Cell Entry of C3 Exoenzyme from Clostridium botulinum

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Abstract Clostridium botulinum C3 is the prototype of C3-like ADP-ribosyltransferases that selectively ADP-ribosylate the small GTP-binding proteins RhoA/B/C and inhibit their downstream signaling pathways. It is used as pharmacological tool to study cellular Rho functions. In addition, C3bot harbors a transferase-independent activity on neurons to promote axonal and dendritic growth and branching. Many bacterial protein toxins interact specifically with proteins and/or other membrane components at the surface of target cells. Binding enables access to the appropriate cellular compartment so that the knowledge of the receptor allows essential insight into the mechanism of these toxins. Unlike other bacterial protein toxins (such as the clostridial C1 and C2 toxins from C. botulinum), C3 exoenzyme is devoid of a binding and translocation domain, with which toxins usually initiate receptor-mediated endocytosis followed by access to the intact cell. To date, no specific mechanism for internalization of C3 exoenzyme has been identified. Recently, vimentin was identified as membranous C3-binding partner involved in binding and uptake of C3. Although vimentin is not detected in neurons, vimentin is re-expressed after damage in regenerating neurons. Reappearance of vimentin allows C3 to get access to lesioned neurons/axons to exhibit axonotrophic and dentritotropic effects.

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1 Introduction

C3 exoenzyme is an ADP-ribosyltransferase which was first described in Clostridium botulinum (Aktories et al. 1988; Rubin et al. 1988). Now, eight different C3 isoforms with similar molecular weights are identified, which are from Clostridium limosum (C3lim), Bacillus cereus (C3cer), Staphylococcus aureus (C3stau), and Paenibacillus larvae (C3larvin) (Aktories and Just 2005; Just et al. 1992, 1995; Wilde et al. 2001; Inoue et al. 1991; Krska et al. 2014).

The prototype of the C3-like ADP-ribosyltransferase family is C3 from C. botulinum (C3). It is produced as a single-chain protein harboring a signal sequence of 40 amino acids, which mediates secretion from the vegetative form of the clostridium. During this process, the signal peptide is cleaved off. C3 shows a strong basic isoelectric point (pI > 9) and exhibits a molecular weight of about 23.5 kDa. It is a mere exoenzyme, devoid of a receptor binding and translocation domain, with which classical protein toxins mediate their cell entry. So far, little is known about the binding of C3 to eukaryotic cells and its uptake. It was assumed that the exoenzyme is internalized via non-specific processes such as pinocytosis. The reported requirement of high concentrations of C3 in the μM range and extended incubation times seem to support this notion (Boquet et al. 1998).

C3 cleaves the intracellularly ubiquitous nicotinamide adenine dinucleotide (NAD⁺) into nicotinamide and ADP-ribose and transfers the ADP-ribose moiety onto Rho GTPases (Sekine et al. 1989). The C3 transfers the ADP-ribose moiety N-glycosidically to the acceptor amino acid asparagine 41 (Asn41) of the GTPases RhoA/B and C (Aktories et al. 1989; Chardin et al. 1989; Vogelsgesang et al. 2007; Wilde et al. 2001). ADP-ribosylation renders Rho inactive by inhibition of GEF-mediated activation and by stabilizing the inactive Rho-GDI complex (Genth et al. 2003; Sehr et al. 1998). This leads to redistribution of the actin filaments and depolymerization of stress fibers in cells (Barth et al. 1999; Chardin et al. 1989; Paterson et al. 1990; Wiegers et al. 1991). In addition to the Rho-ADP-ribosylating activity of C3, also non-enzymatic functions are reported, namely a direct interaction with the GTP-binding protein RalA (Wilde et al. 2002). C3 forms a high affinity complex with Ral, however, without transferring ADP-ribose. This complex formation with RalA results in inhibition of the transferase activity of C3 like a GDI complex (Pautsch et al. 2005).
The delimited substrate specificity of C3 (only three GTPases out of about 170 GTPase of the Ras superfamily) is the basis of its use as tool to study cellular function of Rho GTPases.

2 C3 Exoenzyme from Clostridium Botulinum

2.1 C3 Exoenzyme as Virulence Factor

The role and importance of C3 as virulence factor is largely unknown. C3 exoenzyme has no translocation domain, compared to classical bacterial toxins, and could act as ligand. An interesting hypothesis to explain how C3 exoenzyme acts as virulence factor is the intracellular release of C3 by S. aureus bacteria that invaded host cells. During their intracellular habitation in the host cell cytosol (Menzies and Kourteva 1998), bacteria release the C3 (C3stau) which immediately ADP-ribosylates Rho. This mechanism is, to date, only found for S. aureus (Madden et al. 2001). Inhibition of RhoA/B/C results in actin depolymerization accompanied by significant morphological (Wiegers et al. 1991; Paterson et al. 1990; Rohrbeck et al. 2012) and functional changes. Indeed, treatment of macrophages with C3 results in inhibition of phagocytosis (Park et al. 2003) and migration (Rotsch et al. 2012). Therefore, macrophages are no longer capable of eliminating pathogens. Additionally, the functional inactivation of RhoA in human monocytes leads to a flattening shape and formation of neurite-like extensions (Aepfelbacher et al. 1997). C3 treatment of natural killer cells or cytotoxic T lymphocytes causes inhibition of their cytolytic function (Lang et al. 1992). Invasion of T lymphoma cells into fibroblast cells is inhibited by C3 (Stam et al. 1998). Furthermore, leukocyte adhesion is inhibited by C3 (Laudanna et al. 1996). Thus, it is conceivable that C3 disables immunological defense processes such as reduction of cytotoxicity of immune cells, inhibition of macrophage phagocytic activity, and inhibition of migration of immune cells. Both the innate and the acquired immune system are affected. Conversely, C3 exoenzyme thereby enhanced the survival of C3-producing microbes in the host organism.

2.2 C3 Exoenzyme as Cell Biological Tool

Based on the selective inactivation of RhoA/B/C GTPases out of approximately 170 low molecular weight GTP-binding proteins (Aktories and Just 2005; Just and Boquet 2000), C3 is used as cell biological tool, even in the era of knockout techniques. C3 has a clear advantage over the usage of RhoA-siRNA. Application of RhoA-siRNA is always accompanied by massive expression and activation of RhoB, which at least partially replaces cellular RhoA functions (Ho et al. 2008). As
RhoA suppresses the rhoB promoter, RhoA inactivation results in deinhibition and thus in RhoB expression (Huelsenbeck et al. 2007). C3 also causes strong RhoB expression, but RhoB is immediately inactivated by C3-mediated ADP-ribosylation. Thus, application of C3 represents at this time the only approach to effectively inhibit RhoA without concomitant RhoB activation (Just et al. 2011). Although C3 lacks a cell binding and translocation domain, it is widely used as tool to study RhoA signal pathways. For two decades, C3 was thought to be internalized into cells through non-specific pinocytosis. But Fahrer et al. 2010 claimed a selective and specific uptake process of C3. The C3-induced morphological changes, which occurred significantly delayed after Rho-ADP-ribosylation, were used as readout for a successful uptake of C3. To accelerate C3 uptake and thus cytoskeletal reorganization, C3 has been introduced in eukaryotic cells by microinjection (Ridley and Hall 1992; Olson et al. 1998), electroporation (Tokman et al. 1997), or permeabilization with digitonin or streptolysin O (Mackay et al. 1997; Fensome et al. 1998; Koch et al. 1993). Moreover, chimeric fusion toxins, in which C3 was fused to the binding domain of the C2 toxin (Barth et al. 1998) or diphtheria toxin (Aullo et al. 1993), were used. Furthermore, a membrane-permeating form of C3 was created by fusing Tat (trans-activating transcription factor) transduction domain of human immunodeficiency virus (Frankel and Pabo 1988) to the C3 amino terminus (Tan et al. 2007). Tat domain is known to cross cell membrane even when fused with large heterologous protein (Fawell et al. 1994). The Tat-protein transduction domain improves the uptake of C3 into NIH3T3 fibroblasts and resulted in disruption of actin stress fibers after 16 h (Sahai and Olson 2006). However, in this study, no direct comparison between ADP-ribosylation of RhoA by C3 and Tat-C3 was performed. In our study, treatment of murine hippocampal HT22 cells and J774A.1 mouse macrophages with C3 or Tat-C3 caused a time-dependent ADP-ribosylation of RhoA finally resulting in degradation of ADP-ribosylated RhoA. In both cell lines, C3 and Tat-C3 induced a multinucleated phenotype and the disappearance of actin stress fibers after same time. Thus, no differences in uptake kinetics between C3 and Tat-C3 were detected in both cell lines. In this context, the mechanism responsible for Tat-dependent membrane translocation is controversially discussed. Some studies demonstrated that Tat-protein rapidly crosses the plasma membrane of cells (Frankel and Pabo 1988; Mann and Frankel 1991). Other data suggest that membrane translocation of Tat-protein is mediated by binding to cell surface-expressed heparan sulfate glycosaminoglycans (HPSGs) (Rusnati et al. 1999; Suzuki et al. 2002) and that Tat-protein promotes cellular uptake of cargo through glycosaminoglycan receptor-mediated endocytosis (Richard et al. 2003; Console et al. 2003). Perhaps more C3 enters the cells as C3-Tat-protein, but it seems that this does not increase the biological effect of C3. Meanwhile, it is accepted that C3 enters eukaryotic cells despite the absence of a binding and translocation domain. In addition, recent findings show that C3 causes ADP-ribosylation of RhoA in a short time and at nanomolar concentrations (Ahnert-Hilger et al. 2004; Fahrer et al. 2010; Rohrbeck et al. 2015). The development of alternative application techniques in fact prevented a detailed study of binding and uptake of C3.
2.3 C3 Exoenzyme and Enzyme-Independent Effects on Neuronal Function

A number of studies have demonstrated a role of RhoA in mediating neurite retraction (Wahl et al. 2000; Gallo 2006). Pharmacological inhibition of RhoA or expression of dominant negative RhoA resulted in neurite outgrowth of neuronal cell lines (Nishiki et al. 1990; Albertinazzi et al. 1998; Sebö et al. 1999). In neuronal cells, inhibition of Rho by C3 exoenzyme led to increased axonal and dendritic growth and branching (Kozma et al. 1997; Winton et al. 2002; Ahnert-Hilger et al. 2004). C3 promoted axonal elongation from chick DRG neurons (Jin and Strittmatter 1997). Additionally, C3 treatment of crushed optic nerves resulted in significant axonal growth passing the lesion site into white matter (Lehmann et al. 1999). Interestingly, this axonotrophic activity of C3 is independent from its enzymatic activity (Ahnert-Hilger et al. 2004). The enzymatically inactive mutant form C3-E174Q and an enzyme-deficient C-terminal peptide fragment covering amino acids 156–181 of C3 increased dendritic as well as axonal growth and synaptic connectivity of neurons in organotypic cultures in vitro (Höltje et al. 2009; Loske et al. 2012). Furthermore, treatment for acute spinal cord injuries in mice with enzyme-deficient C3-peptide significantly improved axonal and functional regeneration (Höltje et al. 2009). Furthermore, a single injection of the 26mer C3-peptide into the nerve repair sites (rat sciatic nerve lesion model) increased axonal elongation and maturation which finally resulted in better functional motor recovery than in NGF reference-treated animals (Huelsenbeck et al. 2012). Notably, the C3lim and C3stau from C. limosum and S. aureus, respectively, have no influence on axonal growth although they are homologues of C3bot (Ahnert-Hilger et al. 2004). Recently, a recombinant cell-permeable variant of C3 (VX-210® formerly Cethrin®) has been evaluated in a clinical trial for safety in the treatment of human acute spinal cord injury (Fehlings et al. 2011).

3 Binding and Uptake of C3 Exoenzyme

3.1 Effect of Posttranslational Modifications on the Binding of C3

Many bacterial protein toxins interact with proteins and/or other membranous structures at the surface of target cells. The binding to specific structures initiates the cellular uptake and mediates access to the appropriate cellular compartment, so that the knowledge of the receptor allows essential insight into the uptake mechanism. Posttranslational modifications such as glycosylation or phosphorylation can influence the interactions with other proteins and the localization of receptors within membrane domains (lipid rafts) (Dennis et al. 2009; Gu et al. 2012). The majority of plasma and membrane proteins of mammals are glycoproteins. Glycosylation is
the most common posttranslational modification of proteins in eukaryotic cells and in terms of the modified amino acids also the most diverse structures (Moremen et al. 2012). Several studies described that carbohydrates can affect ligand–receptor binding. Thus, a highly glycosylated form of human granulocyte-macrophage colony-stimulating factor (hGM-CSF) resulted in a lower receptor affinity compared to less glycosylated or non-glycosylated form (Cebon et al. 1990). Another example of the influence of carbohydrates on ligand binding to receptors is the observation that an increased sialylation resulted in a reduced receptor binding of erythropoietin (EPO) (Darling et al. 2002). Also pathogens and bacterial toxins use glycosylated proteins at the cell surface as a receptor. However, despite some studies, so far no specific receptor for C3 has been identified.

C3 overlay binding assays, in which murine hippocampal HT22 cell lysates were immobilized by blotting onto nitrocellulose membrane, revealed that C3 bound within the 55-kDa protein band. This indicates that C3 recognizes a carbohydrate structure because denatured proteins are present in the overlay assay (i.e., proteins with altered secondary and tertiary structure due to the sample preparation with SDS and 95 °C boiling) and bound C3 can still be detected. And in fact, an elimination of carbohydrates with glycosidase F caused a reduced binding of C3 in overlay assay. Interestingly, this finding was not confirmed with intact cells. After cleavage of carbohydrate moieties with glycosidase F, the opposite was observed, namely an increased binding of C3 suggesting that removal of carbohydrates in the context of native proteins resulted in more binding sites (e.g., proteins or lipids) for C3.

In addition to glycosylation, the phosphorylation state of membrane proteins also influences binding of C3. Phosphorylation is the covalent attachment of a phosphate group to serine (S), threonine (T), or tyrosine (Y) residues of proteins and is catalyzed by kinases (Manning et al. 2002). However, the cleavage of phosphate moieties by phosphatase (CIP, calf intestinal phosphatase) resulted in a decreased binding of C3 within the 55-kDa protein band. By contrast, the stabilization of phosphate moieties by adding phosphatase inhibitors such as ortho-vanadate caused an increased binding of C3. There are few reports for extracellular phosphoproteins and their influence on binding of ligands. In the past five years, extracellular phosphoproteins were identified in the cerebrospinal fluid (Bahl et al. 2008), in serum (Zhou et al. 2009), and in the extracellular matrix (Yalak and Vogel 2012; Tagliabracci et al. 2015) by mass spectrometric approaches. Recently, extracellularly phosphorylated membrane proteins were identified in human umbilical vein endothelial cells (HUVECs) and K562 cells (Burghoff et al. 2015). The importance and influence of these identified membranous and secreted phosphoproteins on the interaction of bacteria or bacterial toxins with their target cells is not yet clear. Interestingly, phosphorylation commonly occurs at the same serine or threonine residues which are glycosylated. Thus, phosphorylation can compete with glycosylation at the same residues (Comer and Hart 2000). On the other hand, both modifications may be necessary for protein–protein interaction. For example, phosphorylation and O-glycosylation both affect binding of insulin-like growth
factor-binding protein-5 (IGFBP-5) to heparin (Graham et al. 2007). However, our binding assays demonstrate that C3 binds to an extracellular posttranslationally modified membrane structure. These findings argue against the hypothesis that C3 is taken up into cells by unspecific processes.

3.2 Uptake of C3 Exoenzyme into Cells

It was assumed for long time that C3 enters cells by non-specific processes or processes involving endosomal uptake (Fahrer et al. 2010) and that cell accessibility of C3 is generally low. Recently, it was shown that C3 enters different cells within few hours (Rotsch et al. 2012). In hippocampal cells (HT22), murine fibroblasts (NIH3T3), and human intestinal cells (HT29), C3 treatment resulted in morphological changes, which is a clear indicator of internalization of C3. Slight morphological changes occurred only after 24 h. Initially, some rounded cells with neurite-like extensions were observed increasing with longer incubation time (>24 h). In addition to these morphological changes (rounded cells with bipolar neurites), cells became large, amorphous, and multinucleated after 48 h (Rohrbeck et al. 2012; Rotsch et al. 2012). However, an appreciable ADP-ribosylation of RhoA was detected already after few hours. In the majority of reports, the morphological changes are viewed as a clear indicator of a successful cellular uptake of C3 (Chardin et al. 1989; Wiegers et al. 1991; Just et al. 1992; Miura et al. 1993; Krska et al. 2014; Slauson et al. 2015). Since morphological changes were detectable not before 12–24 h (depending on the cell type), the majority of cell lines were classified as relatively insensitive to C3 (leading to the above-mentioned diverse delivery techniques of C3). In contrast, macrophages have been postulated as C3 sensitive because C3 reached the cytosol of these cells within 2–3 h, and after 4–6 h, morphological changes were observed (Fahrer et al. 2010). But also in the hippocampal HT22 cells, a significant proportion of the cellular pool of RhoA is inactivated by ADP-ribosylation after 4 h. But significant morphological changes were only detectable after 48 h. This divergence is of particular importance because the morphological cell changes, e.g., cell rounding (as easily representable alteration of cytoskeleton), are regarded generally as an indicator of the C3 effect and thus as an indicator of the sensitivity of cells to C3. Since the morphological changes occur after 24–48 h, almost all cell lines were considered as insensitive to C3. However, the findings indicate rather the opposite; most cell lines are in fact sensitive. The detection system (cell rounding) is therefore inappropriate. The appropriate detection for internalization of C3 is measuring the rate of intracellular ADP-ribosylation of RhoA. Moreover, the relatively rapid and efficient internalization of C3 in J774A.1 cells (2 h) or HT22 cells (4 h) points to a specific internalization of C3.
3.3 Classical Endocytosis Mechanism and Internalization of C3 Exoenzyme

Different routes of internalization are reported for toxins such as cholera toxin (Torgersen et al. 2001). Involving clathrin- (Parton 1994) and caveolae-dependent (Schwitzer et al. 1996) pathways as well as endocytosis via lipid rafts (van Deurs et al. 1993). Although various chemical inhibitors of endocytosis as well as substances altering the cytoskeleton were used to clarify the uptake mechanism of C3, no distinct endocytotic mechanism has been identified.

Lipid rafts play an important role in uptake of numerous protein toxins. However, filipin and methyl-beta-cyclodextrin (MBCD) did not show any significant effect on uptake of C3, although MBCD was effective in inhibiting the uptake of *C. difficile* toxin B (Rohrbeck et al. 2015). Therefore, the uptake mechanism of C3 exoenzyme seems to be independent of cholesterol. The cytoskeleton has a prominent role in endocytosis and trafficking of endocytotic vesicles. Microtubules are also involved in uptake and are therefore disrupted by nocodazole (Hasegawa et al. 2001). The function of F-actin is inhibited by latrunculin B, which sequesters G-actin and prevented F-actin assembly (Helal et al. 2013). Disruption of both cytoskeletal structures (actin and microtubule filaments) did not result in any alteration of C3 uptake. C3 was internalized even if the formation of the clathrin-coated vesicles is blocked by acidification of the cytosol (Sandvig et al. 1987) or when clathrin assembly and disassembly are inhibited by chlorpromazine (Wang et al. 1993). This indicates that clathrin-coated pits did not play a role in the uptake of C3 (Rohrbeck et al. 2015). Also, bafilomycin A1, an inhibitor of the vacuolar proton ATPase, did not inhibit the uptake of C3. It should be noted that one contrary result was reported (Fahrer et al. 2010). This study revealed that bafilomycin A1 reduced the uptake of C3 into J774A.1 cells and human promyelocytic leukemia HL-60 cells but only partially. In addition, bafilomycin A1 only inhibited the uptake at low C3 concentration and showed no effect at high C3 concentration. However, in our studies, bafilomycin A1 did not significantly alter the uptake of C3 even at low concentrations, although it efficiently blocked the uptake of *C. difficile* toxin B (Rohrbeck et al. 2015). Toxin B is known to enter cells via receptor-mediated endocytosis involving acidic endosomes. Experiments with dynasore, which blocks dynamin-dependent endocytosis by inhibition of the dynamin GTPase activity, showed a clear-cut reduction in the uptake of C3 into HT22 cells. These results suggest that the dynamin-dependent pathway of endocytosis may participate but is not the exclusive route of internalization of C3. Dynamin was originally noted for its role in severing vesicles from the plasma membrane in clathrin-dependent and caveolae-mediated endocytosis (Guha et al. 2003). There is evidence for clathrin- and caveolae-independent but dynamin-dependent endocytosis (Benlimame et al. 1998; Damm et al. 2005; Mayor and Pagano 2007). Several studies reported the involvement of dynamin but not of caveolae in the formation of non-coated vesicles at the plasma membrane (Nabi and...
Indeed, for rotavirus, it was described that internalization of viruses is not mediated by either clathrin- or caveolae-dependent endocytosis, but it is dependent on the function of the large GTPase dynamin (Sánchez-San Martín et al. 2004). The coronavirus, feline infectious peritonitis virus, enters monocytes through a clathrin- and caveolae-independent pathway that strongly depends on dynamin (Van Hamme et al. 2008). This endocytosis mechanism (clathrin- and caveolae-independent but dynamin-dependent) has so far not been described for bacterial toxins. It is known that bacterial toxins can use different mechanisms of endocytosis. For example, the internalization of cholera toxin involves different entry routes such as clathrin-dependent (Nichols 2003), caveolae-mediated (Orlandi and Fishman 1998), Arf6-dependent (Jobling and Holmes 2000), and dynamin-independent (Massol et al. 2004). However, the findings for C3 indicate that the internalization of C3 does not strictly require either clathrin-coated vesicle or the caveolae-like pathways of endocytosis, but the endocytosis mechanism is dynamin-dependent.

4 Vimentin Mediates Binding and Uptake of C3

4.1 Identification of Vimentin as Binding Partner of C3

To identify the membrane interaction partner of C3, different binding assays with subsequent mass spectrometric analyses of the bound proteins were performed and the intermediate filament vimentin was identified (Rohrbeck et al. 2014). It is known that vimentin is not exclusively intracellularly localized, but it is also expressed at the cell surface (Podor et al. 2002; Mor-Vaknin et al. 2003; Mitra et al. 2015). Furthermore, it is involved in endocytosis (Kim and Coulombe 2007). Vimentin is a filament protein that can be both glycosylated (Rho et al. 2009) and phosphorylated (Eriksson et al. 2004). These posttranslational modifications reportedly affect the binding to C3. Indeed, vimentin has a glycogen synthase kinase 3 (GSK3)-regulated O-GlcNAcylation site at S54 (Wang et al. 2007). Moreover, phosphorylation of vimentin regulates stability, organization, and function of vimentin (Hyder et al. 2008). Phosphorylation of intermediate filaments typically occurs at the head and tail domains. Phosphorylation sites at the central helical rod domains have been predicted by mass spectrometry but not validated by other methods (Hornbeck et al. 2004). Therefore, vimentin has been studied in detail as a possible interaction partner of C3. The direct interaction of C3 with vimentin was confirmed by pulldown with recombinant His-tagged vimentin fragments in a cell-free system. Further evidence that vimentin interacts with C3 resulted from the immunoprecipitation and colocalization. C3 is not the only pathogen to bind as also *Pasteurella multicaoda* toxin (Shime et al. 2002) as well as *Salmonella* virulence protein StpP (Salmonella protein tyrosine phosphatase) (Murli et al. 2001) binds directly to vimentin.
Biotinylation of cell surface proteins with subsequent mass spectrometric analysis and flow cytometry assay evidenced that the intermediate filament vimentin in fact appears at the cell surface. The observation that the type III intermediate filament vimentin, which is a cytoskeletal component, is present at cell surface is surprising. However, recent studies revealed that vimentin is also present at the cell surface (Steinmetz et al. 2011; Bryant et al. 2006; Satelli et al. 2015; Mitra et al. 2015) and it is even secreted into the extracellular milieu (Mor-Vaknin et al. 2003). Vimentin interacts when located at the cell surface with several pathogens (Table 1).

Recent data indicate that vimentin interacts directly with the severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein and serves as a putative coreceptor involved in the entry of this virus (Yu et al. 2016). Moreover, vimentin presented at endothelial cell surface binds specifically to both a peptide-25 (Leu132 to Arg152 in the latency-associated peptide molecule) (Nishida et al. 2009) and a 12 amino acid SP-peptide (SAHGTSTGVWP) (Glaser-Gabay et al. 2011). The exact mechanism how vimentin reaches the plasma membrane and reenters the cytosol

| Pathogen                                | Vimentin mediates                        | References                  |
|-----------------------------------------|-----------------------------------------|-----------------------------|
| Human immunodeficiency virus type 1     | Virus binding and infection              | Thomas et al. (1996)        |
| Vaccinia virus                          | Virus binding                           | Risco et al. (2002)         |
| Cowpea mosaic virus                     | Virus binding and invasion               | Koudelka et al. (2009)      |
| Theiler’s murine encephalomyelitis virus | Virus binding                           | Nedellec et al. (1998)      |
| Enterovirus 71                          | Virus binding and invasion               | Du et al. (2014)            |
| Blue tongue virus                       | Virus binding and invasion               | Bhattacharya et al. (2007)  |
| Porcine reproductive and respiratory syndrome virus | Virus binding and invasion           | Kim et al. (2006)          |
| Japanese encephalitis virus             | Virus binding                           | Das et al. (2011)           |
| SARS-coronavirus                        | Virus binding and invasion               | Yu et al. (2016)            |
| *Escherichia coli*                      | Bacterial binding and invasion           | Zou et al. (2006), Chi et al. (2010) |
| *Shigella flexneri*                     | Bacterial binding                       | Russo et al. (2016)         |
| *Salmonella enterica*                   | Bacterial invasion                      | Murli et al. (2001)         |
| *Streptococcus pyogenes*                | Bacterial binding                       | Bryant et al. (2006), Icenogle et al. (2012) |
| *Mycobacterium avium* subspecies hominissuis | Bacterial binding                   | Babrak et al. (2015)       |
| *Trypanosoma cruzi*                     | Bacterial binding and invasion           | Teixeira et al. (2015)      |
after ligand binding remains unanswered. In this context, Bhattacharya et al. (2009) demonstrated that β3-integrin regulates recruitment and interaction of vimentin with the cell surface at the site of focal adhesions in several different cell types. Another hypothesis is that the surface recruitment of vimentin is carried out by phosphorylation-mediated disassembly of vimentin (Ise et al. 2012). Recently, it was shown that vimentin filaments are recomposed by filament severing and reannealing in a phosphorylation-independent way (Robert et al. 2015). Short filaments of vimentin such as squiggles and particles were found in the cell periphery (Yoon et al. 1998) and were actively transported bidirectionally along microtubules (Hookway et al. 2015). Additional analysis will be required to explore this mechanism in more detail.

Depletion of vimentin by use of vimentin siRNA led to increased binding of C3. This finding is amazing, but it is explained by immunohistochemical studies of siRNA-transfected cells. Although vimentin siRNA resulted in a significant reduction of intracellular vimentin, vimentin fragments were at the cell periphery and also at the cell surface to a greater extend, which resulted in an increased binding to C3. This observation is supported by Chou and Goldman (Chou 2000). They identified that small vimentin fragments moved in the cytoplasm and preferably accumulated at the cell periphery. This hypothesis was confirmed with the intermediate keratin filaments. Soluble keratin oligomers dissociate from keratin filaments and diffuse through the cytoplasm to the cell periphery (Robert et al. 2016).

The extracellular addition of recombinant vimentin caused a biphasic effect which is dependent on the vimentin concentration. Low extracellular concentrations of vimentin led to increased binding of C3, whereas high concentrations decreased binding of C3 (Rohrbeck et al. 2014). One explanation is that vimentin forms oligomers in the culture medium at high concentrations, which allows binding of C3 to vimentin oligomers. C3 was trapped and could not bind vimentin at the cell surface. At low concentrations, vimentin was monomeric; monomeric vimentin binds to the membrane and enhances the interaction with C3 at the cell membrane. Although it is not yet understood how vimentin arrives at the plasma membranes and gets to extracellular leaflet of the membranes, it clearly works as receptor for C3.

### 4.2 Role of Vimentin in the Uptake of C3

Vimentin is not only involved in the binding to C3 but also in its internalization. Compared to the scramble siRNA-transfected cells (control cells), a significantly stronger signal band of unmodified RhoA was detectable after vimentin depletion. In control cells, C3-mediated ADP-ribosylation of RhoA, which increased the susceptibility of ADP-ribosylated RhoA to proteasomal degradation, caused a faint signal band of RhoA in Western Blot. This difference of the signal intensity of RhoA indicates a delayed internalization of C3. Further evidence that vimentin is
involved in the uptake of C3 resulted from acrylamide experiments. Acrylamide at low concentrations caused a resolution of intermediary filament networks, whereas neither the actin nor microtubule cytoskeleton was influenced (Eckert 1985; Sager 1989; Aggeler and Seely 1990; Miller and Hertel 2009). Acrylamide-induced alteration of vimentin system in cells showed that vimentin is involved in both the binding and in the internalization of C3. The findings are reasonably supported by the cellular functions of the vimentin filament network in endocytosis, vesicular membrane traffic, and localization of cell organelles like Golgi apparatus (Faigle et al. 2000; Gao and Sztul 2001; Styers et al. 2004, 2005). The GTPases Rab7 and Rab9 directly interact with filamentous and soluble vimentin (Walter et al. 2009; Cogli et al. 2013). Both Rab-proteins regulate aggregation and fusion of late endocytic structures/lysosomes (Bucci et al. 2000; Ganley et al. 2004). Interaction with vimentin caused sequestration of Rab-GTPase positive endosomes and inhibition of endocytosis (Walter et al. 2009). So far, the involvement of vimentin in the binding and uptake of pathogens has been described several times (Table 1).

However, preincubation of cells with recombinant vimentin resulted in an increased internalization of C3, as evidenced by a more rapid ADP-ribosylation of RhoA. Preincubation of primary astrocytes with vimentin caused a significant acceleration of C3-induced morphological changes. Interestingly, the study of Bonfiglio supports this finding. Bonfiglio and coworkers reported that vimentin translocated to the membrane in HT22 cells and associated with dynamin by the internalization of the cortico-releasing hormone receptor-β-arrestin 2 complex (Bonfiglio et al. 2013). This report supports our finding that vimentin and dynamin are required for internalization of C3.

### 4.3 Significance of Vimentin as a Binding Partner of C3

The importance of vimentin in binding and uptake of C3 was supported by a proof of principle study at primary vimentin-knockout neurons. The vimentin knockout was verified by genotyping and Western Blot analysis. In primary vimentin-knockout hippocampal neurons, C3 induced only very slight axonal and dendritic elongation. Additionally, vimentin-knockout neurons showed a very weak cytosolic C3 signal in fluorescence microscopy and ADP-ribosylated RhoA was significantly reduced compared to the amount in the wild-type neurons (Adolf et al. 2016). Thus, vimentin-knockout neurons were almost insensitive to C3, whereas primary wild-type neurons were highly sensitive. These findings clearly demonstrate that vimentin is functionally involved in the uptake of C3.

Although the exact mechanism of C3 uptake and the functional impact on neurons are not yet clear, it mediates neuro-regenerative effects in primary hippocampal neurons and animal models. Thus, C3 is used in the treatment of spinal cord injuries, both in animals (Dergham et al. 2002; Ellezam et al. 2002) and in humans (clinical phase II trial, Fehlings et al. 2011; McKerracher and Anderson 2013). C3 induced axonotrophic effects in neurons although vimentin was not
detectable in neurons under physiological conditions. These findings raise the important question how C3 is taken up into the neurons. Vimentin is (re-)expressed after neuronal lesions so that the receptor of C3 is present at the cell surface under this condition. In fact, upregulation of vimentin was shown in mice after neural lesions (Takano et al. 2004; Perlson et al. 2005) and after optic nerve lesion (Martin et al. 2003). It was also discussed that the lack of vimentin caused significantly slowed progression of axonal regeneration after sciatic nerve lesion (Berg et al. 2013). Down regulation of vimentin expression in dibutyryl cAMP-differentiated neuroblastoma cells is accompanied by a slowing down axonal elongation and re-expression of vimentin promotes axonal growth again (Dubey et al. 2004). The findings indicate a participation of vimentin in neuro-regeneration. Neurons and axons in a regenerative context are thus much more sensitive to C3 than in a non-damaged state.

5 Conclusion

In the last twenty years, several molecular and cell biology standard methods have been applied to study the internalization and effects of C3 (Just et al. 1992; Aullo et al. 1993; Olson et al. 1998; Barth et al. 1998; Marvaud et al. 2002; Park et al. 2003; Fahrer et al. 2010; Lillich et al. 2012; Rohrbeck et al. 2015). Yet there are substantial gaps in our knowledge about uptake and molecular effects of C3. However, recently, it was clearly shown that intermediate filament protein vimentin is crucial for binding and uptake of C3 into neuronal cells (Rohrbeck et al. 2014; Adolf et al. 2016). The mechanism how vimentin reaches the cell surface is unknown so far. Additionally, it is not clear whether there are other binding partners which act as coreceptor together with vimentin. Therefore, further research is needed to evaluate whether further binding partner for C3 does exist and how C3 induces the observed enzyme-independent effects. The availability of alternative binding partners suggests further uptake mechanism of C3, if the receptor or receptors are widely distributed among tissues, allowing C3 to act on a variety of cell types. In the other case, when the receptors are specific for certain cell types such as neuronal cells, it is possible to administer C3 in a cell-type-specific manner.

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