Cleavage of Neuregulin-1 by BACE1 or ADAM10 Protein Produces Differential Effects on Myelination*

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Neuregulin-1 (Nrg1) is encoded by a single gene and exists in naturally secreted and transmembrane isoforms. Nrg1 exerts its signaling activity through interaction with its cognate ErbB receptors. Multiple membrane-anchored Nrg1 isoforms, present in six different membrane topologies, must be processed by a protease to initiate a signaling cascade. Here, we demonstrate that BACE1 and ADAM10 can process type I and III Nrg1 at two adjacent sites. Our cleavage site mapping experiments showed that the BACE1 cleavage site is located eight amino acids downstream of the ADAM10 cleavage site, and this order of cleavage is the opposite of amyloid precursor protein by these two enzymes. Cleavages were further confirmed via optimized electrophoresis. Cleavage of type I or III Nrg1 by ADAM10 and BACE1 released a signaling-capable N-terminal fragment (ntf), either Nrg1-ntfα or Nrg1-ntfβ, which could similarly activate an ErbB receptor as evidenced by increased phosphorylation of Akt and ERK, two downstream signaling molecules. Although both Nrg1-ntfα and Nrg1-ntfβ could initiate a common signaling cascade, inhibition or down-regulation of ADAM10 alone in a co-culture system did not affect normal myelination, whereas specific inhibition of BACE1 impaired normal myelination. Thus, processing of Nrg1 by BACE1 appears to be more critical for regulating myelination. Our results imply that a significant inhibition of BACE1 could potentially impair Nrg1 signaling activity in vivo.

Members of the neuregulin (Nrg)3 family of proteins contain an EGF-like domain that mediates cell-cell signaling functions via interaction with cognate ErbB receptors (1, 2). The Nrg protein family consists of four members (Nrg1–Nrg4), with Nrg1 being the most well characterized. Because of multiple splicing events, mammalian Nrg1 has a total of 33 isoforms that can be distinguished by six different membrane topologies (types I–VI) (3). Mice with a genetic deletion of Nrg1 are embryonic-lethal due to circulatory failure and defects in neural and cardiac development (4), indicating the importance of Nrg1 in normal growth. As a critical signaling molecule, Nrg1 has also been linked to developmental abnormalities and pathogenesis of multiple diseases. For example, Nrg1 polymorphism has been identified as a risk factor for schizophrenia (5). Mouse genetic studies also demonstrate that type III Nrg1 is a master regulator of myelination; its level in axons is correlated with the thickness of the myelin sheath (6, 7).

Most of the 33 Nrg1 isoforms are membrane-anchored ligands, and proteolytic cleavage of these membrane-bound Nrg1 isoforms is required for the secreted Nrg1 fragment to bind to its cognate receptor, an ErbB2/ErbB3 heterodimer or ErbB4 homodimer (8–10). In our recent study of Alzheimer β-secretase (BACE1), we demonstrated that membrane-anchored type I and III β1 Nrg1 isoforms are cleaved by BACE1 at the site between residues EF and ME, ~10 residues away from the transmembrane region (11). In BACE1-null mice, the cleavage of type I and III β1 Nrg1 isoforms is abolished, but this lost cleavage does not completely prevent the processing of Nrg1 by other proteases, as cleaved Nrg1 fragments remain detectable (12). Consequently, this reduced processing of Nrg1 correlates with reduced myelin sheath thickness of axons in both the peripheral and central nervous systems during developmental stages (11, 13). In nerve crush experiments, we further demonstrated that abolished cleavage of Nrg1 by BACE1 causes delayed remyelination in the adult (12). This result also suggests that Nrg1 cleavage by BACE1 is required for optimal remyelination. Consistent with our observation, ablation of axonal Nrg1 causes severe impairment in remyelination upon sciatic nerve crush (14).

Because the cleaved N-terminal fragments (ntfs) are not completely abolished in BACE1-null mice (11), we asked what enzymes cleave Nrg1 at a site in close proximity to the BACE1 site. Proteolytic inhibitory profiling suggests that Nrg1 can also be processed by a metalloprotease (12, 15, 16). In this study, we investigated the potential role of ADAM10 (a disintegrin and metalloproteinase 10) and ADAM17 in cleaving Nrg1. Our data show that ADAM10 cleaves Nrg1 effectively to produce a fragment that matches the size seen in BACE1-null mice, whereas ADAM17 likely has a significantly weaker effect on the same cleavage. Mapping of the cleavage sequence of Nrg1 by
ADAM10 further confirmed this cleavage. Hence, BACE1 and ADAM10 will shed Nrg1 at adjacent sites to release their respective Nrg1-ntfs (i.e. Nrg1-ntfβ and Nrg1-ntfα). Although Nrg1-ntfβ and Nrg1-ntfα can similarly activate ErbB receptors to increase phosphorylation of Akt and ERK, inhibition of BACE1 or ADAM10 surprisingly exhibits differential effects on myelination in an in vitro myelination model. Our results demonstrate that processing of Nrg1 by BACE1 rather than ADAM10 is more critical for the regulation of myelination.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Stable cell lines expressing type I or III Nrg1 were generated by stably transfecting the indicated construct in HEK-293 cells as described previously (12). A cell line stably expressing BACE1 (HM cell line) was previously generated by expressing HA-tagged BACE1 in HEK-293 cells (17). Anti-myelin basic protein (MBP) antibody was purchased from Sternberger Monoclonal Inc. (Lutherville, MD). Antibodies against actin, calnexin, TuJ1, and the amyloid precursor protein (APP) C terminus (A8717) were purchased from Sigma. Anti-Nrg1 antibodies recognizing the Nrg1 N or C terminus were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ADAM10 and anti-ADAM17 antibodies were purchased from Millipore (Billerica, MA). Anti-Akt and anti-phospho-Akt (Ser-473) antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Generation of Expression Constructs—The cDNAs encoding full-length human type I and III Nrg1 were PCR-amplified and inserted into the pcDNA3.1-Myc/His vector. Plasmid DNA expressing type I β1 Nrg1 mutated at either the BACE1 or ADAM10 cleavage site was generated by site-directed mutagenesis according to the Stratagene mutagenesis protocol. Type III β1 Nrg1 was used for inserting a stop codon after either BACE1 or ADAM10 cleavage site to produce Nrg1-ntfβ and Nrg1-ntfα. Knockdown of ADAM10 or ADAM17 expression in HEK-293 cells was achieved by expressing siRNA duplexes targeting ADAM10 and ADAM17 (purchased from Invitrogen). Each siRNA duplex has the following RNA sequence: ADAM10-Si1, CCA UGC CCA UGG AGG ACA U; ADAM10-Si2, CCA AUG GAA GAC AUU UCA A; ADAM17-Si1, CCA GAG ACU CGA GAA GCU U; and ADAM17-Si2, AAC AAA UCU CCA UGG CC.

ADAM10 Cleavage Site Mapping—Soluble recombinant Nrg1 protein spanning Met-1 to Lys-245 was purchased from R&D Systems. One μg of Nrg1 protein was incubated with 1 μg of purified recombinant ADAM10 in a protease assay as described previously (18). The result suggested that the cleavage site was near the C terminus, and two peptides, CKSFYKHGIEF (peptide 1; M, 1472), which was initially designed for producing peptide antibody, and CKNVYMASFYKHGLGIEFMEAEE (peptide 2; M, 2768), were subsequently synthesized and HPLC-purified for mapping the cleavage site by ADAM10. After incubation of the peptide with ADAM10 for 24 h at room temperature, the reaction mixtures were first assayed by HPLC to determine the cleavage, and the cleaved mixtures were then further analyzed by MALDI-TOF mass spectrometry.

RESULTS

Cleavage of Nrg1 by ADAM10—To determine which metalloprotease would cleave Nrg1, we hypothesized that ectodomain shedding of Nrg1 might resemble the cleavage of APP by α- and β-secretases. BACE1 is the only protease that cleaves APP at the β-secretase site, whereas three proteases (ADAM9, ADAM10, and ADAM17) are reported to process APP at an identical α-secretase site (22, 23). To test our hypothesis, we incubated either ADAM10 or ADAM17 protease with a recombinant protein spanning the entire extracellular domain of human Nrg1 in an enzymatic assay. Under the optimal in vitro cleavage conditions, ADAM10 efficiently cleaved recombinant Nrg1 protein at one site by producing one 30-kDa fragment (Fig. 1A). This cleavage appeared to be similar to the cleavage of the same recombinant Nrg1 protein by BACE1 because BACE1 cleavage of this recombinant Nrg1 protein produces a single fragment similarly migrating near 30 kDa (12). Unexpectedly, ADAM17 cleaved recombinant Nrg1 protein at multiple sites to produce multiple bands between 25 and 28 kDa (Fig. 1B). Although the 30-kDa fragment was not present in ADAM17-
cleaved products, this experiment alone could not exclude the possibility that these smaller fragments are derived from additional cleavage of the original 30-kDa fragment by ADAM17 under this *in vitro* enzymatic condition. Increasing or decreasing the cleavage of recombinant Nrg1 via increasing or reducing the concentration of ADAM10 or ADAM17 in the reaction did not significantly change the cleavage patterns (data not shown). Hence, ADAM10 cleavage of Nrg1 appears to shed Nrg1 in a similar manner as BACE1, whereas ADAM17 perhaps processes Nrg1 at multiple sites.

**Mapping the ADAM10 Cleavage Site**—To further confirm our initial observation, we incubated ADAM10 with several synthetic peptides to map the cleavage site. One peptide, CKSYKHLGIEF (peptide 1; $M_r$, 1472), which was initially synthesized for generating antibody to recognize a BACE1-cleaved neoepitope, was effectively cleaved by ADAM10. Cleavage of peptide 1 by ADAM10 produced two separable peaks by HPLC purification (Fig. 2A), whereas ADAM17 barely processed this fragment (data not shown). Analysis of the cleaved products by MALDI-TOF mass spectrometry revealed that the cleavage site is located between residues SF and YK (Fig. 2, B and C). To confirm this cleavage, we synthesized another longer peptide, CKQNYVMAFSYKHLGIEFMEAEE (peptide 2; $M_r$, 2768), which also spans this cleavage site. ADAM10 (but not ADAM17) effectively cleaved peptide 2 to produce two fragments (CKQNYVMAF ($M_r$, 1190.2) and YKHLGIEFMEAEE ($M_r$, 1595.4); similar mass spectrum not shown), further confirming the above cleavage site. Our mapping results indicate that ADAM10 and ADAM17 have different Nrg1 cleavage patterns *in vitro*. The ADAM10 cleavage site is located eight residues upstream of the BACE1 cleavage site. The close proximity
Neuregulin Signaling via BACE1 and ADAM10 Cleavage

Table 1: Cleavage sites of BACE1 substrates

| Protein substrate | Substrate recognition site | Ref. |
|-------------------|---------------------------|------|
| APP               | KM (for APP cleavage site) | 33   |
| ST6Gal-1          | ASGMAVKEQSKPMQFEKAQ (for ST6Gal-1 cleavage site) | 35   |
| PSGL-1            | VTHKGMASNL (for PSGL-1 cleavage site) | 34   |
| Nrg1 (type I and III β1a) | GDRQNYVMASF (for Nrg1 cleavage site) | 12   |
| Nrg3              | FLKTDILSPTDLH (for Nrg3 cleavage site) | 12   |
| IL-1 receptor-2   | APP K (for IL-1 receptor-2 cleavage site) | 12   |
| Navβ2             | IMNPPDRHGGKHIH (for Navβ2 cleavage site) | 12   |

FIGURE 3. Cleavage of Nrg1 variants by BACE1 and ADAM10. A, type I Nrg1 and its various mutants were transiently expressed in HM cells stably expressing BACE1 for 48 h, and prepared lysates were resolved by electrophoresis. Under these conditions, Nrg1-ctfs were separated into two bands on Western blots. Mutation of the BACE1 cleavage site in Nrg1, such as deletion of residues ME (Nrg1-ΔME) or replacing F (Nrg1-FK) with K (Nrg1-ΔAA) or A (Nrg1-ΔAA), reduced the faster migrating bands; whereas the slower migrating band, specified as Nrg1-ctfl, was increased. Nonspecific bands are indicated by the arrowhead. B, in separate transfection experiments, HM cells were treated with BACE1 inhibitor IV (β-i) or GM6001 (α-i) during the transfection. BACE1 inhibition essentially abolished the production of Nrg1-ctfl in the case of Nrg1-ΔAA, further confirming that the faster migrating band is due to BACE1 cleavage. BACE1 inhibition also elevated the levels of full-length Nrg1 in cells transfected with both wild-type and mutant Nrg1.

of the cleavages by these two endopeptidases also explains why the Nrg1-nft is not abolished in BACE1-null mice.

In comparison with the cleavages of APP by BACE1 and ADAM10 in producing APP-CTF99 (99 amino acids) and APP-CTF83 (83 amino acids) fragments, BACE1 surprisingly cleaved Nrg1 at a site downstream (rather than upstream) of the ADAM10 cleavage site (Table 1). To ensure the authenticity of the Nrg1 cleavages produced by these two proteases, we optimized electrophoresis conditions to resolve proteins in the 40–80-kDa range to separate these two cleaved fragments on an SDS gel. Consistent with our expectations, the ADAM10-cleaved Nrg1 C-terminal fragment (Nrg1-ctfβ) migrated slower on the gels because Nrg1-ctfα is slightly longer than BACE1-cleaved Nrg1-ctfβ. Type I Nrg1 was chosen for this extensive characterization due to the structural similarity to APP, and constructs expressing either wild-type Nrg1 or type I Nrg1 variants with mutations surrounding the BACE1 cleavage site were generated for analyses in HM cells, which were generated by stably expressing HA-tagged BACE1 in HEK-293 cells (17). We observed that the C-terminal fragments of wild-type Nrg1, detected by an antibody recognizing the Nrg1 C terminus, could be separated on Western blots under our optimized conditions (Fig. 3). Because of the high BACE1 activity in HM cells, wild-type Nrg1 was more favorably processed by BACE1, and the faster migrating band near 51 kDa was therefore predominant (Fig. 3A, second lane). This fast migrating band was not easily discernible if wild-type Nrg1 was transected in HEK-293 cells, which express very low levels of endogenous BACE1 (data not shown). When the BACE1 cleavage site was mutated in three cases as shown in the third through fifth lanes, this faster migrating band was evidently decreased, whereas slower migrating fragments became apparent, indicating that these Nrg1 variants with disrupted BACE1 cleavage sites were favorably cleaved by α-secretase, perhaps mainly by ADAM10. On the other hand, when the ADAM10 cleavage site SFV was replaced by four Ala residues, this Nrg1 variant was favorably processed by BACE1 to yield a faster migrating band corresponding to the BACE1-cleaved product (Fig. 3A, sixth lane).

To further confirm this, wild-type and mutant Nrg1 plasmids were transfected in HM cells, which were then treated with either GM6001 (for metalloprotease inhibition) or BACE1 inhibitor IV (for BACE1 inhibition). On these gels, the levels of the faster migrating BACE1-cleaved C-terminal fragment (Nrg1-ctfβ) were clearly diminished when HM cells were treated with BACE1 inhibitor (Fig. 3B). On the other hand, this inhibition of BACE1 activity significantly increased both Nrg1-ctfα and full-length Nrg1 (Fig. 3B). Treatment of cells with the metalloprotease pan-inhibitor GM6001 further reduced or altered Nrg1-ctfα.

Clearly, processing of Nrg1 by BACE1 and ADAM10 is competitive, and this competitive processing is similarly observed with APP cleavage (24). Taken together, our results provide biochemical evidence that Nrg1 is indeed shed by ADAM10 at a site upstream of the BACE1 cleavage site, as the ADAM10-cleaved C-terminal fragment migrated slower than the BACE1-cleaved C-terminal fragment on a Western blot (Fig. 3).

Knockdown of ADAM10 Expression—To determine whether ADAM10 is the protease that cleaves Nrg1 in vivo, we knocked down ADAM10 expression by treating HEK-293 cells stably expressing the type I β1 Nrg1 isoform with specific siRNA duplexes. Fig. 4 shows that ADAM10 and ADAM17 siRNA duplexes specifically knocked down the levels of mature

FIGURE 3. Cleavage of Nrg1 variants by BACE1 and ADAM10. A, type I Nrg1 and its various mutants were transiently expressed in HM cells stably expressing BACE1 for 48 h, and prepared lysates were resolved by electrophoresis. Under these conditions, Nrg1-ctfs were separated into two bands on Western blots. Mutation of the BACE1 cleavage site in Nrg1, such as deletion of residues ME (Nrg1-ΔME) or replacing F (Nrg1-FK) with K (Nrg1-ΔAA), reduced the faster migrating bands; whereas the slower migrating band, specified as Nrg1-ctfβ, was increased. Nonspecific bands are indicated by the arrowhead. B, in separate transfection experiments, HM cells were treated with BACE1 inhibitor IV (β-i) or GM6001 (α-i) during the transfection. BACE1 inhibition essentially abolished the production of Nrg1-ctfβ in the case of Nrg1-ΔAA, further confirming that the faster migrating band is due to BACE1 cleavage. BACE1 inhibition also elevated the levels of full-length Nrg1 in cells transfected with both wild-type and mutant Nrg1.
ADAM10 and ADAM17, respectively. Full-length Nrg1 in ADAM10 siRNA duplex-treated samples was clearly elevated, whereas the cleaved Nrg1-ctf was reduced in comparison with control samples treated with nonspecific siRNA duplexes (Fig. 4). ADAM17 siRNA duplexes showed a relatively weak effect on the cleavage of Nrg1, as changes in both full-length Nrg1 and cleaved C-terminal fragments were relatively smaller (Fig. 4), consistent with the mapping result that ADAM17 is less effective at this site. Consistently, the level of CTF83, the APP C-terminal fragment released after ADAM10 cleavage, was significantly reduced. Because the endogenous protease activities of ADAM10 and ADAM17 are much higher than that of BACE1 in HEK-293 cells, it is not surprising that both BACE1-cleaved Nrg1 and APP fragments were not easily detectable. Taken together, these data indicate that ADAM10 can shed Nrg1 in a fashion similar to APP in cultured cells.

**FIGURE 4.** Knocking down the expression of ADAM10 and ADAM17. A, HEK-293 derivative cells stably expressing type I Nrg1 were treated with the indicated siRNA duplex targeting either ADAM10 (ADAM10-Si) or ADAM17 (ADAM17-Si). Each siRNA duplex has specific sequences, with two targeting ADAM10 and two targeting ADAM17. Forty-eight h after transfection, equal amounts of prepared lysates were examined by Western blot analysis. The effect of lower expression of ADAM10 or ADAM17 on APP processing was detected using APP C terminus-specific antibody (A8717), whereas Nrg1 C terminus-specific antibody was used to assess Nrg1 processing by ADAM proteases. Nonspecific bands are indicated by the arrowhead. B and C, the relative levels of Nrg1-ctf were calculated from three independent transfection experiments, and the results reflect the average of two treated siRNA oligonucleotides. *, p < 0.05; **, p < 0.01 (Student’s t test). Nrg1-fl, full-length Nrg1; con-Si, control siRNA.

ADAM10 and ADAM17 are much higher than that of BACE1 in HEK-293 cells, it is not surprising that both BACE1-cleaved Nrg1 and APP fragments were not easily detectable. Taken together, these data indicate that ADAM10 can shed Nrg1 in a fashion similar to APP in cultured cells.

Signaling Activity of N-terminal Fragments—Although it was clear that both BACE1 and ADAM10 could cleave type I and III β1 Nrg1, it was unclear whether the shed fragments would similarly activate ErbB receptors and initiate signaling cascades. To address this, we expressed type I and III β1 Nrg1 (illustrated in Fig. 5A) in breast cancer MCF-7 cells, which express the ErbB4 receptor (25). Expression of either type I or III Nrg1 activated Akt and ERK, as the levels of phosphorylated Akt and ERK were elevated in transfected cells (Fig. 5B). However, the signaling activity of type I Nrg1 was significantly weaker compared with that of type III Nrg1. This weak signaling effect is likely due to the fact that type I Nrg1-ntf and Nrg1-ntfβ are released into the conditioned medium, whereas type III Nrg1-ntf and Nrg1-ntfβ remain tethered on the membrane. The juxtacrine signaling activity of membrane-tethered type III Nrg1-ntf and Nrg1-ntfβ may be more effective than the paracrine signaling activity of type I Nrg1-ntf and Nrg1-ntfβ.

To determine whether type III Nrg1-ntf and Nrg1-ntfβ would similarly activate ErbB4 receptors, we inserted a stop codon after the ADAM10 or BACE1 cleavage site in a type III Nrg1 expression construct. Expression of these mutant constructs produced either Nrg1-ntfα or Nrg1-ntfβ (illustrated in Fig. 5A). Overexpression of either Nrg1-ntfα or Nrg1-ntfβ in the human breast cancer cell line MCF-7 showed clear elevation of both phosphorylated Akt and phosphorylated ERK, whereas overexpression of full-length type III β1 Nrg1 appeared to be even more potent in activating Akt (Fig. 5C). These results indicate that both Nrg1-ntfα and Nrg1-ntfβ are capable of binding to ErbB receptors and activating the signaling molecules Akt and ERK.

**BACE1 and ADAM10 Inhibition in Myelination**—In BACE1-null mice, the reduced levels of type III β1 Nrg1-ntf were pos-
tulated to cause hypomyelination (11, 13). Because ADAM10 can process Nrg1 at a site near the BACE1 cleavage site, we asked whether inhibition of ADAM10 activity would affect myelination. To address this, we employed an in vitro myelination model to assess the role of ADAM10 in myelination. The in vitro myelination model was established by co-culturing Schwann cells and DRG neurons isolated from embryonic rats according to published procedures (19–21). We generated a lentiviral expression vector carrying siRNA targeting ADAM10. Transfection of this lentiviral expression vector in co-cultures for 48 h showed a clear reduction of ADAM10 protein levels, and this reduction remained evident when lentivirus-infected co-cultures were examined 5 weeks later (Fig. 6A). Although there was a significant reduction of ADAM10 expres-

FIGURE 5. Activation of signaling molecules by the BACE1- or ADAM10-cleaved Nrg1-ntf. A, schematic illustration of the processing of type III β1 Nrg1. The cleavage sites of Nrg1 by ADAM10 and BACE1 are specified by arrows; the corresponding N-terminal fragments (type III Nrg1-ntfα or type III Nrg1-ntfβ) are indicated. The putative cleavage site of Nrg1 by γ-secretase is likely within the lipid bilayer based on its cleavage of APP. CRD, cysteine-rich domain. B, human breast cancer MCF-7 cells were transfected with type I or III Nrg1 expression constructs. After 48 h of transfection, protein lysates were prepared for Western blot analyses with antibodies to total and phosphorylated (p) Akt and ERK. C, human breast cancer MCF-7 cells were transfected with plasmid DNA expressing type III β1 Nrg1-ntfα, Nrg1-ntfβ, or full-length (fl) Nrg1 for 48 h, and cell lysates were prepared for Western blot analyses. Nrg1-ntfα and Nrg1-ntfβ were not easily separable under these electrophoresis conditions, likely due to glycosylation. Like full-length Nrg1, both fragments similarly activated Akt and ERK by increasing their phosphorylation. Anti-Nrg1 antibody also recognizes bands in all cells, and these nonspecifically reacted proteins served as natural loading controls.
sion in the treated co-cultures, the myelination in 6-week treated co-cultures was not significantly different from that in the sham-treated co-cultures based on the staining of myelin by an antibody specific to MBP (Fig. 6B). This observation is consistent with a recently published study (26).

In addition, we also explored the in vitro effect of a BACE1 inhibitor or a pan-ADAM inhibitor (GM6001) on myelination. During the 6-week co-culture procedure, axons of cultured DRG neurons were visibly myelinated by purified Schwann cells (Fig. 7). However, this in vitro myelination of DRG axons was significantly reduced when the same co-cultures were treated with BACE1 inhibitor IV for 5 weeks (n = three independent experiments) (Fig. 7). BACE1 inhibitor IV is a commonly used potent inhibitor of BACE1 (27, 28), and it can effectively reduce the processing of overexpressed Nrg1 by BACE1 (12). The inhibited myelination in BACE1-treated co-cultures was not due to cellular toxicity because large number of Schwann cells could contact DRG axons, although myelination occurred sporadically (Fig. 7). Hence, BACE1 inhibitor IV does not appear to inhibit the growth of Schwann cells and DRG neurons but instead causes delayed or ineffective myelination in co-cultures. In contrast, myelination was comparable to control levels when co-cultures were treated with the metallopeptase inhibitor GM6001 (Fig. 7). GM6001 is known to inhibit a broad spectrum of metalloproteinases and has been previously shown to inhibit the processing of Nrg1 (12, 15). Collectively, it appears that specific down-regulation of ADAM10 or inhibition of metaloproteases is not sufficient to alter normal myelination, in stark contrast to the inhibition of BACE1.

**DISCUSSION**

Nrg1 exerts its signaling activity by binding to its cognate receptors, ErbB2/ErbB3 heterodimers or ErbB4 homodimers. For membrane-bound Nrg1, proteolytic cleavage is required to release either the soluble type I Nrg1-ntf or the membrane-tethered type III Nrg1-ntf. The soluble Nrg1-ntf freely binds to the ErbB receptor on the cell surface, whereas the membrane-tethered Nrg1-ntf may bind to the ErbB receptor in a juxtacrine fashion. Despite the presence of many isoforms, only type I and III β1 Nrg1 isoforms are processed by BACE1 at a site in close proximity to the transmembrane domain (12). These Nrg1 isoforms can also be processed by ADAM10 at a site eight residues upstream of the BACE1 cleavage site (Table 1); both cleavage sites are completely conserved among vertebrate Nrg1 orthologs (see Fig. 8 in Ref. 12). In our experiments, ADAM10 cleaves type I and III β1 Nrg1 at only one site, as no additional N-terminal fragments were detectable by either Coomassie Blue staining (Fig. 1) or Western blotting (Fig. 2) after this cleavage. Upon either ADAM10 or BACE1 cleavage, the released EGF-like domain-containing Nrg1-ntf or Nrg1-ntfβ can activate ErbB receptors to initiate signaling cascades.

Although ADAM10 and BACE1 cleave Nrg1 at adjacent sites to release signaling N-terminal fragments, inhibition or down-regulation of these two proteases displays differential effects on normal myelination. We found that inhibition of BACE1 activity by the potent BACE1 inhibitor IV could delay or reduce myelination in an in vitro myelination model (Fig. 6). However, inhibition of ADAM activity by GM6001 or significant reduction of ADAM10 protein levels via an RNA silencing approach had no obvious effect on myelination. These in vitro observations are consistent with results obtained from ADAM10 knock-out animal models. BACE1-null mice exhibit hypomyelination of axons in both the peripheral and central nervous systems (11, 13), whereas ADAM10-null mice display no obvious alteration of myelination (29). Several possible explanations may account for this differential effect. First, BACE1 is perhaps the sole protease shedding Nrg1 at the F↓M site, whereas several ADAM proteases perhaps shed Nrg1 at the F↓Y site. Although we demonstrated that ADAM10 was more potent than ADAM17 in shedding Nrg1 and a recent study also demonstrates that ADAM10 is a physiologically relevant α-secretase that processes APP constitutively (30), we cannot exclude the compensatory processing of Nrg1 by ADAM17 or other ADAM proteases. Second, both ADAM10 and ADAM17 can function as an α-secretase (22, 23), an observation similar to that obtained in this study. Despite the fact that ADAM10 is a more abundant protease in the axon (26), the presence of ADAM17 and other not yet characterized ADAM proteases may compensate for the loss of ADAM10 in ADAM10-null mice. Third, the treatment of co-cultures with 10 μM BACE1 inhibitor IV (IC$_{50}$ = 15 nM for BACE1 and 230 nM for BACE2) is perhaps potent enough to inhibit most of the BACE1 activity (31), whereas the treatment of co-cultures with 20 μM GM6001 (K$_{i}$ = 27–500 nM for various metalloproteinases) is perhaps not potent enough to completely block ADAM activity. In cultured cells, treatment with BACE1 inhibitor IV can reduce BACE1 activity by >85% (32). On the other hand, we found that GM6001-treated cells showed only a small reduction of the Nrg1-ntf (12). Applying high doses of GM6001 causes cellular toxicity. Finally, neurons express high levels of BACE1, especially during early developmental stages (13). The high BACE1 activity, together with other ADAM candidates, may be sufficient to compensate for the loss of ADAM10.

In this study, we have provided biochemical evidence that Nrg1 is processed near the BACE1 cleavage site and releases a signaling Nrg1-ntf when processed by ADAM10. Although ADAM10 is expressed in high abundance in the peripheral nerve (26), it is not critical for normal myelination, as inhibiting ADAM10 activity does not affect myelination. In contrast, BACE1-null mice display hypomyelination in response to the reduced cleavage of Nrg1. We therefore conclude that the proteolytic activity of BACE1 is more important than that of ADAM10 in the control of myelination. Because the Nrg1-ErbB receptor pathway is implicated in various neuronal functions, this study further highlights the importance of understanding BACE1-dependent Nrg1 signaling, especially in the case of significant BACE1 inhibition in vivo.

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**REFERENCES**

1. Burden, S., and Yarden, Y. (1997) Neuron 18, 847–855
2. Gassmann, M., and Lemke, G. (1997) Curr. Opin. Neurobiol. 7, 87–92
3. Mei, L., and Xiong, W. C. (2008) Nat. Rev. Neurosci. 9, 437–452
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4. Meyer, D., and Birchmeier, C. (1995) Nature 378, 386–390
5. Stefansson, H., Sigurdsson, E., Steinhorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Gudmundsdottir, S., Bjornsdottir, S., Ivarsson, O., Chou, T. T., Hjaltason, O., Birgisdottir, B., Jonsson, H., Gudnadottir, V. G., Gudmundsdottir, E., Bjornsdottir, A., Ingvarsson, B., Ingason, A., Sigfusson, S., Hardardottir, H., Harvey, R. P., Lai, D., Zhou, M., Brunner, D., Mutel, V., Gonzalez, A., Lemke, G., Sainz, J., Johansson, G., Andresson, T., Gudbjartsson, D., Manolescu, A., Frigge, M. L., Gurney, M. E., Kong, A., Gulcher, J. R., Petursson, H., and Stefansson, K. (2002) Am. J. Hum. Genet. 71, 877–892
6. Nave, K. A., and Salzer, J. L. (2006) Curr. Opin. Neurobiol. 16, 492–500
7. Nave, K. A., and Trapp, B. D. (2008) Annu. Rev. Neurosci. 31, 535–561
8. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Nave, K. A., and Salzer, J. L. (2006)
9. Hu, X., He, W., Diaconu, C., Tang, X., Kidd, G. J., Macklin, W. B., Trapp, B. D., and Yan, R. (2003) J. Biol. Chem. 278, 21918–21926
10. Kramer, R., Bucay, N., Kane, D. J., Martin, L. E., Tarpley, J. E., and Theill, L. E. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 4833–4838
11. Hu, X., Hicks, C. W., He, W., Wong, P., Macklin, W. B., Trapp, B. D., and Yan, R. (2008) FEBS J. 22, 2970–2980
12. Hu, X., He, W., Diaconou, C., Tang, X., Kidd, G. J., Macklin, W. B., Trapp, B. D., and Yan, R. (2008) FASEB J. 22, 2970–2980
13. Willem, M., Garratt, A. N., Novak, B., Citron, M., Kaumann, S., Ritter, A., DeStrooper, B., Saftig, P., Birchmeier, C., and Haass, C. (2006) Science 314, 664–666
14. Fricker, F. R., Lago, N., Balarajah, S., Tsantoulas, C., Tanna, S., Zhu, N., Fageiry, S. K., Jenkins, M., Garratt, A. N., Birchmeier, C., and Bennett, D. L. (2011) J. Neurosci. 31, 3225–3233
15. Taveglia, C., Zanazzi, G., Petroylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R. M., Loeb, J. A., Shager, P., Chao, M. V., Falls, D. L., Role, L., and Salzer, J. L. (2005) Neuron 47, 681–694
16. Ozaki, M., Itoh, K., Miyakawa, Y., Kishida, H., and Hashikawa, T. (2004) J. Neurochem. 91, 176–188
17. Yan, R., Han, P., Miao, H., Greengard, P., and Xu, H. (2001) J. Biol. Chem. 276, 36788–36796
18. Tomasselli, A. G., Qahwash, I., Emmons, T. L., Lu, Y., Leone, J. W., Lull, J. M., Fok, K. F., Bannow, C. A., Smith, C. W., Bienkowski, M. J., Heinrikson, R. L., and Yan, R. (2007) J. Neurochem. 84, 1006–1017
19. Lee, X., Yang, Z., Shao, Z., Zoll, S., Xiong, Y., Lai, D., Zhou, M., Brunner, D., Mutel, V., Gonzalez, A., Lemke, G., Sainz, J., Johansson, G., Andresson, T., Gudbjartsson, D., Manolescu, A., Frigge, M. L., Gurney, M. E., Kong, A., Gulcher, J. R., Petursson, H., and Stefansson, K. (2002) Am. J. Hum. Genet. 71, 877–892
20. Dhashashilvili, Y., Zhang, Y., Galinska, J., Lam, I., Grumet, M., and Salzer, J. L. (2007) J. Cell Biol. 177, 857–870
21. Kiyry-Seo, S., Ohno, N., Kidd, G. J., Komuro, H., and Trapp, B. D. (2010) J. Neurosci. 30, 6658–6666
22. Allinson, T. M., Parkin, E. T., Condon, T. P., Schwager, S. L., Sturrock, E. D., Turner, A. J., and Hooper, N. M. (2004) Eur. J. Biochem. 271, 2539–2547
23. Arai, H., Hattori, C., Szabo, B., Sasagawa, N., Maruyama, K., Tanuma, S., and Ishiura, S. (2003) Biochem. Biophys. Res. Commun. 301, 231–235
24. Neve, R. L. (2003) Trends Neurosci. 26, 461–463
25. Aqelan, R. I., Donati, V., Gaudino, E., Nicoloso, M. S., Sundvall, M., Korhonen, A., Lusin, J., Sudo, M., Joensuu, H., Croce, C. M., and El nieuys, K. (2007) Cancer Res. 67, 9330–9336
26. Jangouk, P., Dhemel, M., Meyer Zu Hörste, G., Ludwig, A., Lehmann, H. C., and Kieseier, B. C. (2009) Glia 57, 1765–1774
27. Brady, S. F., Singh, S., Crouthamel, M. C., Holloway, M. K., Coburn, C. A., Garisky, V. M., Bogusky, M., Pennington, M. W., Vacca, J. P., Hazuda, D., and Lai, M. T. (2004) Bioorg. Med. Chem. Lett. 14, 601–604
28. Pietrak, B. L., Crouthamel, M. C., Tugusheva, K., Lineberger, J. E., Xu, M., DiMuzio, J. M., Steele, T., Espeseth, A. S., Stachel, S. J., Coburn, C. A., Graham, S. L., Vacca, J. P., Shi, X. P., Simon, A. J., Hazuda, D. J., and Lai, M. T. (2005) Anal. Biochem. 342, 144–151
29. Freese, C., Garratt, A. N., Fahrenholz, F., and Endres, K. (2009) FEBS J. 276, 1568–1580
30. Kuhn, P. H., Wang, H., Dislich, B., Colombo, A., Zeitschel, U., Ellwart, J. W., Kremmer, E., Rossner, S., and Lichtenthaler, S. F. (2010) EMBO J. 29, 3020–3032
31. Stachel, S. J., Coburn, C. A., Steele, T. G., Jones, K. G., Lichenthaler, S. F., Gregro, A. R., Rajakapake, H. A., Lai, M. T., Crouthamel, M. C., Xu, M., Tugusheva, K., Lineberger, J. E., Pietrak, B. L., Espeseth, A. S., Shi, X. P., Chen-Dodson, E., Holloway, M. K., Munshi, S., Simon, A. J., Kuo, L., and Vacca, J. P. (2004) J. Med. Chem. 47, 6447–6450
32. Sankaranarayanan, S., Price, E. A., Wu, G., Crouthamel, M. C., Shi, X. P., Tugusheva, K., Tyler, K. X., Kahana, J., Ellis, J., Jin, L., Steele, T., Stachel, S., Coburn, C., and Simon, A. J. (2008) Pharmacol. Exp. Ther. 324, 957–969
33. Kitazueme, S., Tachida, Y., Oka, R., Kotani, N., Ogawa, K., Suzuki, M., Dohmns, N., Takio, K., Saio, T. C., and Hashimoto, Y. (2003) J. Biol. Chem. 278, 14865–14871
34. Lichtenthaler, S. F., Dominguez, D. I., Westmeyer, G. G., Reiss, K., Haass, G. C., Saftig, P., De Strooper, B., and Seed, B. (2003)
35. Kuhn, P. H., Wang, H., Dislich, B., Colombo, A., Zeitschel, U., Ellwart, J. W., Kremmer, E., Rossner, S., and Lichtenthaler, S. F. (2010) EMBO J. 29, 3020–3032
36. Gersbacher, M. T., Kim, D. Y., Bhattacharyya, R., and Kovacs, D. M. (2010) Mol. Neurodegener. 5, 61