Sensory and Motor Neuron-derived Factor Is a Transmembrane Heregulin That Is Expressed on the Plasma Membrane with the Active Domain Exposed to the Extracellular Environment*

(Received for publication, March 12, 1998, and in revised form, August 7, 1998)

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The β-heregulin sensory and motor neuron-derived factor (SMDF) has been suggested to be an important regulator of Schwann cell development and proliferation. In the present study, human SMDF was expressed in cultured cell lines. The cells and the recombinant protein were used to examine the membrane association and biological activity of the growth factor. Transfection of cells with SMDF cDNA constructs bearing FLAG epitope tags at either the amino- or carboxyl-terminal ends of the polypeptide resulted in expression of anti-FLAG immunoreactive polypeptides of approximately 44 and 83 kDa. The 83-kDa polypeptide was the major form expressed on the cell surface, as demonstrated by sensitivity to proteolysis in intact cells and surface biotinylation. SMDF was tightly associated with membranes isolated from transfected cells but was solubilized by Triton X-100. Immunofluorescent staining and immunoprecipitation experiments using cells expressing amino- or carboxyl-terminal tagged SMDF revealed that only the carboxyl-terminal end of the protein is exposed on the cell surface. Membranes from SMDF-transfected cells stimulated tyrosine phosphorylation of the β-heregulin receptor ErbB3 in Schwann cells. Conditioned medium from transfected cells contained a similar activity, suggesting that SMDF is subject to proteolytic release from the plasma membrane. In contrast with other β-heregulin isoforms, SMDF failed to bind heparin. Stimulation of Schwann cell ErbB3 receptor phosphorylation by SMDF was not affected by inhibition of Schwann cell heparan sulfate proteoglycan synthesis. These results demonstrate that SMDF is a type II transmembrane protein. This orientation places the active epidermal growth factor homology domain, which is located near the carboxyl-terminal end of the polypeptide, on the cell surface, where it can function as a membrane-anchored growth factor.

Proliferation of Schwann cells during embryonic and early postnatal development is necessary to provide sufficient numbers of Schwann cells to ensheath and myelinate peripheral axons. A series of studies carried out with purified cultures of Schwann cells and sensory nerve cells established that the mitogenic signal that drives Schwann cell proliferation is associated with the axons of these nerve cells (1–4). This mechanism, in which the Schwann cell mitogen is immobilized on the axonal surface, ensures proper matching of Schwann cell numbers and axons during development.

Although the axon-associated mitogen has not been purified, there is evidence that it is a member of the heregulin family of polypeptide growth factors (5, 6). The heregulins form a subset of the epidermal growth factor (EGF) family of polypeptide growth factors. At least 12 heregulin isoforms have been described, all of which appear to be generated by alternative splicing of a single gene (7, 8). All heregulin isoforms contain a domain of approximately 50 amino acids that is homologous to the active domain of EGF and is responsible for receptor binding (9). Heregulins exert their biological activities by binding and activating receptor tyrosine kinases, called ErbB2, ErbB3, and ErbB4, that bear structural similarity to the EGF receptor (10–12). ErbB receptors undergo tyrosine autophosphorylation upon ligand-dependent activation. This leads to activation of cytoplasmic signaling pathways, such as the mitogen-activated protein kinase pathway, which accounts for their mitogenic activity.

Results of in situ hybridization studies suggest that the principal form of β-heregulin expressed by sensory nerve cells is sensory and motor neuron-derived factor (SMDF) (13). Analyses of mouse embryos with targeted heregulin gene mutations provide indirect evidence that SMDF is important for early Schwann cell development (8). SMDF contains a β-heregulin EGF homology domain near the carboxyl-terminal end of the protein (13). With the exception of this domain, however, the rest of the SMDF sequence appears to be unique to this heregulin isoform.

The structural variation among isoforms produces multiple molecular forms of heregulins. Some heregulins contain amino-terminal signal peptides that can mediate secretion or membrane insertion of the polypeptides (7). Other isoforms lack such sequences (10, 14). There is evidence that both structural varieties can associate with membranes, however, and/or be released in soluble form into the medium of cultured cells expressing these proteins. These properties could result from the utilization of an internal hydrophobic sequence, present in most heregulins, to mediate membrane insertion and/or function as a membrane-spanning domain. The available data on membrane-associated forms of heregulins suggest these proteins are oriented with the amino-terminal ends exposed to the extracellular space and the carboxyl-terminal ends in the cytoplasm (14, 15). This orientation places the EGF homology domain of these heregulins on the external side of the plasma membrane. SMDF lacks an apparent amino-terminal signal

* This work was supported by National Institutes of Health Grant RO1 NS21925. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: EGF, epidermal growth factor; SMDF, sensory and motor neuron-derived factor; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; DME medium, Dulbecco's modified Eagle's medium.
peptide, but contains an internal hydrophobic sequence that could serve as a membrane insertion site. The structure of SMDF differs from most other herculein isoforms, however, in that it contains a cysteine-rich domain in place of the Ig domain. In SMDF, as well as some other herculein isoforms, the EGF homology domain of SMDF is positioned near the carboxyl-terminal end of the polypeptide. Thus, the nature of the association of SMDF with the plasma membrane would have important consequences for its biological activity.

Several herculeins have also been shown to bind to heparin and heparan sulfate (10, 16–18). Binding of some growth factors, such as basic fibroblast growth factor, to cell surface heparan sulfate molecules has been shown to have important consequences for the activities of the factors (19, 20). There is evidence that heparan sulfate-dependent interactions are involved in Schwann cell mitogenesis (18, 21, 22). The ability of glial growth factor-2 to activate ErbB receptors and stimulate Schwann cell proliferation is inhibited by exogenous heparin or heparan sulfate or by incubation of the Schwann cells in medium containing an inhibitor of proteoglycan synthesis (18).

The present study was carried out to address several specific questions related to SMDF structure and function. The data presented demonstrate that SMDF is a cell surface transmembrane protein that is oriented in the plasma membrane with the carboxyl-terminal EGF homology domain exposed to the extracellular compartment. Membrane-associated SMDF activates ErbB receptors on Schwann cells. In contrast with some herculein isoforms, SMDF does not bind heparin, and its activity is not dependent on Schwann cell heparan sulfate molecules.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression of SMDF—** Human SMDF cDNA was cloned by nested PCR amplification from a human brain stem cDNA library (LMC2, American Type Culture Collection, 57432). The primers were based on the published human SMDF cDNA sequence (13) and are shown in Table I. Conditions for PCR reactions were 94 °C for 1 min (denaturation), 60 °C for 2 min (annealing) and 72 °C for 10 min (extension) for 30 cycles. The resulting PCR product was cloned into pCR1 (Invitrogen). The insert was excised from this vector by digestion with EcoRI and subcloned into the plasmid pCMVneo. For the addition of FLAG epitope tags, the insert was reamplified by PCR using primers that overlapped the ends of the protein coding sequence and were extended on their 5′-ends by sequence coding for the FLAG epitope sequence. These products were cloned into the T/A expression plasmid pCR3.1 (Invitrogen). Sequences of expression plasmids were verified by DNA sequence analysis.

**Receptor Phosphorylation Assays—** Schwann cells were prepared from neonatal rat sciatic nerves and cultured as described previously.

**Antibody Preparation—** A 52-amino acid peptide corresponding to the EGF homology domain of β-herculein (9) was synthesized in the Weis Center for Research Core Molecular Biology Laboratory and purified by reverse phase high pressure liquid chromatography. The peptide was used to immunize rabbits using a synthetic adjuvant system (Ribi Immunocchemicals). Anti-β-herculein antibodies were purified by affinity chromatography using the synthetic peptide covalently coupled to CNBr-activated Sepharose.

**Immunofluorescence Microscopy—** Cells were used for immunofluorescence microscopy were grown on glass slide chambers. For surface staining, the cells were incubated with primary antibody solution before fixation and without permeabilization. For intracellular staining, the cells were fixed with 3% paraformaldehyde, 0.15 mM NaCl, 50 mM sodium phosphate, pH 7.4, and then permeabilized by incubation for 2 min in 0.05% Triton X-100 before incubation with the primary antibody. Blocking and washing steps were carried out as described previously (23). Bound antibodies were detected by incubation with Texas Red-conjugated secondary antibodies and visualized using a Nikon Optiphot microscope equipped for epifluorescence.

| Primer | Sequence |
|--------|----------|
| First round Sense | GCCCTTCGCTGTTGATTTGAGC |
| Antisense | AAGTGTCTTAGCCGACAGGC |
| Second round Sense | CTTCTGGGTTGAGCGGCTG |
| Antisense | AAGSAGACACCAACTGAGCAT |
Sensory and Motor Neuron-derived Factor

Expression of SMDF—Human SMDF cDNA was cloned as described under “Experimental Procedures.” The sequence of this cDNA matched the published human SMDF sequence reported previously (13) and contained coding information for a polypeptide with a predicted Mₚ of approximately 32,000.

The SMDF cDNA was used to generate several full-length expression constructs, including forms with FLAG epitope tags (hatched boxes) above. These results suggest that the 83-kDa form of SMDF is expressed on the cell surface and that the 43-kDa form is intracellular. This conclusion was further supported by results of cell surface labeling of SMDF-expressing cells. Surface proteins were covalently labeled with biotin by incubation of intact cells with a membrane-impermeant protein-modifying reagent. Immunoprecipitation of lysates from these cells with anti-FLAG antibodies and detection of biotin-labeled polypeptides with an avidin-conjugated probe revealed mainly the 83-kDa product. An alternative possibility that SMDF undergoes some type of processing or modification to produce the 83-kDa product. An alternative possibility that SMDF undergoes some type of processing or modification to produce the 83-kDa product. An alternative possibility that SMDF undergoes some type of processing or modification to produce the 83-kDa product.

To provide additional evidence for membrane association of SMDF, transfected cells were subjected to subcellular fractionation. When CHO cells that were stably transfected with NT-SMDF were lysed in the absence of detergent and subjected to differential centrifugation, the bulk of SMDF was present in the low speed pellet that was enriched in plasma membranes (Fig. 3A). Only small amounts of SMDF were present in the high speed pellet, composed mainly of membranes of intracellular organelles. SMDF was not detected in the soluble fraction.
analysis and stained with anti-FLAG antibody. soluble (as indicated. The samples were recentrifuged to produce pellet (EGF homology domain of SMDF is located near the carboxyl-
 of the polypeptide within the plasma membrane, however. The cent cells. This property would be dependent on the orientation factor molecules that can activate heregulin receptors on adja-
 the plasma membrane has the potential to generate growth

\[ \text{transiently transfected with CT-SMDF were lysed in the absence of} \]

\[ \text{NT-SMDF were lysed in the absence (Fig. 6A).} \]

\[ \text{SMDF were stained with anti-FLAG antibodies as either intact} \]

\[ \text{membrane fragments. Immunoblot analysis of medium condi-

\[ \text{ditioned medium were significantly lower than the amount} \]

\[ \text{of SMDF-derived peptides in} \]

\[ \text{from cells transfected with a plasmid that contained the SMDF} \]

\[ \text{activated Schwann cell ErbB receptors was tested.} \]

\[ \text{Experiments with a synthetic } \beta\text{-heregulin EGF homology} \]

\[ \text{domain peptide revealed that Schwann cells express functional} \]

\[ \text{on interactions with heparan sulfate.} \]

\[ \text{Cell Surface Orientation of SMDF—Expression of SMDF on} \]

\[ \text{the plasma membrane has the potential to generate growth} \]

\[ \text{factor molecules that can activate heregulin receptors on adja-
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\[ \text{cent cells. This property would be dependent on the orientation of} \]

\[ \text{the polypeptide within the plasma membrane, however. The} \]

\[ \text{EGF homology domain of SMDF is located near the carboxyl-} \]

\[ \text{terminal end of the protein (Fig. 1A). To determine the orien-
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\[ \text{tation of SMDF within the plasma membrane, CHO cells that} \]

\[ \text{were transiently transfected with either NT-SMDF or CT-} \]

\[ \text{SMDF were stained with anti-FLAG antibodies as either intact} \]

\[ \text{or permeabilized cells. As shown in Fig. 4, staining of SMDF on} \]

\[ \text{the surface of intact cells was observed only with CT-SMDF (A)} \]

\[ \text{and not with NT-SMDF (B). Staining of both forms of} \]

\[ \text{SMDF was observed when fixed cells were permeabilized with Triton} \]

\[ \text{X-100 (Fig. 4, C and D). Identical results were obtained in 293} \]

\[ \text{cells (data not shown). These results demonstrate that SMDF is} \]

\[ \text{oriented in the plasma membrane with the carboxyl-terminal} \]

\[ \text{end exposed to the extracellular space. This orientation is} \]

\[ \text{consistent with the utilization of an internal, uncleaved signal} \]

\[ \text{peptide, which would direct the insertion of SMDF as a type II} \]

\[ \text{transmembrane protein. Furthermore, this orientation places} \]

\[ \text{the active EGF homology domain of SMDF on the extracellular} \]

\[ \text{side of the plasma membrane.} \]

\[ \text{Additional evidence for this membrane orientation was pro-
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\[ \text{vided by results of immunoprecipitation experiments. Cells} \]

\[ \text{that were transfected with either CT-SMDF or NT-SMDF were} \]

\[ \text{incubated with anti-FLAG antibodies. The unbound antibodies} \]

\[ \text{were removed, and the cells were lysed without use of deter-
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\[ \text{gent and used to prepare membrane fractions. The membranes} \]

\[ \text{were dissolved in immunoprecipitation buffer, and the FLAG} \]

\[ \text{antibodies were isolated by adsorption to protein A-Sepharose.} \]

\[ \text{As shown in Fig. 5A, the 83-kDa form of CT-SMDF, but not} \]

\[ \text{NT-SMDF, was isolated by this procedure. In contrast, when} \]

\[ \text{membranes were isolated from CT-SMDF- or NT-SMDF-transfected} \]

\[ \text{cells and then incubated with anti-FLAG antibodies and} \]

\[ \text{purified by adsorption to Protein A-Sepharose, both the 83-} \]

\[ \text{and 43-kDa forms of CT-SMDF and NT-SMDF were isolated (Fig.} \]

\[ \text{5A). These results indicate that the carboxyl-terminal, but not} \]

\[ \text{the amino-terminal, end of SMDF is exposed to the extracellu-
}

\[ \text{lar environment. They also demonstrate that the amino-termi-
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\[ \text{nal FLAG epitope is accessible to the antibody in the absence of} \]

\[ \text{detergent. Finally, these results provide additional evidence} \]

\[ \text{that the 83-kDa SMDF is the cell surface form.} \]

\[ \text{Essentially identical results were obtained when CT-SMDF-} \]

\[ \text{and NT-SMDF-transfected cells were incubated with anti-} \]

\[ \text{FLAG antibodies and then lysed with detergent before the} \]

\[ \text{addition of protein A-Sepharose (Fig. 5B). Significant amounts} \]

\[ \text{of SMDF were immunoprecipitated only from cells expressing} \]

\[ \text{the CT-SMDF.} \]

\[ \text{SMDF Activates ErbB Receptors on Schwann Cells—} \beta\text{-} \]

\[ \text{heregulins exert their biological activities by binding to and} \]

\[ \text{activating the receptor tyrosine kinases ErbB2, ErbB3, and} \]

\[ \text{ErbB4 (11, 12). Since SMDF is produced by nerve cells that are} \]

\[ \text{ensheathed by Schwann cells (8, 13), the ability of recombinant} \]

\[ \text{SMDF to activate Schwann cell ErbB receptors was tested.} \]

\[ \text{Experiments with a synthetic } \beta\text{-heregulin EGF homology} \]

\[ \text{domain peptide revealed that Schwann cells express functional} \]

\[ \text{ErbB2 and ErbB3 receptors, but not ErbB4.2 As shown in Fig.} \]

\[ \text{6A, membranes purified from cells that were stably transfected} \]

\[ \text{with SMDF stimulated tyrosine phosphorylation of ErbB3. No} \]

\[ \text{activation of ErbB3 was observed with membranes isolated} \]

\[ \text{from cells transfected with a plasmid that contained the SMDF} \]

\[ \text{coding sequence in the reverse orientation. These results dem-
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\[ \text{onstrate that membrane-associated SMDF is biologically ac-
}

\[ \text{tive. A 52-amino acid peptide corresponding to the EGF homol-
}

\[ \text{gy domain of SMDF also stimulated ErbB3 tyrosine} \]

\[ \text{phosphorylation when added to Schwann cell culture medium} \]

\[ \text{(Fig. 6B).} \]

\[ \text{Concentrated conditioned medium obtained from SMDF-} \]

\[ \text{transfected cells also stimulated ErbB3 tyrosine phosphoryla-
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\[ \text{tion (Fig. 6B). Medium from cells transfected with the anti-
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\[ \text{sense orientation SMDF vector had no activity. The activity in} \]

\[ \text{conditioned medium remained soluble after centrifugation for} \]

\[ \text{1 h at 100,000 } \times g \text{ (Fig. 6B, compare lanes 3 and 4), demon-
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\[ \text{strating that it was not derived from particulate cell debris or} \]

\[ \text{membrane fragments. Immunoblot analysis of medium condi-
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\[ \text{tioned by CT-SMDF-transfected cells revealed anti-FLAG immu-
}

\[ \text{nunoreactive polypeptides with } M_c \text{ values of 27,000, 34,000,} \]

\[ \text{and 72,000 kDa, which were smaller than SMDF present in} \]

\[ \text{membranes (Fig. 6C). Anti-FLAG immunoreactive polypep-
}

\[ \text{ptides were not detected in medium from NT-SMDF-transfected} \]

\[ \text{cells (not shown). The amounts of SMDF-derived peptides in} \]

\[ \text{conditioned medium were significantly lower than the amount} \]

\[ \text{associated with the cells, however (data not shown). These} \]

\[ \text{results suggest that SMDF can be released from the cell sur-
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\[ \text{face in soluble form, apparently by proteolytic shedding. In the} \]

\[ \text{A. Schroering and D. J. Carey, manuscript in preparation.} \]
cell types used for these experiments, however, this process is not highly active.

**SMDF Does Not Bind Heparin**—Earlier studies have shown that some forms of β-heregulin bind heparin or heparan sulfate (10, 15, 17, 18) and that heparan sulfate binding influences β-heregulin activity (17, 18). To test whether SMDF binds heparin, SMDF extracted from membranes of CHO cells stably transfected with NT-SMDF was subjected to heparin-agarose affinity chromatography. As shown in Fig. 7, all of the SMDF was recovered in the column flow-through and wash fractions. No SMDF was eluted by the NaCl step gradient. The heparin column was functional, based on the observation that other cell-derived proteins bound to the column under the loading and wash conditions used and were eluted by the NaCl step gradient (not shown). Thus, in contrast to other forms of β-heregulin, SMDF does not bind heparin.

**Activation of Schwann Cell ErbB3 Receptors by SMDF Is Not Dependent on Heparan Sulfate**—Biological activity of the β-heregulin GGF2 has been shown to be dependent on the presence of endogenous Schwann cell heparan sulfate molecules (18). The effect of endogenous heparan sulfate molecules on SMDF activity was examined by measuring ErbB3 tyrosine phosphorylation in Schwann cells that were treated with agents that perturb heparan sulfate synthesis. 4-Methylumbellifer-β-D-xyloside inhibits glycosaminoglycan chain initiation on proteoglycan core proteins. As we have shown previously, 1 mM β-xyloside inhibits the synthesis of heparan sulfate proteoglycans by Schwann cells by approximately 90% (24). Sodium chlorate inhibits sulfation of glycosaminoglycans (25). 30 mM sodium chlorate inhibited Schwann cell proteoglycan sulfation by approximately 90% (not shown). As shown in Fig. 8, incubation of Schwann cells in medium containing 30 mM sodium chlorate (lane 2) or 1 mM β-xyloside (lane 4) had no effect on SMDF-stimulated ErbB3 tyrosine phosphorylation.

Soluble heparin is a competitive inhibitor of heparan sulfate-dependent interactions. As shown in Fig. 8, heparin in the range of 0.03–3 μg/ml (lanes 5–7) had no effect on SMDF-stimulated ErbB3 tyrosine phosphorylation. Taken together, these results demonstrate that endogenous heparan sulfate molecules do not affect SMDF-dependent activation of Schwann cells.

**DISCUSSION**

The data presented demonstrate that the heregulin growth factor SMDF is synthesized as a tightly membrane-associated protein. SMDF is expressed on the plasma membrane and is oriented with the carboxyl terminus, but not the amino terminus, exposed to the extracellular space. This orientation is consistent with the use of an internal, uncleaved membrane insertion signal, and identifies SMDF as a type II transmembrane protein. This orientation places the EGF homology domain of SMDF, which is located near the carboxyl terminus, on the extracellular face of the plasma membrane (Fig. 9). This domain is sufficient to stimulate phosphorylation of Schwann cell ErbB2 and ErbB3 heregulin receptors and activate Schwann cell proliferation. Furthermore, recombinant membrane-associated SMDF stimulates Schwann cell ErbB3 phosphorylation. Thus, SMDF expressed on the plasma membrane can function as a membrane-anchored mitogen.

Other heregulin isoforms are also membrane-associated. At least some of these appear to have membrane orientations that differ from that of SMDF, since they are oriented in the membrane with their amino-terminal ends exposed to the extracellular space (14, 15). Interestingly, this orientation also places the EGF homology domains of these heregulins on the extracellular face of the plasma membrane. Thus, heregulin gene products appear to use at least two distinct mechanisms for generating membrane-associated growth factors.

As described here, SMDF exhibits anomalous migration on SDS-polyacrylamide gels. The two SMDF products that were observed migrated as polypeptides of 83 and 43 kDa, both of which are larger than the molecular weight predicted from the
The adsorbed proteins were subjected to immunoblot analysis with anti-FLAG antibodies. The protein A-adsorbed proteins were subjected to immunoblot analysis with anti-FLAG antibodies. For lanes 1 and 2, the cells were incubated in medium containing anti-FLAG antibodies for 30 min on ice. The cells were then washed and lysed in hypotonic medium, and membranes were isolated. The membranes were dissolved in immunoprecipitation buffer, and the FLAG antibodies were isolated by adsorption to protein A-Sepharose. The protein A-adsorbed proteins were subjected to immunoblot analysis with anti-FLAG antibodies. For lanes 3 and 4, membranes were isolated from transfected cells and then incubated with anti-FLAG antibodies. The membranes were dissolved in immunoprecipitation buffer, and the FLAG antibodies were isolated by adsorption to protein A-Sepharose. The adsorbed proteins were subjected to anti-FLAG immunoblot analysis as for lanes 1 and 2. B, 293 cells were transiently transfected with CT-SMDF or NT-SMDF. For lanes 1 and 2, the cells were incubated in medium containing anti-FLAG antibodies for 30 min on ice. The cells were then lysed in immunoprecipitation buffer, and the FLAG antibodies were isolated by adsorption to protein A-Sepharose. The protein A-adsorbed proteins were subjected to immunoblot analysis with anti-FLAG antibodies. For lanes 3 and 4, the transfected cells were first lysed in immunoprecipitation buffer, and then anti-FLAG antibodies were added. The FLAG antibodies were isolated by adsorption to protein A-Sepharose, and the adsorbed proteins were subjected to anti-FLAG immunoblot analysis.

cDNA sequence (32,000). Polypeptides with identical apparent sizes were detected, however, when SMDF molecules bearing FLAG epitope tags at either end of the molecule were expressed. Thus, there was no evidence for the presence of stable, proteolytic fragments of SMDF, as has been described for some other heregulin isoforms (15). Results of surface labeling and immunoprecipitation experiments indicated that the 83-kDa polypeptide is the form of SMDF that is expressed on the cell surface. The nature of the modification that is responsible for generating this product is not known. The fact that this form is, within experimental error, twice the apparent size of the smaller polypeptide suggests that the cell surface molecules are dimers of full-length SMDF polypeptides. The amino-terminal domain of SMDF is rich in cysteine residues, which could produce dimers via formation of interchain disulfide bonds. Reduction of the recombinant SMDF with β-mercaptoethanol or dithiothreitol failed to convert the 83-kDa form to smaller products, however.

There is evidence that SMDF is an important regulator of Schwann cells during development of the peripheral nervous system. Studies with primary cultures of sensory nerve cells and Schwann cells have demonstrated that axonal membranes provide the mitogenic signal that drives Schwann cells proliferation that occurs before the cells undergo terminal differentiation (1, 3). Although this activity has not been purified, it appears to be a member of the heregulin growth factor family. This is based on the finding that preincubation of the nerve cells with anti-heregulin antibodies or treatment of Schwann cells with anti-ErbB3 antibodies significantly inhibits the axonal contact-induced Schwann cell proliferation (5). In situ hybridization experiments have shown that SMDF is the major form of heregulin expressed in sensory nerve cells. The importance of SMDF in Schwann cell development is also suggested by the observation that in embryos of mice that bear a targeted mutation that eliminates expression of the known heregulins except for SMDF, Schwann cell precursors can be detected in cranial and spinal nerves (8). Schwann cell precursor cells are absent from embryos of mice bearing a mutation in the EGF homology that abolishes expression of all heregulins. The heregulin mutant embryos do not develop beyond embryonic day 10.5, precluding analysis of the role of SMDF in later stages of Schwann cell development.
from the membrane to produce a soluble mitogen. SMDF can be released by proteolytic cleavage on the outside of the cell. The protein is oriented within the plasma membrane, which places the active EGF-homology domain sequence that is unique to SMDF. The model is based on the data presented in the paper. The solid box represents the hydrophobic sequence that functions as both internal uncleaved signal peptide and transmembrane domain. The gray shaded area indicates sequence that is unique to SMDF. The protein is oriented within the membrane with the COOH-terminal end exposed on the external face of the plasma membrane, which places the active EGF-homology domain on the outside of the cell. SMDF can be released by proteolytic cleavage from the membrane to produce a soluble mitogen.

The tight membrane association of SMDF demonstrated here is consistent with the properties of the sensory nerve cell Schwann cell mitogen. The axon-associated Schwann cell mitogenic activity of rat embryo sensory nerve cells is solubilized by a combination of octyl glucoside and 0.5 M sodium chloride (22). In contrast, a similar activity can be solubilized from neonatal rat brain membranes in the absence of detergent. The brain-derived activity also bound to a heparin-affinity column and was eluted at a sodium chloride concentration of greater than 0.5 M, indicating tight binding to heparin (22). Based on the data presented here, it is likely that the brain-derived activity is due to heparulins other than SMDF. Heparulin receptors are widely expressed in the developing brain, including several forms, such as glial growth factor and acetylcholine receptor-inducing activity (ARIA), that bind heparin (17, 18). The model is consistent with these findings.

The mitogenic activity of the solubilized brain-derived factor is inhibited by soluble heparin (22), similar to the mitogenic activity of glial growth factor. As shown here, SMDF does not bind heparin. Moreover, its ability to activate Schwann cell ErbB3 heregulin receptors was not affected by inhibition of Schwann cell heparan sulfate proteoglycans or by the addition of soluble heparin. SMDF is similar in these respects to the sensory nerve cell mitogenic activity, which is also inhibited by heparin (22) or inhibition of Schwann cell heparan sulfate proteoglycan synthesis (21). Interestingly, the mitogenic activity of axonal membranes is reduced by inhibition of nerve cell heparan sulfate proteoglycan synthesis (21). A potential explanation for this finding is that a neuronal heparan sulfate proteoglycan binds the active mitogen to the axonal membrane. This mechanism is not consistent, however, with the properties of SMDF reported here.

Our data also demonstrated that some SMDF was released in a soluble form from cells expressing the growth factor. The immunoreactive polypeptides that were observed were similar to the membrane-associated form, consistent with proteolytic cleavage. Since the soluble forms retained the ability to stimulate phosphorylation of Schwann cell ErbB3 heregulin receptors, the cleavage site must be located between the internal hydrophobic sequence and the EGF homology domain. The anomalous migration of SMDF on SDS-polyacrylamide gels makes it difficult to identify the site of cleavage based on the apparent Mr values of the products observed. Proteolytic shedding of membrane-associated growth factors has been well documented (15, 26–28). A membrane-associated metalloproteinase has been identified that mediates cleavage of membrane-associated tumor necrosis factor-α to generate the soluble factor (29, 30). At least in the cells types used in this study, however, SMDF was cleaved at a fairly slow rate, since only small amounts of soluble SMDF polypeptides were observed relative to the amounts associated with the cell membranes.

Except for the EGF homology domain, most of the SMDF molecule consists of protein sequence that is unique to this form of heregulin. Data presented here indicate that one function of this unique structure is to anchor the growth factor in the membrane. Membrane anchoring restricts spatially the range over which SMDF can act and would limit stimulation of mitogenesis to Schwann cells that are in direct contact with axons. This provides an appealing mechanism for regulating Schwann cell numbers relative to the quantity of axonal surface area that must be myelinated and ensheathed by these Schwann cells. Heparulins have also been suggested to function as survival factors for Schwann cells (6, 31). Thus, Schwann cells that have lost direct contact with axons would undergo apoptosis as a result of failure to receive SMDF-dependent activation.

A number of interesting questions pertaining to SMDF structure and function remain. What factors regulate SMDF expression by nerve cells? Do the unique structural domains of SMDF carry out other functions? What are the signaling mechanisms used by SMDF and ErbB heregulin receptors to control Schwann cell activity? These and other questions are the subjects of current investigation.

REFERENCES

1. Wood, P., and Bunge, R. P. (1975) Nature 256, 662–664
2. Salzer, J. L., and Bunge, R. P. (1980) J. Cell Biol. 84, 739–752
3. Salzer, J. L., Bunge, R. P., and Glaser, L. (1980) J. Cell Biol. 84, 767–778
4. Salzer, J. L., Williams, A. K., Glaser, L., and Bunge, R. P. (1980) J. Cell Biol. 84, 783–796
5. Morissey, T. K., Levi, A. D., Ouijena, A., Sliwkowski, M. X., and Bunge, R. P. (1995) Proc. Natl. Acad. Sci. U. S. A 92, 1431–1435
6. Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Misky, R., and Jessen, R. R. (1995) Neuro 15, 585–596
7. Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-Moensog, O., Kirk, C., Hendricks, M., Danely, P., Misumi, S., Suidhalter, J., Takenashi, K., Wrablewski, D., Lynch, C., Baldassare, M., Hiles, L., Davia, J. B., Hauan, J. J., Totty, N. F., Otus, M., McBurney, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D. (1985) Nature 312, 312–318
8. Meyer, D., Yamaai, T., Garry, A., Reithmacher-Sonnenberg, E., Kane, D., Theil, L. E., and Birchemieer, C. (1997) Development 124, 3575–3586
9. Barbacini, E. G., Guarino, B. C., Streif, J. G., Singleton, D. H., Rosnack, K. J., Hsuan, J. J., Totty, N. F., Otus, M., McBurney, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D. (1985) Nature 312, 312–318
10. Meyer, D., Yamaai, T., Garry, A., Reithmacher-Sonnenberg, E., Kane, D., Theil, L. E., and Birchemieer, C. (1997) Development 124, 3575–3586
11. Barbacini, E. G., Guarino, B. C., Streif, J. G., Singleton, D. H., Rosnack, K. J., Hsuan, J. J., Totty, N. F., Otus, M., McBurney, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D. (1985) Nature 312, 312–318
12. Pinkas-Kramarski, R., Shelly, M., Glathe, S., Rathkin, B. J., and Yarden, Y.
Sensory and Motor Neuron-derived Factor

(1996) J. Biol. Chem. 271, 19029–19032

13. Ho, W.-H., Armanini, M. P., Nuijens, A., Phillips, H. S., and Osheroff, P. L. (1995) J. Biol. Chem. 270, 14523–14532

14. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden Y. (1992) Cell 69, 559–572

15. Burgess, T. L., Ross, S. L., Qian, Y., Brankow, D., and Hu, S. (1995) J. Biol. Chem. 270, 14523–14532

16. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden Y. (1992) Cell 69, 559–572

17. Loeb, J. A., and Fischbach, G. D. (1995) J. Cell Biol. 130, 127–135

18. Sudhalter, J., Whitehouse, L., Rusche, J. R., Marchionni, M. A., and Mahanthappa, N. K. (1996) Glia 17, 28–38

19. Yayon, A., Klagesbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) Cell 64, 841–848

20. Rapraeger, A. C., Krufta, A., and Obwin, B. B. (1991) Science 252, 1705–1708

21. Ratner, N., Bunge, R. P., and Glaser, L. (1988) J. Cell Biol. 111, 744–754

22. Ratner, N., Hong, D., Lieberman, M. A., Bunge, R. P., and Glaser, L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6992–6996

23. Carey, D. J., and Stahl, R. C. (1990) J. Cell Biol. 111, 2053–2062

24. Carey, D. J., Rafferty, C. M., and Tod, M. S. (1987) J. Cell Biol. 105, 1013–1021

25. Greve, H., Culy, Z., Blumberg, P., and Kresse, H. (1988) J. Biol. Chem. 263, 12886–12892

26. Higashiyama, S., Lau, K., Besmer, G. E., Abraham, J. A., and Klagesbrun, M. (1992) J. Biol. Chem. 267, 6205–6212

27. Arrisius, J., Cordly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S., and Ruoslahti, E. (1996) J. Biol. Chem. 271, 11376–11382

28. Reuning, A. J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, A. H., Drummond, A. H., Galloway, W. A., Gilbert, R., Gordon, J. L., Leber, T. M., Mangan, M., Miller, K., Naye, P., Owen, K., Patel, S., Thomas, W., Wells, G., Wood, L. M., and Woolley, K. (1994) Nature 376, 555–557

29. Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Welless, M. F., Castner, B. J., Stokking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Pei, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Ceretti, D. P. (1997) Nature 385, 729–733

30. Mass, M. L., Jin, S. L., Mills, E. A., Bickett, D. M., Burkhardt, W., Carter, H. L., Chen, W. J., Clay, W. C., Didsbury, J. R., Haussler, D., Hoffman, C. R., Kost, T. A., Lambrecht, M. H., Lees, G., McGeehan, G., Mitchell, J., Moyer, M., Pahl, G., Roesch, W., Overton, L. K., Schoenen, F., Seaton, T., Su, J. L., Warner, J., Willard, D., and Beck, J. B. (1997) Nature 385, 733–738

31. Syroid, D. E., Maycox, P. R., Burrola, P. G., Liu, N., Wen, D., Lee, K.-F., Lemke, G., and Kilpatrick, T. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9229–9234