How reggies regulate regeneration and axon growth

Claudia A. O. Stuermer

Abstract The microdomain-forming proteins reggie-1 and reggie-2 (alias flotillins) were found to be upregulated in axon-regenerating fish retinal ganglion cells (RGCs). They were subsequently shown to be indispensable for axon regeneration and neurite extension in fish and mammals. Our current concept proposes that reggies—often together with the cellular Prion protein (PrP)—regulate the turnover of membrane and specific membrane proteins at the growth cone, which is the prerequisite for neurite elongation and guidance.

Keywords Axon regeneration · Mammalian CNS · Reggie upregulation · Targeted delivery · Membrane proteins

Introduction

In the search for the mechanisms underlying success and failure of axon regeneration in the central nervous system (CNS), it has been recognized that extrinsic (environmental) as well as intrinsic factors decide whether or not neurons regrow axons (Liu et al. 2011). It has meanwhile become clear that neuron-intrinsic conditions that would allow powerful axon regrowth decline during the transition from embryonic to adult stages of neural development in mammals and that the sensitivity to inhibitors increases in postnatal neurons. Extrinsic factors are provided by glial cells at the site of lesion in form of inhibitors. Nogo-A made by oligodendrocytes is one of the strongest inhibitors of axon growth, which can collapse growth cones (Caroni and Schwab 1988; Chen et al. 2000). Many other non-permissive molecules are provided by the

glial scar and prevent axon elongation (Silver and Miller 2004). The importance of the extrinsic inhibitors and neuron-intrinsic properties (Benowitz and Yin 2007) was impressively illustrated by experiments from A. Aguayo's laboratory some 30 years ago: when the optic nerve of rats was replaced by a nerve graft from the growth permissive peripheral nervous system (PNS), retinal ganglion cell (RGC) axons, which would not spontaneously regenerate, grew over substantial distances and reached the brain (Richardson et al. 1980). Surprisingly, only 5% of the neurons extended long axons into the graft, whereas the majority (95%) in the same retina would still not do so. It is not clear why 5% regrow but it must be connected to the differential ability of RGCs to upregulate specific growth-related proteins, which reflects their growth supportive intrinsic properties.

Curiously, CNS neurons in lower vertebrates (fish, amphibians) are highly successful in regenerating axons and repairing CNS fiber tracts after lesion (Gaze 1970), which correlates with the fact that all fish RGCs are able to upregulate the growth-related proteins (found in the 5% rat RGCs) again emphasizing the significance of the neuron-intrinsic properties (Stuermer and Leppert 2000). Fish RGCs are perhaps the best example to demonstrate that neuron-intrinsic properties do not have to be so negative as in mammals. In contrast to neurons in rats, all fish RGCs upregulate mRNA and protein expression in response to axotomy and regenerate their axons. These neurons are able of coordinately generating cytoskeletal elements and of re-expressing cell adhesion molecules, receptors for growth factors and guidance cues and molecules for target recognition and synapse reformation—as if fish RGCs had a “master switch” turned on by axotomy to activate the gene expression program required to regrow an axon and restore appropriate connections.

The fish visual system is, therefore, a model system for the exploration of the cellular and molecular factors making up

C. A. O. Stuermer (✉)
Department of Biology, University of Konstanz,
78467 Konstanz, Germany
e mail: claudia.stuermer@uni-konstanz.de

the cell-intrinsic properties underlying axon regeneration and, as discussed here, for identifying candidate factors for a forced upregulation in mammals to trigger regeneration.

The search for molecules being upregulated during axon growth in fish RGCs led to the discovery of two proteins, reggie-1 and reggie-2 (Schulte et al. 1997), which were later shown to be indispensable for axon growth and regeneration—in fish as well as in mammals (Munderloh et al. 2009). Here, we will present a survey of the molecular and biochemical properties of reggie-1 and reggie-2, describe their role for axon growth and discuss the gradual elucidation of the mechanisms that reggies seem to activate and control.

The identification of the reggie/flotillin proteins

Reggie-1 and -2 were discovered as two 48-kD proteins being upregulated in goldfish RGCs after optic nerve lesion (Schulte et al. 1997) and in some rat RGCs after PNS graft application (Lang et al. 1998). They were independently identified as lipid raft proteins, which become enriched in the floating fraction after sucrose density centrifugation of molecules insoluble in the nonionic detergent Triton-X100 and were named flotillins (Bickel et al. 1997). Immuno-electron microscopy-analyses from our work (Lang et al. 1998; Stuermer et al. 2001) showed that reggies/flotillins are not constituents of caveolae as had been suggested by Bickel and colleagues (Bickel et al. 1997) but instead form their own type of microdomains—the reggie microdomains. Consistent with this notion is the fact that reggies are expressed at particularly high levels in neurons (Schulte et al. 1997; Bickel et al. 1997) and lymphocytes (Stuermer et al. 2004) representing cell types that do not possess caveolae (Fra et al. 1994). Reggies are associates of lipid rafts/microdomains at the cytoplasmic face of the plasma membrane and at membranes of specific types of vesicle that can be subsumed under trafficking vesicles; for instance, phagosomes in mammalian macrophages (Dermine et al. 2001) and Dictyostelium (Wienke et al. 2006), post-Golgi vesicles in HeLa, Jurkat T cells and neurons (Morrow et al. 2002; Langhorst et al. 2008a) and glucose transporter 4 (Glut4) containing special cargo vesicles in adipocytes (Baumann et al. 2000). Thus, we suggest that reggies at specific types of vesicles participate in cargo transport (Stuermer 2010). Their widespread distribution implies that they subserve this function not only in neurons but in every single cell type examined to date. Incidentally, reggies are present in basically all species (except *C. elegans*), are evolutionarily highly conserved from fly to man and exist as reggie-like proteins in plants and bacteria (Hinderhofer et al. 2009; Borner et al. 2005). They are members of the so-called SPFH (stomatin, prohibitin, flotillin and bacterial HflK/C) protein family, which also includes podocin (a protein of kidney podocytes) and erlin (Morrow and Parton 2005; Browman et

al. 2007). These proteins share the so-called SPFH or head domain in their amino-terminal end containing hydrophobic stretches and residues for palmitoylation and myristoylation (Morrow et al. 2002; Neumann-Giesen et al. 2004) and mediate the association of reggie with the membrane. At their C-terminal end, reggies possess a predicted alpha helical coiled-coil tail domain promoting homo- and hetero oligomerization and thus formation of reggie clusters or reggie coats (Solis et al. 2007).

Reggie/flotillin at trafficking vesicles and in lysosomes

Due to their property to cluster at specific vesicles, reggies/flotillins were considered as the molecules responsible for the lipid raft-dependent, clathrin-, caveolin-, dynamin-independent endocytosis of certain GPI-anchored proteins (Glebov et al. 2006; Ait-Slimane et al. 2009). However, this view was challenged since other investigators could not find a reggie-dependent endocytosis of GPI-anchored proteins nor was the emergence of caveola-like indentations—reported to be formed in HeLa cells after reggie-1 and -2 overexpression for endocytosis (Frick et al. 2007)—observed by other laboratories (Kirkham et al. 2008; Langhorst et al. 2008a; Lundmark et al. 2008; Schneider et al. 2008; and our own unpublished data). Also, the recently published reggie-2 knock-out mouse did not show the expected defect (Ludwig et al. 2010). From our point of view, several observations speak for a reggie-mediated cargo transport (which includes the recycling compartment) towards the plasma membrane. Evidence in support of this view was the implication of reggie in Glut4 translocation to the cell membrane in adipocytes in association with the exocyst (Baumann et al. 2000; Kioka et al. 2002; Chang et al. 2007). In T lymphocytes, reggies form a so-called preformed cap (Rajendran et al. 2003), a circumscribed region of the T cells where the T cell receptor complex (TCR) accumulates upon stimulation. Capping precedes full T cell stimulation and can be induced by antibody-mediated crosslinking of GPI-anchored proteins such as Thy-1 (Friedrichson and Kurzchalia 1998; Simons and Ehehalt 2002) and the cellular prion protein (PrP). Our results show that Thy-1, as well as PrP, co-cluster with reggie in the cap, an event that activates src and MAP kinases and Ca^{2+} signaling and which leads to the recruitment of the TCR to the reggie cap (Stuermer et al. 2004). Movies of vesicle trafficking in HeLa cells showed that reggie vesicles rapidly shuttle between the plasma membrane and regions further down in the cell, suggesting a function in delivery of components associated with and contained in these vesicles (Langhorst et al. 2008a; Katanaev et al. 2008). Other studies have implicated reggie/flotillin in trafficking of the cholesterol transporter (Ge et al. 2011) in muscle cells and the endocytosis and microdomain localization of DAT (dopamine active transporter) in

neurons (Cremona et al. 2011), respectively. In macrophages, reggies were, furthermore, found to interact with the motor protein KIF9, which participates in the delivery of vesicles with specific proteases into the podosomes (Cornfine et al. 2011).

Recent evidence suggests a role of reggie in N-cadherin delivery in conjunction with the accumulation of PrP at cell contacts and in the growth cone periphery in neurons (Bodrikov et al. 2011). Thus, reggies seem to play a role in the transport of specific proteins to very specific sites of the cell.

The other type of vesicle at which reggies are compellingly abundant are multivesicular bodies (MVB) and lysosomes. Immuno EM analysis has demonstrated that reggies are localized within MVBs and lysosomes (Stuermer et al. 2001, 2004; Langhorst et al. 2008a) and are most likely subjected to degradation—which complies with the fact that the concentration of the reggies within the cell is tightly regulated. Our own unpublished work has demonstrated that expression of dominant-negative forms of specific Rho-GTPases, which co-exist with reggies on specific tubules and vesicles and regulate vesicle sorting and trafficking, causes loss of reggie from these tubules/vesicles and promotes delivery of reggie to lysosomes. It has, moreover, been observed that downregulation of reggie-1 in HeLa and N2a cells by siRNAs leads to the degradation of reggie-2 (Solis et al. 2007) while others have reported vice versa (Ludwig et al. 2010). Together with the fact that reggies form homo- and hetero-oligomers, their joined up- and downregulation suggests that they are perhaps scaffolds of a dynamic tubulo-vesicular compartment whose abundance and efficacy seems to depend on the available concentration of reggie-1 and reggie-2.

Reggies are essential for axon growth and regeneration

In neurons in culture, both reggies were found to be localized along the entire axon and to be enriched in growth cones and filopodia. This predicts that reggies might regulate aspects of growth cone function and elongation (Munderloh et al. 2009), which was tested by using RNAi- based downregulation, first in embryonic mouse hippocampal neurons and then in the more complex in vivo environment of the adult visual system.

Downregulation of reggie-1 in hippocampal neurons by siRNA transfection severely impaired neuronal differentiation and blocked process formation. The neurons remained alive but appeared abnormally small (Fig. 1). They produced ruffling lamellipodia-like membranous veils but were unable to extend neurites. Occasional neurons with axons carried club-shaped endings instead of growth cones, which failed to elongate (Langhorst et al. 2008b). Most siRNA-transfected neurons neither formed axons nor dendrites. The fact that process extension was partially recovered (in 60% of the cells) in the parallel rescue experiments (in which reggie siRNAs and reggie-siRNA-resistant-RNA were co-transfected) supports the proposition that the absence of neurites is a reggie-specific loss-of-function phenotype (and not a siRNA-artifact) (Munderloh et al. 2009).

The severeness of this phenotype demonstrates that reggies are necessary for axon growth and for neuronal differentiation. To prove their role in regeneration, a method, pioneered by Becker et al. (2004), was employed that allows the downregulation of the expression of specific proteins in RGCs within the adult animal with the modified siRNAs, commonly known as morpholinos (from work with zebrafish embryos). We applied reggie morpholinos in adult fish to the optic nerve at the time of nerve transection by means of a piece of gelfoam.

Fig. 1 Hippocampal neurons after si RNA mediated downregulation of reggie fails to differentiate and to extend neurites. **a** Control si RNA; **b, c** si RNA against reggie; **d** rescue with reggie RNA; **e** quantification (from Munderloh et al. 2009). Scale bar 20 μ m

The morpholinos, being fluorescently labeled, are taken up by the severed axons and are retrogradely transported to the RGC of origin where they are identified by their fluorescent tag. Two methods were employed for the evaluation whether reggie downregulation would interfere with axon regeneration *in vivo*: 4–6 days after morpholino application, the retina was isolated and divided in $200 \times 200 \mu\text{m}$ miniexplants that extend axons (under control conditions). The number of axons from reggie morpholino-treated retinas was reduced to 30–50% compared to controls. In the second *in vivo* assay, RGCs—after optic nerve transection and morpholino application—were allowed to regenerate their axons for 7 days. Then, a green fluorescent dye (Alexa 488) was applied a few mm behind the original lesion to the axons that had succeeded to cross the lesion site. The retrograde transport along the axon delivers the dye specifically to the RGCs that had regenerated their axons. Comparison between the control morpholino (right) and reggie morpholino-treated side (left) showed a 70% reduction in number of Alexa 488-labeled RGCs after reggie downregulation, demonstrating that reggie downregulation robustly reduces the ability of fish RGCs *in vivo* to regenerate their axons (Munderloh et al. 2009).

The lack of differentiation of reggie siRNA-treated hippocampal neurons and the impaired regeneration of RGC axons *in vivo* after reggie downregulation are compelling results and suggest that reggies must perform a very basic function in neurons, a function that is needed for the formation of the growth cone and its elongation (Stuermer 2010), axonal transport, vesicle trafficking, cargo supply, or delivery (see below).

Experimentally induced upregulation of reggie in rat RGCs allows axon regeneration in mammals

The blockage of differentiation in mouse hippocampal neurons had already suggested that reggies do play an important role for axon growth not only in fish but also in mammals. Therefore, we examined reggie expression and regulation in the RGCs of rats. We used rats because a small fraction of RGCs was shown to be able to regenerate axons when the optic nerve with its inhibitory glial cells and CNS myelin is replaced by a PNS graft (Richardson et al. 1980). Reggie-1 and -2 are highly expressed across the RGCs in the rat embryo by *in situ* hybridization (Lang et al. 1998) but were not detected by this technique in the retina of adults, either normal or after optic nerve lesion. However, specifically, those RGCs that had been able to extend an axon into the graft and that were retrogradely labeled after HRP application, expressed increased levels of reggie mRNAs (Lang et al. 1998). These observations suggest that reggies are needed for axon regeneration in the mammalian CNS.

It has become possible to induce the expression of specific proteins by adeno-associated viral vector (AAV)-mediated transformation of rat RGCs (Planchamp et al. 2008). In a collaboration between M. Bähr's and our laboratory, the AAV transformation of RGCs with reggie-1-EGFP in adult rats was performed in combination with an optic nerve crush (and in parallel to controls receiving AAV-EGFP). The AAV-reggie-1-EGFP rats possessed many regenerating RGC axons in the optic nerve that had crossed the lesion and several had extended 5 mm through the nerve towards the brain—a result rarely seen in controls (unpublished). This nicely confirmed that the forced upregulation of reggie enables neurons even in adult mammals to extend axons through their old pathways and in spite of the glial cell inhibitors. This, together with evidence showing that downregulation of reggie in neurons with spontaneous high reggie expression levels (fish RGCs, mouse embryonic hippocampal neurons) impairs axon extension, highlights the importance of reggie for axon growth and regeneration and also speak for the right choice in naming them “reggie”.

How reggies might trigger axon growth

Several reggie/flotillin interaction molecules were discovered in adipocytes in the context of research aimed at elucidating how glucose transporter (Glut) 4 is delivered to the plasma membrane after insulin stimulation. Reggie/flotillin binds fyn (Liu et al. 2005), as well as the adaptor protein CAP (c-cbl-associated protein), which interacts with c-cbl upon activation of the insulin receptor (Baumann et al. 2000). c-cbl acts on the one hand as ubiquitin ligase but activates on the other hand through CrkII, the adaptor protein C3G, the GEF for the small cdc-42-related GTPase TC10 (Kioka et al. 2002). TC10 is not only important in adipocytes, where it participates together with the GTPase RalA in the exocyst-dependent delivery of cargo (Glut4) to the plasma membrane (Feig 2003; Chen et al. 2006; Chang et al. 2007), it and the exocyst are also involved in axon growth (Pommereit and Wouters 2007; Dupraz et al. 2009), which provides a link to a function of reggie in neurons.

As discussed earlier in this article, reggies represent platforms for PrP clustering and signaling in T cells, which suggested to us that the PrP-reggie interaction might influence aspects of axon growth. Meanwhile, we have demonstrated that PrP transinteraction (PrP on one cell with PrP on the opposing cell) is required for the delivery of E-cadherin from internal vesicles to cell contact sites in zebrafish embryos and various mammalian cells (Malaga-Trillo et al. 2009; Chiesa and Harris 2009). In the absence of PrP, E-cadherin remains intracellularly. Ongoing work in epithelial cell lines, designed to test this hypothesis, shows that reggie as well as PrP are involved in cell contact formation and adhesion and both are needed for the proper function of E-cadherin in adherens junctions in HeLa and A431 cells (Solis et al. submitted).

Based on this information, we argued that PrP-reggie interactions might also affect the delivery of important cargo proteins in neurons, i.e., N-cadherin and influence neurite elongation. More precisely, would PrP and reggie—under participation of TC10 and the exocyst—mediate the delivery of N-cadherin to the plasma membrane of axons and growth cones? Since E-cadherin recruitment to contact sites in epithelial cells required PrP-PrP transinteraction, we activated PrP on the surface of neurons by adding the soluble PrP-Fc fusion protein thus mimicking PrP-PrP transinteraction (Bodrikov et al. 2011). This caused clustering of PrP on the axon of hippocampal neurons and, as hypothesized, led to increased co-clustering of activated PrP with reggie. This elicited an increased reggie-dependent activation of fyn and MAP kinase and led to a TC10 and exocyst-dependent (100%) increase of neurite length. Importantly, PrP activation resulted in the recruitment of N-cadherin vesicles to PrP-reggie co-clusters and to the relative enrichment of PrP, reggie and N-cadherin in the growth cones.

The existence of PrP knock-out mice allowed us to show that PrP on the axon is indeed important for these signaling events and for N-cadherin recruitment. Most impressively, while PrP wt neurons more than doubled the length of their axons after stimulation by PrP-Fc—in correlation with the increased recruitment of N-cadherin vesicles to the growth in association with reggie, this gain in axon length did not occur in PrP knock-out neurons and was paralleled by the failure of N-cadherin association with reggie (Bodrikov et al. 2011).

These results are relevant in several respects: they show (1) that PrP activation increases growth cone elongation, (2) that PrP activates reggie-associated signaling molecules and (3) that reggie, by co-clustering with PrP, transduces signals that result in the recruitment of N-cadherin to reggie-PrP co-clusters in the growth cone. Here, N-cadherin can trigger axon growth by its associated signaling partners including alpha and beta catenin, p120 and adaptor proteins for the regulation of the actin cytoskeleton. This work shows that reggie-PrP interaction is responsible for the recruitment of specific membrane proteins to specific sites of the cell: N-cadherin into the growth cone, E-cadherin to cell contact sites and the TCR to the T cell cap (Das et al. 2004). This assigns a general cell biological function to reggie and its associates/interacting proteins, which complies with its widespread expression and evolutionary conservation and accounts for the fact that reggies play a role for specific but at the same time very different, membrane proteins in cells as diverse as adipocytes, T cells, macrophages and neurons.

Are reggies involved in the turnover of the growth cone membrane and specific membrane proteins?

For its elongation with dynamically adjusting spatial cues, the growth cone requires the continuous supply of

membrane and membrane proteins, which is, on the one hand, provided by the anterograde axonal transport from the neuron's biosynthetic activity. On the other hand, it has been recognized that, when the growth cone moves forward, not only new membrane plus proteins are added at its edge but membrane plus proteins are simultaneously subjected to endocytosis and recycling (Shao et al. 2002; Falcone et al. 2006). It will therefore be interesting to examine whether reggie and PrP participate in the local recycling and sorting of membrane proteins (such as N-cadherin) in the growth cone (and the axons) (Bodrikov et al. 2011). This view fits several observations including the impaired migration of leucocytes (Rossy et al. 2009), also observed in the reggie-2/flotillin-1 knock-out mouse (Ludwig et al. 2010).

We propose that reggies as intracellular proteins receive signals from the cell surface by communicating with molecules of the extracellular side of the plasma membrane, PrP being one of them. Furthermore, PrP is known to undergo rapid recycling and is, in fact, one of the most rapidly recycled membrane protein (Shyng et al. 1993). PrP clustering, for instance by PrP transinteraction, increases signaling via reggie and spurs the delivery of cargo contained in reggie containers. This implies that the cell surface proteins and their preferential accumulation in the growth cone are causing the preferential delivery of cargo (N-cadherin)-vesicles associated with reggie to the growth cone (Fig. 2).

Given that the lesion-induced upregulation of reggie in fish RGCs is a major determinant of successful axon growth and regeneration and that the reggie-dependent cargo

Fig. 2 Reggies regulate the macroendocytic recycling and targeted re delivery IN cadherin at the elongating growth cone. Growth cone elongation involves the macroendocytic uptake of membrane proteins such as N cadherin, local recycling and the re delivery to the growing portion of the growth cone. This process involves reggie at cargo vesicles, membrane and membrane proteins such as N cadherin, the interaction of reggie with PrP and the PrP and reggie dependent activation of TC10, fyn and Map kinase. (reggie, red triangle, N cadherin, blue rod, blue star, TC10, fyn and Map kinase, yellow dot, PrP)

delivery and recycling machinery is a necessary condition/tool for axon growth and regrowth, it will be crucial, on the one hand, to further dissect the exact cell biological mechanisms governed by reggie, such as identification of the mechanism of cargo vesicle selection by reggie and of reggie interacting molecules. Are reggie vesicle-scaffolding proteins and specific for rapid membrane and cargo recycling events? On the other hand, a major challenge for future work is to uncover regulators of reggie expression. It has been suggested that p53 regulates reggie expression in PC12 cells (Santamaria et al. 2005) but this has not been pursued further. The goal would be to induce re-expression of reggie when needed and to enable a significant proportion of neurons in the mammalian CNS to regenerate axons after lesion.

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