Doppel (Dpl) is a glycosylphosphatidylinositol-anchored protein expressed in the testis. It exhibits 26% sequence identity with the prion protein (PrP) but lacks the octarepeat region implicated as the major copper-binding domain. Contrary to expectations, Cu(II) induced a 26% reduction in the intrinsic fluorescence of Dpl(27–154) and a calculated \( K_d \) for a single-site model of 0.16 ± 0.08 \( \mu \)M. Other metals had minimal effects on fluorescence quenching. Matrix-assisted laser desorption ionization mass spectrometry of a Dpl peptide revealed binding of copper (but not other metals) to the helical \( aB/B'-loop-aC \) subregion of Dpl. Fluorescence quenching and equilibrium dialysis analyses of this Dpl(101–145) peptide were compatible with a binding site of \( K_d = 0.4 \) \( \mu \)M. Diethylpyrocarbonate footprinting (Qin, K., Yang, Y., Mastrangelo, P., and Westaway, D. (2002) J. Biol. Chem. 277, 1881–1900) of Dpl(27–154) defined one residue/molecule was protected by copper from diethylpyrocarbonate adduct formation, and reiteration of this analysis with Dpl(101–145) suggested that His\(^{131} \) may contribute to Cu(II) binding. Taken together, our data indicate that the \( \alpha \)-helical region of mouse Dpl possesses a selective copper-binding site with a submicromolar \( K_d \) and perhaps one or more lower affinity sites. Although metallated forms of Dpl might exist in vivo, analyses of Tg(Dpl)10329 mice were inconsistent with reports that Dpl expression is associated with increased carbonylation and nitrosylation of brain proteins. Thus, rather than comprising an important source of free radical damage, copper binding may serve to modulate the activity, stability, or localization of the Dpl protein.

The PrP-like Protein Doppel Binds Copper

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From the ‡Centre for Research in Neurodegenerative Diseases, the §Mass Spectrometry Laboratory, Molecular Medicine Research Centre, the †Institute for Biomaterials and Biomedical Engineering, the **Department of Medical Biophysics, and the ‡‡Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5S 3H2, Canada, the ††Institute for Neurodegenerative Diseases, University of California, San Francisco, California 94143, and the ¶¶Boston Biomedical Research Institute, Watertown, Massachusetts 02272

Doppel (Dpl) is a glycosylphosphatidylinositol-anchored protein expressed in the testis. It exhibits 26% sequence identity with the prion protein (PrP) but lacks the octarepeat region implicated as the major copper-binding domain. Contrary to expectations, Cu(II) induced a 26% reduction in the intrinsic fluorescence of Dpl(27–154) and a calculated \( K_d \) for a single-site model of 0.16 ± 0.08 \( \mu \)M. Other metals had minimal effects on fluorescence quenching. Matrix-assisted laser desorption ionization mass spectrometry of a Dpl peptide revealed binding of copper (but not other metals) to the helical \( aB/B'-loop-aC \) subregion of Dpl. Fluorescence quenching and equilibrium dialysis analyses of this Dpl(101–145) peptide were compatible with a binding site of \( K_d = 0.4 \) \( \mu \)M. Diethylpyrocarbonate footprinting (Qin, K., Yang, Y., Mastrangelo, P., and Westaway, D. (2002) J. Biol. Chem. 277, 1881–1900) of Dpl(27–154) defined one residue/molecule was protected by copper from diethylpyrocarbonate adduct formation, and reiteration of this analysis with Dpl(101–145) suggested that His\(^{131} \) may contribute to Cu(II) binding. Taken together, our data indicate that the \( \alpha \)-helical region of mouse Dpl possesses a selective copper-binding site with a submicromolar \( K_d \) and perhaps one or more lower affinity sites. Although metallated forms of Dpl might exist in vivo, analyses of Tg(Dpl)10329 mice were inconsistent with reports that Dpl expression is associated with increased carbonylation and nitrosylation of brain proteins. Thus, rather than comprising an important source of free radical damage, copper binding may serve to modulate the activity, stability, or localization of the Dpl protein.

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we reasoned that Dpl, a PrP-like protein lacking octarepeats, would comprise a useful control. Unexpectedly, as described below, Dpl was found to exhibit selective Cu(II) binding with an affinity comparable with that of PrP.

**Copper Binding to Dpl**

**MATERIALS AND METHODS**

Dpl Peptide and Proteins—Mouse Doppel 27–154 (MoDpl(27–154)) refolded by a copper catalysis method (3) and mouse Doppel 26–157 (MoDpl(26–157)) refolded in the presence of glutathione (32) were prepared as described previously. Intrinsically copper content was determined by atomic absorption spectrophotometry. A MoDpl helix-loop-helix peptide was synthesized by standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry. This peptide corresponds to residues 101–145 of the mouse doppel and includes the interrupted α-helix B (α/β), the α-helix C (αC), and the loop between these two helical structures (32). The disulfide bond was introduced by treating the peptide overnight in an aerated solution of ammonium bicarbonate. Protein concentrations were calculated by amino acid hydrolysis (33). Far-UV CD measurements, endoproteolysis, and S-carboxamidomethylation of cysteines to determine the presence of integrity of disulfide bonds were carried out as described previously (3, 33).

Fluorescence Spectroscopy and Titrations—Steady-state fluorescence spectra were recorded on a Photon Technology International QM-1 spectrophotometer. Emission spectra were collected from 310 to 400 nm (λex = 290 nm, band pass = 1 nm). For Cu(II) binding experiments, MoDpl(101–145), MoDpl(27–154) or MoDpl(26–157) were diluted to a final concentration of 0.7 μM in 25 mM N-ethylmorpholine, pH 7.4, 150 mM KCl (NEMO-KCl buffer). The sample volumes were 1 ml, and the experiments were performed at room temperature. The protein was titrated with a concentrated Cu(II) stock solution such that sample dilution was never more than 1.7%. To detect metal binding selectivity, 1, 2, or 3 μM CuCl₂, ZnCl₂, NiSO₄, MnCl₂, CaCl₂, or MnCl₂ was mixed with 0.7 μM MoDpl(27–154). The emission spectrum was collected after each CuCl₂ addition, and fluorescence intensities from 310 to 380 nm were integrated.

Equilibrium Dialysis—MoDpl(101–145) Peptide Prior to MALDI-MS analysis, MoDpl helix-loop-helix (90 μM) was incubated with 10-fold molar excess CuCl₂, ZnCl₂, NiSO₄, MgCl₂, CaCl₂, MnCl₂, or FeSO₄ in 25 mM NEMO-KCl buffer, pH 7.4, at room temperature for 1 h. Sequencing grade chymotrypsin (Roche Molecular Biochemicals) was used to digest the intact peptide and copper-peptide complex for 2 h at room temperature (the ratio of enzyme:protein was 1:20). In some cases, before chymotrypsin digestion, the samples were incubated with 5-fold molar excess diethylythiocarbamate (DEPC) at room temperature for 30 min. The samples were then analyzed by using MALDI-MS as described previously (1).

Equilibrium Dialysis—MoDpl(101–145) and control protein (Hu-PrP(28–386), Hu) were prepared in NEMO-KCl buffer, pH 7.4. Chelate solution (Gly:Cys = 1:1) was prepared by mixing CuSO₄ solution with a 2-fold molar excess of glycine and then adjusting the pH to 7.4. The equilibrium dialysis experiments were carried out in multiple 5-cell equilibrium dialyzers (Spectrum Industries). One ml of protein plus chelated Cu(II) mix (retentate) and 1 ml of buffer (dialysate) were placed on opposite sides separated by a 1-kDa cut-off cellulose ester membrane (Spectrum Industries) in a dialysis chamber. For a given experiment, the concentration of protein was held constant, and the concentration of chelated Cu(II) was varied over a Cu(II):protein ratio from 0.5:1 to 40:1. The system was rotated to come to equilibrium for 108 h at 4 °C (verified by analysis of “blank reactions” not containing protein), and the concentration of all forms of copper in each half-chamber was measured by atomic absorption spectroscopy. 2.5 μM protein was used in all of the equilibrium dialysis experiments.

**RESULTS**

Recombinant Dpl Proteins and a Synthetic Dpl Peptide—To exclude trivial or idiosyncratic effects pertaining to expression and purification of Dpl proteins from *Escherichia coli*, we used two recombinant polypeptides prepared by different methods (3, 32) (in the absence of the N-terminal sequences of mature Dpl isolated from mammalian cells, the lengths of these two proteins, Dpl(27–154) and Dpl(26–157), merely reflect ambiguities in assigning N- and C-terminal signal peptides by different algorithms). For Dpl(27–154) prepared by oxidative refolding in catalytic concentrations of Cu(II), we used atomic adsorption spectroscopy to quantify residual metal content (<3 × 10⁻⁴ mol of copper/mol of dialyzed protein). Extrapolating from studies attributing special significance to the helix B/B′-loop-helix C region of Dpl (34), we hypothesized that this area of Dpl may comprise a site with biological activity and synthesized a corresponding peptide, Dpl(101–145) (Fig. 1A). This peptide encompasses one of the two disulfide bonds present in full-length Dpl, the “inner” Cys₁₀⁹-Cys₁¹³ linkage located at an equivalent position to the single disulfide bond of a corresponding peptide, Dpl(27–154). To detect metal binding experiments, MoDpl(101–145), MoDpl(27–154) or MoDpl(26–157) were diluted in the presence of glutathione (32) were prepared as described previously (7). Mice were between 15 and 20 weeks of age at the time of analysis.

Antibodies—The anti-Dpl rabbit polyclonal antibody E6977 raised against recombinant mouse doppel (7) was used as described below. The anti-nitrotyrosine antibody was from Cayman Chemical (a gift from N. Cashman), whereas antibodies against 2,4-dinitrophenylhydrazine were obtained from Intergen.

**Detection of Nitrotyrosine Residues**—A positive control for the nitrosylation blot was made just prior to SDS-PAGE. Peroxynitrite was added to 50 μg of a wild type mouse brain homogenate in sodium phosphate buffer while vortexing to create a final concentration of 15 mM peroxynitrite. After 2 min, 2× gel loading buffer was added to quench the reaction, and the sample was kept on ice. The positive control as well as the sample homogenates of 50 μg of total brain protein were subjected to Western blot analysis with a monoclonal anti-nitrotyrosine primary antibody (Intergen).

**Detection of Reactive Carbonyl Groups**—The amount of carboxyl groups introduced into the total protein of the brain homogenates via oxidative modification was detected using the OxyBlot protein oxidation detection kit (Intergen). Samples of 15 μg of total brain proteins in 0.32 mM sucrose and 2% 2-mercaptoethanol were denatured and then derivatized with 2,4-dinitrophenylhydrazine, electrophoresed, and electroblotted as per the manufacturer’s instructions. The blots were blocked with 1% (w/v) bovine serum albumin in phosphate-buffered saline (pH 7.4, 0.05% Tween 20 for 1 h at room temperature and then probed with a rabbit anti-DNP primary antibody, used at a concentration of 1:150 in blocking solution overnight at 4 °C. Following incubation, a horseradish peroxidase-conjugated secondary antibodies were used. The blots were visualized using ECL detection reagents (Amersham Biosciences) and exposed on X-Omat AR film (Eastman Kodak Co.).

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present in PrP. Measured in NEMO-KCl buffer at pH 7.4, Dpl(101–145) exhibited a CD spectrum with minima at 208 and 222 nm, indicative of a high α-helical content (Fig. 1B). MALDI-MS analysis of Dpl(101–145) peptide showed a single charged peptide signal at m/z 5162.9 (Fig. 2A), in excellent agreement with the calculated molecular mass of 5162.9 Da as [M+H]+. S-Carbamidomethylation (3, 33) was used to verify the presence of appropriate disulfide linkages in the recombinant polypeptides (not shown) and Dpl(101–145) peptide (see Fig. 6A) used for the following analyses.

**MALDI-MS Analysis of Dpl(101–145)—**Because MALDI-MS provided some of the first evidence in favor of copper binding by PrP (20), this technique was applied to Dpl(101–145). After incubation with 10× molar excess of CuCl2 in NEMO-KCl buffer, pH 7.4, at room temperature for 1 h, Cu(II)-peptide complexes were detected. In addition to intact protein at m/z 5162.9, additional peaks at m/z 5225.4 and 5286.7 were observed in the presence of copper (Fig. 2B). Averaged over three experiments, the starting material and derivatives were established as 5162.63 ± 1.42, 5225.90 ± 0.78, and 5287.23 ± 1.09 Da, yielding individual mass increments of 63.3 and 61.3 Da, respectively, and an averaged figure of 62.3 Da. These data are consistent with up to 2 mol of copper (mass 63.5 Da) added per mole of peptide under the conditions of this assay, although intrinsic errors for the mass determinations do not permit conclusions as to how many protons are expelled from the protein commensurate with copper binding. To detect whether other divalent ions bound to Dpl, MoDpl(101–145) was incubated with 10× molar excess of Zn(II), Ni(II), Mg(II), Ca(II), Mn(II), or Fe(II) in NEMO-KCl buffer, pH 7.4, at room temperature for 1 h prior to MALDI-MS analysis. However, in agreement with fluorescence quenching studies presented in Fig. 3, no signals corresponding to metal-peptide complexes were detected in these other analyses (Fig. 2, C–H).

**Copper-Protein Interactions Assessed by Fluorescence Quenching—**Fluorescence quenching analysis for recombinant mouse Doppel (recDpl) was undertaken exploiting the intrinsic properties of four tryptophan residues at positions 35, 85, 136, and 151, and five tyrosine residues at positions 78, 79, 84, 91, and 92 (present within both Dpl(27–154) and Dpl(26–157)). To confirm that these residues undergo a change in environment, we measured the change of fluorescence intensity of Trp and Tyr residues upon Cu(II) addition. The diminutions in intrinsic fluorescence (integrated from 310 to 380 nm) induced by Cu(II) addition were 26% for Dpl(27–154) and 30% for Dpl(26–157). However, the change in the intensity was not accompanied by a significant shift in the wavelength position of the maximum (λmax), suggesting that the solvent accessibility of Trp residues are not affected significantly by copper binding. Also, CD spectra were not notably altered by copper addition (not shown), suggesting that copper binding is not accompanied by a significant change in the secondary structure of recDpl. MoDpl(101–145) contains a single tryptophan residue at position 136 (Fig. 1A), facilitating similar fluorescence spectroscopic studies to those performed for the aforementioned full-length recDpl molecules.

Binding affinities and stoichiometries for copper binding to the various proteins and peptides were quantified by fitting of the conventional model expressed in Equations 1 and 2 to the fluorescence data in Fig. 3,

\[ F = F_0 + \Delta F \frac{[\text{bound}]}{[P]} \]  
(Eq. 1)

where \( F \) is the measured fluorescence, normalized by taking the difference in the first and last data point as 1, \( F_0 \) is the fitted fluorescence before addition of copper, and \( \Delta F \) is the fitted change in fluorescence. The concentration of the Dpl-copper complex, \([\text{bound}]\), is described in Equation 2,

\[ [\text{bound}] = (K_d + [\text{Cu}_\text{tot}]) \frac{[P] - \text{sqr}t([K_d + [\text{Cu}_\text{tot} - [P]^2] - 4([\text{Cu}_\text{tot}] [P])/2]}{[\text{bound}]} \]  
(Eq. 2)

where \( K_d \) is the dissociation constant of the copper-binding site(s), \([\text{Cu}_\text{tot}]\) is total copper concentration, and \([P]\) is protein concentration.
concentrations set at the measured values of 0.7 μM. B, D, and F, the change in fluorescence intensity integrated over a wavelength range of 310–380 nm plotted against copper concentration (abscissa); thus, 1.0 on the ordinate indicates 100% (maximal) observed fluorescent change. The binding curves represent the best fits of Equations 1 and 2 (main text) to the data with the polypeptide–Dpl(101–145), 1:1 binding, 0.006; and 2:1 binding, 0.022.

Residual plots and chi-squared values for the best fits to the data are shown in Fig. 4. Graphing [bound] versus [Cu]tot yielded a plateau at about 2.5 μM, the same concentration as the protein used in the experiments. Fitting Equation 2 to the data gives \( K_d = 0.4 \pm 0.2 \mu M \), the same within experimental error as that determined by fluorescence titration, and \([\text{protein}] = 2.49 \pm 0.13 \mu M\), very similar to the concentration of the protein used in the experiment, indicating a 1:1 correspondence between protein monomers and copper-binding sites. Equilibrium dialysis therefore confirms that there is one copper-binding site or of a second binding site associated with a metal-peptide complex with different fluorescent properties cannot be excluded based solely upon these fluorescence titrations.

To evaluate metal binding selectivity of the Dpl protein, we calculated the fluorescence intensity change of 0.7 μM Dpl(27–154) in the presence of 3 μM divalent cations, e.g., Cu(II), Zn(II), Ni(II), Mg(II), Ca(II), and Mn(II). Whereas no significant changes of Trp and Tyr fluorescence signals were observed for other cations (1.18 ± 0.40% for Zn(II), 2.85 ± 0.40% for Ni(II), 2.60 ± 0.20% for Mg(II), 2.60 ± 0.18% for Ca(II), and 1.91 ± 0.10% for Mn(II)) (Fig. 3G, first column). Although we cannot exclude that Dpl complexes (which do not result in fluorescent changes) with metals other than copper exist, we note that similar selectivity was found in MALDI-MS analyses (Fig. 2). Also, these data are strikingly reminiscent of prior studies of selective interactions between copper and hamster PrP (22).

**Copper Binding to Dpl(101–145) Assessed by Equilibrium Dialysis—**In the equilibrium dialysis experiments, Cu(II) was presented to 2.5 μM MoDpl(101–145) as a glycine chelate. Binding data are shown in Fig. 4. Graphing [bound] versus [Cu]tot yielded a plateau at about 2.5 μM, the same concentration as the protein used in the experiments. Fitting Equation 2 to the data gives \( K_d = 0.4 \pm 0.2 \mu M \), the same within experimental error as that determined by fluorescence titration, and \([\text{protein}] = 2.49 \pm 0.13 \mu M\), very similar to the concentration of the protein used in the experiment, indicating a 1:1 correspondence between protein monomers and copper-binding sites. Equilibrium dialysis therefore confirms that there is one copper-binding site.
binding site/monomer and that fluorescence provides a reliable signal for the rapid measurement of copper binding to Dpl.

**DEPC Footprinting of Dpl(27–154)**—In a further technique to interrogate copper binding by Dpl(27–154), we exploited the ability of copper to protect certain reactive amino acid residues (predominantly histidine) from chemical modification with DEPC (1). Upon incubation of native protein (15 μM) (Fig. 5A) with DEPC, up to nine DEPC adducts were detected per mole of Dpl(27–154) (Fig. 5B), assuming an increment of 72.06 Da/mono-carbethoxylated adduct. In the case of protein preincubated with a molar excess of copper, there was a shift of 1 in the spectrum of adducts (Fig. 5C), indicating that one DEPC-reactive residue was protected from modification by the presence of copper.

**Copper Binding in the Region of Dpl(122–136)**—Chymotrypsin digestion was used to map the location of Cu(II)-binding site(s) detected by the prior MALDI-MS analysis of Dpl(101–145). In the absence of Cu(II) (Fig. 6A), a prominent peak at m/z 1910.05 correspond to a partially digested chymotryptic peptide containing amino acid sequences 122–136 (C3/C4/C5/C6, calculated 1910.03 Da). An additional peak at m/z 1032.70 corresponds to 137–145 (C7/C8, calculated 1032.58 Da). In the presence of Cu(II), two new peaks were observed at m/z 1973.50 and 2036.07 Da, indicating that up to two copper ions can bind to the area between amino acids 122 and 136. This chymotryptic peptide has the sequence SREKQDSKLHQRVLW. Mass increments upon addition of the first and second Cu(II) ions were 62.69 and 62.57 Da, respectively (Fig. 6B), yielding a net increment of 125.26 Da, in close agreement with the figure of 124.60 Da obtained for intact Dpl(101–145) peptide.

**Histidine-dependent Copper Binding in Dpl(101–145)**—DEPC footprinting was also used to assess the histidine-dependent copper coordination within the Dpl(101–145) peptide. Dpl(101–145) (90 μM) in NEMO-KCl buffer, pH 7.4, was incubated with Cu(II). The DEPC footprinting spectrum of native Dpl(27–154) (Fig. 5A) is shown, along with the spectra of samples subsequent to preincubation with Cu(II) (Fig. 5B). Note the “additional” peaks adjacent to the peak at m/z 1910.81, compatible with one or two copper adducts (1Cu and 2Cu, respectively) and invariance of the disulfide cross-linked chymotryptic fragments. C, chymotryptic digest of DEPC-reacted peptide. The prominent peak at m/z 1910.08 in A and B is diminished in amplitude, and two new peaks of mono-carbethoxylated peptide (1m and 2m) are apparent at m/z 1982.09 and 2055.08. D, Cu(II)-protected DEPC reacted peptide analyzed with chymotrypsin. The intensity of the 1m peak is diminished concomitantly with the reappearance of the 122–136 peptide fragment and 1- and 2-Cu-derived complexes.
bated with 5-fold excess DEPC and then digested with chymotrypsin. Compared with the signal in Fig. 6A, the signal at \( m/z \) 1910.09 corresponding to unmodified residues 122–136 (C3 + C4 + C5 + C6, calculated 1910.03 Da) was reduced in amplitude (Fig. 6C). Two new peaks were found at \( m/z \) 1982.09 and 2055.08 and indicated one mono- and two mono-carbethoxylation of fragment 122–136 (calculated 1982.09 and 2054.14 Da, respectively). A different mass spectrum was obtained when Dpl(101–145) peptide was preincubated with 10-fold excess Cu(II) prior to reaction with DEPC (Fig. 6D). Here the major peaks were at \( m/z \) 1910.34, 1973.28, 2036.21, 1982.38, and 2046.31. Diminution of the mono-carbethoxylated peak at 1982.09 and an increase in the peak corresponding to unmodified 122–136 C1–H11001 C2–H11001 C3–H11001 peptide (compare with Fig. 6D), indicated that Cu(II) did not interact with this fragment.

Fine Mapping DEPC Modification of His\(^{131}\) within Dpl(122–136)—The post-source decay technique of tandem analysis was used to verify DEPC modification and copper protection of His\(^{131}\). Partial post-source decay spectra of fragment 122–136 and its counterpart in DEPC- and copper-DEPC-treated samples are presented in Fig. 7. Fig. 7A shows the scheme of nomenclature for peptide post-source decay fragment ions of chymotryptic peptide Dpl(122–136). In unmodified samples, a peak at \( m/z \) 109.9 corresponds to an immonium histidine ion (His \(^{131}\)) (calculated 110.0 Da) (Fig. 7B). After DEPC treatment, a new peak at \( m/z \) 181.7 indicated one mono-carbethoxylated histidine (calculated 182.0 Da) (Fig. 7C). Incubation of the sample with Cu(II) prior to DEPC treatment resulted in the disappearance of one monocarbethoxylated histidine and indicates that Cu(II) coordination to this histidine protected the imidazole ring from DEPC modification (Fig. 7D). It is also of note that peaks at \( m/z \) 128.7,
Copper Binding to Dpl

**DISCUSSION**

**Metal Binding to Dpl In Vitro**—Many studies have documented binding of one copper ion/histidine containing octarepeat within PrP, when measured at neutral pH (Ref. 36 and references therein). In addition to these four sites, there is growing agreement upon the existence of a fifth copper site (1, 37–39). Although sequence alignments reveal that none of these residues have an equivalent in Dpl, our in vitro studies have revealed, nonetheless, that Dpl also possesses copper binding properties.

It is unlikely that the copper binding detected in our studies has a trivial origin. Although contaminants from E. coli capable of binding copper can be hypothesized, they would have to be present in substantial quantities to account for the observed stoichiometry of ≥1 mol of copper/mol of polypeptide. We also note that similar fluorescence quenching properties were produced by two forms of recDpl prepared by different chromatographic procedures. Furthermore, a Dpl(101–145) peptide synthesized in vitro also had the ability to bind copper (Fig. 2), as did a Dpl(122–136) proteolytic derivative of this peptide (Fig. 6). Another notable feature of the interaction with copper is the degree of specificity. Assessed by fluorescence quenching, the Dpl substrates exhibited no discernable affinity for other divalent cations, a pattern of selectivity reminiscent of PrP itself (1, 20, 22). Although it is possible that peptide complexes formed by metals other than copper have fluorescent properties similar to that of Dpl apo-protein and thus appear not to interact in this assay, the similar pattern of metal specificity derived from MALDI-MS analysis (Fig. 2) tends to argue against this interpretation and in favor of an intrinsic metal selectivity.

Our experiments demonstrate that Cu(II) binding to recDpl can be largely attributed to the αB/C-loop of C-domain; using fluorescence quenching, $K_d$ values for full-length protein and Dpl(101–145) differ only by a factor of two. Binding assessed directly by equilibrium dialysis and indirectly by fluorescence...
Copper Binding to Dpl

quenching analysis can be accounted for by a single-site model, with the additional site detected by MALDI-MS analysis, perhaps reflecting contribution of a low affinity site only filled by high concentrations of Cu(II). In the NMR structure for Dpl (32), the αB/β-loop-aC domain lies at the opposite end of the molecule from the free C terminus (the site of the glycosylphosphatidylinositol anchor addition) and may therefore be displayed toward the extracellular environment in vivo. The notion of copper-binding sites within the α-helical domain of a cellular prion protein has a precedent from studies of PrP (40). Of potentially greater importance, this region of the Dpl molecule encompassing a kinked helix B (αB/β) contributes to a triangular hydrophobic pocket with no exact equivalent in PrP (32). It will be of interest to determine the details of how amino acid residues in this vicinity contribute to copper binding. The observation that a cluster of fCJD mutations in the helix B-loop-helix C region of PrP recapitulate conserved residues in Dpl provides further impetus for deciphering the biological properties of this region of the protein (34).

Regarding the issue of binding affinity, some of our analyses use copper in the form of CuCl₂. Such solutions contain oxy and hydroxy polymers of copper, which may be kinetically inert (41). Accordingly, some studies of PrP have instead used copper presented in the form of glycine- or histidine-chelated complexes. These experiments still yield binding at micromolar concentrations of copper, but back-calculations based upon the dissociation constants of the metal-amino acid complexes can be used to derive a concentration for free ionic metal and protein-metal binding constants estimated in the range of 10⁻¹⁴ M (38). Our equilibrium dialysis binding analysis of Dpl(101–145) was performed with glycine-chelated copper and a Kᵣ value for a high affinity site of 0.4 ± 0.2 μM. Irrespective of the validity of back-calculating ionic copper concentrations from experiments using copper chelates (where ternary complexes of protein/copper/amino acid complexes might also have to be considered (42)), our numerical data (i) are nonetheless quite similar to estimates of binding constants derived from “uncorrected” binding data for PrP obtained under similar conditions (21) and (ii) illustrate that Dpl, like PrP, can extract copper from an amino acid chelate, a form that might better approximate presentation in biological fluids than in solutions of copper salts. “Exchangeable” copper in plasma bound to amino acids has been estimated at 3.6 μM, whereas the total copper levels in plasma and seminal fluid are measured at 15.5 ± 9 and 5.9 ± 3.7 μM, respectively (43, 44) and are thus about 1 order of magnitude above the Kᵣ values presented herein. These data strongly suggest that metallated forms of Dpl could exist in vivo, which is of potential relevance to physiological function(s) in spermatogenesis and also to the pathological effect of central nervous system expression.

Central Nervous System Expression of Dpl: Oxidative Damage and Neurotoxicity—Based upon prior connections between copper-polypeptide complexes and the generation of reactive free radical species, we investigated indicators of oxidative damage in Tg(Dpl) mice (35). Ectopic expression of Dpl in the brain is known to be toxic, and genetically engineered mice with this property succumb to an ataxic syndrome characterized by apoptotic death of cerebellar cells (2, 6, 7). Our studies exploited the Tg(Dpl)10329 line of mice (7). In contrast to another study (35), we found no evidence of enhanced oxidative damage to brain proteins (Fig. 8). This discrepancy cannot be attributed to the penetrance of the disease phenotype in the Tg(Dpl)10329 mice studied here. Tg(Dpl)10329 mice develop an ataxic syndrome at 375 ± 8 days, as compared with R6/1 mice, which develop ataxia much later in life at 611 ± 12 days of age. Furthermore, the Tg(Dpl)10329 mice at age of sacrifice in the analyses of oxidative markers described here were approximately twice as old as R6/1 mice analyzed for markers of oxidative damage (35). It is possible that the discrepancy between our findings and those of Wong et al. (35) reflects a contribution of modifier loci deriving from different genetic backgrounds. Nonetheless, because the Tg(Dpl)10329 mice described here have levels of protein carbonyl and nitrotyrosine formation no different from those seen in non-Tg mice (yet are certainly prone to loss of cerebellar cells), it seems quite unlikely that the neurotoxic properties of Dpl depend upon causing oxidative damage to proteins.

Competition between Dpl and PrP²⁻: a Role for Cu?—Despite our failure to implicate oxidative damage in the neurotoxic action of Dpl, it remains plausible that an ability to bind copper ions is necessary for neurotoxicity proceeding by a different, but as yet undefined, mechanism. The studies presented here were not designed to elucidate what this mechanism might be. Nonetheless, by revealing that the ability to bind copper is a property of both Dpl and PrP²⁻, they may speak to “competition” between the PrP²⁻ and Dpl proteins in modulating this pathologic process (7). Specifically, because expression of PrP²⁻ can nullify the neurotoxicity of Dpl to cerebellar cells, the question now arises as to whether the competing actions of these two glycosylphosphatidylinositol-linked proteins somehow derive from a shared predilection for this particular transition metal. Experiments to recapitulate Dpl-mediated neurotoxicity in a tractable system may allow us to appraise this possibility.

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