membrane upon platelet activation (Holada, *et al* 1998). Starke et al. confirmed this proposal by revealing PrPc in α-granules both by means of confocal microscopy and immunoelectron microscopy, moreover, they localized PrPc also in open canalicular system of platelets (Starke, *et al* 2005). Finally, our recent data have shown failure in up-regulation of PrPc on Grey platelet syndrome platelets, which lack α-granules, as followed by flow cytometry (Holada, *et al* 2006).

Not all species have the same pattern of platelet PrPc – for example PrPc in sheep platelets seems to reside only in intracellular compartments (Halliday, *et al* 2005). Interestingly PrPc expression on platelets differs even among primates, only the chimpanzee expression is similar to humans (Holada, *et al* 2007).

The current study extends the recent data that localized PrPc to α-granules. Starke et al. used for immunolabeling of PrPc anti-PrP MAb 6H4 while in the current study 6H4 was used in the mixture with another anti-PrP MAb 3F4. This arrangement of antibodies was chosen in order to enhance the signal of PrPc. We have also set the immunolabeling protocol using fixation with PFA and treatment with methanol, which led to the best signal of PrPc from tested approaches. PrPc signal was very weak and blurred when only fixation with PFA was done. Interestingly it became even worse if platelets were permeabilized with 1% Triton X-100, suggesting that PrPc might be washed out the sample by this process. Starke et al. observed fixed platelets without any permeabilization; therefore although they used confocal microscope we do not consider their PrPc signal to be convincing.

By immunofluorescence method we demonstrated that α-granular proteins CD62P and TSP colocalize. Surprisingly, when CD62P was co-labeled with PrPc we did not detect any colocalization in resting platelets. Nevertheless, when platelets were activated the colocalization appeared in the central area, which belongs to the clustered platelet granules. Following platelet activation organelles fuse individually with the channels of the surface-connected canalicular system, which serves as a channel for their secretion. This might be the source of open canalicular system associated PrPc which was detected by Starke et al. As Starke at al. also suggested the existence of cytoplasmic pool of PrPc, we performed the co-labeling of PrPc and cytoplasmic actin and did not found any PrPc in the same compartment as actin. With regards to the cytoplasmic membrane, we detected PrPc pool which did not colocalize with CD62P suggesting a different membrane localization for each. Based on this observation and published studies we proposed that PrPc is localized within microdomains of cytoplasmic membrane called lipid rafts.

Platelet PrPc associates with lipid rafts

PrPc and other GPI-anchored proteins are known to localize in lipid rafts of different cell types. With the use of different detergents we have demonstrated incorporation of PrPc

into lipid rafts of platelets whereas no CD62P was detected in raft fractions (Brouckova and Holada 2009). This is in agreement with different pattern of fluorescence signals of these proteins on the membrane area of activated platelets as shown before. Platelet raft marker CD36 was significantly enriched in isolated raft fractions confirming their identity (Dorahy, *et al* 1996). The isolation of PrPc containing rafts with Brij 98 at 37°C confirmed that PrPc association with rafts is not artifact of isolation at 4°C (Drevot, *et al* 2002, Chamberlain 2004). Moreover, we have proved that PrPc association with lipid rafts strongly depends on cholesterol content, as its depletion with saponin as well as with MβCD totally reduced buoyancy of PrPc. The PrPc containing rafts could be also disrupted with dodecyl maltoside.

It has been suggested that a basic raft unit may consist of a protein surrounded by a sphingolipid/cholesterol-rich 'lipid shell' and detergent solubilization of cells can be used to purify compositionally distinct lipid raft domains (Chamberlain 2004). Thus, the number of physically distinct lipid raft domains in living cells is likely to be enormous. They were suggested to be distinguished by different detergents. The most commonly used detergent for rafts studies is Triton X-100. In agreement with study of Schuck et al., who assessed Triton X-100 to have the highest solubilization strength (Schuck, *et al* 2003), we have found that Triton X-100 was less effective in PrPc recruiting to rafts comparing to NP-40. On the opposite site, in the case of RBC, more PrPc was recruited to rafts when Triton X-100 was used for cell lysis. This suggests that platelet PrPc resides in lipid rafts of different composition then that in RBC. In contrast to PrPc in RBC, the amount of raft associated CD59 was independent on type of detergent suggesting that it resides in different rafts then PrPc. Recent study based on confocal microscopy of erythroblasts revealed that PrPc and CD59 have different distribution in the plasma membrane what supports our hypothesis (Griffiths, *et al* 2007). The differences between platelet and RBC raft associated PrPc might be also strengthened by studies on PrPc and tetraspanin CD63 localization. Thus, in RBC PrPc was shown to colocalize with CD63 (Griffiths, *et al* 2007). On the other hand, in platelets PrPc was shown to up regulate on the surface of activated platelets from different intracellular compartments then CD63 (Holada, *et al* 2006) suggesting that they are likely to be deposited also in different membrane compartments. To conclude we propose that platelet and RBC PrPc containing rafts may differ in their composition, but this assumption must be confirmed by independent studies.

Then, we focused on quantity of raft associated PrPc in platelets. We expected PrPc to be present in rafts in higher amounts and thus we searched for the reason of low yield in our initial experiments.

Assuming that PrPc resides predominantly in soluble membranes, platelet activation may trigger its redistribution into rafts, as was previously demonstrated for collagen receptors GPVI and TIIICBP, vinculin, and G protein subunit alpha q (Bodin, *et al* 2001, Locke, *et al* 2002, Maurice, *et al* 2006). On the other hand, PLCγ2, Syk, Src or Lyn incorporation into rafts was reported not to be affected by platelet activation (Gousset, *et al* 2004). Nevertheless, we did not found any effect of platelet activation on the amount of raft associated PrPc. Similarly, neither CD36 nor CD59 association with rafts was influenced by platelet activation. For CD36 this is in agreement with previous studies (Gousset, *et al* 2004, Maurice, *et al* 2006).

Low yield of raft associated PrPc might be also explained by the existence of so called "heavy rafts" which have a higher protein/lipid ratio and are hidden in the bottom fraction of the gradient (Horejsi 2005). In order to reveal possibly hidden heavy raft-PrPc pool we used step sucrose gradient, however, this approach did not reveal another pool of raft associated PrPc.

Finally we hypothesized that small amount of raft associated PrPc in platelets could be caused by binding of PrPc containing rafts to cytoskeleton, which might prevent raft flotation in the flotation assay. Therefore platelet cytoskeleton was analyzed. Platelet cytoskeleton consists of spectrin based membrane skeleton, a tubulin coil and a rigid actin cytoskeleton that fills the cytoplasmic space of the cell. We found that PrPc associated with both membrane skeleton and cytoplasmic actin cytoskeleton. Interestingly, the association with the latter dramatically increased upon platelet activation. Approximately one-fifth of PrPc remained soluble and not associated with the platelet cytoskeleton. The most likely explanation for this increase is cross-linking with the membrane skeleton containing additional PrPc (Hartwig and DeSisto 1991). The same was described for integrin $\alpha_{IIb}\beta_3$ and the tyrosine kinase Src suggesting the role of this phenomenon in signaling events of activated platelets (Fox, *et al* 1993). Neither raft marker CD36 nor CD62P association with cytoskeleton was influenced by platelet activation implying that PrPc resides in separate membrane domains interacting with cytoskeleton.

Important finding of our study was that cytoskeleton depolymerization led to flotation of significant amount of raft associated PrPc. A small pool of PrPc remained in

the bottom fractions of the flotation gradient even after cytoskeletal depolymerization, suggesting that part of PrPc pool associates with soluble membranes. In contrast, CD36 and CD59 were entirely found to be raft associated. Our findings suggest the existence of an interaction between platelet cytoskeleton and PrPc containing rafts. Again, the amount of raft associated PrPc in actin depolymerized platelets was not influenced by platelet activation. Based on our observations we propose model of platelet PrPc localization in different membrane domains (Fig. 34): we suggest that cytoskeleton associated PrPc comprises of two pools: raft associated pool and non raft pool, with the later being up regulated upon platelet activation. We also conclude that the amount of raft associated PrPc interacting with cytoskeleton is constant regardless of platelet status.

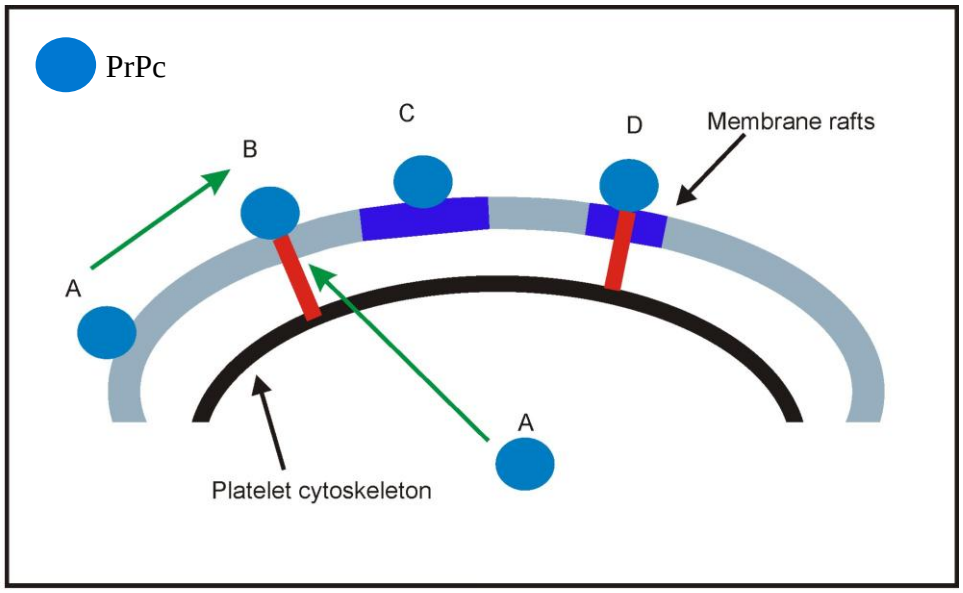


Figure 34. Model of different pools of PrPc.
We suggest that platelet PrPc consists of four pools (PrPc is shown as a blue circle). A – The pool which is either intracellular or membrane in resting platelets and upon their activation associates with cytoskeleton (platelet activation is illustrated by 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.

Here, it is the first evidence of PrPc association with the cytoskeleton in platelets. In other cells the interaction of PrPc with cytoskeletal components such as actin, alpha

actinin, and tubulin has been proposed by immunoprecipitation and cross-linking studies (Nieznanski, *et al* 2005, Petrakis and Sklaviadis 2006). A transmembrane form of PrPc may be engaged in a direct interaction with the cytoskeleton (Hachiya, *et al* 2004); however, transmembrane forms of PrPc are pathogenic rather than physiologic. In addition, the presence of GPI-anchorage of platelet PrPc was confirmed by treatment with 45 % hydrofluoric acid. Thus some other connection between PrPc containing rafts and cytoskeleton is likely to exist in platelets. One such candidate might be dystroglycan, which was shown to interact with PrPc (Keshet, *et al* 2000) as well as with platelet cytoskeleton (Cerecedo, *et al* 2005).

To conclude, we have found that there is a significant pool of raft associated PrPc in platelets, with most of it linked to platelet cytoskeleton. The size of this pool is relatively stable regardless of platelet activation status, although activation leads to its increased association with the actin cytoskeleton. In addition, smaller pools of soluble PrPc and cytoskeleton-associated nonraft PrPc also seem to be present in platelets. Protein association with either lipid rafts or cytoskeleton is supposed to be critical for platelet signaling, as it allows signaling molecules to interact. Our results may help in revealing PrPc function in platelets; however, more studies need to be done in this field. Since raft associated PrPc might serve as a receptor for PrPtse or as a substrate for PrPtse conversion, our findings are also 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* 2002b). 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 PMCA (Jones, *et al* 2007). Blood platelets were also shown to have ability to transfer the infectivity when transfused from CWD deer to healthy deer and healthy mice (Mathiason, *et al* 2010).

The study on PrPc involvement in platelet physiological function

Since PrPc is a GPI-anchored protein localized in lipid rafts which serve as molecular scaffolds for signal transduction, PrPc role in signaling has been tackled. Thus, there are now a number of studies suggesting that PrPc can activate transmembrane signaling pathways involved in several different phenomena, including neuronal survival,

neurite outgrowth, and neurotoxicity. Several studies also suggested PrPc role in signal transduction in leukocytes (Krebs, *et al* 2006, Mazzoni, *et al* 2005).

Tyrosine phosphorylation of a number of proteins is observed when platelets are activated by certain agonists such as thrombin and collagen. Platelets also contain an abundance of tyrosine kinases Fyn and Src which were suggested to be functionally connected with PrPc (Mouillet-Richard, *et al* 2000) and also have been shown to reside in lipid rafts (Dorahy and Burns 1998, Gousset, *et al* 2004). In order to follow possible PrPc role in platelet signaling we examined tyrosine phosphorylation upon stimulation with anti-PrP MAbs and upon their cross-linking. In monocytes Krebs et al. detected increase in tyrosine phosphorylation upon stimulation with PrP tagged to Fc. In platelets upon stimulation with MAb 3F4 we detected only an increase in phosphorylation of minor double band around 42 kDa, however no additional phosphorylation was detected when MAb was cross-linked. We consider this to be too small change in phosphorylation comparing to unstimulated samples so we did not follow the identity of phosphorylated double band. Regarding PrPc cross-linking using MAbs 3F4 or SAF 32, no other changes in proteins phosphorylation were observed.

We also tested the influence of PrPc blocking and of PrPc cross-linking on platelet aggregation. Platelet aggregation is a key event in the process of thrombus formation and results from a coordinated series of cell adhesion events. It is mediated by the binding of fibrinogen or von Willenbrand factor to integrin $\alpha_{IIb}\beta_3$, which conformation essential for aggregation is regulated by the signal from inside the cell. Thus, failure in signal transduction prevailingly leads to disruption of aggregation. Platelet aggregation was shown to be decreased by cholesterol depletion suggesting the role of lipid rafts in this platelet physiological function (Grgurevich, *et al* 2003). This is in agreement with the observation, that different signaling molecules are enriched within rafts. In previous part of this study we have proved that PrPc is localized in rafts. It is therefore plausible that it might interact with some signaling molecules localized within the same compartments, either with a transmembrane receptor or through some interactor with cytoplasmic kinases. We tested hypothesis, that PrPc might be possibly a part of signaling complex and so its blocking might influence platelet aggregation. We used anti-PrP MAbs directed against different regions of PrPc molecule: N-terminus (SAF 32), intermediary region (3F4), core protein region (AH6) and C-terminus (GE8) and thus whole protein sequence was covered. However, none of the anti-PrP MAb tested has any effect on the aggregation. Next, we

followed the effect of PrPc cross-linking on aggregation. It has been observed that cross-linking of rafts component induce raft aggregation and in that way the reorganization of other raft interacting molecules (Janes, *et al* 1999). Neither PrPc cross-linking caused any change in platelet aggregation. We have also evaluated the possible involvement of PrPc in disaggregation of platelets induced by promethazine (Mondoro and Vostal 2002), however without any interesting result.

In our study, to induce aggregation we used collagen or the combination of ADP and EPI, which bind to different receptors. Recently, Robertson used as agonist either U46619 or collagen and did not find any effect of anti-PrP antibodies on platelet aggregation. To conclude, it results from our study as well as from the literature that PrPc is not involved in platelet aggregation (Robertson 2005).

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 35.

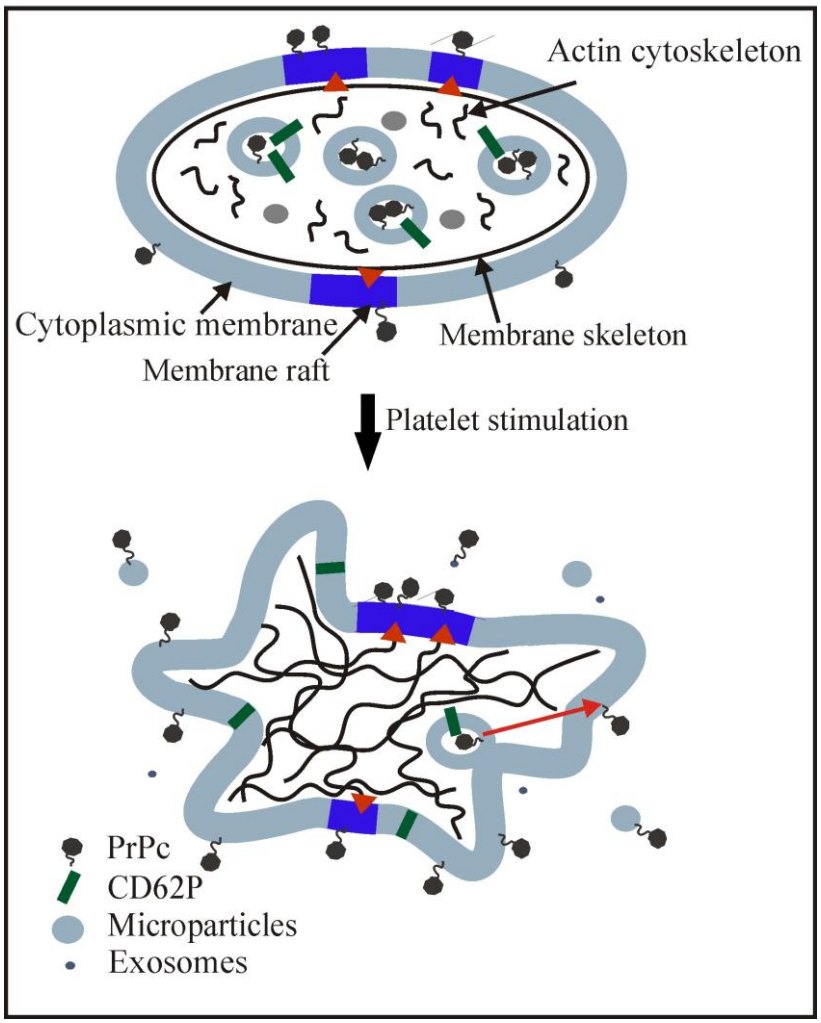


Figure 35. Model of PrPc localization in platelets.
The majority of PrPc is localized in the intracellular compartment of resting blood platelets, namely in α-granules. Upon platelet activation PrPc up regulates on the platelet surface due to organelles fusion with cytoplasmic membrane (illustrated by 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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Supplements

Publications with impact factors

1. 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*

2. **Broučková A.**, Holada K.: Cellular prion protein in blood platelets associates with both lipid rafts and the cytoskeleton. Thrombosis and Haemostasis **2009**, Nov;102(5):966-74. *IF 4,45*