Normal neurogenesis and scrapie pathogenesis in neural grafts lacking the prion protein homologue Doppel

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Received January 25, 2001; revised March 1, 2001; accepted March 2, 2001

The agent that causes prion diseases is thought to be identical to PrP^{Sc}, a conformer of the normal prion protein PrP^C. Recently a novel protein, termed Doppel (Dpl), was identified that shares significant biochemical and structural homology with PrP^C. To investigate the function of Dpl in neurogenesis and in prion pathology, we generated embryonic stem (ES) cells harbouring a homozygous disruption of the *Prnp* gene that encodes Dpl. After *in vitro* differentiation and grafting into adult brains of PrP^C-deficient *Prnp*^{0/0} mice, Dpl-deficient ES cell-derived grafts contained all neural lineages analyzed, including neurons and astrocytes. When *Prnp*-deficient neural tissue was inoculated with scrapie prions, typical features of prion pathology including spongiosis, gliosis and PrP^{Sc} accumulation, were observed. Therefore, Dpl is unlikely to exert a cell-autonomous function during neural differentiation and, in contrast to its homologue PrP^C, is dispensable for prion disease progression and for generation of PrP^{Sc}.

INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders which occur naturally in man and in a variety of animals (Aguzzi *et al.*, 2001). One of the hallmarks of TSEs is the accumulation in the brain of a protease-resistant protein, PrP^{Sc}, which is a conformer of a normal host-encoded protease-sensitive isoform, designated PrP^C (Prusiner, 1982). PrP^C is attached by a glycolipid anchor to the cell surface and is expressed by many cell types, including neurons and glia (Cashman *et al.*, 1990; Moser *et al.*, 1995).

To elucidate the physiological function of PrP^C, several mouse lines deficient for PrP^C were independently generated, however, they exhibited a puzzling discrepancy in their phenotypes: Zrch

Prnp^{0/0} and Edbg *Prnp*^{-/-} show only minor electrophysiological and circadian rhythm defects (Büeler *et al.*, 1992; Collinge *et al.*, 1994; Tobler *et al.*, 1996); whereas Ngsk *Prnp*^{-/-} mice develop ataxia with advancing age, due to cerebellar Purkinje cell degeneration (Sakaguchi *et al.*, 1996; Moore *et al.*, 1999). Two additional *Prnp* knockout lines, Zürich II and Rcm0, confirmed the observations on the Nagasaki line (Moore *et al.*, 1999; Rossi *et al.*, 2001).

Sequence analysis of a cosmid containing *Prnp* revealed a novel gene encoding a protein of 179 residues, christened Dpl ('downstream of the *Prnp* locus' or 'doppel', german for 'double'), 16 kb downstream of the murine *Prnp* gene. The predicted protein showed ~25% identity with the carboxy-proximal two thirds of PrP^C (Moore *et al.*, 1999). Dpl may contain three alpha helices (as does PrP^C) and two disulphide bridges between the 2nd and 3rd helix, (Lu *et al.*, 2000; Silverman *et al.*, 2000). Dpl mRNA is expressed at high levels in testis, less in other peripheral organs and at very low levels in brain of adult wild-type mice. However, significant *Prnp* mRNA transcripts were detected during embryogenesis and in the brains of newborn mice, arguing for a possible function of Dpl in brain development (Li *et al.*, 2000).

In the brain of ataxic Ngsk but not of healthy Zrch and Edbg *Prnp*^{-/-} mice, an intergenic splicing event places the Dpl locus under the control of the *Prnp* promoter, probably due to the deletion of the *Prnp* intron 2 sequence including its splicing acceptor. This causes overexpression of Dpl in neurons and possibly Purkinje cell degeneration. Taken together, these results show that ectopic Dpl expression rather than functional loss of PrP^C may be responsible for neuronal degeneration in ataxic *Prnp*^{-/-} mice. However, Ngsk *Prnp*^{-/-} mice were rescued from the

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A. Behrens et al.

neuronal degeneration by introduction of a *Prnp* transgene. This suggests that Dpl toxicity may be dose-dependent and can be antagonized by PrPC (Nishida, 1999). Interestingly, overexpression of an amino-proximally truncated transgene encoding PrPC devoid of the octa repeats and the conserved 106–126 region (PrPΔ32–135) into Zürich I *Prnp*^{0/0} mice caused ataxia and degeneration of the cerebellar granule cell layer within weeks of birth, and introduction of a single intact PrPC allele prevented the disease (Shmerling et al., 1998). Therefore PrPC and truncated PrPC interact genetically. Because Dpl resembles truncated PrPC (Moore et al., 1999), it might cause disease by the same mechanism.

In this study we have investigated the role of Dpl in neurogenesis and in prion pathogenesis. We induced neural differentiation of embryonic stem (ES) cells carrying a homozygous null mutation of the *Prnd* locus, but a normal *Prnp* locus. Dpl-deficient ES cells were found to undergo normal neurogenic differentiation and were capable of giving rise to all neural cell lineages when transplanted into host brains. After inoculation with scrapie prions, Dpl-deficient neural grafts showed spongiosis, gliosis and unimpaired accumulation of PrP^{Sc} and infectivity similar to wild-type neuroectodermal grafts. We conclude that Dpl is not required for neural differentiation and that *Prnd*-deficiency does not prevent prion pathogenesis in neural grafts.

RESULTS AND DISCUSSION

To inactivate *Prnd* in ES cells, the *Prnd* genomic locus was cloned and used to generate a targeting construct (termed *PrndKO*), in which the Dpl open reading frame (ORF) was replaced with a neomycin resistance gene flanked by loxP sites. A diphtheria toxin α (*DTα*) chain gene was added for selection against random integrants (Figure 1A). After electroporation of *PrndKO* into E14.1 ES cells and G418 selection, 1/382 ES cell clones had undergone homologous recombination at the *Prnd* locus as assessed by PCR and Southern analysis, yielding the *Prnd*^{neo} allele (Figure 1C and D). In order to generate ES cells deficient for Dpl production, the second *Prnd* allele was inactivated in *Prnd*^{+/neo} heterozygous cells by selection with high concentrations of G418 (Figure 1B). At 4 mg/ml G418, 22 ES cell clones out of 10⁶ cells plated survived selection, two of which (clone *Prnd*HB6 and *Prnd*HB8) were homozygous for *Prnd*^{neo} (Figure 1C and D). Undifferentiated *Prnd*^{neo/neo} ES cell clones were similar in morphology to *Prnd*^{+/neo} heterozygous and wild-type control cells (data not shown).

To investigate the role of Dpl in neural development, *Prnd*^{neo/neo}, *Prnd*^{+/neo} and wild-type ES cells were differentiated into neuroectoderm *in vitro* following a procedure that was established previously (Benninger et al., 2000) and transplanted into the brains of adult mice *Prnd*^{+/neo} ES cell clones that had also undergone high G418 selection were used as controls to ensure that gene targeting, high G418 selection and prolonged *in vitro* passaging had no adverse effect on neural differentiation efficiency. Twenty-nine days after transplantation, grafts deficient for *Prnd* (*Prnd*^{neo/neo}) and control grafts (*Prnd*^{+/neo}, *Prnd*^{+/+}) consisted of neural tissue with neurons, astrocytes and oligodendrocytes as identified by immunostaining for MAP2, NeuN, synaptophysin, GAP43, GFAP and S-100 protein (Figure 2 and data not shown). These results indicate that Dpl is not required for the differentiation of ES cells into neural cells, and

Fig. 1. Generation of Dpl-deficient ES cells. (A) Schematic representation of the targeting strategy to generate ES cells harbouring a heterozygous mutation of *Prnd*. Exons of the *Prnd* gene are represented by rectangles; thin lines represent intronic regions of the *Prnd* locus. The Dpl ORF is represented as a dark gray rectangle. The neomycin resistance gene (*PGK-Neo*) and the diphtheria toxin alpha gene (*DTα*) are represented by light gray rectangles; loxP sites are shown as black triangles. The location of the *ApaI* restriction sites, the *ApaI* restriction fragments, and the PCR primers used for genotyping are indicated. (B) Schematic representation of the gene conversion event generating *Prnd* homozygous mutant ES cells selected for with high G418 concentration. (C) PCR analysis of genomic DNA from the parental ES cell line E14.1 (*Prnd*^{+/+} CTR), one targeted *Prnd*^{+/neo} clone (*Prnd*^{+/neo} CTR) and four ES cell clones that had undergone selection with 4 mg/ml G418 (*Prnd*^{+/neo} and *Prnd*^{neo/neo}). (D) Southern blot analysis of genomic DNA from the parental ES cell line E14.1 (*Prnd*^{+/+}), one *Prnd*^{+/neo} and one *Prnd*^{neo/neo} ES cell clone.

argue against a cell-autonomous function for Dpl during neurogenesis. To detect a possible influence of the genotype on the development of glial or neuronal cells, we have assessed the cellular density and the contribution of astrocytes, oligodendrocytes and neurons to the graft tissue and found no difference between *Prnd*^{neo/neo} and *Prnd*^{+/neo}, *Prnd*^{+/+} control grafts (Figure 3).

Next, we investigated whether Dpl is required for the development of spongiform pathology and for accumulation of PrP^{Sc} and infectivity. *Prnd*-deficient and control ES cells were differentiated *in vitro* and transplanted into the caudoputamen of *Prnp*^{0/0} mice. Inoculation with RML prions was carried out 35–45 days after transplantation, and mice were kept for up to 211 days after

Fig. 2. Histological characterization of non-infected and Scrapie-infected *Prnd*^{neo/neo} neuroectodermal grafts. Histological analysis of non-infected *Prnd*^{+/+} (A–D), *Prnd*^{+/neo} (E–H) grafts and *Prnd*^{neo/neo} grafts (I–L). Intraventricularly or intraparenchymally placed ES cell-derived grafts of all genotypes showed no difference in their differentiation pattern, as they contained astrocytes (GFAP immunostain, B, F, J) and showed regular immunoreactivity for synaptophysin (C, G, K) and MAP-2 (D, H, L). Inoculation with mouse prions (185 days) resulted in typical histopathological hallmarks of scrapie, such as vacuolation (M) and astroglia (N) but not yet significant loss of neuropil, as indicated by normal staining pattern for synaptophysin and MAP-2. Scale bar: 500 μ m (A, E, I, M) and 200 μ m (all other panels).

inoculation. In previous experiments we had shown that this time period is sufficient to allow the formation of PrP^{Sc} in grafts (Brandner *et al.*, 1998).

One *Prnd*^{neo/neo} and two *Prnd*^{+/neo} mice were killed 52 days after inoculation and were analyzed for the presence of disease-associated PrP^C. In these grafts, we found very small amounts of proteinase K-resistant prion protein by histoblotting, as was expected from the short incubation time and infectivity, as determined by transmission into *tga20* indicator mice. Instead, prolonged incubation for 195–203 days in two additional *Prnd*^{neo/neo} and two *Prnd*^{+/neo} mice led to significant accumulation of PrP^{Sc} in all transplants irrespective of their genotype (Figure 4), and transmission into indicator mice confirmed the presence of infectivity (Table I). Analysis of paraffin sections of long-term infected grafts confirmed typical histopathological hallmarks of scrapie, i.e. vacuolation and astroglia (Figure 2M and N) that were strictly confined to the grafts as described earlier (Brandner

et al., 1996a, 1998). Therefore, the absence of *Prnd* in neural tissue does not affect disease progression of experimental prion pathogenesis, at least in the neurografting paradigm.

In this study we have investigated the role of the PrP^C homologue Dpl in neural differentiation and prion pathogenesis. Both *Prnp* and *Prnd* are expressed during embryogenesis and in the brains of newborn mice, suggesting a role for these proteins in neurogenesis. However, neuronal development proceeds normally in mice lacking *Prnp*. Likewise, in the absence of Dpl, the differentiation of ES cells into neurons and astrocytes is not impaired. Several similarities between both proteins may indicate similar biological functions. The subcellular localization of PrP^C and Dpl is similar: both are GPI-anchored membrane proteins. In addition, the primary amino acid sequence is 23% identical between both proteins, and the structure of Dpl is predicted to share significant homology with PrP^C (Mo *et al.*, 2001). Thus a possible role of PrP^C and Dpl during neuronal development may

A. Behrens et al.

Fig. 3. Quantitation of cell populations in *Prnd^{+/+}*, *Prnd^{+/neo}* and *Prnd^{neo/neo}* grafts. Neurons, astrocytes and oligodendrocytes were counted on an area of $400 \times 400 \mu\text{m}$ (as described in Benninger, 2000). There was no apparent difference in distribution, ratio and number of these cell types in grafts of either genotype.

be masked by functional redundancy. To address this question it will be necessary to generate ES cells and mice lacking *Prnp* as well as *Prnd* and to study whether the lack of both PrP^C and Dpl will result in impaired neurogenesis.

The ability of Dpl-deficient ES cells to give rise to normal neural grafts enabled us to investigate a potential role of Dpl in prion disease. The typical histological features of scrapie were observed, including spongiosis and gliosis. Also, typical PrP^{Sc} depositions developed in neuroectodermal grafts devoid of *Prnd*. The Dpl protein resembles an N-terminally truncated PrP^C protein lacking the octamer repeats, a version of PrP^C that is capable of PrP^{Sc} propagation (Flechsiger et al., 2000). *Prnd* transcription was not detected in adult neurons, although *Prnd* mRNA was reported to be present in endothelial cells of the brain (Li et al., 2000). Likewise, we have not detected Dpl protein in the brains of terminally ill scrapie mice (data not shown).

Recently, two groups have searched for possible linkage disequilibrium of *Prnd* alleles in human prion diseases. Four polymorphisms in *Prnd* were detected, but no strong association was found between any of these polymorphisms and human prion diseases (Mead et al., 2000; Peoc'h et al., 2000). Taken together, these findings further argue against an important function of Dpl in neurons during prion disease. On the other hand, the experimental approach described here does not rule

Fig. 4. Detection of disease-associated PrP^{Sc} in intraparenchymal grafts derived from *Prnd^{+/neo}* and *Prnd^{neo/neo}* ES cells. (A–D) Frozen sections with intraparenchymally located grafts (outlined with asterisks). Histoblots of these grafts without (E–H) and with (I–L) proteinase K treatment. After short incubation time (52 days), minute amounts of proteinase resistant PrP were detectable in both, *Prnd^{neo/neo}* and *Prnd^{+/neo}* grafts, while prolonged incubation time (195–203 days) led to strong accumulation of proteinase resistant PrP^{Sc}. The central irregular shaped region in the *Prnd^{+/neo}* graft (H, L) results from regressive changes and calcifications which are occasionally observed in ES cell-derived neuroectodermal grafts of all genotypes.

Table 1. Determination of prion infectivity by transmission of graft tissue into *tga20* indicator mice

Genotype of graft	Prion inoculum	incubation time (days)	Latency of scrapie in <i>tga20</i> indicator mice
<i>Prnd</i> ^{neo/neo}	3 × 10 ⁶ LD ₅₀	195	63 ^a , 68 ^a , 78 ^a , 86 ^a
<i>Prnd</i> ^{+/neo}	3 × 10 ⁶ LD ₅₀	203	65 ^a , 65 ^a , 67 ^a , 68 ^a
<i>Prnd</i> ^{neo/neo}	3 × 10 ⁶ LD ₅₀	52	86 ^b , 86 ^b , 86 ^b , 86 ^b
<i>Prnd</i> ^{+/neo}	3 × 10 ⁶ LD ₅₀	52	81 ^a , 84 ^a , 86 ^a , 86 ^a
<i>Prnd</i> ^{+/neo}	3 × 10 ⁶ LD ₅₀	52	74 ^a , 86 ^b , 86 ^b , 86 ^b
<i>Prnd</i> ^{neo/neo}	mock	188	>86 ^c , >86 ^c , >86 ^c , >86 ^c
<i>Prnd</i> ^{neo/neo}	mock	201	>86 ^c , >86 ^c , >86 ^c

Long-term infection of *Prnd*^{neo/neo} and *Prnd*^{+/neo} grafts (195–203 days) leads to accumulation of high titres of infectivity, as inferred from short incubation times of indicator mice. Short-term infection of *Prnd*^{neo/neo} and *Prnd*^{+/neo} grafts (52 days) led also to accumulation of infectivity, but at lower levels than in long-term infected grafts, resulting in longer incubation times of indicator mice. Transmission of mock-infected graft tissue (188–201 days after mock-infection) did not result in scrapie.

^aIndicator mouse with terminal scrapie disease.

^bIndicator mouse with incipient scrapie disease at the time of writing.

^cIndicator mouse with no sign of scrapie disease.

out a role for Dpl in peripheral prion pathogenesis and PrP^{Sc} transport to the brain. The latter possibility is intriguing, because *Prnd* is expressed in the spleen, a major peripheral reservoir of PrP^{Sc} and prion infectivity (Li *et al.*, 2000). Mice lacking *Prnd* derived from the ES cells described here will doubtlessly help in addressing this question.

METHODS

Cells and tissue culture conditions. E14.1 ES cells (derived from 129 Ola/Ola mice) were cultured on γ-irradiated (30 Gy) mouse embryo fibroblast feeder layers. Culture medium was Glasgow minimal essential medium (Gibco 21710-025) supplemented with non-essential amino acids (Gibco 11140-025), 1 mM pyruvate (Gibco 11360-070), 0.1 mM β-mercaptoethanol (Sigma), 15% fetal calf serum (FCS; Sigma) and leukemia inhibitory factor (LIF 10³ U/ml; Gibco 13275).

Induction of differentiation. Subconfluent ES cell cultures from 75 cm² culture area were trypsinized and transferred into Petri dishes with 12 ml differentiation medium, where they spontaneously formed embryoid bodies (EB) which did not attach to the dishes. Serum-free medium formulation Neurobasal (Gibco 21103) was supplemented with B27 (Gibco 17504) and G5 supplement (Gibco 17503) containing: insulin, transferrin, selenite, biotin, hydrocortisone, basic fibroblast growth factor epidermal growth factor and glutamine (73.5 mg/l). Medium was replaced on days 1, 2, 3, 4, 6 and 8 by entirely removing EB from Petri dishes and transferring them into a 15 ml Falcon tube where they sedimented by gravity. Transplantation was carried out after 8 days of *in vitro* differentiation.

Generation of Dpl-deficient ES cells. The genomic *Prnd* locus was cloned from genomic DNA of E14.1 ES cells by PCR. In the targeting vector, the *Prnd* ORF was replaced with a floxed neomycin resistance selection cassette. A DTα gene was inserted for selection against random integrants.

Subconfluent ES cells (20 cm²) were trypsinized, spun and resuspended in 200 μl PBS containing linearized targeting vector (20–40 μg). Electroporation conditions were 0.8 kV, 3 μF, in

0.4 cm Bio-Rad electroporation cuvettes with Bio-Rad GenePulser. Cells were then transferred to a gelatin-coated tissue culture dish with culture medium containing LIF. G418 was added after 24 h at 0.3 mg/ml. After 8 days, surviving colonies were transferred to 96-well plates and expanded. Southern blot analysis was carried out to confirm homologous recombination of the construct into the *Prnd* locus. Genomic DNA of ES cells was digested with *Apal* and an external probe radioactively labeled with ³²P was used to confirm integration by homologous recombination. The frequency of homologous recombination was 1/384 clones. High G418 selection of the *Prnd*^{+/neo} ES cell clone was performed with 4.0 mg/ml G418.

Animals and transplantation procedure. Adult male or female *Prnp*^{0/0} mice (genetic background C57BL/6 × 129 SV) were used as graft recipients. For transplantation, mice were anaesthetized according to published protocols and placed in a stereotaxic frame (Narishige SR 6N). The coronal suture was identified after a longitudinal incision of the scalp. The syringe containing the EB was positioned at 2.5 mm paramedian and then advanced 3 mm through the coronal suture into the caudoputamen region and EB were injected (Coordinates in relation to bregma: anterior 0 mm, lateral 2 mm, ventral 2.5 mm). Injection of embryoid bodies was done as follows: a 10-μl Hamilton syringe (model 701RN) with electrotapered 26G pointstyle needle was back-filled with intact EB. After insertion of the plunger, 3–5 μl EB suspension was injected. Passage of the EB through the narrow needle resulted in dispersion upon injection into the brain. Twenty-three mice received *Prnd*^{neo/neo} and 16 mice received *Prnd*^{+/neo} grafts as a control.

Preparation of the infectious inoculum and inoculation procedure. Inoculation with mouse prions derived from the Chandler strain was performed according to published protocols (Brandner *et al.*, 1996a). The RML5 (Rocky Mountains Laboratory) inoculum was prepared from brains of terminally ill CD-1 mice.

We inoculated 17 grafted *Prnp*^{0/0} mice intracerebrally with 30 μl of the scrapie prion inoculum 6 weeks after the grafting procedure. For control experiments, four grafted mice did not receive any inoculum. For determination of infectivity in grafts,

we collected six frozen sections (10 µm thickness) at the level where sections for histoblots were obtained, and processed them as described earlier (Brandner *et al.*, 1996a), and inoculated them into groups of four *tga20* mice intracerebrally.

Histological evaluation. Histological analysis of mature grafts was carried out 29–252 days after transplantation of embryoid bodies. For analysis of growth and differentiation, grafts were analyzed between 29 and 45 days after transplantation, while analysis of scrapie- and mock-infected grafts was carried out up to 211 days after inoculation.

For routine sections, brains were removed, immersion-fixed in 4% buffered paraformaldehyde for at least 4 h, cut in coronal slices of ~2 mm thickness and dehydrated through graded alcohols. After paraffin embedding, sections of 3 µm nominal thickness were cut and mounted on coated slides (Super Frost). Hematoxylin & Eosin (H&E), and Luxol-H&E stains as well as immunocytochemical stains were performed. Immunostains were carried out as described (Benninger *et al.*, 2000).

Histoblots. Histoblots were carried out according to published protocols (Taraboulos *et al.*, 1992; Brandner *et al.*, 1996b). Frozen sections of 8 µm thickness were mounted on uncoated glass slides, immediately pressed on a nitrocellulose membrane wetted in lysis buffer and air-dried for at least 24 h. For detection, they were rehydrated in Tris buffered saline, and limited proteolysis was performed using proteinase K concentrations of 20, 50 and 100 µg/ml at 37°C for 4 h. The sections were then denatured in 3 M guanidinium thiocyanate for 10 min and blocked for 1 h in 5% non-fat milk serum. The primary antibody (anti-PrP, 6H4, monoclonal, Prionics Zürich) was used at a dilution of 1:5000 in 1% non-fat milk overnight at 4°C. Detection was accomplished with an alkaline phosphatase-conjugated goat anti-mouse antibody at a dilution of 1:2000. Visualization was achieved with nitroblue tetrazolium using 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride (Boehringer, Mannheim) according to the manufacturer's protocols.

ACKNOWLEDGEMENTS

We thank Roland Hardegger, Mauri Peltola and Petra Schwarz for technical assistance and Charles Weissmann for critical reading of the manuscript. This work was supported by grants from the Swiss National Foundation to A.A. N.G. is fellow of the Koetser foundation.

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DOI: 10.1093/embo-reports/kve088