Oligomerization of the Sensory and Motor Neuron-derived Factor Prevents Protein O-Glycosylation*

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The sensory and motor neuron-derived factor (SMDF) is a neuregulin that promotes Schwann cell proliferation and differentiation. Hence, understanding axon myelination is important to unveil the mechanisms involved in SMDF biogenesis, membrane delivery, and compartmentalization. SMDF is a type II membrane protein expressed as two distinct polypeptides of ~40 and 83 kDa. Whether the 83-kDa polypeptide results from posttranslational modifications of the protein monomers or protein dimerization remains unknown. Here we have addressed this question and shown that the 83-kDa polypeptide is an O-glycosylated form of the protein. Deletion of the N-terminal domain fully abrogates the SMDF O-glycosylation, indicating that incorporation of O-glycans occurs in the intracellular domain of the protein. Notably, O-glycosylated forms are excluded from partitioning into lipid raft microdomains. In addition, we found that heterologously expressed SMDF monomers interact in intact living cells as evidenced from fluorescence resonance energy transfer of cyan fluorescent protein/yellow fluorescent protein-SMDF fusion proteins. A stepwise deletion approach demonstrated that SMDF self-association is primarily determined by its transmembrane segment. Notably, biochemical analysis revealed that SMDF multimers are exclusively composed of the 40-kDa polypeptide. Collectively, these findings indicate that the 40-kDa form corresponds to unmodified SMDF, which may be present as monomers, whereas the 83-kDa polypeptide is a monomeric O-glycosylated form of the protein. Furthermore, our observations imply a role for oligomerization as a potential modulator of the distribution in membrane domains and O-glycosylation of the protein.

Transcription of the neuregulin 1 (NRG-1) gene produces 15 different splicing forms (1, 2). Structurally, this protein family displays an epidermal growth factor (EGF)-like domain, which endows the members with their specific biological activity. Based on the cellular processing of these proteins, three fundamental modes of action have been assigned to the NRG-1 protein family. (i) Proteins like glial growth factor II (GGFII) are secreted to the extracellular medium and freely diffuse toward its receptors. (ii) Neuregulins such as acetylcholine receptor inducing activity (ARIA) are inserted into the plasma membrane, where they are enzymatically processed and shed to the extracellular medium. (iii) Type III neuregulins are placed in close contact with their receptors expressed in neighboring cells such as Schwann cells and oligodendrocytes (1–3). The distinct processing mode appears to be a modulator of the neuregulin biology. For example, the activation of erbB receptors in Schwann cells by membrane-attached neuronal neuregulins evokes a differentiation and axonal myelination response. In marked contrast, secreted neuregulins induce cellular proliferation and block differentiation (4).

Neuregulin receptors are members of the epidermal growth factor receptor family erbB. Four types of erbB receptors (erbB1–erbB4) that recognize distinct ligands and have different modes of action have been described previously (5). erbB1 binds EGF, whereas erbB3 and erbB4 recognize the EGF-like domains of the neuregulin family. Ligand binding to the erbB3 and erbB4 receptors can induce the formation of homodimers or heterodimers with the erbB2 receptor subtype. erbB2 displays the presence of tyrosine kinase activity, but it does not interact with ligands (6). Structural data revealed that two EGF molecules bind to the extracellular binding domains present in dimerized erbB1 (7). A similar signaling mechanism may underlie the activity of erbB3 and erbB4 homodimers or heterodimers containing the erbB1 receptor, but not the erbB2 isoform.

SMDF is a type III neuregulin expressed in the nervous system with a fundamental role in the development and proliferation of Schwann cells (8). Structurally, the protein displays the presence of three distinct domains: (i) an N-terminal cytosolic region containing putative O-glycosylation sites, (ii) a transmembrane cysteine-rich domain, and (iii) an extracellular C terminus containing the EGF-like domain and potential N- and O-type glycosylation sites (1, 8). Furthermore, an additional membrane-anchoring domain has been proposed to be present in the C terminus of the protein (9). Biochemical analysis of the protein shows the presence of two polypeptides of distinct electrophoretic mobility, namely a fast migrating protein; benzyl-GalNAc, benzyl 2-acetamido-2-deoxy-α-N-galactopyranoside; WGA, wheat germ agglutinin; SBA, soybean agglutinin; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; TRPV1, transient receptor potential vanilloid type 1.

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The abbreviations used are: NRG, neuregulin; EGF, epidermal growth factor; GGFII, glial growth factor II; ARIA, acetylcholine receptor inducing activity; SMDF, sensory and motor neuron-derived factor; O-glycosylation; FRET, fluorescence resonance energy transfer; TRPV1, transient receptor potential vanilloid type 1.

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40-kDa protein and a slow migrating 83-kDa form (10). The 83-kDa polypeptide may arise either from posttranslational modification of the monomeric 40-kDa protein or from multimerization of SMDF monomers. N- or O-type glycosylation may occur in one or several of the putative consensus motifs that incorporate glycans and are present in the SMDF protein. Similarly, monomer self-association could be mediated by the transmembrane cysteine-rich domain of the protein through intermolecular disulfide bond formation. In addition, SMDF dimerization may play a role in the juxtacrine mode of signaling, expressed by type III neurotrophins. Indeed, SMDF homodimerization using the anti-axonal membrane, may directly interact with the dimeric neuregulin receptors expressed at the plasma membrane of the Schwann target cells (10).

Here we have studied the molecular identity of the two SMDF forms, and we report that the 83-kDa polypeptide corresponds to O-glycosylated SMDF with GlcNAc as the terminal sugar. O-Glycosylation occurs in the cytosolic N terminus of the protein. Notably, O-glycosylated SMDF is not segregated into lipid rafts. In addition, we found that 40-kDa SMDF monomers self-associate in cells through the transmembrane cysteine-rich domain. Taken together, our results are consistent with a model in which the oligomerization state of SMDF influences lipid raft segregation and posttranslational modification, thus providing a potential mechanism for the activation of differential subsets of erbB receptors in target cells.

EXPERIMENTAL PROCEDURES

Materials—Primers, Dulbecco’s modified Eagle’s medium, fetal bovine serum, antibiotics, Optiprep, and pcDNA3.1 were obtained from Invitrogen. Pfu turbo DNA polymerase was from Stratagene. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG second antibodies as well as monoclonal antiphosphotyrosine clone PT-66 were obtained from Sigma. pEYFP-C1, pECFP, and the anti-GFP monoclonal antibody or the anti-HRGβ3, anti-GAP43, or anti-GFP antibodies as indicated by the supplier. After 24 h, the medium was removed and substituted with phosphate-buffered saline at room temperature. Cells were observed with a fluorescence-inverted Axiovert 200 microscope and a CCD ORCA camera (Hamamatsu). Cells were excited at 440 nm with a mercury lamp, with exposition times of 200 ms and light recorded at 480 and 535 nm using the FRET set of filters and dichroic mirror for CFP/YFP (λ ≥ 455 nm, CHROMAS). A computer-controlled filter wheel (Sutter Instruments) allowed the recording of the CFP emission and YFP emission with a short delay. Images were obtained for each wavelength, and the relative amount of light for each cell was calculated using the Aquacosmos software package. Fluorescence emission at 535 nm (F) was plotted as a function of fluorescence emission at 480 nm (D), giving rise to a linear distribution that was fitted to a straight line. The non-interacting, free CFP and YFP give rise to a linear distribution that can be described by a straight line with a slope of 0.60 (± 0.02 (n = 5). Thus, a slope > 1.0 was taken as an indicator of the occurrence of FRET between the donor and acceptor pairs. Data are given as mean ± S.E., with n as the number of experiments performed. The Tukey test was used for statistical analysis.

RESULTS

SMDF Is an O-Glycosylated Protein—Electrophoretic analysis of SMDF exposes the presence of two distinct forms of 40 and 83 kDa. The molecular nature of the 83-kDa form is not yet known. Analysis of the primary sequence with the neuronal network predictor NetOGlyc (www.cbs.dtu.dk/services/netOGlyc) and YingOYang 1.2 (www.cbs.dtu.dk/services/YinOYang) servers reveals the presence of several mucin-type GalNAc and non-mucin-type GlcNAc O-glycosylation sites in SMDF. To investigate whether the protein is O-glycosylated in cells, we incubated the SMDF-transfected COS cells with the sugar analogue benzyl-GalNAc, an inhibitor of glucosaminyltransferases (12). As illustrated in Fig. 1A, treatment of cells expressing the SMDF protein with the O-glycosylation inhibitor prevented the appearance of the 83-kDa polypeptide without affecting the mobility of the 40-kDa form. Note that the benzyl-GalNAc augmented the mobility of the 83-kDa form to that of the 68-kDa polypeptide, suggesting that the inhibitor did not fully block the O-glycosylation of the protein or, alternatively, that SMDF may suffer another type of posttranslational modification. A similar result was obtained when the YFP-SMDF fusion protein was used; namely, the benzyl-GalNAc increased the electrophoretic mobility of the 110-kDa form to that of the 95-kDa polypeptide, without altering the migration properties of the 68-kDa polypeptide (Fig. 1B). Thus, these results indicate the 83-kDa form of SMDF and the 110-kDa form of YFP-SMDF correspond to an O-glycosylated form of the protein.

Amino acid sequence analysis displays the presence of O-glycosylatable residues throughout the protein. We first evaluated whether the O-glycosylation occurs in the N terminus of

saline/Tween (0.3%), incubated with a horseradish peroxidase-conjugated secondary antibody, and developed with the ECL Plus system.

Trition X-100 flotation experiments—Analysis of detergent-insoluble complexes in flotation gradients was performed as described by Cabedo et al. (9). Briefly, about 2.5 × 10⁵ transiently transfected cells were cooled on ice, washed with phosphate-buffered saline, and scraped off in buffer A (150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 20 mM Hepes, pH 7.4). The cells then were passed 10 times through a 29-gauge needle, extracted at 4°C for 30 min, and brought to 35% Optiprep. One ml of the extract was sequentially overlaid with 8 ml of 30% Optiprep in 0.5× buffer A and 400 μl of buffer A in a SW41 tube. After centrifugation (178,000 × g at 4°C for 4 h), 10 fractions were collected from the top to the bottom of the gradient. 200 μl of the three top fractions (rafts) or three bottom fractions (non-rafts) were pooled and precipitated with trichloroacetic acid, pH-neutralized with 0.1 M NaOH, and analyzed by immunoblotting using the anti-HRGβ3, anti-GAP43, or anti-GFP antibodies as indicated.

FRET Experiments—We used a ratiometric method modified from Ruiz-Velasco and Ikeda (11). Briefly, COS-7 or PC12 cells were cultured on 24-well plates at 2.5 × 10⁵ cells/well and transfected with equimolar amounts of the indicated plasmids with LipofectAMINE as recommended by the supplier. After 24 h, the medium was removed and substituted with phosphate-buffered saline at room temperature. Cells were observed with a fluorescence-inverted Axiovert 200 microscope and a CCD ORCA camera (Hamamatsu). Cells were excited at 440 nm with a mercury lamp, with exposition times of 200 ms and light recorded at 480 and 535 nm using the FRET set of filters and dichroic mirror for CFP/YFP (λ ≥ 455 nm, CHROMAS). A computer-controlled filter wheel (Sutter Instruments) allowed the recording of the CFP emission and YFP emission with a short delay. Images were obtained for each wavelength, and the relative amount of light for each cell was calculated using the Aquacosmos software package. Fluorescence emission at 535 nm (F) was plotted as a function of fluorescence emission at 480 nm (D), giving rise to a linear distribution that was fitted to a straight line. The non-interacting, free CFP and YFP give rise to a linear distribution that can be described by a straight line with a slope of 0.60 (± 0.02 (n = 5). Thus, a slope > 1.0 was taken as an indicator of the occurrence of FRET between the donor and acceptor pairs. Data are given as mean ± S.E., with n as the number of experiments performed. The Tukey test was used for statistical analysis.
the protein. The experimental strategy considered the use of deletion mutant YFP-SMDFΔ1–64, which lacks most of the N terminus of SMDF. Notably, truncation of the SMDF N terminus completely abolished the appearance of the higher molecular weight, O-glycosylated forms of the protein (Fig. 1C), in accordance with the tenet that O-glycosylation of SMDF occurs in its N end. Note that the lack of O-linked glycans in the truncation mutant YFP-SMDFΔ1–64 cannot be attributed to a defect in the protein biogenesis and membrane delivery because this protein is normally produced and trafficked to the cell surface (9). Therefore, this observation implies the absence of O-glycosylation in the putative sites present in the C terminus of the protein.

To unveil the type of sugars attached to the O-glycosylated protein we took advantage of the sugar specificity of two agarose-immobilized lectins. Triticum vulgaris lectin (WGA) binds to β-GlcNAc-modified proteins, whereas SBA (a lectin from Glycine max) interacts with both α- and β-GalNAc-glycosylated proteins. Whole cell extracts from COS cells expressing SMDF were incubated with WGA- or SBA-agarose, and the presence of SMDF in the precipitates was evaluated by Western immunoblot with an anti-HRG β3 antibody. As depicted in Fig. 1D, O-glycosylated SMDF was specifically purified by the WGA lectin, with no detectable presence of the modified protein in the SBA precipitated. The non-modified 40-kDa form of the protein did not interact with either immobilized lectin. Therefore, these results demonstrate that SMDF is mainly a β-GlcNAc-glycosylated protein.

The O-Glycosylated Form of SMDF Does Not Partition into Lipid Raft Microdomains—We have shown previously that SMDF segregates into lipid rafts (9). Because partition in these membrane microdomains may be modulated by posttranslational modification of the proteins (12), we questioned whether O-glycosylation of SMDF influences its insertion into membrane rafts. To address this issue we investigated whether inhibition of O-glycosylation affected the distribution of the protein in raft and non-raft microdomains. COS-7 cells were transfected with YFP-SMDF, incubated with the inhibitor where indicated, and extracted with 1% Triton X-100 at 4 °C. Cell extracts were centrifuged in Optiprep gradients as described previously (9), and fractions were subjected to Western blot analysis. This experimental paradigm identifies raft-associated proteins in the top fractions, as illustrated for the palmitoylated GAP43 protein, while non-raft proteins such as YFP are present in the bottom fractions (Fig. 2). Fig. 2A depicts that O-glycosylated SMDF was found in the bottom fractions, in agreement with previous findings indicating that this protein form does not segregate into lipid rafts (9). Inhibition of O-glycosylation with 4.5 mM benzyl-GalNAc did not significantly augment the segregation of unmodified SMDF into lipid rafts. Notice that partially O-glycosylated SMDF does not partition into lipid rafts, suggesting that only completely non-O-glycosylated forms are able to localize in these membrane microdomains. Taken together, our results suggest that raft association is inversely related to the O-glycan posttranslational modification of the protein.

SMDF Monomers Interact “in Vivo”—We also evaluated whether SMDF monomers could homodimerize. SMDF self-association is plausible because of the presence of a cysteine-rich domain N-terminally adjacent to the transmembrane segment of the protein that may favor the linkage of monomers by disulphide bridges. To address this question we first compared the electrophoretic mobility of YFP-SMDF in reducing and non-reducing conditions. Fig. 3A depicts that under non-reducing conditions YFP-SMDF migrates as three distinct bands of 68, 110, and 130 kDa. Notice that the 130-kDa band disappears when the reducing agent 2-mercaptoethanol is added to the samples, thus suggesting that it may be a multimer of YFP-SMDF monomers.

To further investigate the multimerization of the SMDF in vivo, we used FRET between YFP and CFP fused to SMDF as a highly sensitive assay of protein-protein interactions. The fluorescent proteins were fused to the N terminus of SMDF giving rise to YFP-SMDF and CFP-SMDF. Fusion of a fluorescent protein to the N terminus of SMDF did not affect the biological properties of SMDF (9). Heterologous expression of both YFP-SMDF and CFP-SMDF produced fluorescent proteins with the spectral properties characteristic of YFP and CFP (data not shown). Fig. 3B shows the occurrence of FRET in COS cells co-expressing both CFP-SMDF and YFP-SMDF but not in cells co-transfected with CFP and YFP (Fig. 3C). FRET is
readily detected as YFP fluorescence (λ = 535 nm) as a result of CFP excitation (λ = 440 nm). The plot of the emission signal measured at 535 nm (Ff) as a function of the signal measured at 480 nm (Df) using the excitation FRET filter (λ = 440 nm) is depicted in Fig. 3D. The experimental data are linearly distributed and may be fitted to a straight line with a slope of 1.25 ± 0.06 (mean ± S.D., n = 5) for the pair CFP/YFP-SMDF and 0.91 ± 0.02 (n = 5) for cytosolic CFP/YFP. Note that the slope value of the CFP/YFP-SMDF proteins was a significant ~35% (p < 0.001) larger than that exhibited by the non-interacting pair CFP/YFP. Thus, the value of the slope (Ff/Df) can be considered an operational measurement of FRET in intact cells, in agreement with results reported by Gordon et al. (13). To further substantiate that Ff/Df > 1.0 is because of FRET, we tagged the subunits of the ionotropic receptor TRPV1 with CFP (CFP-TRPV1) and YFP (YFP-TRPV1), co-transfected the fusion proteins, and obtained the plot Ff versus Df for this donor-acceptor pair (Fig. 3E). The rationale for using the TRPV1 receptor was that this receptor subunit tetramerizes giving rise to ion channels (14). Fig. 3E reveals that the slope of the pair CFP-TRPV1 and YFP-TRPV1 was 1.24 ± 0.05 (n = 4), akin to CFP-SMDF/YFP-SMDF. Taking together, our findings demonstrate that the CFP/YFP-SMDF fusion monomers interact in COS-7 cells and that they are within a distance consistent for the occurrence of FRET (1–10 nm). A similar result was obtained when CFP-SMDF and YFP-SMDF were expressed in the neuron-like PC12 cell line (Fig. 3F), implying that the SMDF monomers also interact in neurons. Therefore, SMDF appears to self-associate in vivo.

The Transmembrane Cysteine-rich Domain Drives SMDF Oligomerization—We next sought to identify the molecular determinants of the SMDF monomer–monomer interaction by a stepwise deletion approach on both CFP-SMDF and YFP-SMDF fusion proteins. For this purpose, the domains comprising amino acids 108–296 (CFP/YFP-SMDF(Δ108–296)) and 65–296 (CFP/YFP-SMDF(Δ65–296)) were removed by inverse PCR (Fig. 4A). As illustrated in Fig. 4B, deletion mutant CFP/YFP-SMDF(Δ108–296) shows the presence of FRET with a slope of 1.38 ± 0.04 (n = 5). In contrast, truncation form CFP/YFP-SMDF(Δ65–296) abrogated almost completely the occurrence of FRET (1.05 ± 0.04 (n = 5)) (Fig. 4C), suggesting that the transmembrane cysteine-rich domain segment of SMDF is a molecular determinant of monomer–monomer interaction. A summary of these results is depicted in Fig. 4D.

SMDF Monomers Self-associate in Cells—The strength of the interaction was evaluated by a co-immunoprecipitation strategy. For this purpose, we co-expressed the full-length SMDF with the deletion protein Δ108–296 fused to YFP. Protein complexes SMDF-YFP-SMDF(Δ108–296) were immunopurified with anti-GFP antibody and separated by SDS-PAGE, and the presence of full-length SMDF was probed with an anti-HRGβ3 antibody. Fig. 5 depicts that the SMDF protein was immunoprecipitated with the anti-GFP antibody when co-expressed with YFP-SMDF(Δ108–296). Note that only the 40-kDa form of SMDF was present in the immunoprecipitates, although similar amounts of both the 40-kDa and 83-kDa forms were noticeable in the cell extracts. Collectively, these findings are consistent with the tenet that SMDF self-association is mediated by its transmembrane domain. In addition, the lack of the 83-kDa O-glycosylated polypeptide in the immunoprecipitates implies that only the unmodified 40-kDa form of the protein self-assembles in cells.

DISCUSSION

SMDF is a type II membrane protein expressed on the plasma membrane with the active EGF-like domain in the C terminus oriented to the extracellular space (10). Transfected cells expressed two distinct forms of SMDF, a fast migrating ~40-kDa protein and a slow migrating ~83-kDa protein, with distinct membrane compartmentalization (9). While the fast migrating polypeptide is located in both non-raft and raft microdomains, the higher molecular mass form does not segregate into lipid rafts. These observations imply that the distinct molecular composition of both SMDF polypeptides may be an...
important modulatory strategy of protein biogenesis, trafficking, and/or function. Our findings indicate that the 40-kDa form represents monomeric unmodified SMDF. In contrast, the 83-kDa polypeptide is an O-glycosylated form of SMDF monomers. Inhibition of O-glycosylation with the sugar analogue benzyl-GalNAc increased the electrophoretic mobility of the 83-kDa glycoform to that of the 68-kDa polypeptide, indicating a partial inhibition of the protein O-glycosylation. In contrast, deletion of the N terminus of SMDF resulted in full abrogation of the incorporation of O-linked glycans. Thus, these results imply that O-glycosylation occurs specifically and exclusively in the intracellular N terminus of the protein. Two major types of O-glycosylation have been described: (i) the usually intracellular O-GlcNAc type of modification and (ii) the mucin-like O-GalNAc type, which generally appears in the extracellular domains of membrane proteins (15). However, the O-GalNAc kind of O-glycosylation has also been documented to occur in cytosolic domains (16). The fact that O-glycosylation of SMDF

**Fig. 4. The transmembrane domain drives SMDF oligomerization.** A, constructs used in this study. B, deletion mutant YFP-SMDF(Δ108–296) retains the self-associating ability as demonstrated by FRET; au, arbitrary units. C, deletion of transmembrane and extracellular domains in the deletion mutant YFP-SMDF(Δ65–296) abrogates FRET. D, the slopes of independent experiments in COS-7 cells were averaged, and data are given as mean ± S.E., with n ≥ 5.

**Fig. 5. O-Glycosylated SMDF is excluded from oligomers.** A co-immunoprecipitation strategy was used to confirm the oligomerization of SMDF. COS-7 cells were co-transfected with pcDNA3-SMDF and YFP-SMDF(Δ108–296), lysed, and immunoprecipitated with anti-GFP antibody. Immunoprecipitates were blotted with the anti-HRGβ3. Control experiments were performed with cells transfected only with the pcDNA3-SMDF construct. Pre-immunoprecipitation cell extracts were also blotted (first and second lanes). As shown, only the non-O-glycosylated form of SMDF is able to oligomerize. The schematic on the left shows the experimental strategy used for the co-immunopurification of SMDF heteromultimers. IB, Western immunoblotting; IP, immunoprecipitation.
is partially sensitive to benzyl-GalNAc, a widely used inhibitor of the O-GalNAc type of O-glycosylation (12), suggests that SMDF is O-GalNAc-modified. However, by using the sugar-specific binding lectins SBA and WGA, we found that the attached sugar is mainly GlcNAc. Nonetheless, because of the selectivity of these lectins for the terminal sugar, we cannot rule out the presence of non-exposed GalNAc residues in the protein. Because intracellular O-glycosylation has been shown to play diverse physiological roles ranging from enzyme activity to control of protein-protein interactions (15–18), our observations imply that SMDF O-glycosylation may modulate the assembly of the protein monomers. Alteration of monomer self-association will, in turn, influence SMDF subcellular localization and trafficking through regulation of its compartmentalization in membrane domains.

In the course of these studies, we obtained compelling evidence that SMDF monomers self-associate in cells through the transmembrane cysteine-rich domain of SMDF. First, FRET measurements in COS-7 and PC12 cells revealed an interaction between CFP-SMDF and YFP-SMDF. Structure-function analysis by a stepwise deletion strategy identified the transmembrane segment of the protein as the fundamental determinant of the molecular interaction. It is interesting to mention that the single transmembrane domain of the erbB receptor self-associates in cell membranes (19), suggesting a parallelism between the oligomerization of neuregulin ligands and that of receptors. Second, multimers composed of SMDF and YFP-SMDF(Δ108–296) could be immunopurified with an anti-GFP antibody, suggesting that these proteins strongly interact in cells. Notably, only the 40-kDa polypeptide was present in the immunopurified complex. The lack of O-glycosylated protein in the oligomers is consistent with the notion that multimerization modulates the protein O-glycosylation or vice versa. Similar linkage between intracellular domain O-glycosylation and protein-protein interactions has been described for other single span transmembrane proteins (20). Because only the fast migrating protein segregates into lipid rafts, our findings also imply that SMDF multimerization may be favored in these membrane microdomains as has been shown previously for other proteins (21, 22). However, we found that cholesterol depletion of lipid rafts with methyl-β-cyclodextrin does not abrogate FRET of the donor-acceptor pairs CFP/YFP-SMDF or CFP/YFP-SMDF(Δ108–296) (data not shown). Moreover, the strong FRET detected for CFP/ YFP-SMDF(Δ108–296), a deletion mutant that does not partition into membrane rafts (9), evidences that SMDF self-association also occurs in non-raft domains. These findings are similar to those reported for the urokinase-type plasminogen activator receptor (uPAR/CD87), a protein that dimerizes in both raft and non-raft microdomains (22). uPAR/CD87 dimerization is also insensitive to cholesterol depletion (22). Collectively, our findings are consistent with the tenet that SMDF multimerization may occur in both raft and non-raft microdomains. Further experimental data are required to unveil the specific mechanism underlying the protein multimerization.

A fundamental question arises; what could be the biological relevance of these findings? Cumulative evidence shows that compartmentalization of proteins in distinct membrane domains is important for precise signaling (23). For instance, in neurons some axon-located proteins are distributed in lipid rafts, whereas those delivered to soma and dendrites are placed in non-raft domains (24). Our results are consistent with the notion that the raft-segregated 40-kDa polypeptide may be preferentially stationed in the axon of the neuron, where it may serve as a juxtaclirine signal for Schwann cells thus inducing myelination of the axon (Fig. 6). Juxtaclirine signaling by type III neuregulins is a currently held hypothesis based on evidence showing that signaling by membrane-bound neuregulins can differ from signaling by soluble NRGs (1, 25). Because active erbB receptors are homo- or heterodimers of the different family members, dimerization of SMDF in lipid rafts may be important for its juxtaclirine signaling. Yen et al. (26) reported a differential biological role mediated by distinct erbB homo- and heterodimers. It is tempting to hypothesize that a dimer of ligands could bind preferably to erbB3 or erbB4 homodimers instead of erbB2 heterodimers, thus providing a mechanism of cell signaling able to discriminate diverse biological responses in target cells expressing erbB receptors. In contrast to the 40-kDa oligomeric form, the O-glycosylated 83-kDa polypeptide may be primarily targeted to soma and/or dendrites, where upon cleavage it would shed a bioactive ectodomain fragment that could act as a paracrine signal. Thus, O-glycosylation and oligomerization of SMDF could be important determinants of the signaling mode of this type III neuregulin. Future experiments in co-cultures of neurons and Schwann cells will address the proposed polarized distribution for the unmodified and O-glycosylated forms of SMDF and will elucidate its contribution to the neuregulin signaling mechanism.

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