Myelin biogenesis
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The objective of the study described in this thesis was to obtain insight into cell-biological processes that are operative in the biogenesis and maintenance of myelin. In particular, the characteristics and mechanisms of protein and lipid trafficking in oligodendrocytes were investigated. Oligodendrocytes are the cells that form myelin in the central nervous system (CNS). Myelin itself is most important, as it is necessary for the saltatory conduction of the nerve pulse. In humans and animals, the absence of myelin, resulting e.g. from a mutation in a myelin protein, such as PLP or MBP, is lethal. Demyelinating disorders, including multiple sclerosis, also lead to neurological dysfunctions (see chapter 1). Therefore, it is of great importance to understand the key factors involved in the process of myelin formation and the metabolism and trafficking of its constituents under physiological conditions, in order to devise (biomedical) means for repair or, preferably, to prevent damage to the myelin membrane.

Myelination is a very complex and extremely tightly regulated process. Oligodendrocytes are derived from precursor cells that can differentiate into either a type of astrocyte or into an oligodendrocyte, depending on culture conditions. Maturation of the cells is accompanied by the sequential expression of myelin components, which can be identified by a panel of monoclonal antibodies. The first myelin components to appear after induction of oligodendrocyte maturation are the glycosphingolipids sulfatide and galactosyl cerebroside (GalCer), and within 2 to 3 days the cells start to express the myelin specific proteins, CNPase, PLP, MBP and MAG, respectively. Most of the myelin components are synthesized in the cell body, and are subsequently inserted locally into the plasma membrane at the distal end of the oligodendrocyte processes. For a better understanding of the mechanisms that target each protein to its site of function, it is important to know the intracellular pathways that these proteins traverse on their way from their site of synthesis to their destination.

**Golgi Outstations**

One of the special adaptations of oligodendrocytes which may have developed to meet the high demands of protein and lipid transport are intracellular structures, which we identified as Golgi outstations. Chapter 2 describes the presence of these Golgi stacks that extend into the cell’s primary processes as revealed by labeling of cultured oligodendrocytes with a fluorescent ceramide analogue, representing a vital Golgi stain. The Golgi-nature of this compartment was further supported by transmission electron microscopy and the observation that also transferrin (presumably) recycles via these oval shaped structures. Moreover, in oligodendrocytes that were cocultured with neurons, many structures were present all over the processes. These outstations have not been observed in any other cell type. Interestingly, electron-microscopic photographs also show the presence of endoplasmic reticulum near the Golgi outstations. Because of the excessive amounts of proteins and lipids which, upon myelination, are synthesized in the cell body and have to be transported to the myelin membranes (see chapter 1), oligodendrocytes might be especially effective mechanisms near the proximity to their constituents.

**Transport of myelin components**

The oligodendrocyte membrane constitutes the non-compacted membrane areas, whereas the non-compacted areas, which are distinc from the non-compacted areas, are the non-compacted areas. Most of the myelin components are synthesized in the cell body and inserted locally into the plasma membrane at the distal end of the oligodendrocyte processes. For a better understanding of the mechanisms that target each protein to its site of function, it is important to know the intracellular pathways that these proteins traverse on their way from their site of synthesis to their destination.

**PLP/MAG transport**

In chapter 3 we describe the specializations of the myelin membrane sheath of the oligodendrocytes. It seems to mirror the PLP/MAG transport. PLP is present in the membranes and absent from the myelin membrane, but absent from the myelin membrane. It has been suggested that vesicles containing the major myelin constituents are transported to the trans-Golgi network by the mechanism(s) of the domains.

**PLP/Lipid transport**

It has been suggested that vesicles containing the major myelin constituents are transported to the trans-Golgi network by the mechanism(s) of the domains. However, we have not observed any other cell type.
to obtain insight into the functions and maintenance of protein and lipid constituents of the oligodendrocytes are the cells that create myelin itself is most obvious from a mutation in a single gene, leading to hereditary disorders, including Charcot-Marie-Tooth type 2 disease (CMT2). Therefore, myelin is generated in the process of cell differentiation, regulated by extracellular signals, and by the intracellular machinery that controls the expression of myelin proteins.

Myelin proteins and lipids are sorted into different carrier vesicles prior to their delivery to the myelin membrane. The major myelin proteins MBP and PLP are found in the compacted areas, whereas other proteins like MAG and CNPase are found in the non-compacted areas including periaxonal membranes. The presence of these proteins might be especially advantageous to the oligodendrocyte for local repair of myelin constituents.

Transport of myelin components

Myelin components are transported to the myelin membrane through different processes. PLP/MAG transport is an important process in which the major myelin protein, PLP, is transported to the myelin membrane. PLP is a very hydrophobic protein with four transmembrane domains that interacts with lipids in the plane of the membrane and in the apposing layer in compacted myelin, thus forming the intra-period line (see chapter 1). It would therefore appear logical if PLP and myelin lipids would be transported to the myelin membrane in the same transport vehicles. In fact, other groups have reported that PLP transport depends on cotransport with galactosylceramide (Brown et al., 1993; Pasquini et al., 1989). In chapter 4, however, we show that even in the absence of galactosylceramide, PLP is transported to the plasma membranes of transfected COS cells and of cultured oligodendrocytes.
oligodendrocytes. Insolubility of proteins in detergent after extraction of membranes at 4 °C with detergent is a phenomenon that has first been described in epithelial cells and indicates a strong association of particular proteins and sphingolipids in ‘rafts’ that are directed to the apical membrane. After extraction of oligodendrocyte membranes with the detergent Triton X-100, PLP is almost entirely present in the detergent-soluble fraction and can, moreover, not be chased into or out of the insoluble fraction. This means that although PLP might be transported with glycosphingolipids in the same vesicle, a dependence of PLP transport on lipid cotransport does not exist.

**rMAL/Lipid transport**

Although it had long been assumed that myelin is relatively simple in protein composition, recently a number of novel myelin proteins have been isolated and cloned (Schaeren-Wiemers et al., 1995; Holz et al., 1996; Dyer et al., 1991). One of these is rMAL, a highly hydrophobic, four transmembrane domain protein, which is expressed in CNS myelin and in other tissues. Chapter 5 describes the presence of rMAL in central and peripheral myelin, in kidney epithelial cells and in glandular stomach epithelium. A common feature of cell types which express rMAL is their high content in the glycosphingolipids GalC and SGalC. In chapter 5 we show that rMAL co-purifies with these glycosphingolipids in detergent-insoluble membrane domains. rMAL is present in glycosphingolipid enriched microdomains after extraction of membranes with detergent, whereas PLP, as indicated in chapter 4, is not present in these microdomains, implying that even similarities in the topology of membrane insertion and physiochemical properties do not dictate a protein’s ability to associate with glycosphingolipids during transport and at the plasma membrane. These observations further emphasize that the inclusion of proteins and lipids in microdomains is, indeed, highly specific. The data presented suggest that rMAL plays a role in maintenance of membrane domains by interaction with galactosphingolipids, and thus could contribute to typical features of these membranes, such as insulating properties of compact myelin.

**Isolation of processes**

All data about transport of myelin components in cultured oligodendrocytes have necessarily been qualitative. To obtain more quantitative insight, a technique would be desirable that would allow the separation and isolation of processes from their cell bodies. At the same time, such a possibility would greatly facilitate the study of the kinetics of transport. In chapter 6 we describe a sandwich culture system in which the isolated oligodendrocytes are grown on a filter with known pore size. Oligodendrocyte processes grow through the pores in the filter into a layer of matrix proteins (Matrigel®) to which the filter was attached. Removal of the filter results in the successful separation of processes from their cell bodies. However, the biochemical quantitation of the protein material in the isolated processes retrieved from the filter as suggested by others on isolated oligodendrocytes.

**Future perspectives**

In the isolation of processes an inherent question is the extent to which transport is true to that what is occurring in vivo. As we have demonstrated in oligodendrocytes, myelin sphingomyelin is highly enriched in membranes that contain galactosphingolipids. Perhaps this the case also in vivo, indicating that oligodendrocyte processes contain sphingomyelin that is involved in sphingolipid signaling. Recent studies have provided evidence that myelin modulates sphingolipid metabolism of keratinocytes via interaction with sphingomyelin which results in the reduction of neutral sphingomyelin and the synthesis of ceramide. These findings could be of importance for the understanding of myelin two-leaf processes growth. Further studies of galactosphingolipids in myelin of demyelinating diseases may provide further insights into the role of galactosphingolipids in myelin function.
After extraction of PLP from rat brain, PLP has been described as a myelin-specific protein. After extraction of PLP from rat brain, PLP is almost completely solubilized in different solvents. However, PLP might not be completely solubilized, indicating a strong association of particular proteins and sphingolipids, thus bearing analogy to the similar observation in apical membrane-directed targeting in polarized epithelial cells. The results presented in this thesis explain some of the many aspects of transport and sorting mechanisms in oligodendrocytes. An intriguing question for future research is, whether in oligodendrocytes the same sorting mechanisms are operational as those found in epithelial cells and neurons, or that oligodendrocytes use specific recognition signals to sort proteins. Since the signals present in myelin proteins that are involved in this sorting and transport are still unclear (c.f. de Vries et al., 1998), chimeras of viral proteins with myelin proteins, and more importantly, mutations in myelin proteins expressed by viruses in infected, cultured oligodendrocytes will provide further knowledge on the mechanisms that govern transport and sorting of myelin proteins in cultured oligodendrocytes. An important application of this kind of knowledge obtained in oligodendrocytes in culture, can be expected to lie in myelin repair after injury. In multiple sclerosis, a serious, chronic disease characterized by specific myelin loss mediated by an (auto)immune attack, it would be of prime importance for recovery of patients to induce remyelination of demyelinated areas, once a way is found to halt the immunological breakdown of myelin. From animal models such as transgenic mice which overexpress PLP only twofold (Kagawa et al., 1994; Readhead et al., 1994), and ceramide galactosyltransferase (CGT) knockout mice which do not synthesize any galactosylcerebrosides (Coetzee et al., 1996), it has become clear that the balance between myelin constituents and the presence of galactosphingolipids are important for myelin compaction and stabilization. The discovery and application of factors both intra and extracellular, that influence transport and synthesis of myelin components will be of great importance to induce or facilitate remyelination of demyelinated areas. PLP itself may be such a factor, since the protein has been characterized as an inositol hexakisphosphate (IP₆)-binding protein (Yamaguchi et al., 1996). Inositol high polyphosphates have been postulated to have a role in vesicular trafficking (reviewed by Fukuda and Mikoshiba, 1997). Since the alternative splicing form of PLP, DM20, does not bind IP₆, the residues 115-150

Summary and Concluding Remarks

Future perspectives

In this thesis transport of myelin-specific proteins and lipids in oligodendrocytes, an inherent feature of myelin biogenesis, is described. We have shown that in cultured oligodendrocytes, like in their counterparts in vivo, the membranes are divided into domains with distinct protein content and that different routes for transport of myelin proteins to the processes exist. Moreover, our studies (chapters 4 and 5) and those of others (Krämer et al., 1997; Kim et al., 1995) have demonstrated the presence of membrane domains that are detergent-insoluble in oligodendrocytes, indicating a strong association of particular proteins and sphingolipids, thus bearing analogy to the similar observation in apical membrane-directed targeting in polarized epithelial cells. The results presented in this thesis explain some of the many aspects of transport and sorting mechanisms in oligodendrocytes. An intriguing question for future research is, whether in oligodendrocytes the same sorting mechanisms are operational as those found in epithelial cells and neurons, or that oligodendrocytes use specific recognition signals to sort proteins. Since the signals present in myelin proteins that are involved in this sorting and transport are still unclear (c.f. de Vries et al., 1998), chimeras of viral proteins with myelin proteins, and more importantly, mutations in myelin proteins expressed by viruses in infected, cultured oligodendrocytes will provide further knowledge on the mechanisms that govern transport and sorting of myelin proteins in cultured oligodendrocytes. An important application of this kind of knowledge obtained in oligodendrocytes in culture, can be expected to lie in myelin repair after injury. In multiple sclerosis, a serious, chronic disease characterized by specific myelin loss mediated by an (auto)immune attack, it would be of prime importance for recovery of patients to induce remyelination of demyelinated areas, once a way is found to halt the immunological breakdown of myelin. From animal models such as transgenic mice which overexpress PLP only twofold (Kagawa et al., 1994; Readhead et al., 1994), and ceramide galactosyltransferase (CGT) knockout mice which do not synthesize any galactosylcerebrosides (Coetzee et al., 1996), it has become clear that the balance between myelin constituents and the presence of galactosphingolipids are important for myelin compaction and stabilization. The discovery and application of factors both intra and extracellular, that influence transport and synthesis of myelin components will be of great importance to induce or facilitate remyelination of demyelinated areas. PLP itself may be such a factor, since the protein has been characterized as an inositol hexakisphosphate (IP₆)-binding protein (Yamaguchi et al., 1996). Inositol high polyphosphates have been postulated to have a role in vesicular trafficking (reviewed by Fukuda and Mikoshiba, 1997). Since the alternative splicing form of PLP, DM20, does not bind IP₆, the residues 115-150...
that are spliced out in DM20 molecules, may be responsible for binding of IP₆ PLP has also been suggested to be involved in vesicular transport because of the abnormal transport found in PLP mutants. Whether PLP transport to the myelin membrane is affected by IP₆ is an interesting topic for further research. Recently, Zegers and Hoekstra (1997) have implicated protein kinase C (PKC) and protein kinase A (PKA) activity in the regulation of cell differentiation and have shown a differential effect of stimulation of either protein kinase activity on transport of sphingolipids and membrane flow to the apical cell membrane in HepG2 cells, PKC inhibiting and PKA strongly stimulating transport. Indeed cAMP, which activates PKA, has been shown to stimulate the differentiation of immortalized oligodendrocytes in culture (Jensen et al., 1993), monitored by the expression of myelin specific proteins, such as PLP, and morphological changes. Further investigation into the role of both these kinases on development of oligodendrocyte precursor cells (see also Baron et al., 1998) will provide knowledge that will be useful for remyelination of demyelinated areas.

In conclusion, the study of myelin protein segregation in oligodendrocytes will provide a better understanding of two fields of research. On the one hand, the study of myelin protein segregation in oligodendrocytes serves as a model for intracellular localization of proteins, and will lead to a better understanding of protein-segregation mechanisms in other cells. On the other hand, it will provide insight into the process of myelination during neural tissue development, and may lead to strategies for the prevention of, or enhancing the recovery from, demyelinating diseases.

because of the transport of myelin proteins. Recently, PKC (Protein kinase C) and protein have shown a transport of PKC, which activates the expression of immortalized oligodendrocyte progenitor cells. Further investigation will be needed to understand the role of oligodendrocyte progenitor cells. From one hand, the oligodendrocyte progenitor cells are a model for understanding of oligodendrocyte function and may provide a new treatment for recovery from demyelination in the nervous system. If the target of a new myelin protein: a novel phosphatidylinositol-anchored protein to the myelin sheath in glycosphingolipid-rich complex. J Biol Chem 272:8937-8945

Summary and Concluding Remarks