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Copper(II) Binding to the Human Doppel Protein May Mark Its Functional Diversity from the Prion Protein*

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Doppel (Dpl) is the first described homologue of the prion protein, the main constituent of the agent responsible for prion diseases. The cellular prion protein (PrP^C) is predominantly present in the central nervous system. Although its role is not yet completely clarified, PrP^C seems to be involved in Cu^{2+} recycling from synaptic clefts and in preventing neuronal oxidative damage. Conversely, Dpl is expressed in heart and testis and has been shown to regulate male fertility by intervening in gametogenesis and sperm-egg interactions. Therefore, despite a high sequence homology and a similar three-dimensional fold, the functions of PrP^C and Dpl appear unrelated. Here we show by electron paramagnetic resonance and fluorescence spectroscopy that the *in vitro* binding of copper(II) to human recombinant Dpl occurs with a different pattern from that observed for recombinant PrP. At physiological pH values, two copper(II)-binding sites with different affinities were found in Dpl. At lower pH values, two additional copper(II)-binding sites can be identified as follows: one complex is present only at pH 4, and the other is observed in the pH range 5–6. As derived from the electron paramagnetic resonance characteristics, all Dpl-copper(II) complexes have a different coordination sphere from those present in PrP. Furthermore, in contrast to the effect shown previously for PrP^C, addition of Cu^{2+} to Dpl-expressing cells does not cause Dpl internalization. These results suggest that binding of the ion to PrP^C and Dpl may contribute to the different functional roles ascribed to these highly homologous proteins.

Doppel (Dpl, downstream prion protein-like gene or German for "double") is the first described homologue (1) of the prion protein, PrP^C.[1] PrP^C is a cellular glycoprotein of still enigmatic functions and is expressed in higher amounts in the central nervous system (CNS). If present as a conformational isoform called PrP^{Sc}, it causes a class of diseases known as transmissible spongiform encephalopathies (TSEs) or prion diseases (2, 3). PrP^{Sc} seems to be the main, if not the unique, component of the infectious agent of TSEs, the prion (3). TSEs, such as bovine spongiform encephalopathy (BSE) in cattle, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, and fatal familial insomnia in humans, are fatal, progressive, and neurodegenerative disorders of genetic, sporadic, or infectious origin. After the BSE epidemics in Great Britain, the most alarming TSE member is the new variant Creutzfeldt-Jakob disease, probably caused by the consumption of BSE-contaminated meat products (4, 5).

Dpl can be regarded as an *N*-truncated form of PrP^C. It shares 26% sequence homology and an almost superimposable three-dimensional fold, characterized by three α-helices and two short antiparallel β-sheets, with the structured C-terminal domain of PrP^C (1, 6–9). The structure of Dpl is, however, stabilized by the presence of an additional disulfide bridge, which may also explain the incapacity of Dpl to convert into a PrP^{Sc}-like pathogenic conformer (10). Dpl is not required for prion replication (11), and it is mainly expressed in heart and spermatozoa (12, 13), although only transiently in epithelial cells of the CNS during the 1st week after birth (14). Nevertheless, the ectopic expression of Dpl in the CNS of $PrP^{0/0}$ mice results in ataxia due to loss of cerebellar granules and Purkinje cells. As the healthy phenotype is rescued by reintroducing the wild-type PrP transgene (1, 11), the two proteins may have related, albeit opposite, functions in the CNS. PrP^C and Dpl are both anchored to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) molecule. This peculiar surface location, together with the ability of PrP^C to bind copper(II) (Cu(II)) ions, particularly within the PHGGGWGQ consensus sequence of the N-terminal octapeptide repeats (15–18)), has led to the hypothesis that PrP^C acts as a Cu(II)-removing protein from synaptic clefts (19). An involvement of PrP^C in copper metabolism is also supported by the finding that the ion induces internalization of the protein in cell culture systems (20, 21). Recently, we have shown also that the structured C-terminal domain of the protein is able to bind Cu(II) with high specificity, opening the possibility that Cu(II) binding to this region may have an important functional role (18, 22–23), perhaps related to the proposed antioxidant, superoxide dismutase-like activity of the protein (24).

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[1] The abbreviations used are: PrP, prion protein; PrP^C, cellular PrP; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CNS, central nervous system; bPrP, bovine PrP; GFP, green fluorescent protein; NEM, *N*-ethylmorpholine; CHO, Chinese hamster ovary; TSEs, transmissible spongiform encephalopathies; BSE, bovine spongiform encephalopathy; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; GPI, glycosylphosphatidylinositol; hu, human; m, mouse.

Because PrP^C and Dpl have highly similar globular folds and contain histidine residues that appear to be good candidates for Cu(II) binding, we argued that Dpl could also bind the ion (18). Indeed, during the accomplishment of this work, Westaway and co-workers (25) have shown by mass spectrometry and fluorescence measurements that recombinant mouse Dpl (mDpl) contains a specific Cu(II)-binding site at neutral pH, which probably involves histidine residue 131.

Here, we report on the investigation of the Cu(II) binding behavior of recombinant human Dpl, huDpl-(28–152), by EPR and fluorescence spectroscopy. Assays were carried out in the pH range 3–8 not only to clarify fully the binding characteristics of the ion but also to probe copper binding capacities at those pH values that may be experienced physiologically by a protein (pH 4–6 and 7.4). This study shows that Cu(II) binding to Dpl starts only at pH 4 and that at acidic pH values two types of Cu(II)-binding sites are detected as follows: one is visible at pH 4, and the other is observed in the pH range 5–6. Fluorescence quenching measurements show that none of these sites involve a tryptophan residue. At pH 7.4, both specific and nonspecific Cu(II)-binding sites are identified. Moreover, we tested at this pH value the influence of copper on the secondary structure of Dpl using circular dichroism spectroscopy. Finally, in order to get insight into the functional impact of Cu(II) binding to Dpl, we analyzed whether the metal induces internalization of the protein in cell cultures, as was observed previously for PrP^C. We found that Cu^{2+} addition does not induce massive internalization of Dpl, suggesting that Cu(II) binding to Dpl and PrP^C may have different functional implications.

EXPERIMENTAL PROCEDURES

Protein Cloning and Purification

The sequence encoding human Dpl-(28–152) (huDpl-(28–152)) was amplified by PCR using human placenta DNA as template and the following primers: 5′-AAGAATTCAGCCCCTCTCCAACCAAAACTCG-CAA-3′ and 5′-CCGGATCCAGGGGCATCAAGCACAGAATCAAGTG-3′. The amplified product was cloned in the pRSET A plasmid (Invitrogen) between BamHI and EcoRI restriction sites, whereby the 28–152-residue amino acid sequence of Dpl was N-terminally fused to a polyhistidine tag and to a thrombin cleavage site. The latter consists of residue 28 of huDpl and an Ile-Ser substitution at position 29. The plasmid was verified by double-stranded DNA sequencing (ABI Prism kit, Applied Biosystems, Foster City, CA). Expression was carried out in BL-21(D3) *Escherichia coli* cells grown at 37 °C on Luria broth medium containing 100 mg/liter ampicillin. When cells reached an absorbance of 0.6 at 600 nm, Dpl expression was induced by adding 1 mM isopropyl-β-D-thiogalactoside (Sigma) (4 h, 37 °C). Cells were harvested by centrifugation. huDpl-(28–152) was purified as described by Negro *et al.* (26) with the following minor modifications. After solubilization from inclusion bodies (6 M guanidinium chloride, pH 8.0), the protein was immobilized on a metal affinity Ni^{2+}-nitrilotriacetate-agarose column (Qiagen GmbH, Hilden, Germany), refolded on the column in the presence of refolding buffer (20 mM Tris/HCl, pH 8.0, 50 mM NaCl), detached from the histidine tag by addition of thrombin (10 units, Sigma), and eluted from the column (50 mM sodium acetate, pH 5.8). Purified huDpl was dialyzed twice (24 h) against 50 mM ammonium acetate, pH 4.5, and then against Millipore water (20 h). The protein was stored at −80 °C. Analysis by MALDI-MS of the intact protein and of its proteolytic digests, under oxidized or reduced conditions, proved that Dpl has the expected mass (calculated average M_r of oxidized Dpl, including the Ile-Ser substitution at position 29, 14,312.05; observed M_r, 14,312.5), with two intramolecular disulfide bonds mapped at the predicted positions of the human sequence (between cysteine residues 94–145 and 108–140) (1).

Sample Preparation for EPR and Fluorescence Spectroscopy Measurements—The following buffers were used (all at 10 mM concentration): formic acid/sodium hydroxide, pH 3 and 4; sodium acetate/hydrochloride, pH 5.0; sodium cacodylate/hydrochloride, pH 6; and MOPS/sodium hydroxide pH 7.4 and 8. For the EPR measurements at the different pH values, 1–7 molar eq of $CuCl_2$ were added to a stock solution of recombinant huDpl to obtain a final protein concentration of 100 μM (final sample volume 70–100 μl). Alternatively, the metal-free protein was first dialyzed against the desired buffer solution containing 50 μM $CuCl_2$, and afterward against the same buffer without Cu^{2+}, to remove free Cu^{2+}. To improve the spectral quality, 10% (v/v) glycerol was added as a cryoprotectant. Control measurements were performed to ascertain whether glycerol influenced EPR parameters. Because glycerol changed the Cu(II) buffer signal only at pH 5, it was omitted from all measurements at this pH. For fluorescence spectroscopy measurements, stock huDpl-(28–152) solutions were diluted to a final protein concentration of 0.7 μM, using the above described buffer solutions as well as HEPES and *N*-ethylmorpholine (NEM)-KCl buffers, both at 10 mM concentration, pH 7.4. By using a concentrated $CuCl_2$ solution to minimize the sample dilution (lower than 2%), 3–20 molar eq of $CuCl_2$ were added to the 0.7 μM solution of huDpl. Fluorescence quenching was also studied in the presence of 4 molar eq of $ZnCl_2$, $MgCl_2$, or $CaCl_2$.

Continuous Wave EPR Spectroscopy—EPR spectra were recorded on a Bruker ESP300E spectrometer (microwave frequency 9.43 GHz) equipped with a gas-flow cryogenic system, allowing operation from room temperature down to 2.5 K. All spectra were recorded with a microwave power of 10 milliwatts, a modulation frequency of 100 kHz, and a modulation amplitude of 0.5 millitesla. All experiments were performed at 90 K. The magnetic field was measured with a Bruker ER 035M NMR gaussmeter and was calibrated by using a sample of diphenylpicrylhydrazyl. The EPR parameters were determined by simulation of the EPR spectra using the EasySpin program (www.esr.ethz.ch), whereby the contribution of both copper isotopes (^{63}Cu and ^{65}Cu) were taken into account.

Circular Dichroism Spectroscopy—Far-UV circular dichroism spectra were measured at 25 °C on a Jasco J-715 spectropolarimeter in 0.1-cm quartz cuvettes, accumulated eight times, and corrected for the background. Protein samples of 10 μM huDpl in 10 mM MOPS/NaOH buffer, pH 7.4, in the presence or absence of 1 or 2 molar eq of $CuCl_2$ were analyzed.

Fluorescence Spectroscopy—Steady-state fluorescence spectra were recorded on a Cary spectrophotometer. Emission spectra were collected from 290 to 500 nm (λ_{ex} = 280 nm, 0.5 nm/s, bandpass 5 nm for excitation and emission). Fluorescence intensities were integrated from 290 to 480 nm after subtraction of the background. The incubation time before fluorescence measurements was varied to allow protein-Cu(II) interactions. The protein was incubated at room temperature or at 4 °C. For each experimental condition analyzed, a control sample without Cu^{2+} was measured to correct for degradation of the protein during the incubation time.

Endocytosis Experiments

Plasmid Construction—cDNAs coding for the eukaryotic expression of human PrP and human Dpl fused to the green fluorescent protein (GFP) (huPrP-GFP and huDpl-GFP) were constructed as described previously (27). The plasmid encoding the chimeric GFP-GPI_{PrP} protein was obtained from the plasmid pGFP-bPrP (encoding for bovine PrP (bPrP) fused to GFP) (28), after deletion of bPrP sequence 43–221, using inverse PCR and the following primers: 5′-TCCAGATCTGAGTCCGG-ACTTGTACAGCTC-3′ and 5′-AGAAGATCTCAGGCTTATTACCAAG-GGGGGC-3′. The resulting construct codes for GFP linked to bPrP leader sequence 1–42, and to bPrP sequence 222–256 (which includes the attachment signal for GPI), at the N and C terminus, respectively.

Cell Culture—Wild-type or stably transfected CHO cells were maintained at 37 °C in Ham's F-12 medium, 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen) and 1% penicillin/streptomycin (Invitrogen.), in 75-cm^2 culture bottles in a 5% CO_2 atmosphere. The medium was changed every 2–3 days. The transient transfection of wild-type cells was performed 1 day after plating cells on 24-mm coverslips, using the liposome-mediated method (LipofectAMINE Plus, Invitrogen) and Opti-MEM medium, following the manufacturer's instructions. 1 μg of plasmid was added to each well (10^5 cells/well). To optimize protein expression, 4 h after transfection the medium was changed, and cells were kept at 30 °C for 72 h before starting the endocytosis experiment. CHO cells stably expressing huPrP- or huDpl-GFP were established as described by Negro *et al.* (28). Before use, cells were plated on 24-mm coverslips (10^5 cells/well), grown at 37 °C for 24 h, and then kept for the next 24 h at 30 °C.

Fluorescence Imaging—Copper-induced protein internalization was followed in single live cells by means of the GFP fluorescence (excitation at 488 nm; emission at 509 nm), using a Zeiss Axiovert 100 microscope equipped with a 16-bit digital CCD videocamera (Micromax, Princeton Instruments, Trenton, NJ). Images were taken at 5-min intervals, before and after the addition of 200 or 500 μM Cu(II) acetate (pH 7.4, room temperature), and analyzed using the Metafluor or Metamorph software (Universal Imaging). Alternatively, after 40 min of incubation at 37 °C, in

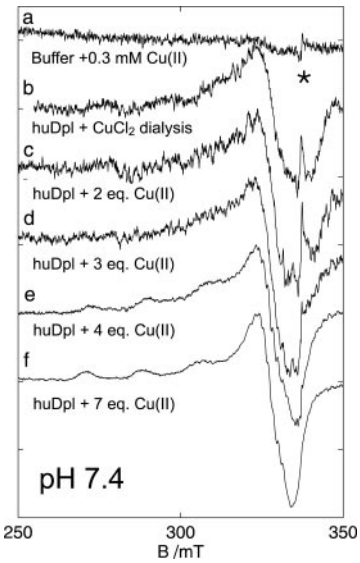

FIG. 1. **X-band EPR spectra of huDpl-(28–152) in the presence of Cu^{2+} at pH 7.4.** EPR spectra of the MOPS buffer solution with 0.3 mM $CuCl_2$ (*a*), 0.1 mM huDpl-(28–152) after dialysis against $CuCl_2$ buffer solution (*b*), 0.1 mM huDpl-(28–152) with 1 molar eq Cu^{2+} added (*c*), 0.1 mM huDpl-(28–152) with 3 molar eq Cu^{2+} added (*d*), 0.1 mM huDpl-(28–152) with 4 molar eq Cu^{2+} added (*e*), and 0.1 mM huDpl-(28–152) with 7 molar eq Cu^{2+} added (*f*).

Ham's F-12 culture medium with or without 500 μM Cu(II) acetate, cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (4 °C, 30 min) and then incubated in chilled methanol (5 min, −20 °C). After washing with phosphate-buffered saline, coverslips were mounted in glycerol for observation under the fluorescence microscope.

RESULTS

EPR Experiments—The EPR spectra of huDpl-(28–152) in the presence of copper (Figs. 1 to Fig. 4) are typical for type 2 protein-Cu(II) complexes (axial g matrix, copper hyperfine splitting, $A_\parallel$ >400 MHz) (29). Type 2 complexes are largely square planar with a possible fifth weak coordination. For type 2 Cu(II) complexes, ($g_\parallel$, $A_\parallel$) values correlate with the type of the equatorially coordinating atoms (30). Unfortunately, the $g_\parallel$ and $A_\parallel$ values depend also on other parameters (such as the charge of the surrounding ligands and a possible fifth ligand). This hampers a clear-cut determination of the first coordination sphere of the Cu(II) on the basis of the CW-EPR data alone. Nevertheless, the $g_\parallel$ and $A_\parallel$ values can be used to get a first idea about the coordinating atoms and are very useful in the comparison of the Cu(II) binding of prion and doppel proteins.

Fig. 1*a* shows the control EPR spectrum of the MOPS buffer, pH 7.4, with 2 mM Cu^{2+}. At pH ≥ 7, aquo-Cu(II) has only low solubility and precipitates largely as EPR silent $[Cu(OH)_2]_n$, so that no Cu(II) signal can be observed in the control. The signal indicated by *, in Fig. 1*a*, is a cavity signal. Fig. 1*b* shows the EPR spectrum of 100 μM huDpl-(28–152) dialyzed against a buffer solution containing 50 μM $CuCl_2$ with subsequent dialysis against the same buffer without Cu^{2+} in order to remove unbound Cu^{2+}. An EPR spectrum typical for a type 2 Cu(II) complex is visible (EPR parameters given in Table I, complex D3). The spectrum is analogous to the one obtained after addition of 1 molar eq of Cu^{2+} to 100 μM of the protein, without dialysis at pH 7.4 (Fig. 1*c*) and at pH 8 (Fig. 2*a*). After addition of more equivalents of Cu^{2+}, a second Cu(II) complex becomes dominant (Fig. 1, *d–f*, Fig. 2*b*, and complex D4 in Table I). Because this second complex does not appear in the dialyzed samples (Fig. 1*b*) and appears only after addition of an excess of copper, the corresponding binding site(s) will have a low binding affinity for Cu^{2+} and is probably of no biological relevance. The increase of the EPR intensity of the D4 contribution with increasing Cu(II) concentration indicates that more than one nonspecific complex with similar structure is formed per Dpl protein (Fig. 1).

The current EPR observations can explain the earlier observations of Qin *et al.* (25). These authors detected with MALDI-MS that two Cu(II) bind per murine recombinant Dpl-(27–154) protein (mDpl-(27–154)). However, their equilibrium dialysis and fluorescence measurements revealed only one Cu(II)-binding site per Dpl peptide with a K_d on the order of 0.16–0.4 μM. Because the MALDI-MS experiments were performed after incubation of the peptide with 10 times molar excess of $CuCl_2$ for 1 h at room temperature, both complexes D3 and D4 can be present. Because our present results show that the D4-type complex is not stable against dialysis, it will not show up in the equilibrium dialysis results. In turn, the fluorescence-quenching experiment of Qin *et al.* (25) only tested the involvement of Trp in the binding sites, and therefore, it does not give information on *all* possible binding sites.

The $g_\parallel$ and $A_\parallel$ parameters of complex D4 are in agreement with four types of Cu(II) ligation, namely ligation to four nitrogens (4N), to three nitrogens and one oxygen (3N1O), to two nitrogens and two oxygens (2N2O), or one nitrogen and three oxygens (1N3O) (30). The EPR parameters of complex D3 agree with ligations 4N, 3N1O, or 2N2O. The observation of a resolved superhyperfine structure in the $g_\perp$ region of the EPR spectrum of D4 (Fig. 1*f*) confirms the direct interaction with different nitrogen nuclei. The EPR parameters of both complexes agree with an involvement of backbone nitrogens in the binding (31).

Because our earlier measurements showed that the C-terminal part of murine recombinant PrP (mPrP-(121–231)) can bind Cu(II) at low pH (pH 3–6) under *in vitro* conditions (18), we also investigate here the Cu(II) binding to huDpl-(28–152) at low pH. At pH 3, no Cu(II) binding to huDpl was observed. At pH 4, huDpl-(28–152) is found to bind Cu(II) (Fig. 3; complex D1, Table I). Upon addition of 2 molar eq of copper, an EPR signal of the copper-bound buffer starts to appear and increases with increasing Cu(II) concentration (Fig. 3). This indicates that huDpl-(28–152) binds no more than two Cu(II) ions per peptide at pH 4. These observations also show that the Dpl protein has a higher affinity for binding Cu(II) than the formate buffer molecules (note that the buffer concentration is 100 times the protein concentration). The binding site was found to be stable against dialysis. The $g_\parallel$ and $A_\parallel$ parameters of complex D1 are in correspondence with the following three combinations: 4O, 3O1N, and 2N2O (30). The EPR parameters of this complex clearly differ from the ones observed for the complexes P1 and P2 in mPrP-(23–231) at the same pH (18) (Table I).

Fig. 4*a* shows that the acetate buffer used at pH 5 is capable of binding Cu^{2+} (Fig. 4*a*), whereas the cacodylate buffer, pH 6, does not bind Cu^{2+} (only a weak EPR signal attributed to hexaquo-Cu(II) is observed (Fig. 4*d*)). Fig. 4, *b* and *c*, shows the EPR spectra of huDpl-(28–152) with 2 molar eq of Cu^{2+} at pH 5 and 6, respectively. In both cases the same spectrum is observed (complex D2, Table I), which differs from the complexes D1 and D3/D4 observed at pH 4 and 7–8, respectively. Because different buffers are used at pH 5 and 6, a ternary Cu(II) complex involving both the huDpl protein and the buffer can be excluded for complex D2. The EPR parameters of complex D2 are in agreement with 1N3O, 2N2O, 3N1O, or 4N ligation (30). At pH 5, the Cu(II)-binding affinity of the Dpl protein ligation clearly higher than that of the acetate buffer molecules. After addition of 3–4 molar eq of Cu^{2+} onward, the EPR spectrum starts to change and contributions of the Cu(II)-buffer complex start to be visible. Similarly, at pH 6, where the cacodylate buffer is not binding copper, the EPR intensity of

TABLE I

EPR parameters of the type 2 Cu(II) complexes observed in copper-containing huDpl-(28–152)

The g values and the principal hyperfine values of ^{63}Cu are given. The given errors apply to the EPR parameters determined in this work. NB indicates no binding. mPrP-(58–91) is not binding at a pH lower than 6 (18). The buffers are abbreviated as follows: F, formic acid/NaOH; A, sodium acetate/HCl; C, sodium cacodylate/HCl; M, MOPS/NaOH; and N, NEM/KCl.

	Complex	$g_\parallel$ (± 0.005)	$g_\perp$ (± 0.005)	$A_\parallel$/MHz (±10)	$A_\perp$/MHz (±20)	pH	Buffer	Ref.
huDpl-(28–152)		NB	NB	NB	NB	3	F	This work
mPrP-(23–231)	P1	2.332	2.068	452	12		F	18
	P2	2.295	2.068	457	20		F	
huDpl-(28–152)	D1	2.320	2.065	490	30	4	F	This work
mPrP-(23–231)	P1	2.332	2.068	452	12		F	18
	P2	2.295	2.068	457	20		F	18
huDpl-(28–152)	D2	2.280	2.058	522	30	5	A	This work
mPrP-(23–231)	P1	2.332	2.068	452	12		A	18
	P2	2.295	2.068	457	20		A	18
huDpl-(28–152)	D2	2.280	2.058	522	30	6	C	This work
mPrP-(23–231)	P1	2.332	2.068	452	12		C	18
	P2	2.295	2.068	457	20		C	18
mPrP-(58–91)	P3	2.270	2.055	520	50		C or N	18, 32
huDpl-(28–152)	D3	2.205	2.045	592	60	7–8	M	This work
	D4	2.260	2.055	560	30		M	This work
mPrP-(23–231)	P4	2.295	2.068	457	20		M	18
	P3	2.230	2.055	495	50		M	18
mPrP-(58–91)	P5	2.270	2.055	520	50		M or N	18, 33
	P3	2.230	2.055	495	50		M or N	18, 33
PrP-(90–101)	P6	2.21		588			N	33
PrP-(92–96)	P6	2.21		588			N	33

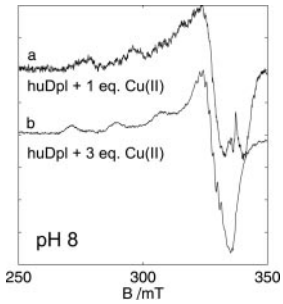

FIG. 2. **X-band EPR spectra of huDpl-(28–152) in the presence of Cu^{2+} at pH 8.** EPR spectra of 0.1 mM huDpl-(28–152) with 1 molar eq Cu^{2+} added (*a*) and 0.1 mM huDpl-(28–152) with 3 molar eq Cu^{2+} added (*b*).

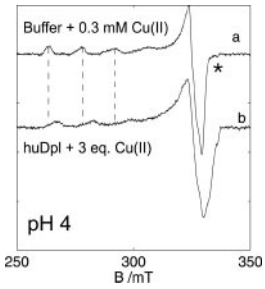

FIG. 3. **X-band EPR spectra of huDpl-(28–152) in the presence of Cu^{2+} at pH 4.** EPR spectra of the pH 4 formate buffer solution with 0.3 mM $CuCl_2$ (*a*) and 0.1 mM huDpl-(28–152) with 2 molar eq Cu^{2+} added (*b*).

complex D2 reaches its maximum after addition of 3 eq of copper, indicating that a maximum of three D2-type binding sites are present in huDpl-(28–152). Comparison of the maximum EPR intensity of D2 (obtained by double-integration of the spectrum after base-line correction) with those of standard solutions of Cu^{2+} in 10 mM imidazole at pH 7.4 indicates that less than two D2-type sites are present per protein molecule. Note, however, that the exact determination of the concentration of bound Cu(II) is very difficult with EPR.

The overall observation that $g_\parallel$ decreases and $A_\parallel$ increases from D1 to D2 to D4 to D3 indicates that there is an increase in the number of nitrogens involved in the Cu(II) binding in the

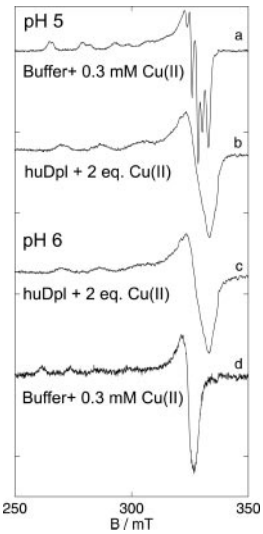

FIG. 4. **X-band EPR spectra of huDpl-(28–152) in the presence of Cu^{2+} at pH 5 and 6.** EPR spectra of the pH 5 acetate buffer solution with 0.5 mM $CuCl_2$ (*a*), 0.1 mM huDpl-(28–152) with 2 molar eq Cu^{2+} added at pH 5 (*b*), 0.1 mM huDpl-(28–152) with 2 molar eq Cu^{2+} added at pH 6 (*c*), and the pH 6 cacodylate buffer solution with 0.4 mM $CuCl_2$ (*d*).

same order. Indeed, at lower pH values, protonation of backbone nitrogens and nitrogen base-type amino acid residues is expected.

Circular Dichroism—The two minima at 208 and 222 nm of the CD spectrum of huDpl-(28–152) at pH 7.4 (Fig. 5, *full line*) show that the protein is recovered with the expected α-helix-rich conformation (8). Most interestingly, however, upon addition of 1 (*long-dashed line*) or 2 eq (*short-dashed line*) of $CuCl_2$, there is an increasing intensity shift of the minimum values, suggesting that copper(II) binding to huDpl enhances the α-helical content of the protein.

Fluorescence-quenching Experiments—Earlier fluorescence measurements showed that one Trp residue is involved in the Cu(II) binding of mDpl at pH 7.4 (25). huDpl-(28–152) contains four Trp residues at positions 35, 84, 133, and 148 and four tyrosine residues at positions 77, 78, 83, and 91.

In order to test whether these residues also undergo a

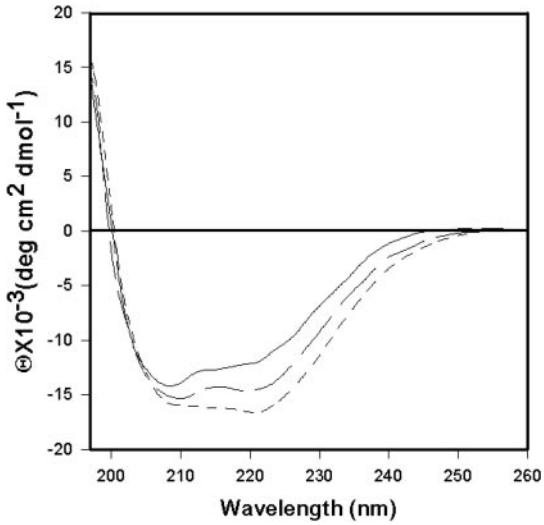

FIG. 5. **CD spectra of huDpl-(28–152).** CD spectra of huDpl-(28–152) (10 μM) were recorded in the absence (*full line*) or in the presence of 1 (*long dashed line*) or 2 molar eq (*short dashed line*) of $CuCl_2$ in 10 mM MOPS/NaOH, pH 7.4.

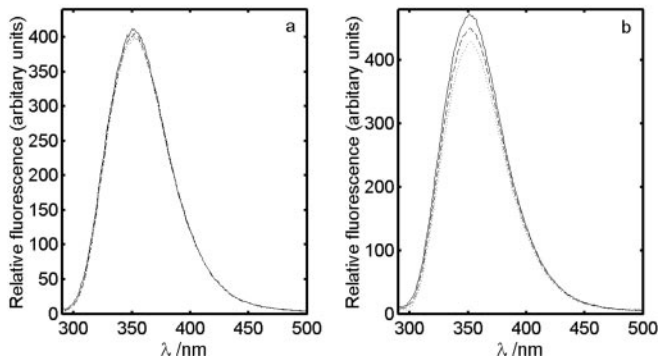

FIG. 6. **Effect of Cu(II) on huDpl-(28–152) proteins measured with fluorescence spectrometry.** Fluorescence quenching by addition of 0 (*full lines*), 6 (*dashed lines*), and 20 molar eq (*dotted lines*) of Cu(II) to 0.7 μM huDpl-(28–152) at pH 4 (*a*) and pH 5 (*b*). The spectra are shown after subtraction of the background.

change in the environment upon Cu(II) binding at pH <7, we measured the change of the fluorescence intensity of the Trp and Tyr residues upon Cu^{2+} addition at pH 4 (formation of complex D1) and at pH 5 (observation of complex D2). The measurements were repeated for different batches, whereby 6–20 molar eq of Cu^{2+} were added.

At pH 4 the fluorescence signal reduction after Cu^{2+} addition is only 2.50% (±1.22%) of the signal intensity in the absence of Cu(II). No significant difference in the quenching was observed even after addition of up to 20 molar eq of copper to the protein (Fig. 6*a*). It can thus be excluded that a Trp residue is taking part in the Cu(II) complex D1.

At pH 5, the fluorescence signal reduction is more dependent on the Cu(II) concentration (Fig. 6*b*). Addition of 6 molar eq of $CuCl_2$ led to a reduction of the fluorescence signal by 4.85% (±1.62%). Addition of a large surplus of Cu^{2+} ions (20 molar eq) led to a quenching of the signal by 10.3% (±1.13%). Because the protein may be less stable upon addition of a large surplus of copper, the latter decrease of the fluorescence signal might be due to protein aggregation. This is corroborated by the fact that the slow quenching does not correlate with the increase of the EPR intensity of complex D2 upon addition of different Cu^{2+} molar equivalents, so it can be excluded that complex D2 formation induces the observed fluorescence change. The change in the intensity was not accompanied by a significant shift in wavelength of the maximum (λ_{max}), indicating that the solvent accessibility of the Trp residue is not affected.

Fluorescence-quenching experiments were also undertaken at pH 7.4 for huDpl in accordance with the analyses of Qin *et al.* (25) for mDpl. The latter experiments were done in NEM-KCl buffer. For additions of less than 2 molar eq of Cu^{2+} ions in this buffer, the fluorescence quenching of huDpl was fully in accordance with the earlier observations for mDpl. However, at higher molar equivalents of copper, the fluorescence signal decreased dramatically. The quenching was found to be temperature-dependent, time-dependent, and buffer-dependent (the maximum quenching observed after addition of 4 molar eq of Cu^{2+} was 46% in MOPS buffer, 40% in NEM-KCl, and 62.2% in HEPES). All these observations indicate that the large decrease of the fluorescence is related to a reduced stability and thus degradation of the protein in the presence of more than 2 molar eq of copper. Furthermore, control experiments with other metal ions showed analogous behavior. In NEM-KCl buffer, the maximum quenching of the fluorescence signal observed after addition of 4 molar eq of Zn^{2+}, Mg^{2+}, and Ca^{2+} was 15.2, 16, and 9.44%, respectively. In contrast, mDpl was found not to bind these metal ions (quenching <3%) (25). Finally, it should be noted that the EPR experiments showed a clear change of the spectrum upon addition of more than 2 molar eq of copper. This change was related to biologically irrelevant binding site(s). huDpl thus appears to be less stable at pH 7.4 in the presence of metal ions than the related murine doppel peptides. This protein instability was not observed at the lower pH values.

Endocytosis Experiments—Copper-induced protein internalization was followed by fluorescence microscopy in CHO cells transiently or stably transfected with a plasmid encoding either huDpl-GFP or huPrP-GFP fusion proteins. The fused GFP has been shown not to interfere with the biochemistry and cellular trafficking of PrP^C and Dpl (27, 28); the GFP fluorescence is thus a useful tool for tracing the movements of a protein within living cells. To exclude that endocytosis after Cu^{2+} addition is a feature common to most GPI-anchored proteins, we also used a GFP-GPI_{PrP} fusion. GFP-GPI_{PrP} is a pertinent control because it is a GPI-anchored protein, sharing the GPI molecule with bPrP.

After addition of 200 μM Cu(II) acetate to CHO cells transiently expressing huPrP-GFP, a pronounced endocytosis process was observed. Indeed, 40 min after Cu^{2+} addition, most of the fluorescence on the plasma membrane is attenuated (Fig. 7), and the fluorescence drop is paralleled by an increased fluorescent signal in the perinuclear region. This result confirms previously reported observations on the ability of copper to induce PrP^C recycling from the plasma membrane to internal compartments in cell culture systems (20, 21). In contrast, 40 min after administration of equal amounts of Cu(II) acetate to huDpl-GFP-expressing cells, there is only a slight fluorescence increase in the perinuclear region, with no relevant reduction of the plasma membrane fluorescence (Fig. 7). This suggests that Cu(II) binding to Dpl does not stimulate endocytosis of the protein as much as it is observed for PrP^C (Refs. 20, 21, and this work). To certify further that Cu(II) is not intervening aspecifically in the trafficking of GPI-anchored proteins, Cu(II) acetate was added to cells transiently expressing GFP-GPI_{PrP}. As evident from the retention of the fluorescent signal on the plasma membrane (Fig. 7), addition of 200 μM Cu(II) acetate does not provoke any movement of the protein inside the cell. As similar results were obtained in CHO cells stably expressing huPrP or huDpl (data not shown), this excludes that the different response of PrP^C and Dpl to copper is dependent on the used transfection protocol. In all cases, similar results were obtained by adding 500 μM Cu(II) acetate (data not shown).

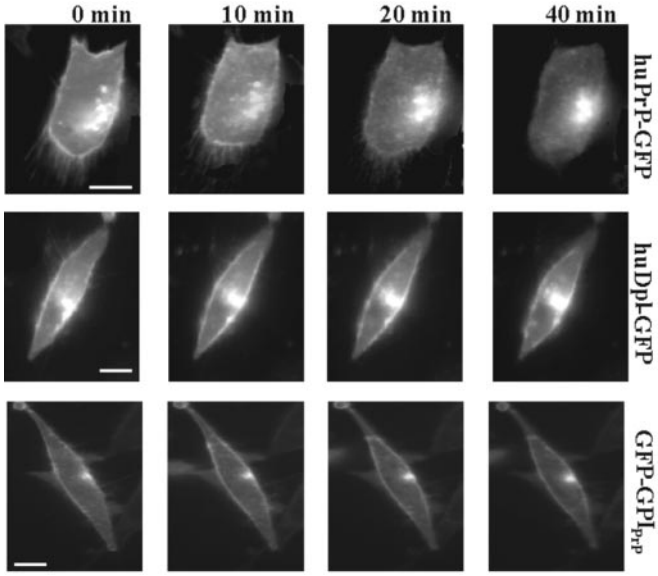

FIG. 7. **Analysis of copper-induced protein internalization in CHO cells.** Fluorescence imaging of CHO cells transiently expressing huPrP-GFP, huDpl-GFP, and GFP-GPI$_{PrP}$. Images are taken before and after 10, 20, and 40 min after the addition of 200 μM Cu(II) acetate, in Ham's F-12 medium at room temperature. The bright intracellular spot present in all pictures corresponds to the Golgi apparatus (27, 28). It should be noted that only in cells expressing huPrP-GFP is there a progressive increase in intracellular fluorescence that correlates with a concomitant fluorescence decrease at the plasma membrane. This indicates that addition of copper induces migration of huPrP-GFP inside the cell. *Scale bar*, 10 μm.

A more quantitative analysis obtained with the cell clones fixed 40 min after incubation, in the presence or in the absence of 500 μM copper, showed that almost all cells expressing huPrP-GFP had a marked perinuclear fluorescence, whereas in 90% of cells with huDpl-GFP the fluorescence signal remained localized at the plasma membrane, similarly to the control cells (data not shown).

DISCUSSION

Although the devastating role of prions in the CNS, resulting in death of neurons and astrogliosis, is well documented, the function of the prion protein in the CNS is still under debate. Among the several proposals, one envisages that PrP^C is essential for the uptake and clearance of Cu^{2+} ions from synaptic clefts, thereby protecting neurons against Cu^{2+} toxicity. Indeed, it has been found that PrP-knock-out neuronal cultures are more sensitive to copper insults than the wild-type counterpart (34). Such an hypothesis is consistent with the capacity of the N-terminal domain of the protein to bind Cu(II) with high affinity but also with the stimulation of PrP^C internalization (hence of the bound metal) found in cell cultures exposed to copper (20, 21). Also dependent on Cu(II) binding is the related function as antioxidant protein attributed to PrP^C. However, we have shown previously (18) that the C-terminal domain of PrP is also able to bind Cu(II) ions *in vitro* at different pH values, an interesting finding with respect to Dpl, which lacks the N-terminal region of PrP^C but shares high biochemical and structural homologies with the C-terminal portion of PrP^C. Dpl is expressed in adults in peripheral tissues and has been shown to play a fundamental role in male fertility (12). Yet if Cu(II) binding to PrP and Dpl would show the same physicochemical characteristics, one could argue that the binding of the ion imposes on the proteins similar modes of action, although performed in different tissues. In this work, we have analyzed this issue by applying sophisticated techniques to the human recombinant full-length Dpl. We have shown by EPR spectroscopy that Dpl also binds Cu(II) and that different binding sites are observed according to the prevailing pH. At pH 7.4–8, for example, we identified two sites (complexes D3 and D4, Table I). However, as complex D4 is removed by dialysis of the Cu(II)-bound protein, its low affinity rules out any biological relevance. The EPR parameters of complex D3 (Table I) indicate a large involvement of nitrogen atoms in the Cu(II) ligation (2N2O to 4N), whereas the comparison with the EPR data of known Cu(II)-peptide complexes suggests that backbone nitrogen(s) may be involved (31). Although the *g* and copper hyperfine values of complex D3 are clearly different from the EPR parameters of the Cu(II)-binding sites found in the C- and N-terminal part of mPrP, they have similarities with the EPR parameters of complex P6 observed in Cu(II)-bound Syrian hamster PrP-(90–101) (GQGGGTHNQWNK) and PrP-(92–96) (GGGTH) (Table I, (33)). In either case, this may be due to the involvement of one histidine and backbone nitrogen. Indeed, diethylpyrocarbonate footprinting of mDpl-(27–154) and mDpl-(101–145) suggested that His-131 is involved in Cu(II) binding at pH 7.4 (equivalent histidine in huDpl, His-128) (25). In the same study, the binding site was also found to involve Trp-136. At Cu(II)/huDpl-(28–152) ratios smaller than 2, we too observed the same fluorescence-quenching behavior as in the murine case (at higher copper concentrations, Cu(II)-induced protein aggregation was observed). This confirms the role of a Trp in the Cu(II)-huDpl complex at pH 7.4, although Trp may not necessarily bind the Cu(II) ion, but indirectly stabilizes the complex via H-bonding, as observed in Cu(II) complexes of HGGGW segments (35).

Because His-128 is located at the beginning of the third helix of huDpl, we also investigated whether the binding of copper impinges on Dpl secondary structure at this pH. Indeed, there was a shift in the intensity of the peak at 222 nm of the protein CD spectrum with increasing copper concentrations (Fig. 5), indicating that such a binding may increase the α-helical content of the protein. Possibly, the effect of copper(II) binding to His-128 is to extend the third helix by allowing Asp-125 to become a coordination partner, whereas the backbone nitrogen(s) may originate from residues positioned in the loop linking the second and third helices. The results obtained with CD spectroscopy also reinforce our previous suggestion that Trp-133 is likely to play a stabilizing role rather than being directly involved in the binding of copper.

At acidic pH values two types of Cu(II)-binding sites are observed. The analysis of binding at acidic pH values is interesting with respect to the possibility that Dpl, like PrP^C, may be sequestered in acidic subcellular compartments like lysosomes (pH 4–6). Complex D1 is observed at pH 4, and the EPR parameters agree with a large involvement of oxygen atoms in the Cu(II) ligation (4O to 2N2O). At pH 5–6 a second complex (D2) is identified, with EPR parameters predicting a larger involvement of nitrogen atoms in the first coordination sphere of the copper. The pH dependence of D2 suggests the involvement of one histidine in the complex. This is corroborated by the fact that the EPR parameters are similar to those observed in Cu(II)-bound mPrP-(58–91) at pH 6 (18, 32), for which histidine binding has been proposed. The lack of complex D2 in the pH 7.4 EPR spectra indicates that the binding site is largely pH-dependent. Possibly, complex D2 converts into complex D3 by increasing the pH and the consequent deprotonation of backbone nitrogens. Our fluorescence-quenching experiments show that none of the binding sites observed at pH <7 involve Trp. D2 thus differs from D3 not only in the number of backbone nitrogens involved in the complex but also in the Trp involvement.

In short, huDpl starts to bind Cu(II) at higher pH values (pH

4) than we have found previously for the C-terminal part of mPrP, which binds Cu(II) already at pH 3. In addition, all Cu(II)-binding sites observed in huDpl have EPR characteristics that clearly differ from the ones reported for the full-length murine PrP. The Cu(II)-huDpl complexes are thus different in number and coordination sphere from those described for the structured C-terminal portion of PrP^{C} (18), suggesting that the Cu(II)-binding sites are not superimposable in the three-dimensional fold. This could result in different structural and/or functional implications for the bound Cu(II) ions, and may tentatively explain, for example, the incapacity of Dpl to convert into a PrP^{Sc}-like isoform, and why PrP^{C} and Dpl exert their prime function in different tissues, *i.e.* in the CNS and in testis, respectively.

Most importantly, these conclusions are in line with the findings that PrP^{C}, but not Dpl, is endocytosed in copper-exposed cells (Fig. 7). This result may exclude that Dpl protects cells from copper toxicity by sequestering the ion from the extracellular space and transporting it into the cell cytoplasm, as in the case of PrP^{C}. In addition, it should be noted that the presence in PrP^{C} of the N-terminal octapeptide repeat region, which can bind as much as six extra Cu(II) in addition to those bound to the C-terminal domain, renders the protein a more efficient Cu(II) transporter than Dpl. Most interestingly, the disordered N-terminal region of PrP^{C} seems indispensable in regulating the proper trafficking of the protein to, and from, the plasma membrane (36, 37), and this may also relate to the presence of the ion in this region.

In conclusion, by applying highly sensitive techniques, we demonstrate in this work that Dpl, like the related C-terminal portion of PrP^{C}, is able to bind Cu(II) ions in a pH-dependent manner but that the bound coppers(II) appear not to fulfill the same structural and functional roles as found for the prion protein.

REFERENCES

1. Moore, R. C., Lee, I. Y., Silverman, G. L., Harrison, P. M., Strome, R., Heinrich, C., Karunaratne, A., Pasternak, S. H., Chishti, M. A., Liang, Y., Mastrangelo, P., Wang, K., Smit, A. F. A., Katamine, S., Carlson, G. A., Cohen, F. E., Prusiner, S. B., Melton, D. W., Tremblay, P., Hood, L. E., and Westaway, D. (1999) *J. Mol. Biol.* **292,** 797–817
2. Prusiner, S. B. (1982) *Science* **216,** 136–144
3. Prusiner, S. B. (1998) *Brain Pathol.* **8,** 499–513
4. Collinge, J., Sidle, K. C., Meads, J., Ironside, J., and Hill, A. F. (1996) *Nature* **383,** 685–690
5. Prusiner, S. B. (1997) *Science* **278,** 245–251
6. Riek, R., Hornemann, S., Wider, G., Glockshuber, R., and Wuthrich, K. (1997) *FEBS Lett.* **413,** 282–288
7. Donne, D. G., Viles, J. H., Groth, D., Mehlhorn, I., James, T. L., Cohen, F. E., Prusiner, S. B., Wright, P. E., and Dyson, H. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 13452–13457
8. Mo, H., Moore, R. C., Cohen, F. E., Westaway, D., Prusiner, S. B., Wright, P. E., and Dyson, H. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 2352–2357
9. Luhrs, T., Riek, R., Guntert, P., and Wuthrich, K. (2003) *J. Mol. Biol.* **326,** 1549–1557
10. Rossi, D., Cozzio, A., Flechsig, E., Klein, M. A., Rulicke, T., Aguzzi, A., and Weissmann, C. (2001) *EMBO J.* **20,** 694–702
11. Moore, R. C., Mastrangelo, P., Bouzamondo, E., Heinrich, C., Legname, G., Prusiner, S. B., Hood, L., Westaway, D., DeArmond, S. J., and Tremblay, P. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 15288–15293
12. Behrens, A., Genoud, N., Naumann, H., Rulicke, T., Janett, F., Heppner, F. L., Ledermann, B., and Aguzzi, A. (2002) *EMBO J.* **21,** 3652–3658
13. Peoc'h, K., Serres, C., Frobert, Y., Martin, C., Lehmann, S., Chasseigneaux, S., Sazdovitch, V., Grassi, J., Jouannet, P., Launay, J. M., and Laplanche, J. L. (2002) *J. Biol. Chem.* **277,** 43071–43078
14. Li, A., Sakaguchi, S., Shigematsu, K., Atarashi, R., Roy, B. C., Nakaoke, R., Arima, K., Okimura, N., Kopacek, J., and Katamine, S. (2000) *Am. J. Pathol.* **157,** 1447–1452
15. Brown, D. R., Qin, K., Herms, J. W., Madlung, A., Manson, J., Strome, R., Fraser, P. E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D., and Kretzschmar, H. (1997) *Nature* **390,** 684–687
16. Stockel, J., Safar, J., Wallace, A. C., Cohen, F. E., and Prusiner, S. B. (1998) *Biochemistry* **37,** 7185–7193
17. Viles, J. H., Cohen, F. E., Prusiner, S. B., Goodin, D. B., Wright, P. E., and Dyson, H. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2042–2047
18. Cereghetti, G. M., Schweiger, A., Glockshuber, R., and Van Doorslaer, S. (2001) *Biophys. J.* **81,** 516–525
19. Herms, J., Tings, T., Gall, S., Madlung, A., Giese, A., Siebert, H., Schurmann, P., Windl, O., Brose, N., and Kretzschmar, H. (1999) *J. Neurosci.* **19,** 8866–8875
20. Pauly, P. C., and Harris, D. A. (1998) *J. Biol. Chem.* **273,** 33107–33110
21. Lee, K. S., Magalhaes, A. C., Zanata, S. M., Brentani, R. R., Martins, V. R., and Prado, M. A. (2001) *J. Neurochem.* **79,** 79–87
22. Van Doorslaer, S., Cereghetti, G. M., Glockshuber, R., and Schweiger, A. (2001) *J. Phys. Chem.* **105,** 1631–1639
23. Cereghetti, G. M., Schweiger, A., Glockshuber, R., and Van Doorslaer, S. (2003) *Biophys. J.* **84,** 1985–1997
24. Brown, D. R., Wong, B. S., Hafiz, F., Clive, C., Haswell, S. J., and Jones, I. M. (1999) *Biochem. J.* **344,** 1–5
25. Qin, K., Coomaraswamy, J., Mastrangelo, P., Yang, Y., Lugowski, S., Petromilli, C., Prusiner, S. B., Fraser, P. E., Goldberg, J. M., Chakrabartty, A., and Westaway, D. (2003) *J. Biol. Chem.* **278,** 8888–8896
26. Negro, A., Meggio, F., Bertoli, A., Battistutta, R., Sorgato, M. C., and Pinna, L. A. (2000) *Biochem. Biophys. Res. Commun.* **271,** 337–341
27. Massimino, M. L., Ballarin, C., Bertoli, A., Casonato, S., Genovesi, S., Negro, A., and Sorgato, M. C. (2004) *Int. J. Biochem. Cell Biol.* **36,** 2026–2041
28. Negro, A., Ballarin, C., Bertoli, A., Massimino, M. L., and Sorgato, M. C. (2001) *Mol. Cell. Neurosci.* **17,** 521–538
29. Messerschmidt, A. (1998) *Struct. Bonding* **90,** 37–68
30. Peisach, J., and Blumberg, W. E. (1974) *Arch. Biochem. Biophys.* **165,** 691–708
31. Szabó-Plánka, T., Peintler, G., Rockenbauer, A., Gyõr, M., Varga-Fábián, M., Institórisz, L., and Balázspiri, L. (1989) *J. Chem. Soc. Dalton Trans.* 1925–1932
32. Aronoff-Spencer, E., Burns, C. S., Avdievich, N. I., Gerfen, G. J., Peisach, J., Antholine, W. E., Ball, H. L., Cohen, F. E., Prusiner, S. B., and Millhauser, G. L. (2000) *Biochemistry* **39,** 13760–13771
33. Burns, C. S., Aronoff-Spencer, E., Legname, G., Prusiner, S. B., Antholine, W. E., Gerfen, G. J., Peisach, J., and Millhauser, G. L. (2003) *Biochemistry* **42,** 6794–6803
34. Brown, D. R., Schmidt, B., and Kretzschmar, H. A. (1998) *J. Neurochem.* **70,** 1686–1693
35. Burns, C. S., Aronoff-Spencer, E., Dunham, C. M., Lario, P., Avdievich, N. I., Antholine, W. E., Olmstead, M. M., Vrielinck, A., Gerfen, G. J., Peisach, J., Scott, W. G., and Millhauser, G. L. (2002) *Biochemistry* **41,** 3991–4001
36. Nunziante, M., Gilch, S., and Schaetzl, H. M. (2002) *J. Biol. Chem.* **278,** 3726–3734
37. Hachiya, N. S., Watanabe, K., Yamada, M., Sakasegawa, Y., and Kaneko, K. (2004) *Biochem. Biophys. Res. Commun.* **315,** 802–807

Copper(II) Binding to the Human Doppel Protein May Mark Its Functional Diversity from the Prion Protein

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