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Cellular roles of the prion protein in association with reggie/flotillin microdomains

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1. ABSTRACT

The prion protein (PrP) has been implicated in many diverse functions, making it difficult to pinpoint its basic physiological role. Our most recent studies in zebrafish, mammalian and invertebrate cells indicate that PrP regulates cell-cell communication, as well cell-matrix interactions at focal adhesions. In addition, we previously have shown that upon antibody-mediated cross-linking, PrP can be induced to cluster in the preformed T-cell cap. Here we review these data and discuss how the spatial link between PrP and the microdomain-forming proteins reggie-1 (flotillin-2) and reggie-2 (flotillin-1) may contribute to PrP signaling, leading to the local assembly of membrane protein complexes at sites involved in cellular communication, such as cell-cell contacts, focal adhesions, the T-cell cap, and synapses.

2. INTRODUCTION

The prion protein (PrP) has received worldwide attention because its misfolding and aggregation are closely associated with the onset of transmissible neurodegenerative diseases in human, cattle, sheep, deer and, possibly, even fish (1, 2). Albeit rare, prion disorders are lethal, incurable, and most crucially, their molecular basis is poorly understood. It is generally recognized that prion-induced neurodegeneration might be caused at least in part by improper PrP function. Paradoxically, it is not clear what the normal function of PrP may be. Although an impressive number of cellular roles have been ascribed to PrP, their physiological or pathological relevance is not certain. These putative functions have been comprehensively summarized previously (2-4) and are therefore not discusssed here in detail. Rather, the intention

of the present article is to highlight some of our data concerning the roles of PrP during zebrafish development and cell-cell contact formation, as well as their functional connection with reggie membrane microdomains. On one hand, our recent work in zebrafish has revealed that PrP-mediated signaling regulates the stability of cell-cell adhesion complexes during early development. In addition, our experiments in mammalian and invertebrate cells suggest that PrP influences cell-cell contact formation, the clustering of T-cell receptor components during lymphocyte activation, the formation of focal adhesions during cell-substrate interaction, and perhaps the accumulation of transmitter receptors at synapses in the central nervous system. For each of these case-studies, the potential contributions of PrP and reggie to the assembly of protein complexes at specialized domains of the plasma membrane will be discussed.

3. STUDYING THE PHYSIOLOGICAL ROLES OF PRP IN ZEBRAFISH EMBRYOS

Analysis of PrP function *in vivo* has been hampered by the lack of suitable animal models. For almost two decades, PrP knockout mice have remained the most commonly used laboratory animal in prion biology. Given the fact that PrP is abundantly expressed in the mouse developing nervous system (5), it came as a surprise that PrP knockout mice developed and behaved rather normally (6). Hence, although lack of PrP renders these animals resistant to prion infection (7) (as predicted by the prion hypothesis), their mild phenotypes had suggested that the physiological role of PrP was either dispensable or replaceable to the organism.

To address this question, we recently took advantage of the zebrafish as an experimental model (8). Unlike mammals, this small teleost fish has two prion proteins, PrP-1 and PrP-2, expressed in the adult brain and during embryonic development (8-10). Despite being considerably divergent in size and amino acid sequence, fish PrPs have the same protein domain architecture found in mammalian PrPs, in addition to being properly glycosylated and attached to the plasma membrane via a GPI-anchor (8, 11, 12). During embryogenesis, zebrafish PrPs are expressed in a tightly regulated manner: PrP-1 is found at its highest levels during blastula and gastrula stages, whereas PrP-2 becomes strongly upregulated somewhat later, during neuronal development (8). From larval stages on, however, both proteins are similarly expressed in the CNS, particularly in the brain region. The expression pattern of PrP in the developing CNS of the zebrafish is remarkably similar to that seen in mouse embryos (5), suggesting that the roles of PrP during ontogeny are conserved among vertebrates.

The development of antisense knockdown techniques has provided a powerful tool to study gene loss-of-function in the zebrafish. For instance, protein translation can be readily blocked by microinjecting morpholino antisense oligonucleotides into early fish embryos (13). Using this methodology, we have shown that zebrafish PrP-1 and PrP-2 play essential roles during distinct phases of embryonic development (8). Specifically, knockdown of PrP-1 produced a lethal embryonic phenotype characterized by developmental arrest at gastrulation stages. In contrast, knockdown of PrP-2 did not affect gastrulation but severely impaired the development of neural structures, particularly the brain and the eyes. While the phenotypes are clearly distinct, the fact that the PrP-1 phenotype could be rescued by expression of exogenous PrP-1 or PrP-2 indicates that the two proteins share the same biological activity. Most interestingly, expression of mouse PrP also partially reverted the PrP-1 phenotype, revealing that this activity of PrP is conserved between fish and mammals. It is intriguing that the loss of such a basic function in PrP knockout mice does not result in dramatic defects as those seen in knockdown zebrafish. Nevertheless, it has been proposed that various factors such as genetic compensation or developmental plasticity might actually mask the knockout phenotype in mice (14, 15), but not become activated in knockdown fish (8, 16).

The cellular and molecular basis of the zebrafish phenotypes have initially been addressed by focusing on the characterization of the PrP-1 phenotype (8). It was established that knockdown embryos became arrested because they fail to carry out an essential gastrulation cell movement known as epiboly. Close examination revealed that the basis for this defect was the gradual loss of adhesion between embryonic cells (Figure 1A, B), which prevented them from migrating in a coordinated fashion to form the germ layers of the embryo. Notably, in rescue experiments, exogenously added PrP localized preferentially at cell-cell contacts (Figure 1C), suggesting that PrP was directly responsible for the regaining of cell-cell adhesion. Through aggregation assays, it was confirmed that PrP-1 positively influences the formation of both Ca^{2+}-independent and Ca^{2+}-dependent cell-cell contacts. On one hand, these experiments suggested that PrP-1 possesses its own intrinsic, Ca^{2+}-independent adhesive properties. On the other hand, since Ca^{2+}-dependent cell-cell adhesion depends on the formation of E-cadherin homophilic interactions at the plasma membrane, these observations led to the conclusion that PrP-1 can modulate the function of E-cadherin and/or its associated molecules (8). Indeed, further biochemical analyses showed that PrP-1 is required for the correct posttranslational cleavage of E-cadherin, and/or for its stability at the cell surface. Accordingly, the normal localization of E-cadherin at the plasma membrane was largely disrupted in knockdown embryos. Instead, E-cadherin was found to accumulate intracellularly in Rab11-positive vesicles identified as recycling/sorting endosomes (Figure 1D) (8).

It has been described that E-cadherin trafficking is regulated during cell-cell contact formation (17-19). For instance, when cell-cell contacts are disrupted in cells that begin to migrate, E-cadherin molecules are rapidly endocytosed via clathrin-coated pits and transported over early endosomes to the Rab11 recycling compartment. Traffic direction is reversed during the re-establishment of contacts between cells leading again to E-cadherin trafficking towards newly formed cell-cell contacts. The

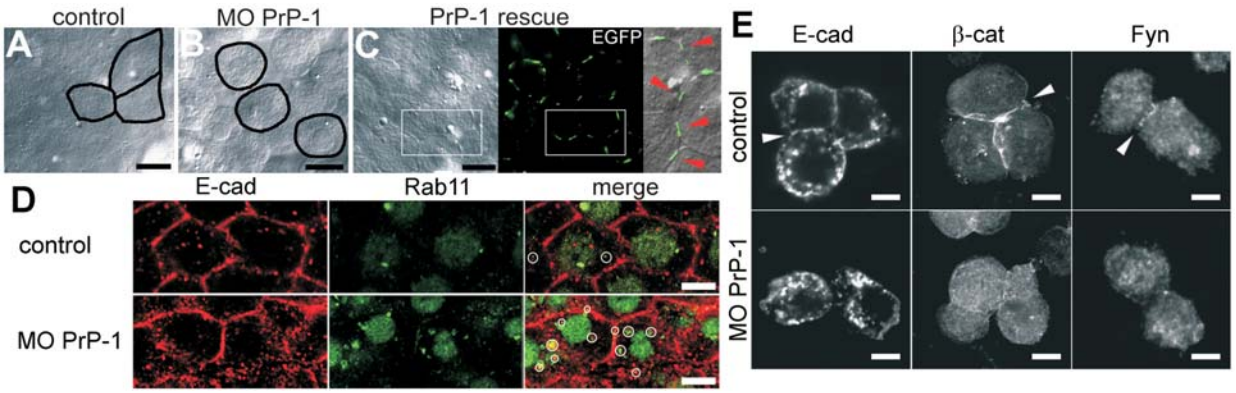

Figure 1. Effect of PrP-1 Knockdown in Embryonic Cell Adhesion. **(**A) Control embryos exhibit normal tissue compactness and polygonal cell shapes at the shield stage (6 hpf). (B) Reduced cell adhesion and rounded cells are evident in PrP-1 morphant (MO PrP-1) embryos. (C) Rescue experiment shows that the local accumulation of EGFP-PrP-1 at cell contacts (right, red arrowheads in detailed overlay view of framed region) reverts cell adhesion defects in embryos. Scale bars: 10 µm. (D) Changes in the number of Rab11-positive vesicles containing E-cadherin (E-cad) between control and PrP-1 morphant embryos (MO PrP-1) were analyzed by immunostaining. Compared to control embryos, PrP-1 morphant embryos exhibit a higher density of E-cadherin/Rab11 double-positive vesicles (white circles). Scale bars: 5 µm. (E) Accumulation of E-cadherin (E-cad), beta-catenin (β-cat) and Fyn tyrosine kinase (Fyn) at cell contacts between primary blastomeres derived from control embryos (white arrowheads) is lost in PrP-1 morphant blastomeres (MO PrP-1). Scale bars: 5 µm. Modified from [8].

findings in knockdown zebrafish embryos indicate that these processes are at least partly controlled by PrP-1 (8). Interestingly, PrP-1 knockdown not only affected E-cadherin distribution in the embryo, but also that of other components of cell adherens junctions, such as beta-catenin and the underlying actin cytoskeleton. Although we established that PrP-1 acts genetically upstream of E-cadherin *in vivo*, co-localization and co-immunoprecipitation data suggest that the two molecules are not necessarily physical interaction partners (8, 20). Instead, our experiments in zebrafish embryonic cells suggest that the regulatory role of PrP-1 over E-cadherin requires the recruitment and local activation of Src-related kinases such as fyn at cell-cell contacts (Figure 1E) (8). In fact, we have shown that fish, amphibian, avian and mammalian PrPs share the intrinsic ability to mediate cell-cell contact formation and trigger intracellular signals (see below). In the early zebrafish embryo, these signals are required to control the stability of adherens junctions, making it possible for cells to remain cohesive and to carry out coordinated morphogenetic movements (8).

Altogether, these studies uncovered important roles of PrP in cell-cell communication *in vivo*, which in the zebrafish are essential for key developmental processes like gastrulation and neuronal differentiation. The identification of some of the molecules influenced by PrP in zebrafish embryos (E-cadherin, beta-catenin, F-actin, Src-kinases) is important for two reasons. On one hand, it may help clarify how PrP exerts its proposed roles in the mammalian brain, such as axonal outgrowth (21), neuritogenesis (22), synaptic activity (23), and myelination (24). On the other hand, it may provide valuable clues about the mechanistic basis of prion-mediated neurodegeneration. Moreover, the PrP knockdown phenotypes of zebrafish offers the exciting opportunity to separately investigate the molecular basis of PrP function (PrP-1 in the early gastrula) and its physiological relevance in the brain (PrP-2 in developing neurons) (16). Such studies would provide a much needed complement to functional analyses of PrP in mammalian cells and knockout mice (25, 26).

4. ROLES OF PRP AT CELL-CELL CONTACTS

A potential involvement of the prion protein in cell-cell adhesion was proposed as early as 1989 (27), mainly based on distinctive biochemical features of PrP like N-glycosylation, GPI-anchorage and cell membrane localization. However, experimental evidence showing that PrP indeed influences cell-cell interactions was not reported until much later. In 2002, Mange *et al.* showed that overexpression of PrP in neuroblastoma N2a cells facilitated cell-cell adhesion and the formation of cell aggregates (28). In addition, Schmitt-Ulms *et al.* identified the neural cell adhesion molecule (NCAM) as a PrP interacting partner, suggesting that PrP may participate in the regulation of NCAM-mediated cell adhesion (29). Indeed, heterophilic PrP *cis*- and *trans*-interactions with NCAM have been reported to regulate NCAM-dependent neuritogenesis via fyn kinase signaling (21). Altogether, these and other early studies pointed to an indirect role of PrP in cell-cell interactions by regulating classical cell-cell adhesion molecules like NCAM. Recently, we provided evidence supporting a more direct role of PrP in cell-cell contact formation (8). The key strategy in these gain-of-function experiments was the use of *Drosophila* Schneider-2 (S2) cells, a well-established non-adhesive cell-line used to characterize cell-cell adhesion molecules (30). It was observed that upon heterologous expression of fish, mouse, chick or frog PrPs, S2 cells acquired the ability to aggregate and accumulate PrP at cell-cell contacts (Figure 2A) (8). Since these cells lack endogenous PrP and do not express adhesion molecules, it was concluded that PrP

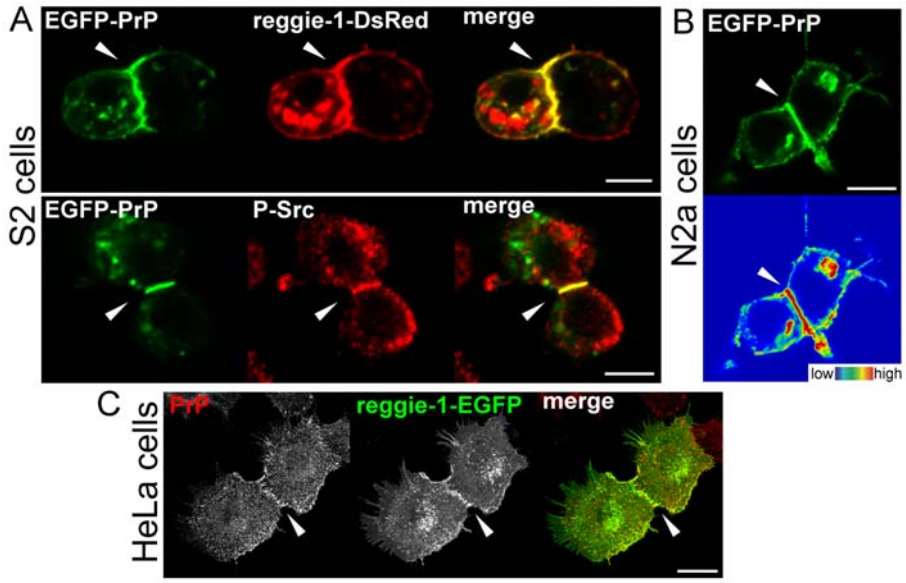

Figure 2. PrP clusters at cell contact sites in various cells in culture. (A) Expression of mouse EGFP-PrP in *Drosophila* non-adhesive S2 cells results in the induction of cell-cell contact formation and local PrP accumulation at cell contacts (white arrowheads). Strong anti-phospho-Src kinase immunostaining (P-Src), as well as accumulation of rat reggie-1-DsRed-monomer colocalize at PrP-mediated cell contacts. Scale bars: 5 µm. (B) N2a cells transfected with mouse EGFP-PrP show local accumulation of the fusion proteins at cell-cell contacts (white arrowheads and fluorescence profiles, below). Scale bars: 10 µm. (C) Endogenous PrP clusters colocalize with reggie-1-EGFP at cell contact sites in HeLa cells (arrowheads). Scale bars: 20 µm. Modified from [8].

itself could mediate the formation of cell-cell contacts via homophilic *trans*-interactions at the plasma membrane. Notably, S2 cell populations separately expressing fish and mouse PrPs were able to form heterologous cell-cell contacts when combined, suggesting that this ability of PrP is strongly conserved throughout all vertebrate classes. These results are in agreement with the striking accumulation of PrP at cell-cell boundaries observed by us and others in various epithelial and neuronal cell lines (Figure 2B) (8, 31), as well as at immunological synapses (32). In particular, co-culture experiments using brain endothelial cells from wild type and PrP-deficient mice have shown that PrP accumulates at cell junctions only between wild type cells (33). However, although experiments in mammalian cells already had suggested a role of PrP in cell-cell communication, the ability of PrP to directly elicit cell-cell contact formation via homophilic *trans*-interactions could not be inferred from these studies because the cells employed –unlike S2 cells- already expressed PrP and many classical adhesion molecules. That PrP homophilic *trans*-interactions may indeed be of physiological relevance is consistent with the current view that PrP exists in a monomer-dimer equilibrium (2), and that PrP dimerization protect cultured cells against prion-induced neurotoxicity (34).

It should be noted that the formation of cell-cell contacts via PrP homophilic *trans*-interactions does not imply that PrP is a *bona fide* cell adhesion molecule. In fact, we have observed that PrP-expressing S2 cells do not aggregate as strongly as cells expressing E-cadherin, which suggests that PrP-mediated cell-cell adhesion is rather weak (our own unpublished data). This is in line with our results in isolated zebrafish blastocytes, where Ca^{2+}-independent, PrP-mediated cell clusters were significantly smaller than Ca^{2+}-dependent cell aggregates (8). Hence, rather than being a classical adhesion molecule *per se*, PrP appears to mediate the formation of weak cell-cell contacts and trigger intracellular signaling, ultimately regulating the formation of strong adhesive interactions. In agreement with this notion, we have observed that the accumulation of PrP at newly-formed cell junctions in S2 cells is concomitant with the co-accumulation of activated Src-kinases, F-actin and reggie proteins (Figure 2A) (8). These data suggest that PrP *trans*-interactions at cell-cell contacts trigger the local activation of signaling molecules at reggie microdomains. The evidence obtained from zebrafish embryos and blastocytes indicates that these signals are further able to regulate the actin cytoskeleton and the function of molecules like E-cadherin (8, 16). Interestingly, the connection between PrP function and Ca^{2+}-dependent cell-cell adhesion is not restricted to the zebrafish embryo, since experiments in intestinal epithelial cells have shown that knockdown of PrP induces an apparent reduction in the levels of E-cadherin and other desmosomal molecules (35). On the other hand, our previous work in various cell lines has shown that several GPI-anchored proteins (including PrP, Thy-1 and F3) co-cluster with reggie membrane microdomains and signal transduction molecules (such as src, fyn and lck) (36). These findings raise the question of whether PrP *trans*-interactions at the plasma membrane may require a spatial association with reggies (which co-accumulate at cell-cell contacts, Figure 2C), in order to

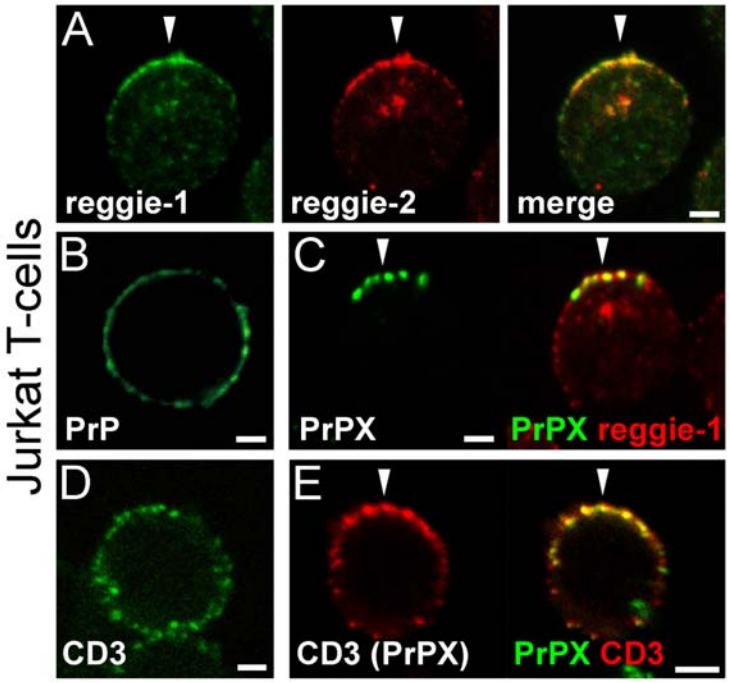

Figure 3. Jurkat T-cell capping induced by antibody-mediated PrP cross-linking. (A) Jurkat T-cells exhibit a pre-formed reggie-cap composed of both reggie-1 and reggie-2 proteins (arrowheads). (B) Endogenous localization of PrP in non-treated cells. (C) Antibody mediated cross-linking of PrP leads to the condensation of PrP at the preformed reggie-cap (arrowheads). (D) Endogenous localization of CD3 in non-treated cells. (D) PrP cross-linking induced a co-clustering of CD3 at the preformed reggie-cap. Scale bars: 5 μm.

favor the clustering of macromolecular signaling complexes (37).

5. REGGIE PROTEINS AS FUNCTIONAL PARTNERS OF PRP

Reggies/flotillins reside at the cytoplasmic face of the plasma membrane within local environments of special lipid composition commonly known as lipid rafts (38). They are linked to the membrane via myristoyl and palmitoyl residues and a stretch of hydrophobic amino acids at their N-terminus (39, 40). Their flotillin (tail) domain is predicted to form coiled-coil structures, which allow for the formation of homo- and hetero-oligomers of reggie-1 and -2 (41, 42). These oligomers are thought to be the building blocks of reggie microdomains ≤ 100 nm in size (36). Available data suggest that co-clustering of PrP and reggies may activate a number of signaling pathways known to be associated with reggie microdomains: src tyrosine kinases (src, fyn, lck and others) (43), MAP kinase activation and Ca^{2+} signaling (including GTPases of the Rho-family) (37, 44, 45). It is generally accepted that GPI-anchored proteins like PrP can trigger intracellular signaling despite their lack of transmembrane domains, owing to their ability to cluster in microdomains. In a biophysical simulation, it was recently shown that GPI-anchored proteins may transduce signals into cells when they are co-clustered with other proteins at the cytoplasmic leaflet of the plasma membrane, without the involvement of transmembrane proteins (Matthias Weiss, DKFZ Heidelberg, personal communication). This concept does not exclude the existence of transmembrane PrP partner proteins, as proposed by Santuccione *et al.* (21), but rather suggests that such partners might not be strictly needed for the initiation of intracellular signaling if PrP is clustered in specific microdomains like those made up by reggie proteins (38).

Notably, previous studies in an inducible neuronal cell line revealed that antibody-mediated cross-linking of PrP can induce signaling via fyn kinase in a caveolin/caveolae-dependent manner (46). In contrast, our studies have shown that in neurons and other cells, PrP co-clusters with reggies, which are not constituents of caveolae but establish their own distinct microdomains (36). Regardless of the cell types and microdomains considered, both views agree that PrP clustering and signaling occurs preferentially in membrane microdomains of special lipid composition.

6. PRP AND REGGIES IN T-CELLS

PrP signaling in association with reggies and its role in the assembly of a protein complex was first demonstrated in T-cells (37). Primary T-cells as well as the Jurkat T-cell line possess a so-called “preformed reggie cap” (Figure 3A) (47). Here, reggies are clustered at one pole of the cell even in quiescent unstimulated cells; however, when subjected to antibody-mediated cross-linking, PrP undergoes capping and co-localizes with the

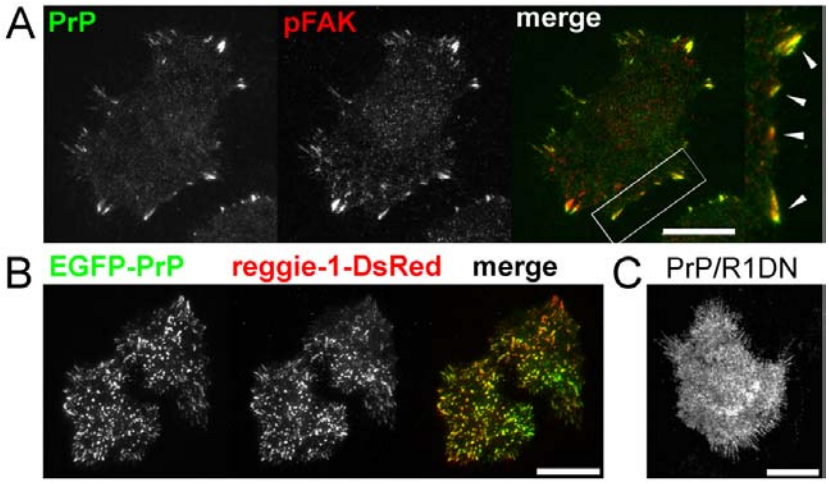

Figure 4. PrP expression at focal adhesions (FA). (A) Total internal reflection fluorescence (TIRF) microscopy shows that endogenous PrP co-localizes with phosphorylated FAK at focal adhesions in HeLa cells (arrowheads in zoom-in field, far right). (B) Formation of FA-like structures upon the expression of EGFP-PrP and reggie-1-DsRed in HeLa cells. (C) Formation of EGFP-PrP FA-like structures is totally abrogated by the co-expression of a reggie-1 dominant negative construct (R1DN). Scale bars: 20 μm.

preformed reggie cap (Figure 3B, C). Our electron microscopic analyses (EM) showed that, at the cap, PrP co-clusters with reggie and lck in ≤ 50 nm microdomains. This finding is consistent with the observation that PrP and reggie can be co-immunoprecipitated with the src-related tyrosine kinases fyn and lck (37). Importantly, co-capping of PrP with reggie results in signal transduction as demonstrated by a Ca^{2+} pulse and phosphorylation of the MAP kinase ERK1/2, leading to the cap association of many T-cell signaling proteins including fyn, lck, LAT and the T-cell receptor (TCR) component CD3 (Figure 3D, E). In addition, blocking of the PrP-evoked Ca^{2+} signal by the membrane-permeable Ca^{2+} chelator BAPTA/AM prevents PrP capping and signaling, as well as capping of the TCR and associated signaling proteins. The reggie cap itself is unaffected by Ca^{2+} downregulation, indicating that reggies represent a preformed platform for signaling (38). That the pre-assembled reggie cap is indeed involved in T-cell signaling was directly demonstrated by misregulating reggies through cell transfection with a reggie-1 dominant-negative construct. This affected Vav activation and impaired T-cell spreading on a substrate of concanavalin A (mimicking capping and T-cell synapse formation) (48). Altogether, our work shows a consistent spatial association of PrP and reggie during lymphocyte stimulation via antibody-mediated PrP clustering. Interestingly, while co-clustering of PrP with reggie led to the assembly of the T-cell receptor components at the cap (38), it did not cause full T-cell activation (37), which would require direct activation of the TCR, a longlasting Ca^{2+} elevation and stronger ERK 1/2 phosphorylation. These data are consistent with a proposed signaling activity of PrP during antigen-driven interactions between T-cells and dendritic cells (32). Accordingly, lymphocyte activation in PrP knockout mice is delayed compared to wild type controls (49, 50). The T-cell response to PrP capping suggests a concrete molecular role of PrP: controlling the assembly of a cell type-specific signaling complex at a functionally important site. It would thus be interesting to study: 1) whether PrP is also involved in the recently described roles of reggies during the polarization and migration of hematopoetic cells (51, 52), and 2) whether these phenomena may be mechanistically related to the PrP-dependent formation of cell-cell contacts (8).

7. PRP AND REGGIES DURING THE ASSEMBLY OF THE FOCAL ADHESION COMPLEX

In a recent study, we reported that the expression of PrP in *Drosophila* S2 cells not only induced the formation of contacts between cells but also promoted adhesion to the substrate and spreading, an abnormal behaviour for S2 cells (53). In N2a cells, downregulation and overexpression of PrP affected the formation of processes and of focal adhesions, which are known to regulate the interaction with the substrate in most cultured cells. Focal adhesions represent a complex of specific proteins with more than 50 members (54, 55) involved in adhesion to substrate, cell migration and the attachment of actin cables (stress fibers).

At focal adhesions, numerous combinations of integrin heterodimers serve as receptors for a vast repertoire of extracellular matrix (ECM) substrates including laminin and fibronectin (56). The intracellular domains of integrins interact with/signal to actin, paxillin, vinculin, focal adhesion kinase (FAK) and many other regulatory components. In particular, the activation of FAK is known to influence the stability and turnover of focal adhesions (57). Our studies revealed that PrP normally accumulates at focal adhesions (Figure 4A), and that its downregulation leads to reduced numbers and increased length of focal adhesions, along with the activation of Src and focal adhesion kinase (FAK) (53). Interestingly, additional structures emerged in PrP transfected HeLa cells (Figure 4B), namely PrP- and reggie-positive streaks with

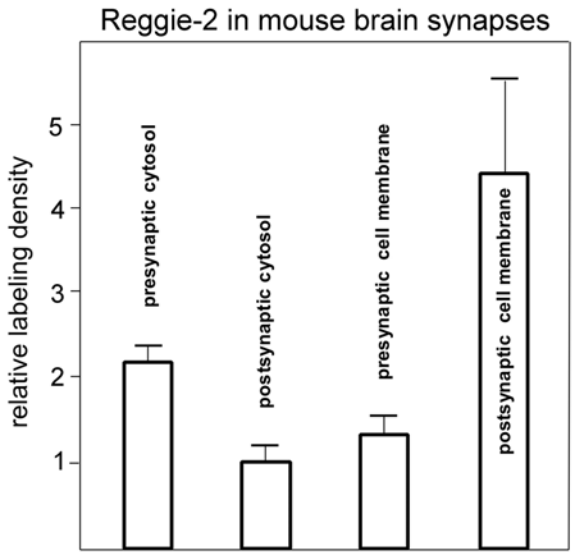

Figure 5. Quantitative assessment of reggie-2 at synapses by immuno-gold labeling and EM analyses. The normalized distribution of gold grain labeling density (5 nm-large gold granules per area size, within $\pm$15 nm wobbling around corresponding to the size of antibody molecules and gold granules) reflecting the binding of specific antibodies against reggie-2 was analysed as described previously (45, 48) on ultrathin hippocampal sections from perfusion-fixed mice brains. Bars = S.E.M. (n = 45 to 54 per column).

morphological resemblance to focal adhesions but which we termed "focal adhesion-like" because they contained none of the established markers of classical focal adhesions, such as paxillin, vinculin or integrin (53). In addition, expression of a reggie-1 dominant-negative construct led to the loss of focal adhesion-like structures, indicating that the accumulation of PrP at these distinct cell-substrate sites requires reggie microdomains (Figure 4C).

It is tempting to speculate that the focal adhesion-like structures observed by us may represent precursor structures where PrP and reggie promote the assembly of protein complexes necessary for the maturation and function of focal adhesions. In fact, we have shown that PrP and reggie affect the phosphorylation state of FAK (44, 45, 53). Thus, PrP and reggie could act as landmarks and signaling platforms for the recruitment and assembly of focal adhesion proteins. It is presently not known which additional factors may be involved in this scenario, or which other cues may be needed for PrP and reggie to co-cluster in focal adhesion-like structures. Since PrP has been shown to interact with the ECM proteins vitronectin and laminin (22, 58), these interactions could well be sufficient to provoke co-clustering of PrP and reggies.

8. ARE PRP AND REGGIES INVOLVED IN THE ASSEMBLY OF SYNAPTIC PROTEINS?

The spatial/functional association of PrP and reggie at cell-cell contacts, focal adhesions and the T-cell cap suggests that the two proteins could be involved in the assembly of further cell type- and membrane-domain-specific protein complexes. Given the fact that PrP has long been implicated in synaptic function (23), we speculate that PrP and reggie may also influence the assembly of signaling complexes that control the formation and maintenance of synapses. In line with this notion, PrP is normally found at synapses (59) and it also has been reported to regulate NMDA receptor function in the mouse hippocampus (60). Concretely, PrP knockout mice suffered from increased excitability, and a receptor subunit was found to co-immunoprecipitate with PrP. In addition, a direct interaction between reggie-1 and -2 with specific NMDA receptor subunits has been recently demonstrated in the hippocampus of rodents (61).

Interestingly, reggies are also located at synapses. Reggie-2, in particular, has been found to be enriched in the membranes of cortical cells (59, 62, 63), as visualized at the EM level using pre-embedding techniques with HRP labeled antibodies. Further, unpublished results from a cooperation with Karl-Heinz Smalla and Eckart D. Gundelfinger (Leibniz Institute for Neurobiology, Magdeburg, personal communication) show both reggie-1 and -2 in a cellular fraction highly enriched in synaptic proteins, a finding consistent with the identification of reggies in synaptic membranes by proteomic approaches (64). Furthermore, after perfusion fixation and processing as previously described (45, 48), we also have localized reggie-2 in synapses *in situ* by post-embedding antibody-gold labeling using quantitative immuno EM evaluation (unpublished work). Our results showed that reggie-2 is present in the cytoplasm of the pre- and postsynaptic neuronal processes, where it is roughly twice as high pre- than postsynaptically (Figure 5). We also found that reggie-2 was most enriched in postsynaptic membrane regions where it was 3 times more enriched than in the presysnaptic membrane domains (reggie-1 has not yet been analyzed by this method). Altogether, the combined information from the different studies suggests that both reggie-1 and -2 are present pre- and postsynaptically, in the cytoplasm and synaptic membranes. This is in line with their known roles during neuronal differentiation (44, 45). A comparison of the spatial distributions of PrP (59) and reggies suggests that they could be associated at synaptic membranes. It remains to be clarified whether they indeed co-cluster and effectively interact at individual synapses. In addition, it will be important to establish whether the formation of synaptic contacts requires PrP-mediated signaling in association with reggies, similarly to the events discussed above. If so, it would be conceivable that PrP and reggie participate in the local recruitment and assembly of proteins required for the initiation of synaptic contacts (such as adhesion and signaling molecules), as well as of transmitter receptors, ion channels and associated proteins involved in synaptic transmission.

9. CONCLUDING REMARKS

Introducing the zebrafish as a new animal model in prion biology allowed us to uncover important roles of PrP as a key modulator of cell-cell communication. In

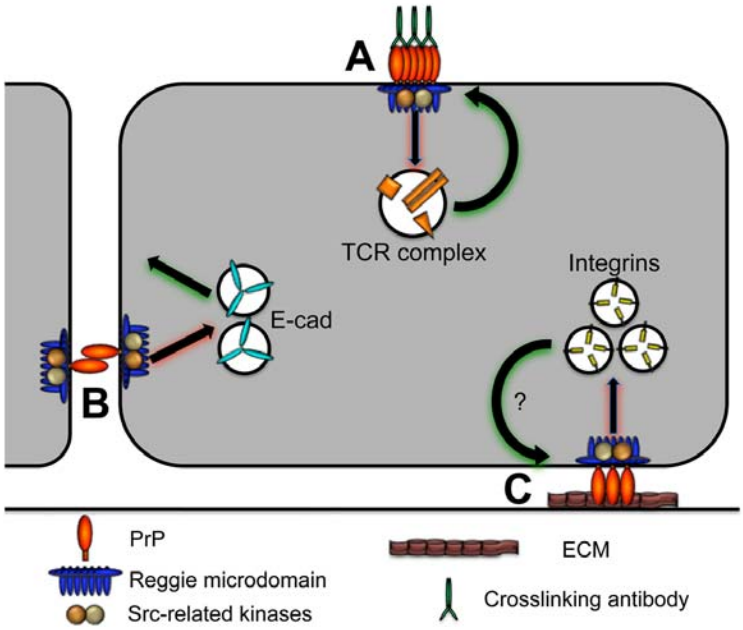

Figure 6. Roles of PrP signaling in association with reggie microdomains. (A) In cultured Jurkat T-cells, antibody cross-linking of PrP induces Src-related signaling via reggie microdomains, resulting in the recruitment and coclustering of PrP and the TCR complex to the preformed reggie-cap. (B) In early zebrafish embryos, PrP homophilic *trans*-interactions trigger clustering of PrP as well as its local accumulation with reggies and Src-related kinases at cell-cell contacts, influencing the targeted delivery of E-cadherin from vesicular compartments to the plasma membrane. (C) At focal adhesions, putative PrP signaling via reggie microdomains regulates focal adhesion turnover, possibly by affecting the trafficking of integrins to the focal adhesion site.

addition, our studies in cultured cells have shown that the formation of signaling PrP clusters at reggie microdomains can be triggered via antibody cross-linking at preformed reggie caps, by PrP *trans*-interactions at cell-cell contacts, and through binding to the ECM at focal adhesion sites (Figure 6). Similar events may occur during synapse formation, although at this stage of research it is not clear whether PrP and reggie co-cluster in the brain and other tissues, and whether these clusters may bear physiological relevance. While the apparent normality of PrP knockout mice suggests that this is not the case, it has been argued that other proteins -perhaps another GPI-anchored molecule- can functionally compensate for the genetic loss of PrP (15). Searching for reggie interaction partners could provide useful information about the putative functional substitutes of PrP at membrane microdomains. Extensive *in vitro* and *in vivo* analysis of PrP-dependent signal transduction and reggie-associated proteins will help clarify our observations concerning the role of PrP in cell-cell and cell-matrix adhesion as well as during T-cell activation and synaptic function.

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