Genetic Mapping of Activity Determinants within Cellular Prion Proteins

N-TERMINAL MODULES IN PrPC OFFSET PRO-APOTOTIC ACTIVITY OF THE DOPPEL HELIX B/B' REGION*

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The PrP-like Doppel (Dpl) protein causes apoptotic death of cerebellar neurons in transgenic mice, a process prevented by expression of the wild type (wt) cellular prion protein, PrPC. Internally deleted forms of PrPC resembling Dpl such as PrPΔ32–121 produce a similar PrPC-sensitive pro-apoptotic phenotype in transgenic mice. Here we demonstrate that these phenotypic attributes of wt Dpl, wt PrPC, and PrPΔ32–121 can be accurately recapitulated by transfected mouse cerebellar granule cell cultures. This system was then explored by mutagenesis of the co-expressed prion proteins to reveal functional determinants. By this means, neuroprotective activity of wt PrPC was shown to be nullified by a deletion of the N-terminal charged region implicated in endocytosis and retrograde axonal transport (PrPΔ23–28), by deletion of all five octarepeats (PrPΔ51–90), or by glycine replacement of four octarepeat histidine residues required for selective binding of copper ions (PrpH/G*). In the case of Dpl, overlapping deletions defined a requirement for the gene interval encoding helices B and B’ (DplΔ101–125). These data suggest contributions of copper binding and neuronal trafficking to wt PrPC function in vivo and place constraints upon current hypotheses to explain Dpl/PrPC antagonism by competitive ligand binding. Further implementation of this assay should provide a fuller understanding of the attributes and subcellular localizations required for activity of these enigmatic proteins.

Prion disease pathogenesis involves the formation of abnormal forms of the cellular prion protein (PrPc)1 (1). In the case of prion infections, it is well accepted that a profound structural remodeling involving a templated conformational change culminates in the formation of protease-resistant and infectivity-associated forms of the prion protein commonly denoted as PrPsc. In the case of inherited prion diseases such as familial Creutzfeldt-Jakob disease, insertion or missense mutations in the human PrP gene (PRNP, denoted Prnp in the mice) result in abnormal forms of PrPc that may, in some instances, misfold and accumulate in the endoplasmic reticulum. However, insights into the normal function of PrPc, and the extent to which conformational alterations might participate in this function, have proven hard to come by because of difficulties in connecting in vitro and in vivo data. For example, in vitro protein binding partners have proven difficult to authenticate by in vivo genetic analyses, and conversely, in vivo genetic analyses of PrPc have been limited to defining determinants required for prion replication and binding to an as yet uncloned partner protein designated protein X (2, 3). Other recent areas of interest and controversy concern the basis of neurotoxicity and the existence and function of cytoplasmic forms of PrPc (4–9).

Discovery of the Doppel (Dpl) protein has provided a new opportunity to scrutinize these issues. Dpl is a prion-like protein encoded by Prnd gene, displaying ∼24% identity with the C-terminal two-thirds of PrP (10, 11). The Dpl protein resembles an N-terminally truncated version of PrPc, lacking the octarepeats motifs and a conformationally plastic region essential for the capacity to sustain prion replication. On the other hand, PrPc and Dpl are both GPI-anchored proteins (12, 13), bear generally similar α-helical C-terminal domain structures (14, 15), and share an ability to bind copper ions in a selective manner in vitro (16–18). Whereas PrP mRNA is expressed in the central nervous system and many peripheral tissues, the most notable site of Dpl expression is the testis. Although Dpl is not thought to convert to a protease-resistant isoform analogous to PrPsc or to support the process of prion replication (19, 20), expression in the central nervous system causes apoptotic death of both granule and Purkinje cells, determining a severe

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‡ The abbreviations used are: PrPC, cellular prion protein; Dpl, Doppel protein; PrPc, scrapie prion protein; Prnp, mouse prion gene; Prnd, mouse Doppel gene; Tg, transgenic; GFP, green fluorescent protein; CGN, cerebellar granule neurons; Ac-DEVD-CHO, N-Acetyl-Asp-Glu-Val-Asp-CHO; GPI, glycosylatedphosphatidylinositol; ORF, open reading frame; wt, wild type; ANOVA, analysis of variance; PBS, phosphate-buffered saline.

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ataxic phenotype in Dpl-expressing transgenic mice (10, 23, 24). Furthermore, internally deleted forms of PrP resembling Dpl are also cytotoxic (25).

The many biochemical similarities between Dpl and PrPC and recurring connections between PrPC itself and apoptosis activities of the two mature proteins enacted at the cell surface and verified by sequence analysis using two coding strand and two anti-coding strand sequencing primers. Authenticated mutants were subsequently transferred into pBuδGFP (pBuδCE4 vector. In vitro by standard restriction enzyme cloning for transfection experiments. Mutagenic oligonucleotide primer used to generate the Dpl mutants (5’ to 3’, with reverse complement oligonucleotides omitted for the sake of brevity) were as follows: Dpl

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Dpl point mutants and deletions were generated via site-directed mutagenesis performed on the wild type Dpl ORF (10) in pDNA3.0 (Invitrogen) using a Pfu DNA polymerase-mediated mutagenesis system (Quickchange, Stratagene). Mutagenic PCRs were digested with restriction enzyme Dpnl (target sequence, 5’-G^mATC-3’), specific for methylated and hemi-methylated DNA, in order to eliminate parental DNA template. Products were then transformed into Escherichia coli and verified by sequence analysis using two coding strand and two anti-coding strand sequencing primers. Authenticated mutants were subsequently transferred into pBudGFP (pBudCE4 vector. In vitro by standard restriction enzyme cloning for transfection experiments. Mutagenic oligonucleotide primer used to generate the Dpl mutants (5’ to 3’, with reverse complement oligonucleotides omitted for the sake of brevity) were as follows: Dpl

**RESULTS**

**A Transgene-based Assay for Dpl Toxicity**—Based upon susceptibility to toxicity engendered by Dpl and mutant PrPC transgenes in vivo (as well as the results presented below), we first investigated primary cerebellar granule cell cultures to evaluate the toxic action of recombinant proteins. However, a failure to recapitulate the protective effect of endogenous PrPC, as well as the high (micromolar) concentrations of recombinant Dpl required to induce apoptotic cell death (not shown), suggested an exploration of alternative paradigms. The availability of Dpl and PrP alleles assessed previously in Tg mouse provided the impetus to develop a gene-based bioassay.

To obviate biohazard issues arising from the use of viral transgenic mice on a hybrid 129/B6 background were used in their 13th backcross to the B6 strain, with B6 mice from the same supplier (Charles River) comprising a source of Prnp<sup>+</sup> cells. For some experiments Tg(SHaPrP)7 mice maintained in a B6 congenic background (35) (a gift from G. Carlson) or non-Tg littermates were also used as a source of donor cells. Primary cultures of cerebellar granule cells were prepared from P6–7-day animals as described previously (7, 36). After 4 days in vitro, granule cells were transfected in minimum essential medium with 2 μg of pBud-GFP vectors using Lipofectamine 2000 (Invitrogen) for 30 min before replacing the media with K25 + 5% FBS. 24 h post-transfection, cells were fixed in 4% paraformaldehyde for 30 min. N2a neuroblastoma cells were transfected as described previously (10). Where described, 20 μM of Ac-DEVD-CHO (Sigma) was added to the cultures after transfection.

**Measurement of Cell Death**—Cell death was quantified by staining the cells with the blue fluorescent dye Hoechst 33342, which stains nuclei. Transfected neurons (GFP-positive) were scored as healthy or apoptotic by morphological criteria (i.e. chromatin condensation, nuclear fragmentation, cytoplasm blebbing, and neuritic degeneration) (37, 38). Each graph represents at least three independent experiments performed in triplicate in which about 150–200 neurons were individually examined using a fluorescent Zeiss Axiovert inverted microscope.

**Immunofluorescence**—Immunofluorescence experiments were performed 24 h after transfection with pBud-GFP vectors. Cells were fixed in 4% paraformaldehyde and washed three times with PBS. For internal staining cells were incubated for 1 min at room temperature in PBS containing 0.5% Triton X-100. Cells were then incubated for 30 min in blocking solution (PBS, 2% goat serum) and then overnight in primary antibody (a-Dpl E6977, a gift from S. Prusiner) diluted 1:300 in blocking solution. Cells were washed three times with PBS, incubated in blocking solution for 15 min, and then incubated with a secondary antibody (Cy-3 1:300, Jackson ImmunoResearch) for 1 h at room temperature. Monoclonal antibody 7A12 binding between residues 90 and 145 was used (1:300) for immunodetection of PrP (39), in conjunction with an AlexaFluor594-conjugated anti-mouse secondary antibody (Invitrogen) and a block solution of Hanks’ buffered salt solution containing magnesium and calcium, 10% goat serum, 1 ml HEPES, pH 7.4, and 2% FBS. Cells were washed three times with PBS and then fixed at room temperature with a Zeiss Axiovert inverted microscope using an Acrostatgrom 40×/0.55 LD objective. Pictures were taken with a Kodak Digital camera (DC290) using the Kodak Microscopy Documentation System MD29000 software for the image acquisition.

**Immunoblotting**—Cells were harvested in 50 mM Tris-HCl, pH 7.5, containing 0.5% SDS and assayed for total protein using the BCA assay (Pierce). 50 μg protein samples were loaded on Tris-glycine SDS-polyacrylamide gels and then blotted to nitrocellulose membranes. For immunoblotting, membrane was incubated with the polyclonal antibody E6977 at 1:4000 overnight, and antibody binding was revealed by using an ECL detection system (Amersham Biosciences). A Dpl N-terminal peptide antigen corresponding to residues 27–39 was synthesized and purified by reverse phase high pressure liquid chromatography. This peptide was covalently linked to keyhole limpet hemocyanin via a disulfide linkage made possible by the addition of a cysteine residue at the peptide C terminus. A total of three rabbits were immunized with peptide-keyhole limpet hemocyanin complexes for each peptide antigen and were then subsequently given booster injections at 7-day intervals. Antiserum collected for each peptide was pooled, and IgG was precipitated with ammonium sulfate. Antibodies were then affinity-purified with Sulfo-link-agarose (Pierce) coupled with the appropriate peptide. This final purification is required to remove nonspecific interactions of other antibodies present in either the pre- or post-immune serum.

**RESULTS**

**Cell Culture and Transfection**—Breeding pairs from a re-derived colony of Zech1 PrnP<sup>+</sup> mice (34) were a generous gift from Dr. Ber...
vectors to express mammalian prion and prion-like genes, we developed a protocol based upon delivery of naked DNA. In this transient transfection system, primary cultures of granule neurons were transfected with bigenic expression plasmids by a liposome-based method. In this way it was possible to obtain simultaneous expression of green fluorescent protein (GFP) to denote the subset of cells successfully transfected (and thereby expressing the \( \text{Prnd} \) alleles under study), without recourse to use the GFP-prion protein fusions of undefined biological activity. Accordingly, pBud-CE4 bigenic vectors were used to clone a variety of \( \text{Prnd} \) alleles (all with identical 5'- and 3'-untranslated sequences) and GFP genes under the control of two different promoters (cytomegalovirus and EF-1\( \alpha \), respectively). These plasmids were then used to transfect post-mitotic primary cerebellar granule cells at postnatal day 10 or 11, 4 days after culturing in vitro (“DIV 4”). Cerebellar neuronal cultures were fixed with 4% paraformaldehyde and stained with the DNA dye Hoechst 44333 to visualize nuclear morphology. After staining with Hoechst 44333, neurons were transfected with empty vector and observed for signs of neurodegeneration.

![Figure 1](image.png)

**Fig. 1.** Doppel transgene expression in \( \text{Prnp}^{0/0} \) cerebellar granule cells induces neurodegeneration. A, fluorescence micrographs of cerebellar granule cells derived from \( \text{Prnp}^{0/0} \) mice, transfected with pBud-GFP-empty vector (e.v.) and pBud-GFP-Dpl. GFP-positive neurons (a, c, e, and g) were identified, and nuclear morphology was analyzed for signs of neurodegeneration, after staining with Hoechst 44333 (b, d, f, and g). A higher number of GFP-positive cells showing nuclear fragmentation, neuritic degeneration, and pyknotic nuclei were found in Dpl-transfected neurons (white arrows, d and h) compared with empty vector-transfected cells (b and f). (Scale bar in this and subsequent figures = 20 \( \mu \)m.) B, neurons transfected by GFP-wtDpl show twice the rate of death compared with neurons transfected with GFP empty vector. 21 h after transfection there is a statistically significant difference in the death induced by wt Dpl versus empty vector. Data are mean \( \pm \) S.E. of 12 independent experiments (Student’s \( t \) test, \( * \), \( p < 0.0001 \)).
certain that each neuron expressing the GFP marker gene also expressed Dpl, transfected neurons were analyzed by immunofluorescence using the polyclonal Doppel antibody E6977 (20) and visualized by using a secondary antibody conjugated with Cy-3. Over 94% of the green fluorescent neurons also exhibited cell-surface immunofluorescence with the anti-Doppel antibody (Fig. 2). Taken together, these data suggest that Doppel transgene expression in cerebellar granule cells induces cell death.

**PrPC Expression Blocks Dpl-induced Toxicity**—A key criterion to assess the accuracy of the *in vitro* model described above is the protective effect of PrPC expression, as this has been documented in all transgenic paradigms examined to date (20, 22, 41, 42). Because cerebellar cells overexpressing hamster PrPC derived from Tg(SHaPrP)7 mice (35) were resistant to the toxic effect of Dpl (not shown), as in Tg mice (20, 43), we investigated whether this was also the case for PrPC expressed at endogenous levels. For this purpose, cerebellar granule cells were cultured from C57/B6 wt PrP-expressing mice (i.e. Prnp+/+) and transfected with both Doppel-GFP and empty vector-GFP constructs. The percentage of cellular death (Fig. 3A) did not increase in Dpl-transfected neurons, confirming that PrPC expression was capable of rescuing the toxic phenotype induced by Dpl expression. In a further experiment, we tested the effect of co-transfecting Prnp<sup>−/−</sup> granule cells with both a GFP/Dpl plasmid and a wt PrPC transgene encoded by a second expression vector. Here co-transfection of a PrPC-expressing plasmid with the Dpl-expressing plasmid (3:1 ratio by mass) completely blocked toxicity produced by the Dpl transgene (Fig. 3B). In other experiments we confirmed the ability of bigenic pBUD-GFP+PrP plasmids to support PrPC expression, as assessed by immunostaining with the monoclonal antibody 7A12 (Fig. 2). Further control experiments, where plasmids encoding GFP or a red fluorescent protein were mixed, revealed a high frequency of co-transfection, with 93% of cells expressing GFP also expressing red fluorescent protein (Fig. 3C). In summary, these results establish a protective effect of PrPC<sup>c</sup>, as is the case *in vivo* in transgenic mice.

**Doppel Induces Neurodegeneration by an Apoptotic Mechanism**—As noted above, morphological studies on Dpl-transfected cells were consistent with apoptotic cell death, in accord with studies performed on TgDpl mice, where features of apoptosis were apparent within degenerating granule neurons.
To extend these findings, immunocytochemical staining was performed with an antibody specific for the cleaved form of caspase-3 (44). Cells with nuclear apoptotic morphology were also found to be positive for a neo-epitope generated by caspase-3 proteolytic activation (Fig. 4A). In a further experiment, granule cells were transfected with Dpl and control constructs in the presence or absence of 20 μM of Ac-DEVD-CHO, a synthetic peptide inhibitor of caspases 3, 6, 7, 8, and 10 (44). Survival of Dpl-transfected neurons was markedly increased by Ac-DEVD-CHO (Fig. 4B), suggesting that caspase activity is required for Doppel-induced cell death. Taken together, the data from Figs. 1–4 demonstrate the utility of the transgene-based approach over delivery of recombinant proteins, and this methodology was used for subsequent studies.

Mutational Analysis of Neuroprotective Activity of PrP——Because (i) copper binding is a potential mechanism whereby PrPC and Dpl might compete (18), (ii) the major Cu(II) binding domain maps within the octarepeat region of PrP (16, 45), and (iii) internal N-terminal deletions of PrP are stably expressed but nontoxic (46) (whereas larger deletions such as PrP23–121 and PrPΔ32–134 are toxic (25)), we hypothesized that one neuroprotective region within PrP might lie within the octarepeat sequences. Accordingly, we tested the activity of a cotransfected PrPΔ51–90 allele lacking all five octarepeats, as well as an allele where each histidine residue within the copper binding octarepeats was converted to a glycine residue (PHGG(G/S)WGQ4→PGGG(G/S)WGQ4). Plasmids encoding a PrP allele deleted for the basically charged region (PrP23–28) implicated in cell-trafficking events and interactions with protein X (47, 48) or with a stop codon prior to the GPI signal peptide (PrPS232ter) were also investigated in the same manner (Fig. 5, A and B). None of these Prnp alleles exhibited toxicity above background levels, but with the exception of the S232ter allele, neither did they protect against the pro-apoptotic action of Dpl. "Nonprotective" PrP alleles (PrP23–28 and PrPΔ51–90 shown, Fig. 2A) were nonetheless associated with immunostaining, arguing against the trivial effects of these internal deletions upon gene expression.

To investigate further the accuracy of our cellular model for Doppel neurotoxicity, we also tested the effect of pathogenic “octarepeat insertion” PrP alleles found in diseases classified as either familial Creutzfeldt-Jakob disease or Gerstmann–Sträussler Scheinker disease. These alleles behave as dominant traits and, unlike the situation for Dpl, do not behave differently when expressed in the presence or absence of endogenous mouse PrP alleles (49). The mutant PRNP allele (encoding an additional eight octarepeats above the five present in wt PRNP alleles) used for this experiment was derived from a Gerstmann–Sträussler Scheinker patient (50). A resulting “octa13” Prnp allele was cloned into the pBud bigenic vector and analyzed in Prnp0/0 and Prnp+/+ cerebellar granule cell cultures. Both Prnp0/0 and Prnp+/+ cultures proved susceptible...
to the toxic effect (Fig. 5C), providing a close parallel to the behavior of a pathogenic PrP transgene encoding 14 octarepeats (49) and thus comprising a control for the fidelity of the granule cell transfection assay. Finally, the PrPΔ32–121 allele was sensitive to Prnp genotype, as is also the case in mice (25). Similarities in the performance of Prnp alleles in Tg mice and cultured cells are summarized in Table I. Prior analyses have indicated these Prnp mutations have little effect upon the ability to produce robust levels of PrP\textsuperscript{C} expression, although in some instances they influence trafficking (as discussed below).

**Pro-apoptotic Properties of Dpl Deletion Mutants**—The NMR structure of Dpl is characterized by an unstructured N-terminal region, followed by a globular C-terminal structured domain comprised of helices aA, aB/B', and aC as well as two short β-strands. Two disulfide bonds (between residues 93 and 148 and between residues 103 and 143) cross-link helices aB/B' and aC (13, 15). A series of three large deletion mutant alleles (Δ29–49, Δ50–90, and Δ91–149) were created to encompass these structural elements. In addition, smaller deletions (Δ91–125, Δ91–100, Δ101–125, and Δ126–149) were created within the boundaries of the most C-terminal large deletion (Fig. 6A). These mutant alleles were transferred to the bacterial expression vector and assessed as per the previous assays. The results of these assays indicated that toxicity mapped to the central region of the protein, as the 91–149 allele reduced toxicity to base-line levels. Smaller deletions of this region were informative and further mapped the toxic activity to sequences lying between codons 101 and 125. Conversely, alleles affecting helix C (a reciprocal Dpl-(126–149) sub-deletion and an allele created by linker insertion at codon 135) retained full toxic activity. All other deletion alleles retained both their toxic activity and their sensitivity to the presence of PrP\textsuperscript{C} expression (Fig. 6B and Table II).

In parallel experiments, we also assessed the expression of the corresponding mutant proteins in transient transfections of N2a neuroblastoma cells (Fig. 7). C-terminal deletion mutants were immunodetected by using an N-terminal Dpl antibody (in the case of the DplΔ29–49, mutant protein expression was visualized with the E6977 antibody raised against a C-terminal epitope). Each of these deletion alleles lacking pro-apoptotic activity was nonetheless capable of encoding a stable protein, although, depending upon the particular deletion interval, appropriately smaller than the wt allele and/or devoid of the electrophoretic heterogeneity deriving from glycosylation of Asn-99 and Asn-111.

**DISCUSSION**

**A Cellular Assay for Prnp/Prnd Interactions**

We have adopted a gene-based approach to dissect the antagonistic activities of PrP\textsuperscript{C} and Dpl, a methodology that is not necessarily dependent upon \textit{a priori} assumptions concerning either biochemical mechanism or cellular site of action. Our assay for activities encoded by the Prnd and Prnp genes is based upon transfection of cerebellar granule cell neurons, one of the target cells for Dpl toxicity observed \textit{in vivo}, and also a target population for an apparently similar syndrome produced by expression of N-terminally truncated forms of PrP\textsuperscript{C} (20, 25, 51, 52). This assay for Dpl toxicity measures apoptosis, as defined by nuclear morphology, the action of a pan-caspase inhibitor and activation of caspase 3, and is blocked by PrP\textsuperscript{C} expression from an endogenous Prnp gene or supplied from a co-transfected transgene. It is important to stress that some Prnp alleles tested here in CGNs have close equivalents that have recently been assessed in Tg mice (43), and results obtained in the two systems are remarkably similar (Table I). In our assay, N-terminal PrP deletions to remove the octarepeats are nontoxic, as is the case in Tg mice (53). Furthermore, Dpl toxicity in granule cells is not blocked by PrP alleles deleted for all five octarepeats (PrP\textsubscript{A351–90}), offering a parallel to an N-terminally deleted Prnp allele (PrP\textsubscript{A123–88}) tested in Tg mice. A second Prnp allele with a larger deletion (PrP\textsubscript{A32–121}) is toxic, yet sensitive to co-expression of wt Prnp, as \textit{in vivo} (25). Finally, an octarepeat expansion familial prion disease allele (octa13) comprised a third internal control. This was pro-apoptotic to an equal or greater extent as Dpl but insensitive to the presence of wt Prnp alleles, as is indeed the case for expression of an octa14 allele (“PG14”) assayed in Tg mice (49). A practical consequence of these findings is that it may be possible to
FIG. 5. *Prnp* alleles analyzed in the CGN assay. A, panel of PrP mutants that have been tested in co-transfection experiments to detect the protective domain of PrP in Dpl-mediated neurodegeneration. Helices A–C are represented as rectangles, and the two short β-strands are represented by rectangles with horizontal shading. Mutants represent an N-terminal deletion protein (PrPΔ23–28), an octarepeat deletion protein (PrPΔ51–90), a molecule where each histidine residue within the copper-binding octarepeats was converted to a glycine residue (PHGG(G/S)WGQ4→PGGG(G/S)WGQ4, “H/G”), and a PrP molecule lacking the GPI anchor (PrPS232ter). B, co-transfection experiments in *Prnp*0/0 cerebellar granule cells were performed to analyze the protective domain of PrP (upper graph). pBud-GFP-wtPrP, pBud-GFP-PrPΔ51–90, pBud-GFP-PrPS232ter, pBud-GFP-PrPΔ23–28, and pBud-GFP-PrP-H/G constructs were transfected alone (data not shown) and in the presence of pBud-GFP-empty vector (e.v.) or pBud-GFP-Dpl. None of the PrP mutants showed intrinsic toxicity when transfected alone (data not shown). Whereas PrPΔ23–28, PrPΔ51–90, and PrP-H/G are incapable of rescuing Dpl-induced neurotoxicity, PrPS232ter is fully protective. ANOVA (#, p < 0.0001 versus empty vector; **, p < 0.0001 versus ev+wtPrP; *, p < 0.0004 versus ev+wtPrP; ***, p < 0.0211 versus ev+wtPrP). C, as a further control for the reliability of the cellular model, both *Prnp*0/0 and *Prnp*−/− cerebellar granule cells were transfected with pBud-GFP-PrPOcta13, an octarepeat insertion PrP mutant bearing eight additional octarepeats and with pBud-GFP-PrPΔ32–121 (“Shmerling syndrome” allele). The PrPOcta13 allele was neurotoxic in both PrP knock-out and PrP−/− expressing cells, behaving unlike Doppel and PrPΔ32–121 as a dominant trait, and thus further confirming the accuracy of our cellular system. ANOVA (**, p < 0.0001; ***, p < 0.0001; #, p > 0.44).
predict neurotoxic properties of novel Prnp alleles by analyses in CGN cells prior to the creation of Tg mice.

While results of our genetic assays are in close accord with in vivo paradigms, we failed to establish a parallel assay based upon treatment of cerebellar cells with natively folded recombinant Dpl and PrP (prepared as described previously (13, 54)). Although Cui et al. (55) have described toxicity for Prnp<sup>151</sup> cells produced by a Dpl-(127–151) peptide at a concentration of 10 μM or more, the significance of these treatments and their pharmacological sequelae is unclear, because toxicity was blocked by PrP-(106–126), an aggregating peptide widely used as a model for PrP-related neurotoxicity (56). Residues 127–151 in the vicinity of helix C were excluded (by three different alleles) from an obligatory role in toxicity in our genetic experiments (Table II).

**Activity Determinants in PrP<sup>C</sup> and Dpl**

PrP<sup>C</sup>—Our data thus far reveal two determinants in PrP<sup>C</sup> that, when deleted or otherwise mutated, result in an inability to protect against Dpl-mediated toxicity. The first is defined by the charged motif KKKRPK at the N terminus of mature PrP<sup>C</sup> (PrPΔ23–28), and the second corresponds to the octarepeat region (PrPΔ51–90). Of note, a similar octarepeat deletion allele failed to protect against Bax-mediated cell death of human neurons (26) and cell death induced by serum deprivation of a Prnp<sup>151</sup> neuronal cell line (57, 58). Missense mutations substituting octarepeat histidine residues for glycine (PHGG/G/H9004, GHWQ/G/H11001) also inactivated the protective effect of PrP<sup>C</sup> (Fig. 5). Not all mutant PrP alleles scrutinized here lead to loss of protective activity, however, arguing that PrP<sup>C</sup> is not indiscriminately sensitive to perturbation. For example, a Prnp allele impaired in its ability to undergo GPI anchor addition exhibited protective activity comparable with a wt allele, as was also the case for a similar allele tested in the context Bax-initiated apoptosis (26). Although the high frequency at which granule cells are co-transfected when exposed to mixtures of two plasmids indicates that transgene-encoded Dpl and PrP<sup>C</sup> proteins likely co-exist within the same cells in our paradigm, it is of interest to note that effects in trans (in a cellular sense) have been noted in other paradigms. In these experiments, wt PrP<sup>C</sup> expressed in cerebellar granule cells was able to abrogate death of Purkinje cells determined by a PrPΔ32–134 transgene expressed from a Purkinje cell-specific "L7" promoter (52). Whether cell-surface shed or donated PrP<sup>C</sup> (59–61), or other GPI anchor-less forms of PrP<sup>C</sup> (which may nonetheless retain membrane association) (62) similar to the protein encoded by the S232ter and PrPΔGPI alleles (this paper and Ref. 26), contribute to neuroprotective effects discerned in Tg mice remains to be established.

With regard to the known properties and functions of the PrP domains defined by genetic mapping, the charged region KKKRPK removed by the PrPΔ23–28 deletion contributes to the action of dominant negative PrP<sup>C</sup> alleles that inhibit conversion of wt PrP<sup>C</sup> to PrP<sup>PSC</sup> (48). This region is also implicated in targeting, in the form of a weak nuclear localization signal (perhaps only germane to the pathogenesis of Prnp alleles encoding truncated forms of PrP<sup>C</sup> (63)), in controlling a transition from raft-like domains to clathrin-coated pits (47), and in dynein-mediated retrograde axonal transport (64). Features of the octarepeat sequences include selective Cu(II) binding in *vitro* (65, 66) and again a relationship to trafficking signals. Thus octarepeat sequences serve to facilitate basal and/or Cu(II)-stimulated endocytosis (67–70) and kinesin-mediated anterograde axonal transport (64). Although there has been recent interest in "cytoplasmic" PrP (5), such species are thought to be neurotoxic rather than neuroprotective in cerebellar neurons. We are currently aware of no data from the granule cell assay that supports action of PrP outside of the compartments of the secretory and endocytic pathways (8, 9).

PrP regions scrutinized to date do not lie in the globular portion of the molecule, and prior studies have indicated they have no overt effect upon stability either in cultured cells and/or the central nervous system (47, 53, 71). Although the effects of these mutations upon PrP<sup>C</sup> half-life in CGNs cannot be excluded, we suggest an influence upon trafficking and delivery is more plausible. In addition to the effect of N-terminal sequences described here, it is likely that yet other determinants of neuroprotective activity map elsewhere within PrP.<sup>2</sup>

Doppel—In contrast to Prnp, deletion analysis of a Prnd cDNA has revealed only major determinants necessary for pro-apoptotic activity. This maps within nucleotides 303–375 of the ORF, corresponding to residues 101–125 encompassing helix B/B'. Landmark features within this interval include cysteine 109 and asparagine 111 (contributing to the N-terminal linkage of the "inner" disulfide bond and an N-linked glycosylation site, respectively), but both Cys-109 and Asn-111 have close equivalents in PrP<sup>C</sup>. Therefore, these particular determinants seem unlikely to contribute to the neurotoxic activity of Dpl. More broadly, the panel of mutant Dpl alleles described here, as well as data deriving from other point mutations,<sup>2</sup> suggests that complete folding and maturation of globular α-helical domain of Dpl is not required for toxic activity.

Although antagonistic actions of Prnp and Prnd (or deleted

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**Table I**

| Prnp allele | Pro-apoptotic activity in Prnp<sup>151</sup> neurons<sup>a</sup> | Pro-apoptotic activity in Prnp<sup>0/0</sup> neurons<sup>b</sup> | Offsets pro-apoptotic effect of Dpl<sup>c</sup> | Ref.* for Tg mice |
|-------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|-------------------|
| wt          | No                                                            | No                                                            | Yes                                                           | 20, 22, 42, 43, 53 |
| Deleted for all 5 octarepeats<sup>3</sup> | No                                                            | No                                                            | No                                                            | This paper        |
| Altered octarepeat histidines<sup>4</sup> | No                                                            | No                                                            | NA                                                            | 25                |
| Deletion of residues 32–121 | NA                                                            | NA                                                            | NA                                                            | 49, 85            |
| Octarepeat expansion | Yes<sup>c</sup>                                                | Yes<sup>c</sup>                                                | Yes<sup>c</sup>                                                |                   |

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<sup>a</sup> For all data regarding apoptosis in CGNs, see this paper.

<sup>b</sup> Prnp allele with a Δ51–90 deletion was assayed in CGNs, and Prnp alleles with Δ23–88 or Δ32–93 deletions were assayed in Tg mice.

<sup>c</sup> Each octarepeat histidine was converted to a glycine residue; see main text.

<sup>d</sup> No overt pathologic abnormalities in Tg mice with 1× endogenous PrP<sup>C</sup> expression were assessed in a Prnp<sup>151</sup> genetic background at 15 months of age (O. Windl and H. Kretzschmar, manuscript in preparation).

<sup>e</sup> NA indicates not applicable.

<sup>f</sup> This result is from an assay of Prnp alleles with a total of 13 octarepeat units.

<sup>g</sup> This result is from an assay of Prnp alleles with a total of 14 octarepeat units.

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<sup>2</sup> B. Drisaldi, manuscript in preparation.
FIG. 6. Neurotoxic properties of Doppel mutants. A, representation of wt Dpl and Dpl mutants representing 7 deletions along the entire coding region of the molecule (DplΔ29–49, DplΔ50–90, DplΔ91–149, DplΔ91–125, DplΔ126–149, DplΔ91–100, and DplΔ101–125), one 5-bp insertion mutant in the middle of αC helix (Dpl-GPS1-1), a stop codon mutant at GPI anchor site 155, and a double termination truncation mutant at site 92. Helices A–C are represented as rectangles, and the two short β-strands are represented by rectangles with horizontal shading. B, Dpl deletion mutants were transfected into Prnp<sup>0/0</sup> granule neurons. Although deletions in both the N-terminal and middle regions of Dpl protein did not change Dpl neurotoxic activity, both deletion of helices αB/B' + αC and subsequently the sub-deletion of the kinked helix αB' only abolished Dpl toxicity (upper graph). ANOVA, *, p < 0.0001 versus empty vector; #, p > 0.36. Of note, Dpl GPI anchor is not required to induce toxicity. C, all deletion mutants were transfected in Prnp<sup>+/+</sup> granule cells in order to confirm the Dpl/PrP<sup>+</sup> paradigm (bottom graph). PrP<sup>+</sup> expression protected neurons from the pro-apoptotic activity associated with wt and mutant Dpl. ANOVA, p > 0.22.
Pro-apoptotic activity of Dpl is dependent upon translation of ORF nucleotides 303–375 and hence upon a biochemical property of the central region of the Doppel molecule.

Distinct from the analogous region of PrP, the NMR structure of this region of Dpl reveals a kink, dividing it into aB and aB′ helices of 16 (residues 101–116) and 9 (residues 117–125) residues, respectively (14, 15, 78, 79). In our genetic analyses, the region including residues 101–125 can exert a pro-apoptotic effect in the absence of aA or aC (Fig. 6), but it is unclear whether residues 101–125 in solution adopt an interrupted helical conformation in the absence of other portions of the protein; for example, in the NMR structure the kinked region involves a hydrogen bond between Asn-117 and Phe-60 in the first β-strand (15), and a synthetic peptide corresponding to the helix B region assessed in phosphate buffer was characterized by a random coil signature (55). The interval defined by the 101–125-residue deletion has also attracted prior interest as overlapping a Cu(II)-binding site defined by fluorescence quenching, equilibrium dialysis binding, and mass spectrometric analysis of a Dpl(101–145) peptide (18). Whether competitive binding of copper contributes to the antagonistic actions of these proteins is uncertain, however, because the Dpl(101–145) peptide used to define Cu binding also includes sequences from helix C. Further analysis of this active region by saturation mutagenesis would appear to be in order.

**Understanding and Manipulating PrPC in Health and Disease**

What are the implications of our findings with respect to prion replication in infectious diseases such as scrapie and bovine spongiform encephalopathy? Dominant negative alleles of PrP comprise an important avenue with regard to targeted therapy, but it is unclear whether trans-dominant Prnp mutations lying within a putative protein X-binding site (3, 80) confer resistance equally to all prion strains. On the other hand, prior experiments strongly suggest that wt Dpl is intrinsically incapable of conversion to a β-sheet pathogenic conformation in the presence of infectious prions (15, 20, 81). By defining a PrndΔ101–125 allele as non-neurotoxic, our studies now provide a basis for the design of hybrid Dpl-PrP molecules resistant to pathologic refolding initiated by PrPSc. Such hybrid molecules might form the basis for new strategies to interfere with prion replication in trans or to create PrP-related proteins that retain important physiological properties of PrPC (82–84), yet are innately resistant to prion infections.

With regard to discerning the active sites and physiological attributes of cellular prion proteins, there has been much interest in the requirements of PrPC for conversion to PrPSc in transformed cells, but comparatively few studies focusing upon...
other biological readouts of activity, and few using primary cultures of adult neurons. To the best of our knowledge, the system described is the first to be calibrated in vivo via the performance of benchmark Prnp alleles tested in Tg mice, and is also compatible with saturation mutagenesis. Our studies thus far have defined a contribution of N-terminal PrP sequences in neuroprotection, and it is of interest to note a close concordance between deletion intervals and features required for anterograde and retrograde axonal transport (64). It is possible that these determinants would have been overlooked in cellular paradigms that do not use differentiated neurons, and that further iterations of the granule cell transfection assay will lead to a genetic definition of structures and subcellular localizations required for the bioactivity of PrPκ. Whether these bioactive forms coincide with the abundant cell-surface displayed molecules detected by routine biochemical and cytological analysis remains to be established.

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22. Bauermann, T., Kourilsky, J.,有一点是，我们已经使用CRISPR-Cas9技术对PrP进行了基因编辑，我们发现PrP在不同器官中的表达并不完全相同，这可能与PrP的功能多样性有关。PrP在大脑中的表达主要与学习和记忆有关，而在肌肉和心脏中的表达则可能与维持肌肉和心肌的结构和功能有关。因此，深入了解PrP的表达和功能对于理解和治疗各种神经系统疾病至关重要。
Genetic Assay of PrP and Doppel

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