Doppel and PrP^C do not share the same membrane microenvironment

Yuval Shaked, Nuha Hijazi, Ruth Gabizon*

The Anges Ginges Center, Department of Neurology, Hadassah University Hospital, Jerusalem, Israel

Received 30 July 2002; revised 9 September 2002; accepted 10 September 2002

First published online 20 September 2002

Edited by Felix Wieland

Abstract Doppel is a paralog of the normal prion protein, PrP^C. It has been suggested that Doppel can compensate for the absence of PrP^C in $PrP^{0/0}$ mice. In this work, we tested whether Doppel and PrP^C share the same cell location, thereby sharing the same neighboring cell components, probably required to share the same cell function. Our results show that, at detergent conditions in which membrane rafts were intact, neither PrP^C and Doppel co-immunoprecipitate with the appropriate antibodies, nor was Doppel retained by a Cu^{2+} IMAC resin, as PrP^C does. This indicates that, although Doppel is a raft-associated protein as is PrP^C, both proteins are not present in the same membrane microenvironment, and they probably do not perform the same function.

Key words: Doppel; PrP; Immobilized metal affinity chromatography; Raft; Prion

1. Introduction

PrP^{Sc} is an essential component of prions, the agent causing transmissible neurodegenerative diseases, such as scrapie and bovine spongiform encephalitis. PrP^C, the normal isoform of PrP^{Sc}, is a glycophosphatidylinositol (GPI)-anchored glycoprotein which differs from PrP^{Sc} by its tertiary structure [1]. As many GPI-anchored proteins, both PrP isoforms are inserted into membrane rafts, which are cholesterol-rich membrane microdomains [2]. The prion proteins are encoded by the *Prnp* gene. Although the function of PrP^C is unknown, it has been shown to bind copper specifically, and it is therefore possible that copper binding might be regarded as a marker for the activity of PrP^C [3–5].

Even in the absence of PrP, some lines of $PrP^{0/0}$ mice present a normal phenotype [6,7]. However, the *Rcm0* and *Ngsk* lines of $PrP^{0/0}$ mice develop a late onset ataxia, probably related to a widespread loss of cerebellar Purkinje cells [8]. It has been suggested that this neurological dysfunction is not associated with the absence of PrP^C, but rather with the overexpression in the brains of these $PrP^{0/0}$ mice of a PrP paralog, denominated Doppel (Dpl) [9]. In wild type (wt) mice as well as in the unaffected $PrP^{0/0}$ mice, Doppel is not expressed in brains, and it can be found mainly in testis. Doppel is encoded by the *Prnd* gene, 16 kb downstream to *Prnp* [9] and expresses a protein of 179 amino acids. The Doppel sequence is approximately 25% homologous with PrP^C, especially in its C-terminal region, and lacks the octarepeat domain present at the N-terminus of PrP [9], which is believed to be the major Cu^{2+}-binding site of PrP [3,4,10–12]. Lately, other copper-binding sites have also been identified in the PrP sequence, one of them in the C-terminal region.

In addition to the sequence homology, PrP^C and Doppel share some biochemical and structural similarities. As PrP^C, Doppel adopts an α-helical conformation [13], forms intramolecular disulfide bonds and has two N-linked oligosaccharides [14]. In addition, it is attached to the cell surface via a GPI anchor [9].

In this work, we compared PrP^C and Doppel for additional properties. We studied both proteins for their raft location and copper-binding properties. We show that, while both Doppel and PrP^C are attached to rafts through their GPI anchor, the populations of rafts to which they are attached are probably different. We also show that, in contrast to PrP^C, Doppel did not bind to an immobilized metal affinity chromatography (IMAC) resin loaded with Cu^{2+} as PrP^C did. These results suggest that Doppel and PrP^C, although similar in their tertiary structure, may perform different functions.

2. Materials and methods

2.1. Tissue samples

Brain homogenate was diluted with 5 volumes of phosphate-buffered saline (PBS) (g/ml) and testis extracts were obtained from FVB (wt mouse control of the $PrP^{0/0}$ Zurich I mice) and $PrP^{0/0}$ [6] mouse lines.

2.2. Antibodies

Monoclonal anti-PrP antibody 6H4 (Prionics) and polyclonal anti-Dpl antibody F0332 or E6997(received from Dr. Prusiner's laboratory at UCSF) were used as described in the text.

2.3. Flotation assays

Flotation of detergent insoluble complexes was performed as described by Naslavsky et al. [15]. Briefly, 100–150 mg of testis tissues or brain were extracted with 700 μl of an ice-cold buffer (150 mM NaCl, 25 mM Tris–HCl, pH 7.5, 5 mM EDTA and 1% Triton X-100). The lysate were loaded onto ultra-centrifuged tubes (TLS 55, Beckman Industry). An equal volume of 70% Nycodenz in TNE (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) was added and mixed with the lysate. An 8–35% Nycodenz linear step gradient in TNE was then overlaid above the lysate. The tubes were spun at 55 000 rpm for 4 h at 4°C in a TLS-55 rotor. Fractions of 200 μl each were collected from the top to the bottom of the tube and immunoblotted with the appropriate antibody.

2.4. Chromatography on IMAC resin

50 mg of brain or testis were extracted with 2.5 ml cold 1% Triton X-100 in PBS and subsequently mixed for 1 h at 4°C with an IMAC resin which was previously loaded with copper ions (Cu^{2+}IMAC) as described [10]. The resin was precipitated by centrifugation and the non-bound extract denominated the flow through. The IMAC resin

*Corresponding author. Fax: (972)-2-642 9441.
E-mail address: gabizonr@hadassah.org.il (R. Gabizon).

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)03430-0

was washed four times with 2.5 ml of Triton X-100/PBS buffer. The attached proteins were eluted from the resin by the addition of 2.5 ml of imidazole at increasing concentrations (0.05 M×2, 0.1 M×2, and 0.2 M×2). Finally, 2.5 ml of 50 mM EDTA were used to release the copper ions from the IMAC resin. All eluted fractions were recovered by centrifugation from the resin and, subsequently, immunoblotted with the appropriate antibody.

2.5. Co-immunoprecipitation assay

Co-immunoprecipitation experiment was performed as described by Keshet et al. [16]. Briefly, anti-PrP antibody (6H4) and anti-Dpl antibody (E6997) were bound to protein A Sepharose beads (Sigma) and cross-linked with dimethylpimedilate. Testis of FVB mice was homogenized with five volumes of PBS+1% Triton X-100. Homogenates were centrifuged at 1000×*g* for 10 min at 4°C. Supernatants were subsequently incubated with 100 μl of Sepharose beads, which bound to the appropriate antibody, for 2 h at 4°C. Then the antibody beads were centrifuged for 1 min at 7000 rpm in room temperature and the elution fractions were collected. The beads washed for several times with PBS+1% Triton X-100. The bound material was eluted off the antibody beads by incubating them for 30 min at 100°C with sample buffer. Samples were immunoblotted with anti-PrP mAb, anti-Dpl rAb, anti-ESA mAb or anti-caveolin rAb (Santa Cruz Biotechnology), as described in the text.

3. Results

3.1. Doppel is a raft-associated protein

As the PrP isoform, Doppel was shown to be a GPI-anchored protein[17]. This suggests that, as PrP^{C}, PrP^{Sc} [18,19] and most other GPI-anchored proteins, Doppel may be inserted into cholesterol-rich membrane microdomains, denominated rafts [20]. Indeed, to be able to perform the function of PrP^{C}, it is conceivable that Doppel must share PrP^{C} membrane location. Since rafts are insoluble in cold Triton X-100, we subjected cold Triton X-100 extracts of testis and brains of FVB and $PrP^{0/0}$ mice to a Nycodenz density gradient and tested the presence of Doppel on the gradient fractions [15]. Under these conditions, the gradient fractions comprising the insoluble cholesterol rafts float to the top of the density gradients. Fig. 1 demonstrates an immunoblot of the gradient fractions developed with either anti-PrP or anti-Dpl antisera. In the testis of wt mice, both PrP and Doppel could be detected in the top fractions, comprising the rafts and their associated proteins. Doppel was also present in rafts in the testis of $PrP^{0/0}$ mice. These results indicate that, like PrP^{C}, Doppel is a raft-associated protein.

3.2. Doppel and PrP^{C} are not localized on the same rafts

The results shown above led us to investigate whether Doppel and PrP^{C} are localized on the same rafts. It was previously shown that PrP^{C} interacts with other components through its raft location [16]. If Doppel can compensate for the absence of PrP^{C}, it may also interact with the same cell components on the same rafts. Fig. 2 shows an immunoblot of a Doppel–PrP co-immunoprecipitation assay. Testis of FVB mice were homogenized with 1% Triton X-100/PBS in order to keep the rafts intact. Under these conditions, an antibody against one raft protein will precipitate the entire raft with its associated proteins [16]. The homogenized samples were subsequently precipitated with Sepharose A beads bound either to anti-PrP antibody (6H4) or to anti-Dpl antibody (E6997). The results of such an experiment show that PrP^{C} precipitates only with PrP beads, in contrast to Doppel, which precipitates only with Doppel beads. The fact that PrP and Doppel did not precipitate with the same beads when rafts were intact indicates that both proteins may not be localized on the same rafts. To reinforce this conclusion, we tested whether proteins known to co-immunoprecipitate with PrP will do so with Doppel. As can be seen in Fig. 2, while ESA co-immunoprecipitated with PrP, this was not the case for Doppel. In contrast, caveolin co-immunoprecipitated with Doppel but not with PrP. These results are consistent with our previous publication on this matter [16]. Our results therefore suggest that Doppel is unlikely to interact with the same membrane components as PrP^{C}, nor be part of any mechanism activated by such proteins. These results suggest it is unlikely that Doppel will compensate for the absence of PrP^{C}.

3.3. Doppel does not bind to a Cu^{2+} IMAC resin

It was recently suggested that PrP^{C} has at least two copper-binding sites [5,10,21,22]. To investigate whether Doppel can bind to copper ions such as PrP^{C}, we looked at the elution profile of Doppel, as compared to PrP^{C}, from a Cu^{2+}IMAC resin. We have shown recently that this method can be used to characterize the C-terminal copper-binding site of PrP. Doppel has been shown to share 25% homology with PrP^{C}, especially in the C-terminal region [23]. Cold Triton X-100 extracts of testis from wt mice were loaded onto a Cu^{2+}IMAC resin as explained in Section 2. Flow-through fractions (not bound to Cu^{2+}IMAC) as well as fractions eluted with increasing concentrations of imidazole and EDTA were immunoblotted either with the anti-Dpl antisera or with anti-PrP mAb 6H4. Doppel, as opposed to PrP^{C}, did not bind to the Cu^{2+}IMAC resin at all, as can be seen from the fact that it was present only in the flow-through (Fig. 3), indicating it may not comprise a copper-binding activity.

Since rafts are insoluble in Triton X-100, raft proteins loaded onto an Cu^{2+}IMAC resin under such conditions can bind either directly to the resin through the copper ion or indirectly through another raft component with copper-binding activity. We also tested the binding of ESA and α-tubulin to the Cu^{2+}IMAC column. Both of these proteins colocalized with PrP on rafts (unpublished results for ESA)[16]. These

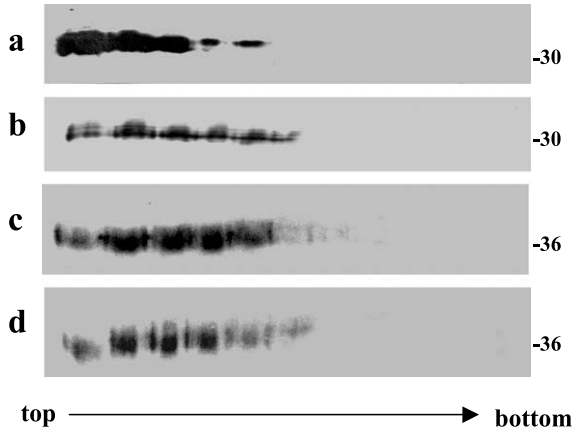

Fig. 1. Doppel is a raft-associated protein. Brain and testis extracts, from either FVB or $PrP^{0/0}$ mice, were subjected to a flotation assay as described in Section 2. Fractions from the Nycodenz gradients were immunoblotted either for anti-PrP (mAb 6H4) or for anti-Dpl (rAb F0332). a: FVB brain fractions were immunoblotted with anti-PrP (mAb 6H4). b: FVB testis fractions were immunoblotted with anti-PrP (mAb 6H4). c: FVB testis fractions were immunoblotted with anti-Dpl (rAb F0332). d: $PrP^{0/0}$ testis fractions were immunoblotted with anti-Dpl (rAb F0332).

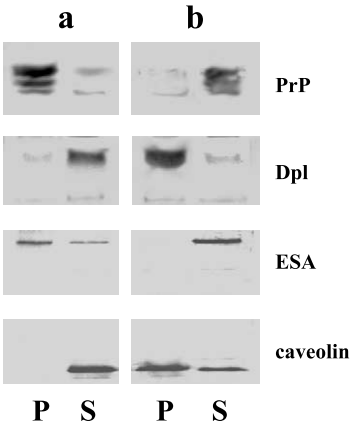

Fig. 2. Doppel does not co-immunoprecipitate with rafts of PrP^C. Membrane samples were prepared from the testis of FVB mice. The extracts were incubated with anti-PrP mAb 6H4 (panel a) or anti-Dpl rAb E6997 (panel b) which were previously attached to Sepharose A beads (as described in Section 2). The proteins attached to the beads (P) as well as the supernatants (S) were immunoblotted with anti-PrP 6H4 mAb, anti-Dpl F0332 rAb, anti-ESA and anti-caveolin.

two proteins were eluted at the same fractions as PrP^C in FVB mice brains but not in $PrP^{0/0}$ mice brains. This suggests that their binding to copper was indirect and resulted from their colocalization with PrP^C in rafts. In contrast, the fact that Doppel did not bind to the resin and therefore was not eluted at the same fractions as PrP^C indicates not only that Doppel does not bind to copper but also, most important, that it is not present on the same rafts as PrP^C, consistent with the co-immunoprecipitation results in Fig. 2.

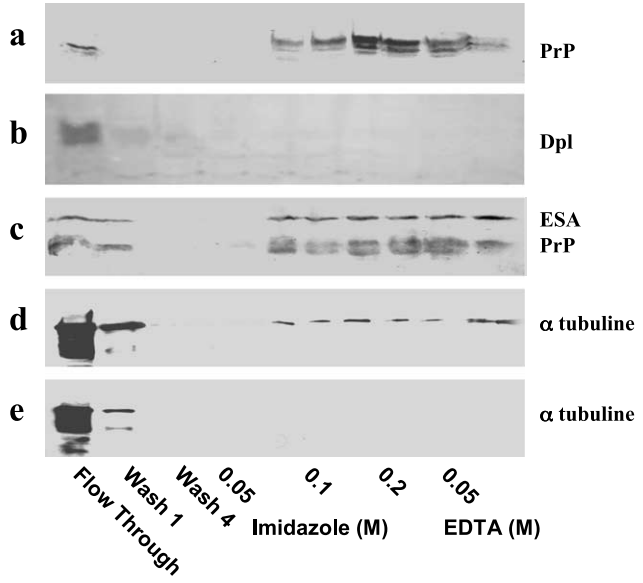

Fig. 3. Doppel does not bind to a Cu^{2+}IMAC resin. Testis and brain (either from FVB or from $PrP^{0/0}$ mice) extracted with cold Triton X-100 were applied to a Cu^{2+}IMAC resin, as described in Section 2. After two washes with the Triton buffer, attached proteins were eluted with increasing concentrations of imidazole (0.05 M, 0.1 M, 0.2 M) and, finally, with EDTA (0.05 M). a and b: Testis extract, loaded onto Cu^{2+}IMAC resin; flow-through, washes and eluted samples were immunoblotted with anti-PrP 6H4 mAb (a) or anti-Dpl F0332 rAb (b). c and d: FVB brain extracts, loaded onto Cu^{2+}IMAC resin; as above, immunoblotted with anti-PrP 6H4 mAb and anti-ESA mAb (c) or anti-α-tubulin rAb (d). e: $PrP^{0/0}$ brain extract, loaded onto Cu^{2+}IMAC resin; as above, immunoblotted with anti-α-tubulin rAb.

4. Discussion

Doppel is encoded by the *Prnd* gene, which is located downstream to the *Prnp* gene. Since Doppel is overexpressed in brain and testis of some lines of $PrP^{0/0}$ mice [17], it was suggested that Doppel may compensate for the absence of PrP in PrP-ablated mice. We hypothesized that for Doppel to substitute PrP^C in its function, it has to share with PrP its cell location and other biochemical properties.

Our findings suggest that PrP and Doppel differ substantially. Although it has been shown that both proteins are present in rafts, are similar in tertiary structure and also have 25% homology in sequence [17,23,24], we show here that both proteins are not present in the same rafts, therefore suggesting that PrP^C and Doppel cannot interact with the same membrane components. We have shown this by demonstrating that an anti-PrP antibody cannot precipitate Doppel in conditions in which rafts are intact and vice versa. We reinforced these results by showing that, in conditions where rafts are insoluble, PrP^C and Doppel can be separated by their different binding properties to a Cu^{2+}IMAC resin. The results obtained from the IMAC experiments also indicate that, unlike PrP^C, Doppel has no affinity whatsoever for copper ions. The fact that Doppel is devoid of a copper-binding N-terminal sequence was not enough to establish that it does not bind to copper since PrP^C has at least one other binding site in its C-terminal [5,21,22].

Our results are therefore inconsistent with the hypothesis that Doppel can compensate for the loss of the function of PrP^C in $PrP^{0/0}$ mice. In agreement with our results, Brown et al. [25] recently claimed that there are multiple biochemical changes between wt and $PrP^{0/0}$ mice, including increased or decreased levels of many different proteins in addition to Doppel, among them NF-κB, Mn^{2+} SOD, COX-IV, p53 and melatonin. If this is the case, the overexpression of Doppel might not result directly from the absence of PrP [25].

It was recently published that $Dpl^{0/0}$ mice are sterile [26] and, since this is definitely not the case for $PrP^{0/0}$ mice, we believe Doppel is a very important protein in the male reproductive system. PrP^C, although present in sperm, is a major brain protein.

We therefore propose that Doppel and PrP, although similar in sequence and structure, do not share enough biochemical properties to indicate that they can perform similar cell functions.

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