Microdomain-forming proteins and the role of the reggies/flotillins during axon regeneration in zebrafish☆

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ABSTRACT

The two proteins reggie-1 and reggie-2 (flotillins) were identified in axon-regenerating neurons in the central nervous system and shown to be essential for neurite growth and regeneration in fish and mammals. Reggies/flotillins are microdomain scaffolding proteins sharing biochemical properties with lipid raft molecules, form clusters at the cytoplasmic face of the plasma membrane and interact with signaling molecules in a cell type specific manner. In this review, reggie microdomains, lipid rafts, related scaffolding proteins and caveolin—which, however, are responsible for their own microdomains and functions—are introduced. Moreover, the function of the reggies in axon growth is demonstrated: neurons fail to extend axons after reggie knockdown. Furthermore, our current concept of the molecular mechanism underlying reggie function is presented: the association of glycosyl-phophatidyl inositol (GPJ)-anchored surface proteins with reggie microdomains elicits signals which activate src tyrosine and mitogen-activated protein kinases, as well as small guanosine 5'-triphosphate-hydrolyzing enzymes. This leads to the mobilization of intracellular vesicles and to the recruitment of bulk membrane and specific cargo proteins, such as cadherin, to specific sites of the plasma membrane such as the growth cone of elongating axons. Thus, reggies regulate the targeted delivery of cargo—a process which is required for process extension and growth. This article is part of a Special Issue entitled Zebrafish Models of Neurological Diseases.

1. Introduction

The zebrafish and goldfish visual pathway has served as a model system for the exploration of rules governing over the development of the retinotopic projection of retinal ganglion cell (RGC) axons onto the optic tectum [1,2]. The restoration of the retinotopic map by regenerating RGC axons after optic nerve lesion has also been intensively analyzed in this system. Because of its remarkable regeneration-supporting properties, the fish optic nerve is, moreover, suited to search for factors underlying successful regeneration of injured CNS fiber tracts per se. It is expected that insights into conditions allowing successful regrowth in fish might provide valuable clues for stimulation of axon regeneration in the mammalian CNS where regeneration does not spontaneously occur to any significant extent. Experiments along these lines have led to the discovery of two proteins, reggie-1 and reggie-2 [3], which turned out to be essential for growth and regeneration [4], hence their name reggie. Evidence for a role of the reggies in axon growth and regeneration, together with our current understanding of the underlying molecular mechanisms, will be reviewed in this article. But before reggies are considered in the context of axon growth, their properties as constituents of "lipid rafts," or better microdomains will be discussed and compared to other microdomains and microdomain-forming proteins in zebrafish. Prior to this, however, the reasons why axons in the CNS of fish regenerate—as opposed to mammals which do not—will be considered in brief.

2. Axon regeneration in the CNS and the identification of the reggie proteins

One reason for successful regeneration in the CNS is the growth-permissive nature of the glial cell environment in the fish optic nerve [5,6]. The mammalian CNS, by contrast, presents several growth inhibiting molecules on glial cells and in the extracellular matrix which block axon growth and prevent regeneration [7]. The second reason for the success of axon regeneration is also dependent on the special neuron-intrinsic properties of nerve cells in fish [8]. Retinal ganglion cells (RGCs), for instance, promptly upregulate growth-associated proteins after optic nerve lesion which enables the neurons to regrow their axons and to provide them with cytoskeletal proteins, cell adhesion molecules, the necessary receptors for guidance cues and, of course, bulk membrane material [4,8,9]. This ability to switch on expression of genes which were active during development but downregulated thereafter is by far less developed in mammals

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although in principle possible [10 and see below]. More than a dozen growth-associated proteins in RGCs have been identified at the molecular level, and these were recently complemented by a proteomics approach in mammalian cortical neurons [11].

The two proteins discovered in this context, reggie-1 and reggie-2 [3,11], are 45% identical to one another and have a molecular weight of 48 kD. Reggies were also identified in mammals [12,13] where they occur in basically all cells examined so far. In the rat retina, they are upregulated in exactly the 3% of RGCs that, as demonstrated by Richardson et al. [10], can regenerate their axon in a growth-permissive environment.

However, it was not immediately clear how reggies regulate regeneration. Unlike cell adhesion molecules which are also upregulated during axon regeneration [14,15] and mediate the interaction of the elongating growth cone with the environment, the reggies were found to reside at the cytoplasmic face of the plasma membrane. They are anchored to the membrane by myristoyl and palmitoyl residues [16,17] and through stretches of hydrophobic amino acids in their N-terminal head domain (Fig. 1). It was also observed that the reggies do not only occur at the plasma membrane but also in association with many intracellular vesicles [9,18,19], including the Rab11 recycling compartment. Consistent with their identity as microdomain proteins, reggies were independently identified as components of the protein fraction known as DRMs (detergent resistant membranes). The DRM contains molecules that are insoluble in non-ionic detergents (such as Triton X-100) and that are regarded as "lipid raft" components. Together with other DRM proteins, the reggies "float" after sucrose density centrifugation [12], and hence, they were named flotillins [12]. Reggie-1 corresponds to flotillin-2, and reggie-2 to flotillin-1. Reggies/flotillins are often used as markers for the lipid raft fraction in biochemical work. We prefer the term "reggie microdomain" (instead of lipid raft) because reggies assemble by oligomerization into clusters of $\leq$100 nm [9,20] and are, therefore, visible at the light microscopic level by immunofluorescence analysis [13,20,21] with specific antibodies. The microdomains appear as dots or puncta at the plasma membrane of cells and along axons, growth cones, filopodia and as densely packed rows of dots, for instance, at cell–cell contacts [21,22] and in the T cell cap [20,23]. This "pearl on a string-like" arrangement in T cell caps and at cell contacts (Fig. 2) is formed by a reduction of the distance between individual microdomains [22].

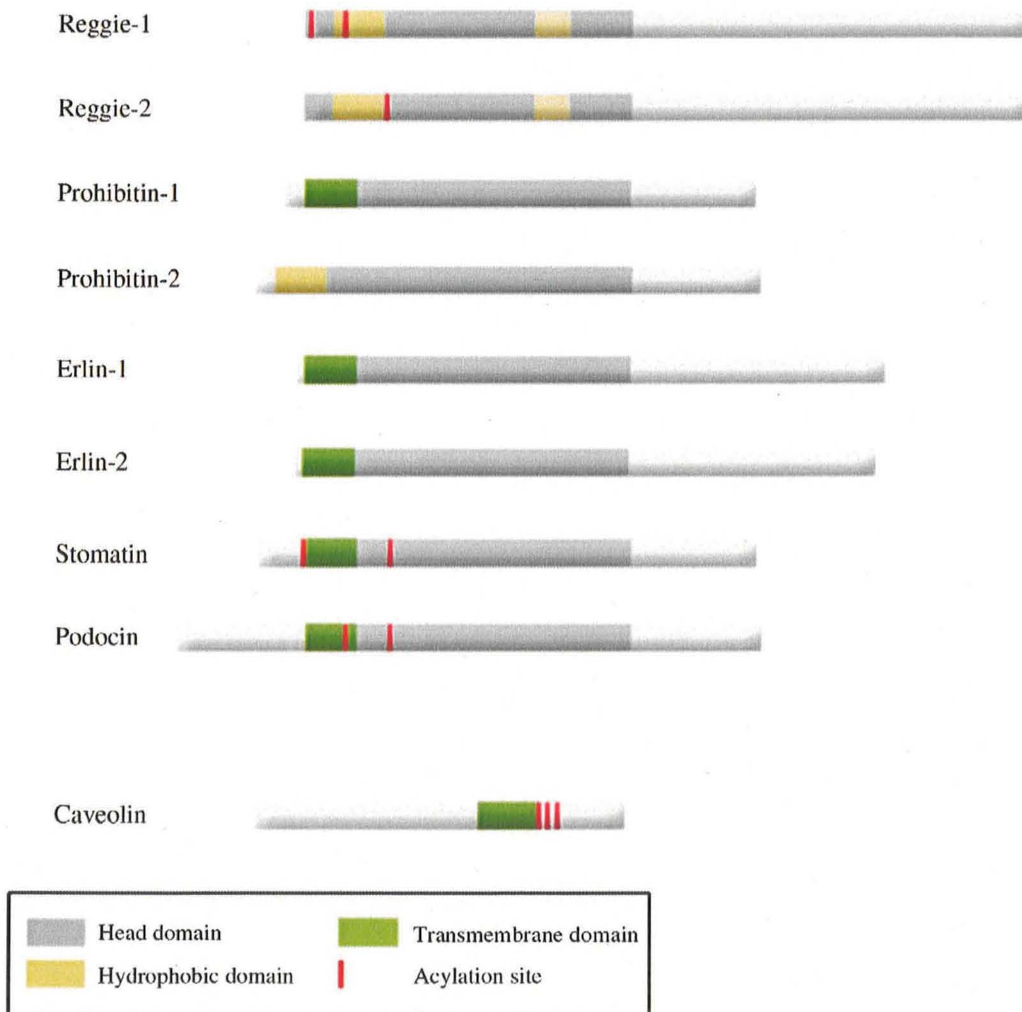

Fig. 1. The microdomain scaffolding proteins and their domains. Reggie-1 and reggie-2 (flotillin) possess an N-terminal head domain (light blue), with two hydrophobic regions (yellowish) and acylation sites (red) for membrane attachment. The tail domain (gray) has strikingly many EA repeats for α-helical coiled-coil formation and oligomerization. The other microdomain scaffolding proteins share some degree of sequence homology with reggie and among each other and also form oligomers.

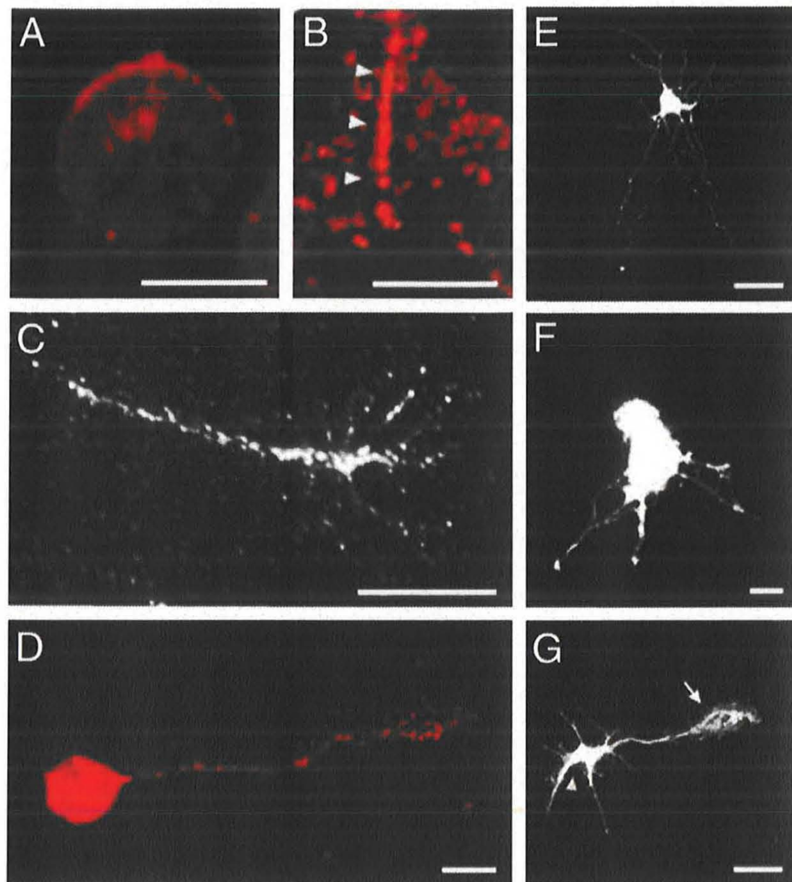

Fig. 2. Distribution and function of reggie microdomains in T cells and neurons. A) In T cells, reggie-1 (and -2)—visualized here by a reggie-1 specific antibody (red)—is clustered at one pole of the cell, the preformed reggie cap which represents a platform for the association of specific GPI-anchored proteins, such as PrP and Thy-1 and signaling. B) Reggie microdomains cluster at cell–cell contact sites (arrowheads) by reducing the distance between individual microdomains (red, immunofluorescence with anti-reggie-1 antibodies). C) The growth cone from a zebrafish RGC axon shows the distribution of reggie microdomains (puncta) at its central region as well as in its filopodia. D) Individual zebrafish RGC neuron and its axon immunostained with a reggie-1 specific antibody and showing the punctate microdomains along the axon. E) Hippocampal neurons differentiate in vitro (E, control siRNA) and extend neurites whereas their counterparts fail to produce neurites after exposure to reggie-1 specific siRNAs (F). G) Hippocampal neurons often produce club-shaped endings where a growth cone should have been, after perturbation of reggie-1 expression. Scale bars: 20 μm.

3. Microdomains and lipid rafts

Microdomains, caveolae and lipid rafts represent functional units in cellular membranes whose existence depends on mainly saturated sphingolipids and cholesterol in a liquid ordered phase and segregated from unsaturated lipids in the liquid disordered phase [24,25]. Rafts range in size from a few nanometer encompassing by estimate six GPI-anchored proteins (nanoclusters) [26] to several hundred nanometers (known as "clustered rafts") [25] are dynamic and generally short-lived. They can be stabilized by the actin cytoskeleton [27] which influences their distribution, half life and size. The existence of lipid rafts has been heavily disputed [28] particularly since they often escape microscopic observation due to their small size [26]. Recent results now demonstrate the phase separation of sphingolipids and cholesterol in giant plasma membrane vesicles of vertebrate cells which support again the raft concept [29].

Microdomains—in contrast to rafts—depend on specific scaffolding proteins that have an affinity for the lipid raft environment and share many raft properties. A family of microdomain proteins with a characteristic membrane interaction domain at their N-terminal "head" domain consists of stomatin, prohibitin, flotillin/reggie, podocin and erlin [9,30–32]. The scaffolding proteins (Fig. 1) possess a region for membrane interaction and stretches of amino acids (tail domain) which promote oligomerization [33]. These protein oligomers, when labeled by antibodies or tags, make microdomains visible at the light microscopic level and amenable to direct analysis of distribution and function.

Lipid rafts and microdomains confer inhomogeneities to the plasma membrane and allow the formation of membrane compartments [25] which are used by specific membrane proteins for cluster formation, protein–protein interactions and signaling [34] as well as for the recruitment and targeted delivery of membrane proteins to specific sites [9]. GPI-anchored proteins are typical cell-surface residents of rafts and microdomains whereas proteins with acyl residues (palmitoylation, myristoylation and others) typically reside at the cytoplasmic face of the microdomain plasma membrane. Certain transmembrane proteins also acquire a preference for the lipid raft and microdomain environment, in particular certain growth factor and transmitter receptors [25].

While being intensively discussed among cell biologist and experts in lipidomics, rafts and microdomains do not play such a central role in research on zebrafish development or disease. Few exceptions are the microdomains scaffolded by the proteins caveolin (i.e. caveolae), podocin and reggie which will be described next and whose function in zebrafish embryos and/or adult fish will be discussed subsequently.

4. Microdomains and caveolae

Among the most intensively studied microdomains are caveolae, 100 nm flask-shaped plasma membrane invaginations, whose formation depends on caveolin [35]. Each of the other scaffolding proteins, reggie/flotillin, stomatin, podocin, prohibitin and erlin, forms its own microdomain without producing membrane indentations or other morphologically conspicuous structures.

There was a debate as to whether reggies represent additional constituents of caveolae which would imply that they serve as a second scaffold of the caveolar invaginations [12]. Reggie proteins, however, are present in basically every cell analyzed so far, whereas caveolin is not. Caveolin, not reggie, is absolutely required for caveola formation. Moreover, caveolae are absent from neurons and lymphocytes [13,20,21,36] which do have reggie microdomains. Even in cells with caveolae, such as astrocytes, caveolae and reggie proteins occupy non-overlapping regions of the plasma membrane [21], so that reggies are clearly not constituents of caveolae. In fact, we are not aware of any report on microdomains with mixed protein scaffolds.

The microdomain scaffolding proteins are evolutionarily highly conserved [32,37] suggesting important functions across kingdoms of life. Accordingly, inspection of databases shows the presence of homologs of caveolin, stomatin, prohibitin, reggie/flotillin, podocin and erlin in zebrafish. Aside from their sequence, however, not much is known on their function. We found no report on zebrafish stomatin, prohibitin and erlin. But work was published on caveolin-1 and caveolin-3 in the early embryo, on podocin during kidney formation, and on reggie-1 and reggie-2 during axon regeneration. The reggies will be considered in greater detail because they were discovered in the fish CNS and functionally characterized in the context of axon regeneration [3,4]. Moreover, results from fish together with insights from various cell types and species has led to a new concept explaining reggie functions [9] but before its presentation published data on zebrafish caveolin-1, caveolin-3 and podocin will be summarized.

5. Zebrafish caveolin and caveolae

In mammals, caveolin and caveolae have been implicated in many functions such as endocytosis, transcytosis, signaling, trafficking of cholesterol (for review see reference 38) so that it came as a surprise that the caveolin-1 knockout mice had at first sight no apparent phenotype. But mice turned out to suffer from pulmonary defects, particularly under the challenge of exercise, and to lack the ability to appropriately adapt their blood pressure [39].

5.1. Caveolin-1

In zebrafish, caveolin-1 and caveolae were shown to exist during the development in neuromasts and in notochord cells (and other structures) [40] which exhibited caveolae in abundance. Morpholino-mediated knockdown to assess the potential role of caveolin in embryogenesis showed that the formation of neuromasts was perturbed and the notochord disrupted [40]. Another publication reports additional defects in the eye and in somites at 12 h post fertilization (hpf) as well as disruption of the actin cytoskeleton [41]. Later defects emerged in the vascular endothelium [41] and in the smaller size of the embryos [40]. In addition, the lateral line developed abnormally most likely because of the defects in neuromasts and notochord [40,41]. The embryos might suffer from other problems as, for instance, reduced cholesterol levels of the plasma membrane since caveolin has been proposed to function as a carrier for cholesterol [42] and cholesterol is needed for the existence of lipid rafts and microdomains [25]. Whether morpholino-treated fish develop severe abnormalities due to imbalanced cholesterol levels has not been reported nor is clear how caveolin-dependent receptor-ligand interaction and signal transduction may change. The enzymes involved in cholesterol synthesis [43] are expressed in zebrafish and in the embryo as maternal messages at the one and two cell stage predicting that cholesterol biosynthesis is crucial already in the early blastocytes. Altogether, it appears that caveolin-1 and caveolae control the differentiation of specific cell types in the embryo, yet the underlying mechanism is not well understood.

5.2. Caveolin-3

Zebrafish also expresses a homolog of mammalian caveolin-3 (cav-3) which is a muscle-specific isoform in mammals and fish [44,45]. Cav-3 in zebrafish was found in the first differentiating muscle precursor cells and later in skeletal and cardiac muscle fibers where expression correlated with the presence of caveolae. Cav-3, however, also appeared in pectoral fin, facial muscle and notochord [44]. Downregulation of cav-3 expression caused severe muscle abnormalities and uncoordinated movement and increased the number of muscle pioneer-like cells adjacent to the notochord. The signaling pathways which depend on cav-3 and caveolae and steer muscle development have not been described and are to this end unknown.

6. Zebrafish podocin

Podocin is expressed by podocytes that are necessary for the blood filtration barrier in the kidney glomerulus [46,47]. Podocytes form the slit diaphragm, a specialized cell–cell adhesion complex at the podocyte foot processes surrounding glomerular blood vessels. Slit diaphragm formation depends on the cell adhesion proteins (of the immunoglobulin superfamily) nephrin and Neph as well as podocin [48]. Much as in higher vertebrates, zebrafish nephrin and podocin are specifically expressed in pronephric podocytes and required for the development of pronephric podocyte cell structures [49]. Morpholino-mediated knockdown of podocin (or nephrin) resulted in loss of a slit diaphragm at 72 and 96 hpf and failure to form normal podocyte foot processes. Absence of normal podocin (or nephrin) expression resulted in defects in glomerular filtration and aberrant passage of high molecular filtrate [49,50]. The exact cellular and molecular mechanism of podocin function during slit diaphragm formation is not known. However, human podocin mutations C-terminal to the membrane association (head) domain resulted in loss of podocin at the cell membrane and aberrant accumulation in ER secretory pathway vesicles [51]. Podocin mutants also resulted in failed delivery of nephrin to the slit diaphragm thus indicating a role of podocin in nephrin trafficking [48].

7. Reggie microdomains

Reggie microdomains are defined by hetero-oligomeric clusters of reggie-1 and reggie-2. To this end, it has not been explicitly examined whether reggie-1 and reggie-2 can perform separate functions. However, it has become clear that reggie-2 undergoes proteasomal degradation when reggie-1 is downregulated (53), for example after siRNA and morpholino-mediated interference with reggie-1 expression levels in neurons, epithelial cells and zebrafish embryo. It is unclear why reggie-2 is so tightly regulated in dependence of reggie-1 nor has it been thoroughly analyzed whether reggie-1 and reggie-2 can subserve separate functions in other cell types. Therefore, we do not distinguish between reggie-1 and -2 in the text when they were not addressed individually.

7.1. Association of the GPI-anchored prion protein and Thy-1 with reggie microdomains

Reggie-1 and -2 were isolated from larval goldfish by co-immunoprecipitation with an antibody against goldfish Thy-1 [3]. This is in line with the fact that Thy-1, as a GPI-anchored protein, associates preferentially with reggie microdomains [20,21,52,53] not only in fish but also in all vertebrate cells studied so far. Moreover, Thy-1 is upregulated in fish RGCs after nerve lesion like reggie [53] and co-localizes with reggie in microdomains in growth cones, along axons, at cell–cell contact sites and in the T cell cap [9]. In the uninjured fish visual system, Thy-1 as well as reggie, is colocalized in the few axons from new RGCs that are constantly added to the fish retina [3]. Thus, Thy-1 seems to cocluster preferentially with reggie microdomains in vitro and in vivo. This applies also to the GPI-anchored cellular prion protein [PrPc; 20] and the zebrafish homologs PrP-1 and PrP-2 [54]. That this association of PrP and Thy-1 with reggie-1 and -2 is functionally important was demonstrated first in T cells as will be described below. Further insights into the role of the reggies came from studies on insulin stimulation of adipocytes (fat cells) [55,56] and on cell–cell contact formation in various types of cells [9].

7.2. Reggie and PrP signaling in T cells

In T cells, reggie microdomains are pre-clustered at one pole of the cell, representing 'the preformed reggie cap' [23] where the T cell receptor complex and the signaling molecules associated with T cell activation coalesce upon stimulation [20]. The preformed reggie cap is important for T cell activation. A dominant-negative reggie construct disrupts the cap and interferes with the formation of the immunological synapse [19]. In T cells, the activation of GPI-anchored proteins by antibody cross-linking is sufficient for TCR complex assembly in the cap [34]. This applies, for instance, to Thy-1 and PrP, whose activation leads to their selective association with the reggie cap [20,21].

When clustered in the reggie cap, PrP elicits the phosphorylation of the MAP (mitogen-activated protein) kinase ERK1/2 and evokes a distinct Ca^{2+} signal leading to the recruitment of CD3 [20,57], the major T cell receptor component, to the membrane in the cap. Yet, CD3 recruitment is not sufficient for the full activation of the T cell receptor complex [20], which would require cross-linking of CD3 or stimulation by antigen. T cell receptor recruitment involves the communication with actin dynamics through src tyrosine kinases (fyn, lck, src), Rho-GTPases and the signaling molecule Vav [19]. The conclusion from these results is that the microdomains consisting of reggie oligomers serve as platforms for PrP cluster formation, signaling, actin rearrangement and the recruitment of CD3 (from internal stores) to the cap [9]. The involvement of reggie in the recruitment of a membrane protein (Glut4) to specific sites of the plasma membrane has previously been observed in adipocytes [55,56] as is discussed next.

7.3. Reggie-associated signaling and the recruitment of Glut4 in adipocytes

In adipocytes (fat cells) flotillin-1/reggie-2 interact with the c-cbl-associated adaptor protein (CAP) and via CAP with a signaling complex that activates the small guanosine 5′-triphosphate-hydrolyzing enzyme (GTPase) TC10 [55] in lipid rafts (reggie/flotillin microdomains). This results in the translocation of the glucose transporter Glut4 from a specific vesicular storage compartment to the plasma membrane [55,56]. This step requires the exocyst and the (Ras-related) GTPase RalA. TC10 and RalA associate with vesicles, such as the recycling compartment and (transport) vesicles en route to the plasma membrane [58,59] which also carry reggie—in agreement with the role of the reggie proteins in activation of TC10 and other small GTPases. It is known that the small GTPases regulate actin dynamics and vesicle trafficking [4,60–62]. Vesicles carrying reggie were observed to shuttle between the plasma membrane and more distant intracellular sites [61] including the recycling compartment. This indicates that reggie microdomains at vesicle membranes participate in the signaling required for vesicle mobilization, trafficking and targeting. These events require the activity of small GTPases that, in turn, have a preference for rafts [9,58,63].

The reggie/flotillin-binding protein CAP/ponsin and its relatives ArgBP2 and vinexin were implied in the interaction with the actin cytoskeleton [56] in which reggie/flotillin is also involved [64] and with substrate and cell adhesion proteins, such as integrins and cadherins. Together, these findings suggest that reggie activates a pathway for polarized delivery of cargo [9]. The type of cargo depends on the cell type and includes, for example, transporters (such as Glut4) [55], cell adhesion molecules (such as cadherins, integrins) [54,56], receptors, ion channels and many other proteins that need to be targeted to specific sites of cells [9]. The sites are "marked" by the clustering of reggie/flotillin microdomains and co-clusters of GPI-anchored proteins, which provides external signals and specificity (Fig. 3). Reggies/flotillins at membranes of intracellular vesicles [18,61] might represent similar signaling centers for clustering of signaling molecules to promote vesicle transport, sorting and targeted delivery of cargo such as cadherin (Fig. 3) [9].

7.4. Reggies, PrP and cadherin recruitment

Reggies are clustered at contact sites between cells [20,21] and so is PrP. PrP–PrP trans-interactions in contacts between mammalian

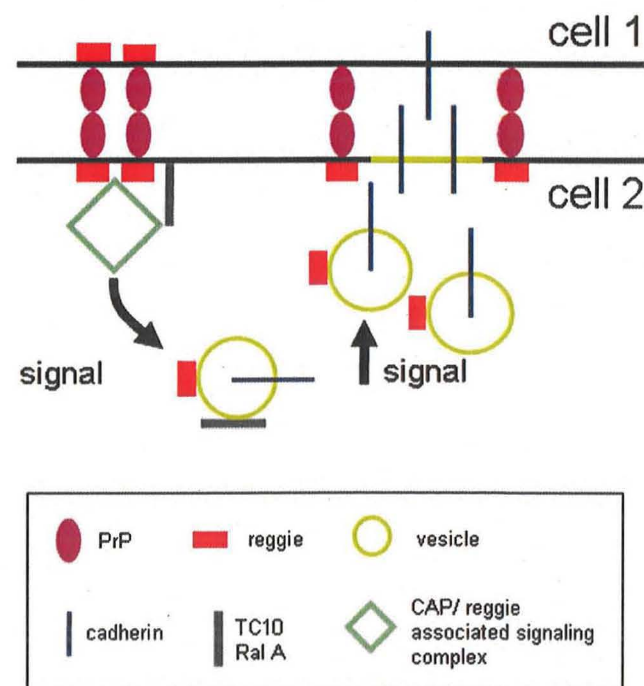

Fig. 3. Recruitment and targeted delivery of membrane and membrane proteins regulated by reggie. PrP is typically associated with reggie microdomains and clustered at contact sites between cells (cell 1 with cell 2). PrP trans-interaction in reggie microdomains leads to signal transduction including the CAP/reggie-associated signaling complex which activates the small GTPase TC10. This leads to the mobilization of cargo (cadherin)-loaded vesicles which also have reggie microdomains, through TC10 and RalA. Vesicles are transported (along cytoskeletal elements) to the plasma membrane and fuse resulting in membrane growth and cadherin delivery at cell contact sites.

epithelial and neuronal cells or zebrafish embryonic blastocytes cause PrP clustering and co-clustering with reggies [9,54]. PrP clusters in reggie microdomains apparently regulate cell contact formation in zebrafish embryos as well as in mammalian cells (Fig. 3) as well as downstream actin rearrangement [54]. In zebrafish embryos, PrP-1 accumulates selectively at contact sites between cells at the blastula and gastrula stage. PrP clustering results in signaling. This signal is necessary for the PrP-dependent recruitment of epithelial (E)-cadherin from the internal vesicle pool to the contacts between cells in zebrafish as well as in various mammalian cells [9,54]. In zebrafish, PrP downregulation by morpholinos has dramatic effects: the cells lose contact and the embryo dies from its failure to proceed through gastrulation [54]. Search for the underlying mechanism showed that E-cadherin was retained in intracellular recycling vesicles instead of emerging at the cell-surface where E-cadherin should be engaged in homophilic adhesion.

How GPI-anchored proteins such as PrP communicate with reggie for the recruitment of cadherins and other membrane proteins remains to be solved. How proteins with lipid anchors confined to one leaflet of the lipid bilayer are capable of signal transduction without associated transmembrane proteins is also unsolved and debated in many reports and reviews dealing with lipid rafts and their function [25]. Recent results from a biophysical simulation approach, however, suggest that cluster formation of GPI-anchored proteins with lipid anchors in the outer leaflet of the plasma membrane is crucial and efficient in influencing the adjacent cytoplasmic leaflet, which offers another cluster of proteins (such as the reggie microdomain) with their own lipid anchors—the myristoyl and palmitoyl residues in reggie (Matthias Weiss, pers. commun.). Moreover, sphingolipids with lipid chains longer than the width of one leaflet are enriched in rafts and microdomains. It is thus conceivable that co-clusters of proteins in the outer and inner leaflet of the plasma membrane can transduce signals into the cell, activating src tyrosine kinases that are also lipid-anchored and able to interact with reggie (such as fyn) [65].

Thus, reggies appear to function as platforms necessary for the assembly of specific cell-surface proteins, for signal transduction and activation of small GTPases to regulate actin dynamics. Specificity and the trigger for signaling appear to come from the cell-surface associates of reggie/flotillin microdomains, which are cell type dependent (insulin receptor in adipocytes, PrP (Thy-1) in T cells and PrP (Thy-1) at cell contact sites in the embryo and cells in vitro) and domain specific (the cap in T cells, contact sites). The cell-surface-derived signals often (or always) target GTPases and actin dynamics, conceivably to activate and recruit specific vesicles from the intracellular pool such as Glut4 and E-cadherin.

Thus, consistent with finding reggie-1 and -2 in basically all cell types and species as distant as flies, fish and mammals [53], their function seems to be of general importance for the communication between cells and for membrane protein sorting, trafficking and delivery. How does this relate to axon growth?

8. Reggies regulate axon regeneration

To examine whether and how reggies might regulate axon regeneration and growth, we applied morpholinos in a piece of gelfoam to the transected right nerve of adult zebrafish and a control morpholino to the left transected nerve [4] following a procedure by Becker et al. [66]. The morpholinos are being taken up by the severed axons and transported retrogradely to the RGCs of origin. The morpholinos are labeled by a fluorescent tag (lissamine) thus allowing identification of morpholino-laden RGCs in retina whole mounts. Miniexplants of retinae of treated fish send out axons. The quantification of axons extending from the explants showed a 45% decrease from morpholino-treated RGCs compared to control. A second experiment gave even more striking effects: a fluorescent dye was applied to the regenerating axons behind the first transection and morpholino application site in the optic nerve which allows to quantify the regenerating axons by the second label in their parent RGCs. This approach showed a 70% reduction of RGCs with regenerating axons in the optic nerve showing that reggie morpholinos block axon regeneration and, in other words, suggest that reggies are necessary for axon growth and regeneration.

This conclusion was supported by individual neurons from the mouse hippocampus in culture in which reggie expression levels were downregulated by reggie-specific siRNAs [4]. These neurons failed to differentiate and did not form axons or failed to elongate them. Axons often showed immobile club-shaped endings where a growth cone should have been and did not grow (Fig. 2). Other neurons had shorter or no processes (Fig. 2), were abnormally large and malformed and produced conspicuous bulges instead of axons and dendrites. The elongation process was apparently blocked because, as we believe, membrane proteins or membrane and proteins from the internal vesicle pool were not appropriately supplied to the prospectively growing tips [9]. Thus, the mechanism of membrane and protein recruitment which is necessary for process growth is controlled by reggies. Therefore, reggies clearly regulate regeneration and axon growth.

Such phenotypes indicate a disturbance in the communication with the cytoskeleton, which was examined in N2a neuroblastoma cells. Reggie mis- and downregulation perturbed the activation levels of the Rho-GTPases Rac, Rho and cdc42 which changed the activation of the downstream effectors WASP, Arp2/3, cortactin and cofilin and of focal adhesion kinase (FAK), p38 and ras [4,62]. This is compatible with the view that the reggie proteins can affect and modify actin dynamics [64], in connection with the recruitment of membrane and proteins (or simply membrane building blocks) from the constitutive secretory pathway and recycling compartment to the growing tips [9].

9. An explanation of reggie functions

Taken together, it seems that reggies are crucial for the recruitment of membrane and specific membrane proteins (or membrane building blocks) to specific regions or domains of the cell (Fig. 3). Neurons with elongating and regenerating axons, where reggies are enriched at growing tips, seem to suffer badly when reggies are missing since blockage of reggie function impairs the delivery of material for growth. Membrane proteins are recruited from vesicular pools (recycling compartment, transport vesicles, Golgi-associated vesicles) and moved along cytoskeletal elements, and this requires the small GTPases of the Rho- and Ras-family and their effectors. Reggie-associated signal transduction and membrane protein recruitment appear to be activated by surface molecules with specific affinities for the reggie microdomain environment such as GPI-anchored proteins and certain receptor types. The surface proteins can be activated by ligands, binding partners (PrP–PrP trans-interaction) or cross-linking antibodies mimicking ligands which activate signaling molecules located in reggie microdomains: src tyrosine kinases, small GTPases, cbl and interacting adaptor complexes. Signaling provokes the recruitment of vesicles and thereby the targeted delivery of membrane building blocks and proteins for axon growth, adhesion, navigation as well as for contact formation between cells and for the establishment of specific membrane domains such as the leading edge, growth cone, its lamellipodia and filopodia. This implies a role of the reggies in the recruitment of specific vesicles and certain membrane proteins from vesicles and stores (transporters, receptors, ion channels and adhesion molecules) [9].

This role of the reggies in the mediation of the recruitment of proteins/membrane from intracellular pools requires their existence in all cells of vertebrates and invertebrates and explains why they are so important for axon growth. Upregulation of reggie expression is a

prerequisite for axon growth/regeneration not only in fish but also in the mammalian CNS [4,13]. In fish, all RGCs express reggie at high levels and regenerate their axons. In the mammalian retina, only 3% of the neurons regenerate their axons and express reggie. When new methods are applied to stimulate the remaining neurons to upregulate reggie, axon regeneration might be successful to an extent that would allow recovery of function in mammals. First results in this direction are encouraging (unpublished results: Jan Koch, Paul Lingor, Matthias Bähr [Göttingen] in collaboration with Gonzalo Solis and C.A.O. Stuermer [Konstanz]). In light of such results it is tempting to speculate that upregulation of reggie in injured neurons after spinal cord lesion might promote axon regrowth and improve the fatal conditions after lesion. Upregulation of reggie and the ensuing growth response might provide neurons with the necessary "power" to resist and counteract effects from neurodegenerative diseases.

This unexpected role of the reggies predicts on the one hand related functions of other microdomain proteins and requires on the other hand identification of all players in the reggie-dependent signaling pathway. Moreover, whether reggies act as molecular shuttles between plasma membrane and intracellular vesicle pools needs to be addressed experimentally. The most crucial issue in the context of axonal regeneration is the identification of the factors that control reggie upregulation in neurons during axon outgrowth and regeneration in fish and in mammals so to stimulate neurons to produce new processes upon injury and resist degeneration.

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