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Effects of Metal Binding on Solubility and Resistance of Physiological Prions Depend on Tissues and Glycotypes

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ABSTRACT

Prion diseases entail the conversion of a normal host-encoded prion protein (PrP^C) into an infectious isoform (PrP^{Sc}). Various PrP^C types differing in banding profiles and detergent solubility are present in different tissues, but only few PrP^{Sc} types have been generated although PrP^C acts as substrate. We hypothesize that distinct PrP^C subtypes may be converted more efficiently to PrP^{Sc} than others. One prerequisite for the analysis is the identification of the PrP^C subtypes present in the protein complexes. Metal binding to PrP^C is one of the most prominent features of the protein which induces increased proteolysis resistance and structural changes which might play an important role in the conversion process. Here we analyzed the metal-induced structural PrP^C transformation of two different Triton X-100 soluble PrP^C types derived from human platelets and brains by changes in protein solubility. We found that zinc and copper rendered approximately half of total PrP^C and mainly un- and low-glycosylated PrP^C to the Triton insoluble fraction. Our results indicate the presence of at least two distinct PrP^C subtypes by metal interactions. The differentiation of high and low soluble metal bound PrP^C offers precious information about PrP^C protein composition and provides approaches for analyzing the transformation efficiency to PrP^{Sc}. J. Cell. Biochem. 114: 2690–2698, 2013.

KEY WORDS: PHYSIOLOGICAL PRION PROTEIN; DETERGENT SOLUBILITY; METAL BINDING; ZINC; COPPER

The infectious agent of transmissible spongiform encephalopathies (TSEs) is proposed to be the prion protein (PrP^{Sc}). TSEs affecting both animals and humans are a group of neurodegenerative disorders, which are characterized by a long incubation period. After onset of the disease it progresses rapidly leading to death. Human prion diseases mainly encompass the sporadic, familial and infectious Creutzfeldt–Jakob diseases (CJD) inclusive the variant (vCJD). The familial CJD arise from mutations within the prion gene sequence while the infectious forms are mainly caused by the use of prion-contaminated biologicals or surgical instruments in medical procedures, The transmission of vCJD to and within the human species has been linked to the consumption of bovine spongiform encephalopathy (BSE)-contaminated cattle meat [Bruce et al., 1997] and to blood transfusions [Llewelyn et al., 2004; Peden et al., 2004]. The presence of prion infectivity in blood has also been demonstrated experimentally in rodent models [Cervenakova et al., 2003].

The key event in the pathogenesis is the conversion of a host encoded physiological prion protein (PrP^C) to an abnormal isoform (PrP^{Sc}) which subsequently serves as a template for the conversion of the remaining PrP^C. The conversion of PrP^C into the infectious PrP^{Sc} form goes along with a dramatic change of the secondary structure of the protein with an increased β-sheet and a decreased α-helix content of the structure [Gasset et al., 1993]. This results in a markedly reduced solubility of PrP^{Sc} in detergents and a high resistance to proteolysis.

The human PrP^C gene is localized on chromosome 20 and encodes for a 254 residue polypeptide. After translation a glycosylphosphatidylinositol (GPI) anchor is added by post-translational processing. The anchor is necessary for the attachment of PrP^C to the outer cell membrane. Two asparagine-linked glycosylation sites located at residues 181 and 197 lead to the occurrence of di-, mono- and non-glycosylated forms of PrP^C. The C-terminal domain contains three α-helices, a short anti-parallel β-sheet [Zahn et al., 2000] and a single disulphide bridge, whereas the N-terminal domain is a flexible unstructured polypeptide chain. It contains a highly conserved region with octapeptide repeats (PHGGGWGQ) that were shown to have a

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high affinity for copper [Stöckel et al., 1998]. Each of the histidine residues in the octapeptide repeat regions is able to bind one copper ion. Copper has a higher affinity to PrP than other divalent ions [Brown et al., 1997a; Hornshaw et al., 1995; Viles et al., 1999]. The outstanding affinity of PrP to copper was the basis for the purification of PrP by immobilized Cu^{2+} affinity chromatography (IMAC-Cu^{2+}) [Pan et al., 1993]. Copper binding is accompanied with changes in the structure of PrP [Stöckel et al., 1998] and with the induction of resistance of PrP against proteases [Quaglio et al., 2001]. However, other metal ions such as Mn^{2+}, Ni^{+}, Pd^{+}, Pt^{+}, and Co^{2+} in sufficient concentrations can also be complexed with PrP^{C} [Gaggelli et al., 2005; Garnett et al., 2006; Pushie et al., 2007, 2009; Walter et al., 2007; Zhu et al., 2008] of which extracellular zinc seems to have a more prominent influence than the other metals.

It has been shown that zinc ions are able to bind to similar regions as copper. Binding of copper and zinc to the peptide PrP106-126 changes the ability of PrP^{C} to aggregate and its peptide toxicity [Jobling et al., 2001]. Both ions are able to induce endocytosis of PrP^{C} in neuroblastoma cells [Pauly and Harris, 1998; Brown and Harris, 2003] and may interact with human PrP^{Sc}, in particular type-1 of sCJD, indicating that both of these metals are likely to play an important role in the development of prion diseases [Wadsworth et al., 1999].

Metal binding is associated with structural transformations in PrP. However, different phenotypes of PrP^{C} are present in platelets and brains [Kuczius et al., 2011a]. Platelet PrP^{C} has an increased solubility in detergents and consists of higher amounts of glycosylated PrP^{C} than brain PrP^{C}. It is an open question whether these differences may lead to variable structural changes of PrP^{C} after metal binding. In this study we analyzed and compared the structural effects of metals on PrP^{C} derived from human platelets and brains. We prepared lysates of platelets and brains with different detergents and incubated them with and without different metal ions. In order to detect changes in solubility of PrP^{C}, we separated a highly soluble PrP-containing protein fraction (supernatant) from a low soluble PrP-containing fraction (pellet) by centrifugation. Furthermore we tested the resistance of metal treated PrP^{C} to protease treatment. High amounts of PrP^{C} were detected in precipitates after copper and zinc binding. The precipitating effect was much lower with other divalent cations. With respect to tissue source or glycotype distinct PrP^{C} subtypes were changed to a reduced solubility, while other PrP^{C} forms remain soluble. This indicates the presence of differential structural changes or differential metal binding efficiencies and effects of the PrP^{C} subtypes.

MATERIALS AND METHODS

ANTIBODIES

The monoclonal anti-PrP antibodies (mabs) SAF34, SAF70 and Pri917 were used for the specific detection of prion proteins in this study. Mabs SAF34 and SAF70 were prepared by immunizing knock-out mice with formic acid denatured hamster scrapie associated fibrils of strain 263K [Demart et al., 1999]. Mab SAF34 binds to the N-terminal octapeptide region within the PrP sequence (amino acids 59–89) whereas mab SAF70 recognizes an epitope at the core protein (amino acids 156–162). Mab Pri917 was raised using a synthetic peptide of human PrP as the immunogen [Morel et al., 2004]. It recognizes an epitope formed by the amino acids 216–221 at the carboxy-terminal region of the prion protein. The antibodies were applied as ascetic fluids derived from mice and were used from a single lot throughout this study. Glycoprotein IIIa (GpIIIa) and neuron-specific enolase (NSE) were used as typical indicator proteins for platelets and brain in this study. GpIIIa and the NSE were detected using purified monoclonal antibodies anti-CD61 (mouse anti-human CD61; Beckmann Coulter, Krefeld, Germany) and anti-NSE (mouse anti-human NSE; Dianova, Hamburg, Germany). Affinity-purified goat anti-mouse IgG conjugated with horseradish peroxidase (HRP; Dianova) served as the secondary antibody.

ISOLATION AND PREPARATION OF PLATELETS

Platelets were isolated from ethylene diamine tetraacetic acid (EDTA)-anticoagulated blood which had been obtained from healthy volunteer blood donors after informed consent. Blood was pooled from ten blood group 0 donors and centrifuged at 240*g* for 10 min. Platelet rich plasma was collected and diluted 1:1 with isotonic saline containing 0.5% (w/v) EDTA. Platelets were spun down at 1,500*g* for 10 min and washed three times with isotonic saline (pH adjusted to 6.5 with Dulbecco PBS (Invitrogen, Karlsruhe, Germany)). The platelet count was adjusted to $4 \times 10^{6}/\mu l$ using a Sysmex K1000 counter (Sysmex, Norderstedt, Germany). Platelets were resuspended in Tris-buffered saline (TBS; 20 mM Tris/HCl and 150 mM NaCl) containing Triton X-100 (1%, "Triton platelet lysate") or *N*-lauroylsarcosine (1%; "sarcosyl platelet lysate"). In a second approach the platelets were lysed in homogenate buffer (TBS containing 0.5% SDS, 0.5% igepal-CA630 and 320 mM sucrose). Platelet suspensions were stored in aliquots at −70°C for up to 6 months.

BRAIN TISSUE PREPARATIONS

Human brain specimens were obtained from six human subjects who were free of prion diseases and other neuropathological alterations [Kuczius et al., 2007]. The brain tissues were obtained from the Institute of Neuropathology Brain Bank at the University Hospital Münster. Consent for autopsy and scientific examination was obtained from the legal representatives (Ethics Committee of the Westphalia Chamber of Physicians and the Faculty of Medicine, University of Münster) for all subjects in accordance with the local institutional review board requirements. The tissue samples, which were not regionally specified, were pooled, weighted and homogenized in nine volumes of TBS using pestles in glass tubes and electric homogenizers (VWR, Darmstadt, Germany). The samples were intensively sonicated for 30–60 s. After centrifugation (900*g*, 5 min) the supernatants were supplemented with Triton X-100 (final concentration 1%) and stored at −20°C for 2–3 weeks until use. Before analysis the lysates were thawed and then centrifuged (16,000*g*, 10 min) in order to remove remaining protein aggregates. The supernatant containing the soluble PrP^{C}-containing protein fraction ("triton brain lysate") was used for further analysis.

ENZYMATIC TREATMENT OF PROTEIN SAMPLES

For proteolytic assays the samples were incubated with proteinase K (PK; Sigma, Taufkirchen, Germany) at a concentration of 25 μg/ml at

37°C for 60 min with gentle agitation. The digestion was stopped by the addition of Pefabloc™ (Roche Diagnostics, Mannheim, Germany) to a final concentration of 1 mM and heating at 99°C for 10 min.

ADDITION OF DIVALENT CATIONS AND EDTA

For binding assays the platelet or tissue lysates were incubated with divalent cation solutions containing $CuCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, $NiSO_4$, or $CaCl_2$ (Sigma-Aldrich, Taufkirchen, Germany) at room temperature for 30 min or over night at 4°C. Fractions of soluble and insoluble PrP^C containing protein were separated by centrifugation at 16,000*g* for 10 min receiving supernatants and pellets.

The metal chelator EDTA (Serva, Heidelberg, Germany) was added to the samples for 30 min prior starting the immunoblot analysis procedure using concentrations from 1 to 5 mM.

IMMUNOBLOT ANALYSIS

Before electrophoresis the samples were resuspended in SDS-loading buffer and denatured at 99°C for 5 min. Proteins were separated using sodium dodecyl sulfate polyacrylamide (13%) gel electrophoresis (SDS–PAGE) in a mini slab gel apparatus (Bio-Rad, Munich, Germany) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Roth, Karlsruhe, Germany) using a semi-dry blotting system (Roth). The membranes were blocked for non-specific binding in TBS with 0.05% Tween 20 (TBST) and 1% (w/v) non-fat dry milk powder for 1 h at room temperature and subsequently incubated with primary antibodies over night. After three washing steps with TBST the membranes were incubated with the secondary HRP-conjugated antibodies (Dianova) at room temperature for 2 h. The membranes were washed again in TBST and the bands were visualized using a chemiluminescence enhancement kit (Thermo Scientific, Bonn, Germany).

QUANTIFICATION AND GLYCOTYPING OF PRION PROTEINS

Immunoblots were scanned on a chemiluminescence photo-imager (Bio-Rad). The signal intensities of the bands of interest were quantified using the QUANTITY ONE software (Bio-Rad). In order to account for differences in SDS–PAGE runs and to minimize experimental artifacts the experiments were performed in triplicates. The signal intensities of the di-, mono-, and non-glycosylated PrP^C were determined in total and were calculated as percentages of the total signals on the immunoblot. After repeated and separated immunoblot analyses the protein intensities were analyzed by calculation of the means. The standard deviations were calculated of the signal intensity means to analyze differences among various PrP^C sources. Processed data were compared by Student's *t*-test. A *P*-value less than 0.05 was considered to be significant.

RESULTS

PrP^C proteins are able to interact with metal ions, in particular with copper and zinc [Jobling et al., 2001]. The binding of metal ions goes along with structural changes and reduce the solubility of the protein in detergent solutions after ultracentrifugation [Quaglio et al., 2001]. However, the effect of metal ion binding to PrP^C obtained from different human tissue sources as well as the effect of metal binding on different PrP^C types has not been analyzed so far. In the present study we demonstrate a novel PrP^C separation dependent on metal binding by high speed centrifugation. An ultracentrifugation is not necessary and more PrP^C subtypes can be identified. The non-polar detergent Triton X-100, which has been used in the purification procedures of a number of platelet proteins [Dubernard et al., 1997; Bednar et al., 1998], was used to generate lysates of platelets isolated from peripheral blood and brain samples. Triton X-100 is not able to solubilize the whole PrP^C. Therefore, in a first step soluble PrP^C types were separated from highly soluble types by high speed centrifugation as described [Kuczius et al., 2011b]. The soluble Triton platelet and Triton brain lysate fractions were incubated with different metal ions, and the change in PrP-protein solubility as an indicator for PrP bound to metals was analyzed.

Proteins of supernatants and pellets were analyzed by immunoblot in order to separate PrP^C soluble and insoluble protein fractions with respect to the amount of PrP^C and the PrP^C glycotype. Proteins on immunoblots displayed three bands. From high to lower molecular mass the bands of two- and onefold glycosylated PrP^C and unglycosylated PrP^C could be distinguished.

After binding of copper or zinc high amounts of PrP^C were determined in the pellets when the detection of PrP^C was performed with mabs SAF34 and Pri917, respectively (Fig. 1). Interestingly, PrP^C incubated with a high copper concentration resulted in a double band in the pellet when determined with mab Pri917. A binding interaction of PrP^C with other metal ions such as Mn, Zn, Ni, Pd, Pt, and Co has been demonstrated by several groups [Garnett et al., 2006; Pushie et al., 2007; Walter et al., 2007; Zhu et al., 2008]. We analyzed the change of PrP solubility after addition of Mn^{2+}, Ni^{+}, and Co^{2+} and found only very low effects when using mab SAF34 for the detection.

However, high amounts of the total PrP^C had been converted from the high to the low soluble fraction after metal binding. The un- and most of the low glycosylated platelet PrP^C and brain PrP^C were found in the pellet. High contingents of the diglycosylated brain PrP^C isoforms and approximately half of platelet PrP^C also showed reduced solubility, however proteins were also detected in the supernatants. This finding indicates a highly efficient metal binding combined with structural changes leading to a low solubility of mainly the un- and low glycosylated isoforms.

The specificity of copper and zinc binding for the structural change leading to a low PrP^C solubility of platelet and brain PrP^C was shown using dilution series of the metals (Fig. 2). Our experiments revealed that the addition of $>100\,\mu M$ (for detection with mab SAF34 in platelets and brain and with mab Pri917 in brain) and $>200\,\mu M$ zinc chloride (platelets with mab Pri917) and $>200\,\mu M$ copper chloride changed PrP^C to low solubility which means that zinc has a stronger influence on the reduction of the solubility of PrP^C.

In order to demonstrate that this effect is due to metal binding, EDTA as metal chelator was applied to the low soluble fraction of metal-treated PrP^C in excess. With respect to the applied metal concentration EDTA in excess reversed the metal specific effect, the proteins became highly soluble and caused PrP^C to be detected in the supernatant (Fig. 3).

It has been shown that the binding of metal ions converts PrP^C to a protease resistant form under in vitro conditions [Qin et al., 2000]. Low soluble PrP^C of platelets after copper binding demonstrated an

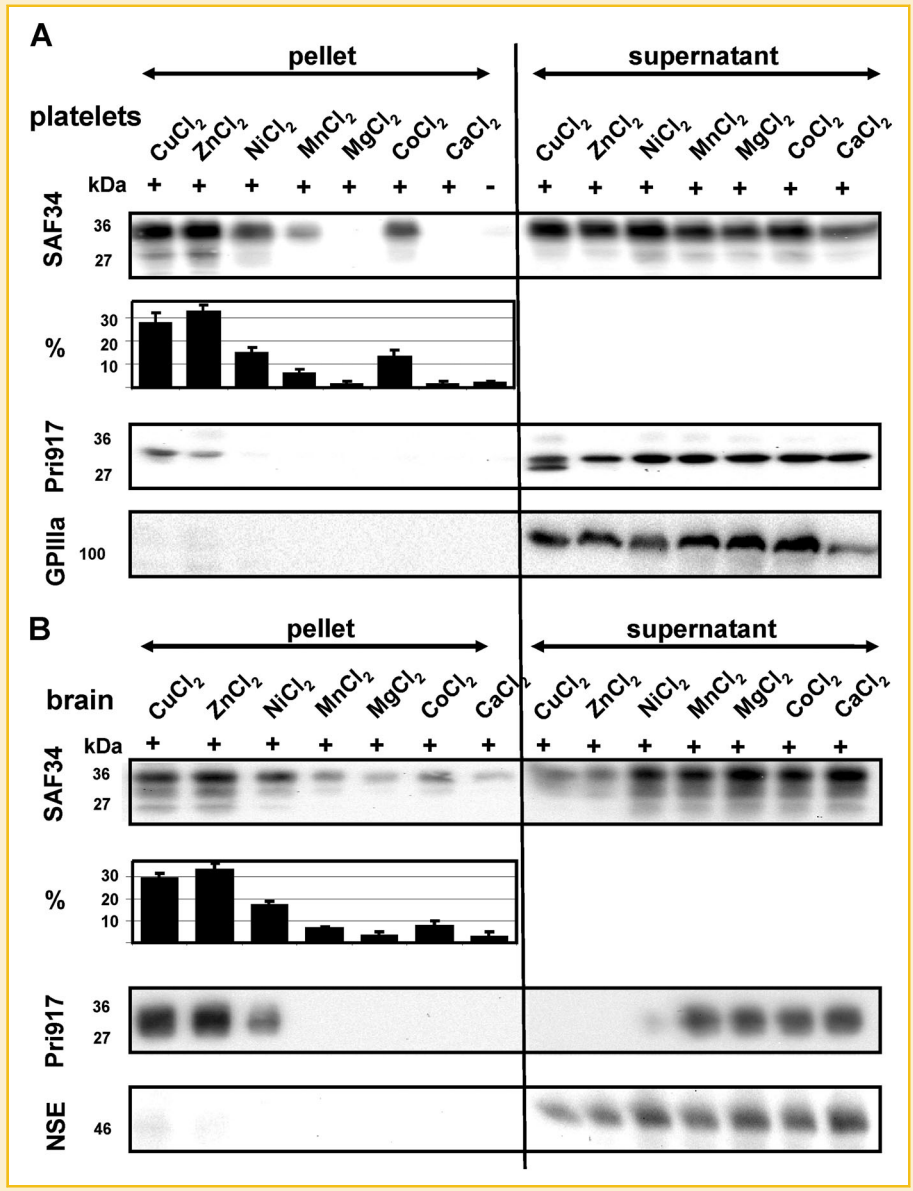

Fig. 1. Copper and zinc bind to platelet and to brain PrP^C. Soluble proteins of platelets (10^7 cells/μl) (A) and of a pooled brain tissue preparation (B; 5 μl of a 10% homogenate loaded) solubilized in TBS with 1% Triton X-100 were pre-incubated with metal ions (1 mM) as indicated. Proteins were separated by centrifugation into high and low soluble protein fractions which were detected in the supernatants and the pellets, respectively. Signals of PrP^C in platelets and in brains were determined using mabs SAF34 (upper blots) and Pri917 (lower blots). Protein GpIIIa and the neuron specific enolase (NSE) were used as indicator proteins of platelet and brain protein suspensions, respectively. GpIIIa and NSE were determined using mabs anti-CD61 and anti-NSE, respectively. Signals of the detected proteins were visualized by a chemiluminescence substrate. Signal intensities of metal bound PrP^C were determined after immunological detection using SAF34. The percentages of the total signal of all pelleted PrP^C were calculated as arithmetic means and standard deviation of the means. The data represent the calculation of three gels each. High contingents of PrP^C changed to the pellet fractions after copper and zinc incubation whereas this effect is not seen in this impact with other metals. The control proteins were always detected in the supernatants.

increased resistance to PK digestion (Fig. 3A). However, copper ions also reduce the enzymatic activity of proteinase K (PK) on PrP^C [Kuczius et al., 2004]. This inhibition was most likely caused by a direct interaction of the cations with the enzyme. This inhibiting effect was not seen for zinc (Fig. 3B).

The effect of metal binding, which goes along with a reduced solubility of PrP^C, was observed with mild and unpolar detergents. The analysis of lysates with Triton X-100 (Fig. 4A) or *N*-octyl-β-D-glucopyranoside (not shown) demonstrated a low solubility of zinc and copper treated PrP^C whereas this effect could not be seen when *N*-lauroylsarcosine (Fig. 4A) or SDS (Fig. 4B) were used as detergents. *N*-lauroylsarcosine and SDS have denaturating effects on proteins. In contrast, Triton X-100 is a non-polar detergent without featuring an important protein denaturation. The ability for metal binding in in vitro conditions is mattered by the choice of the detergent and consequently by the grade of protein denaturation.

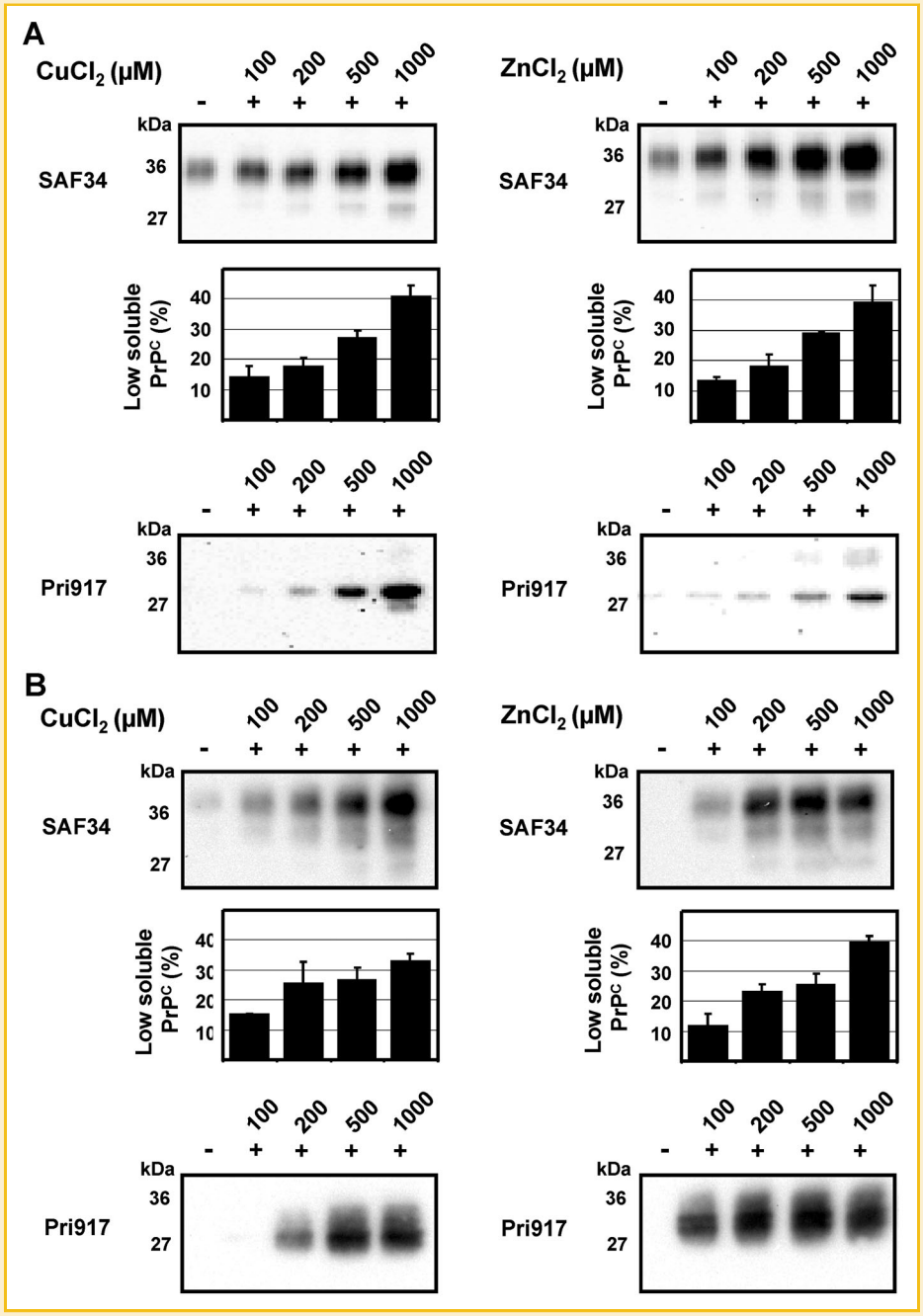

Fig. 2. Copper and zinc pre-incubations reduced PrP^C solubility in platelets and brains. PrP^C of pellet fractions of platelets (A) and brain tissues (B) were detected using mabs SAF34 and Pri917 as indicated and signals were visualized using a chemiluminescence substrate after incubation with HRP-conjugated secondary antibodies. Prior to immunoblotting the protein suspensions were pre-incubated with increasing copper and zinc concentrations as indicated followed by separation using high speed centrifugation. The intensities of the signals were determined for metal bound PrP^C after mab SAF34 detection. The percentages were calculated out of three independent gel runs as arithmetic means and standard deviations of the means. A significant effect was seen with data of 1 mM $CuCl_2$ and $ZnCl_2$ and of 100 μM $CuCl_2$ and $ZnCl_2$. The amounts of PrP^C in the pellets increased with rising metal concentrations indicating a metal specific effect.

DISCUSSION

Physiological prion proteins (PrP^C) are composed of highly heterogeneous isoforms in different tissues and brain areas [Beringue et al., 2003; Kuczius et al., 2009], and human brain PrP^C clearly differs from human platelet PrP^C in expression and banding patterns [Kuczius et al., 2011a]. Furthermore, PrP^C proteins are able to interact with metal ions, in particular with copper and zinc [Jobling et al., 2001]. The effect of metal ion binding to PrP^C and on different PrP^C types obtained from different human tissue sources has not been

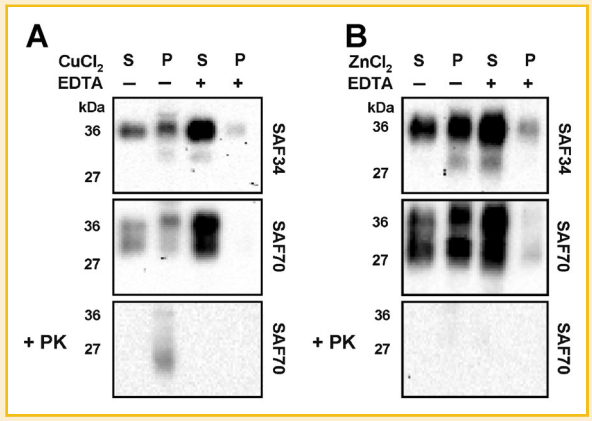

Fig. 3. Effects of chelator EDTA and of protease K treatment to metal bound platelet PrP^C. Triton platelet lysates supplemented with copper (A) and zinc (B) ions each in a concentration of 1 mM were incubated in the absence (–) and presence (+) of EDTA in a concentration of 5 mM. Proteins were separated to high and low soluble protein fractions in the supernatants (S) and the pellets (P), respectively. PrP^C bound by copper and zinc remained in the pellet whereas the application of EDTA reversed the reduced PrP^C solubility to the high soluble protein fraction which was detected using mabs SAF34 and SAF70 as indicated. Signals were visualized with HRP-conjugated secondary antibodies and chemiluminescence substrate development. After metal binding and EDTA supplementation proteins were treated with proteinase K (PK; 25 μg/ml, 37°C, 1 h). Residual proteins of copper bound PrP^C but not of zinc bound proteins were detected using mab SAF70 recognizing the core protein region of PrP.

analyzed so far. Here we demonstrate a novel PrP^C differentiation in protein complexes dependent on metal binding by a high speed centrifugation step. Our results indicate that the binding of copper and zinc to PrP^C induce a change of solubility of the protein recognizable by a high speed centrifugation step. However, the molecular changes are unknown. Several reasons may be responsible for this observation. First the higher solubility may result from the higher content of polar carbohydrate groups in diglycosylated PrP^C. Secondly; the structural change of diglycosylated PrP might have a lower effect on the aggregability of PrP. There may be a partial but not complete binding of cations to all potential binding sites of the protein. A complete or almost complete occupancy of the available cation binding sites may induce a structural change which is associated with low solubility, whereas a partial occupancy may cause an inadequate or minor conformational change, which prevents the protein from aggregation. Thirdly, distinct types of PrP^C have a lower affinity to metal interactions and remain soluble.

Copper binds within the octapeptide region to histidine residues and outside of this region to additional sites localized at His-96, His-111, and His-186 [Jobling et al., 2001; Watanabe et al., 2010]. It has been proposed that copper intercalates in PrP^C molecules by forming defined loop structures of the N-terminal octarepetitive sequences [Viles et al., 1999; Renner et al., 2004] which has been implied with protein misfolding [Cordeiro et al., 2005; Leliveld et al., 2006, 2008]. Accessory copies of repeats can induce spontaneous disease in humans [Owen et al., 1989, 1991; Goldfarb et al., 1991; Van Gool et al., 1995; Laplanche et al., 1999]. Copper binding is highly efficient and the measured K_d for the copper-PrP^C-complex was determined between 6.7 and 14 μM [Hornshaw et al., 1995; Stöckel et al., 1998; Viles et al., 1999]. Zinc is also able to bind to this region. However, with a K_d of approximately 200 μM it has less affinity to PrP^C than copper [Walter et al., 2007]. Zinc is necessary for many functions of the immune system and is required for the activity of several enzymes. It has in many respects an inverse relationship to copper, which means that the activity of the enzyme with zinc goes down while the activity with copper goes up.

Proposed PrP^C functions include a role in transmembrane signaling, development of synapses, adhesion to the extracellular matrix and to the protection against apoptosis [Mouillet-Richard et al., 2000; Schmitt-Ulms et al., 2001; Kanaani et al., 2005]. Specific interactions of PrP^C with cations are suggested to be important for protein transport and for the protection against oxidative stress by superoxide dismutase (SOD) activity [Brown et al., 1997b, 1999]. The SOD contains binding sites for copper and for zinc (Cu-Zn-SOD). The metal binding seems to increase the resistance of neuronal and other cells to oxidative stress.

Most trace elements are bound to proteins and only very low concentrations are available [Wlostowski, 1992]. Therefore, the concentrations of the trace elements copper and zinc vary extremely under physiological conditions. Additional different concentrations were determined in dependence on tissues and fluids used of individuals and on age and gender of the persons [Milne and Johnson, 1993]. The normal ranges of copper and zinc in plasma are 900–1,500 μg/L and 800–1,200 μg/L, respectively [Lee et al., 2000] at which the overall range of plasma copper concentrations were up to 18 μmol/L for men and 11–27 μmol/L for women whereas the concentrations were higher for women taking hormones (up to 32 μmol/L) [Milne and Johnson, 1993]. Furthermore the trace element concentration was significantly affected by the age because persons older than 50 years had higher concentrations than persons below 40 years.

High copper concentrations were determined in the brain in substantia nigra and locus cerelus with up 1,300 μM [Danks, 1989]. In the olfactory bulb of rats zinc was measured up to 27.9 μg/g and copper up to 11.1 μg/g [Ono and Cherian, 1999]. High concentrations of the trace elements were also detected in hypothalamus, hippocampus and corpus stratium [Donaldson et al., 1973; Ono and Cherian, 1999]. Free ions were found in the synaptic space and the concentrations were low with 10 and 60 μM for copper and zinc, respectively [Hopt et al., 2003].

Zinc and copper bindings to proteins have essential roles. Zn is necessary for many functions of the immune system and is required for the activity of several enzymes and copper is essential for the growth of new blood vessels.

PrP^C of platelets and of brains which both are embedded in protein complexes are composed of several subtypes which are dissoluble differentially with various detergents and which demonstrate distinct glycoprotein profiles [Kuczius et al., 2011a]. Irrespective from different glycosylation grades distinct PrP^C types were highly soluble in detergent solutions while others were low soluble and were detected in the pellet fractions. Distinct Triton-soluble PrP^C proteins of both, platelets and brain, were able to bind copper and zinc leading to structural changes detected by a reduction in solubility.

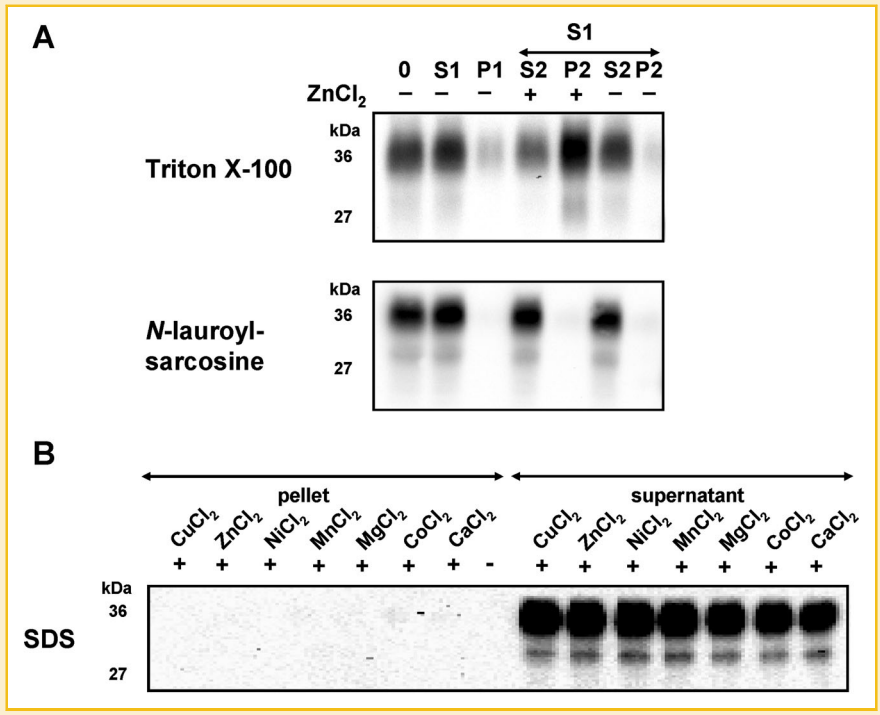

Fig. 4. The type of detergent influences the metal binding effect to platelet PrP^C. A: Proteins of platelets were both treated with Triton X-100 (1%) or *N*-lauroylsarcosine (1%) as indicated and separated to highly (S1) and low soluble (P1) protein fractions by high speed centrifugation. The soluble S1 proteins were incubated in the absence (−) and presence (+) of zinc cations in a concentration of 1 mM. Proteins were separated again to high (S2) and low soluble PrP^C (P2) by centrifugation. Zinc bound proteins were detected in the pellet fraction when proteins were pre-incubated with Triton X-100 but not with *N*-lauroylsarcosine. B: No metal binding effect resulting in a reduced PrP^C solubility was observed when proteins were pre-incubated in TBS buffer containing SDS and igepal C630 each in concentrations of 0.5%. Metals each in a concentration of 1 mM were incubated with platelet proteins for 60 min prior centrifugation. PrP^C in A and B were detected using mab SAF34 following incubation with HRP-conjugated secondary antibodies and chemiluminescence substrate development.

Interestingly, a fraction of PrP^C proteins remained in the soluble fraction. This differential solubility offers a new tool to dissect and differentiate PrP^C types bound in protein complexes. With regard to the "protein-only" hypothesis heterogeneous PrP^C as well as metal-bound and structurally changed isoforms of PrP^C may have different tendencies for PrP^{Sc} conversion and might generate phenotypic PrP^{Sc} variants.

In conclusion, the binding of copper and zinc to PrP^C resulted in a reduced solubility of the un- and monoglycosylated form of PrP in platelets and brains. This effect was observed when proteins were solubilized with non-ionic detergents as Triton X-100 but not with denaturing detergents such as *N*-lauroylsarcosine. We suggest that the un- or low glycosylated form of PrP^C bind metals with higher efficiency than the diglycosylated form, which results in a reduced solubility in non-ionic detergents. As the different PrP types coexist in homogenized solutions our assay would allow the separation of metal bound and unbound prion proteins and accordingly different conformation types by a centrifugation step. The differentiation of PrP^C glycosylation subtypes prior to and after metal binding will provide precious information on the conversion efficiency to PrP^{Sc} by methods such as the protein misfolding cyclic amplification technique (PMCA) [Soto et al., 2002] and the real time quaking induced conversion (RT-QUIC) [Atarashi et al., 2007].

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