Roles of Meltrin β/ADAM19 in the Processing of Neuregulin*

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Meltrin β/ADAM19 is a member of ADAMs (a disintegrin and metalloproteases), which are a family of membrane-anchored glycoproteins that play important roles in fertilization, myoblast fusion, neurogenesis, and proteolytic processing of several membrane-anchored proteins. The expression pattern of meltrin β during mouse development coincided well with that of neuregulin-1 (NRG), a member of the epidermal growth factor family. Then we examined whether meltrin β participates in the proteolytic processing of membrane-anchored NRGs. When NRG-β1 was expressed in mouse L929 cells, its extracellular domain was constitutively processed and released into the culture medium. This basal processing activity was remarkably potentiated by overexpression of wild-type meltrin β, which lead to the significant decrease in the cell surface exposure of extracellular domains of NRG-β1. Furthermore, expression of protease-deficient mutants of meltrin β exerted dominant negative effects on the basal processing of NRG-β1. These results indicate that meltrin β participates in the processing of NRG-β1. Since meltrin β affected the processing of NRG-β4 but not that of NRG-α2, meltrin β was considered to have a preference for β-type NRGs as substrate. Furthermore, the effects of the secretory pathway inhibitors suggested that meltrin β participates in the intracellular processing of NRGs rather than the cleavage on the cell surface.

Various intracellular signaling and adhesion molecules govern the cell-cell interactions during the development of multicellular organisms. The actions of these molecules are regulated not only by transcriptional and translational controls but also by post-translational modifications such as phosphorylations and proteolytic processings. Numerous membrane-anchored signaling molecules are subjected to proteolytic processing to release their extracellular domains. Such modifications may cause qualitative and irreversible changes in the functions of these molecules.

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The abbreviations used are: ADAM, a disintegrin and metalloproteases; EGF, epidermal growth factor; DRG, dorsal root ganglia; TACE, tumor necrosis factor-α converting enzyme; NRG, neuregulin-1; PHS, phosphatase-buffered saline; RF-PCR, reverse transcriptase-polymerase chain reaction; HA, hemagglutinin; PAG, polyacrylamide gel electrophoresis; CM, conditioned medium; GFP, green fluorescent protein; PKC, protein kinase C.

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family. NRGs mediate an array of biological effects, including the synthesis of acetylcholine receptors in skeletal muscle (19) and the stimulation of Schwann cell growth (17). These biological effects of NRGs are mediated by the ErbB family of tyrosine kinase receptors (20, 21). Gene disruption studies indicate that NRGs are essential for early heart and central nervous system development (22). A variety of different protein isoforms are produced from the single NRG gene via alternative splicing mechanisms. All isoforms contain an EGF-like domain sufficient for biological activity. Although alternatively spliced transcripts also generate some secreted isoforms (17), most soluble NRGs are derived from membrane-anchored precursor proteins via proteolytic cleavage of the extracellular region including EGF-like domain. It has been reported that this processing occurs in intracellular organelas (23). However, the nature of the processing enzyme remains elusive.

In this study, we examined whether meltrin β participates in the processing of membrane-anchored NRGs. First, both meltrin β and NRG proteins were expressed in DRG neurons at the same stages of mouse embryogenesis. Next, overexpression of wild-type meltrin β significantly increased the release of soluble NRGs in culture medium and decreased the cell surface expression of the extracellular domains of NRG-β1. Furthermore, the processing of NRGs was abrogated by expression of protease-deficient mutants of meltrin β. Finally, the enhanced processing of NRGs by meltrin β was blocked by the treatment with brefeldin A but not by monensin, which suggested the action of meltrin β in the Golgi apparatus. Taken together, we concluded that meltrin β (or similar ADAM proteases) participates in the cleavage of membrane-anchored NRGs.

**EXPERIMENTAL PROCEDURES**

**Immunohistochemistry**—Anti-meltrin β antisera used in this study was raised in rabbits against a keyhole limpet hemocyanin-coupled peptide (PEYRSQVRGAISSSIK) corresponding to the extreme C-terminal sequence of meltrin β. E12.5 mouse embryos were dissected, rinsed with phosphate-buffered saline (PBS), incubated in PBS containing 20% sucrose at 4 °C overnight, and embedded in OCT compound (Tissue-Tek, Miles Inc.) on a dry ice block. Cryosections (6 μm in thickness) on 3-aminopropyltriethoxysilane-coated glass slides were prepared and fixed for 5 min in acetone at −20 °C. Antibodies were applied overnight at 4 °C in a humidified chamber in PBS containing 10% heat-inactivated normal goat serum at the following dilutions: anti-meltrin β antisera, 1:300; anti-NRG Ab-3 (NeoMarker), 1:500, and anti-neurofilament 160 (NF160, Sigma), 1:500. The slides were washed three times for 10 min each in PBS containing 0.05% Tween 20 (PBST), then incubated with secondary antibodies in PBS containing 10% heat-inactivated normal goat serum for 1 h at room temperature. The slides were then washed three times in PBST and mounted with PERMAFLOUR (ImmunoTech). The anti-meltrin β and anti-NRG immunoreactivities were amplified using biotinylated rabbit-antibody IgG (1:500 or 1:1,000 dilution, Vector Laboratories) and streptavidine-Cy3 (1:500 or 1:1,000 dilution, Jackson Immunoresearch). Fluorescein isothiocyanate-conjugated anti-mouse IgG (1:500 dilution, Jackson Immunoresearch) was used for the anti-NF160 immunoreactivity. Imaging was carried out using a Leica DM IRBE inverted confocal microscope using ×10 and ×40 objectives (Leica) and TCS-NT software (Leica).

**Cell Culture**—P19 rat embryonic carcinoma cells were cultured in minimum essential medium α medium supplemented with 10% fetal bovine serum. Mouse L929 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. All cultures were maintained at 37 °C in the presence of 5% CO2. To induce differentiation, P19 cells were cultured on bacterial grade dishes in growing medium. RT-PCR—mRNA was extracted from P19 cells using Micro-FastTrack 2.0 kit (Invitrogen). Two ng of mRNA was subjected to one RT-PCR reaction. Reverse transcription was carried out using SuperScript II reverse transcriptase (Life Technologies, Inc.). PCR reactions were performed using an annealing temperature of 50 °C for 20 cycles (NRG and meltrin β) or 55 °C for 15 cycles (glyceraldehyde-3-phosphate dehydrogenase). Signals are roughly proportional to the amount of cDNA under these conditions. The following primer pairs were used: 5'-ACATCAATCCACGCTTGGCAGCCACCTCT-3' and 5'-GCAGTAGGCCCCACACACATGTCCTGC-3' (NRG), 5'-GGCGAAATCTGGAGCGGAGACGACTCT-3' and 5'-ACATTGAATAAATA-3' (meltrin β), 5'-ACCAGCTCTTGACCATC-3' and 5'-TCCACACCTTGCTGTGA-3' (glyceraldehyde-3-phosphate dehydrogenase).

**Expression Plasmids and Transfection**—The full-length mouse NRG cDNAs were isolated using the primers corresponding to the nucleotide sequences (5'-GGCCTTGAACATGTCCTGAAAGCCAGC-3' and 5'-TCCCATCTTGCTGGA-3') from mouse neonatal muscle and E12.5 mouse embryo trunk cDNAs. The mouse NRG cDNAs were fused with a synthetic DNA cassette coding for the hemagglutinin (HA)-epitope tag (MYPYDVPDYA) and subcloned into pEF-BOS, which has the promoter region of the human EF-αa chromosomal gene (24), to obtain pEF-BOS-HA-NRGs. To obtain a protease-deficient (E347Q and H346A,H350A) meltrin β cDNAs and a metalloprotease domain-deleted (ΔMP) meltrin β cDNA was constructed by mutagenesis based on a PCR technique using mutated primers. In E347Q mutant meltrin β, glutamine is substituted for the conserved glutamic acid at position 347. In H346A,H350A mutant meltrin β, alanines are substituted for the conserved histidines at positions 346 and 350. In ΔMP meltrin β, amino acid residues 208–430 are deleted. The nucleotide sequences of the mutants were confirmed by direct sequencing. The cDNAs of wild-type and mutant meltrin β were subcloned into pEF-BOS. Wild-type meltrin β and ΔMP meltrin β cDNAs with HA tag were subcloned into pEF-BOS (12), pBIE plasmid was generated by deletion of the human cytomegalovirus promoter region of pIRE2-EGFP (CLONTECH) and replaced by the promoter region of pEF-BOS. Wild-type and ΔMP meltrin β cDNAs were subcloned into pBIE to generate pBIE-meltrin β and pBIE-ΔMP meltrin β, respectively. These plasmids were transfected by the LipofectAMINE PLUS method according to the manufacturer’s instructions (Life Technologies Inc.).

**Western Blot Analysis**—Before harvesting the conditioned medium, the cells were incubated in Opti-MEM (Life Technologies, Inc.) containing CaCl2 (110 μg/ml) for 12 h. The conditioned medium was initially filtered through a sterile filter unit (pore size: 0.2 μm, Millipore) and then concentrated up to 100-fold by centrifugation using a Centriplus 10 (Amicon) concentrator. Cells were extracted in extraction buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Nonidet P-40, 0.1% sodium deoxycholate) containing Complete™ protease inhibitor mixture (Roche Molecular Biochemicals). Extracts were clarified by centrifugation at 10,000 × g for 10 min. Protein concentration was determined using the Bradford method (Bio-Rad). Additionally, 20 μg of protein was loaded into a well. After SDS-PAGE, proteins were electroblotted onto Immobilon (Millipore). Anti-HA mouse monoclonal antibody (1:40 dilution; 12CA5, Roche Molecular Biochemicals), anti-meltrin β rabbit antisemur (1:500 dilution), and anti-C terminus of NRG rabbit polyclonal antibody (1:100 dilution; sc-348, Santa Cruz) were used as primary antibodies. After incubation with primary antibody, the blots were incubated with biotinylated anti-mouse or anti-rabbit IgG (1:2500 dilution, Jackson Immunoresearch) and then horseradish peroxidase-conjugated streptavidin (1:5000 dilution, Amersham Pharmacia Biotech). The blots were developed using the ECL plus system (Amersham Pharmacia Biotech). Prestained protein molecular weight marker was from Bio-Rad.

**Cell Staining**—The cells transfected with pEF-BOS-HA-NRG-β1 together with pBIE, pBIE-meltrin β, or pBIE-ΔMP meltrin β were incubated with anti-HA mouse monoclonal antibody (1.200 dilution; 16B12, Babco) at 4 °C for 30 min, and then washed four times with ice-cold PBS. After fixation with 4% paraformaldehyde in PBS for 15 min, the cells were incubated with Cy3-conjugated goat antibody to mouse IgG (1:400 dilution, Jackson Immunoresearch). The cells were then washed three times in PBS and mounted with PERMAFLOUR (ImmunoTech). Cell cultures were stained using biotinylated anti-rabbit IgG (1:500 or 1:1000 dilution, Vector Laboratories) and streptavidine-Cy3 (1:500 or 1:1000 dilution, Jackson Immunoresearch). Fluorescein isothiocyanate-conjugated anti-mouse IgG (1:500 dilution, Jackson Immunoresearch) was used for the anti-NF160 immunoreactivity. Imaging was carried out using a Leica DM IRBE inverted confocal microscope using ×10 and ×40 objectives (Leica) and TCS-NT software (Leica).

**Metabolic Labeling of Cells**—Cells were starved in medium lacking methionine and cysteine (ICN) for 1 h and pulse-labeled with L-[3S]methionine and -cysteine (EASYTAG express protein labeling mixture, PerkinElmer Life Sciences) at 0.1 μCi/ml. After a 1-h pulse, cells were either extracted in extraction buffer containing Complete™ protease inhibitor mixture or chased with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, the secretary pathway inhibitors, brefeldin A (10 μg/ml, Wako) and monensin (2 μg/ml, Wako), were added during the chase period. After a 5-h chase, cells were extracted in extraction buffer. Cell extracts were clarified by centrifugation at 15,000 × g for 20 min. The supernatants were incubated with anti-HA monoclonal antibody (16B12, Babco) for 30 min on ice. After the addition of protein G-Sepharose beads, the extracts were
incubated for 1 h on ice. Immunoprecipitates were washed six times with extraction buffer and subjected to SDS-PAGE.

RESULTS

Coincidental Expression of Meltrin β and NRGs—We previously reported the expression pattern of meltrin β mRNA during mouse embryogenesis (16). Meltrin β mRNA is markedly expressed in the regions where peripheral neuronal cell lineages differentiate including craniofacial and DRG and ventral horns of the spinal cord. In addition, heart, lung, skeletal muscle, and intestine express meltrin β mRNA transiently. This expression pattern of meltrin β coincides well with that of an EGF family growth factor, NRGs (17, 18). In this study, we further investigated the precise expression sites of NRGs and meltrin β proteins in the developing mouse nervous system. Adjacent transverse sections through mouse E12.5 embryo were coimmunostained with antibodies against neuronal marker, neurofilament 160 (NF160), and meltrin β (A-C) or NRGs (D-F). Asterisks and arrows indicate the neural tube and the DRG, respectively. A and D, anti-NF160 staining (green). B, anti-meltrin β staining (red). E, anti-NRGs staining (red). C, overlay of NF160 and meltrin β immunostaining. F, overlay of NF160 and NRGs immunostaining. Bar, 50 μm.

FIG. 1. Expression of meltrin β and NRGs proteins in dorsal root ganglia during mouse embryogenesis. Adjacent transverse sections through forelimb of E12.5 mouse embryos were coimmunostained with antibodies against neurofilament 160 (NF160) and meltrin β (A-C) or NRGs (D-F). Asterisks and arrows indicate the neural tube and the DRG, respectively. A and D, anti-NF160 staining (green). B, anti-meltrin β staining (red). E, anti-NRGs staining (red). C, overlay of NF160 and meltrin β immunostaining. F, overlay of NF160 and NRGs immunostaining. Bar, 50 μm.

and then the cells were dissociated and plated in the absence of retinoic acid. Cells were harvested at the indicated times, and the cell extracts were subjected to Western blotting using antibodies against the markers of neurons (microtubule-associated protein-2), glial cells (glial fibrillary acidic protein), and smooth muscle cells (smooth muscle actin). The expression of microtubule-associated protein-2 was increased at day 6 and then decreased gradually (data not shown). On the other hand, the expression of glial fibrillary acidic protein was increased during the differentiation period (data not shown). To examine the expression level of NRGs and meltrin β mRNAs, total mRNAs were prepared from cells at the indicated times, and subjected to RT-PCR (Fig. 2). NRGs and meltrin β mRNAs were both expressed at very low levels at day 0. The transcripts of these genes began to appear at day 6, then reached a maximum level at day 8. This similarity in the transcriptional profiles indicates a plausible interaction between meltrin β and NRGs. Many NRGs are derived from membrane-anchored precursor proteins via proteolytic cleavage. We therefore investigated whether the metalloprotease activity of meltrin β is involved in the processing of NRGs.

Proteolytic Processing of NRGs by Meltrin β—Alternative splicing of a single gene gives rise to multiple isoforms of NRG. Many of these encode transmembrane, glycosylated precursors of soluble NRGs. In this study, we used three transmembrane isoforms of NRG (α2, β1, and β4) and the domain structure of the NRGs used here is shown schematically in Fig. 3A. The extracellular portion of these NRGs contains an immunoglobulin motif (Ig), a glycosylated spacer domain (Glyco.), and an EGF-like domain (EGF) (25). The two major classes of NRGs diverge in the C terminus of the EGF-like domain giving rise to the α- and β-isoisofoms. Additional variation is seen in the juxtapembrane region following the EGF domain by the insertion of one of three different sequences (numbered 1, 2, or 4). To detect the ectodomains of NRGs released into the culture medium, the N terminus of NRGs was tagged with HA epitope.

Since DRG neurons express meltrin β and NRG simultaneously (Fig. 1), we first examined the processing of a neuronal type of NRG, NRG-β1. In this study, we used mouse L929 fibroblast which expresses a low level of endogenous meltrin β (data not shown). L929 cells were transfected with an expression plasmid encoding NRG-β1 and then the conditioned medium (CM) was subjected to Western blotting using anti-HA antibody. Released soluble NRG-β1 (~46 kDa) was detected in the CM of NRG-β1 expressing cells (Fig. 3B, lane 2). This released polypeptide could induce the tyrosine phosphorylation of ErbB2 and -3 when added to differentiated muscle cells, C2C12 (data not shown), which shows that this 46-kDa polypeptide is a functionally mature NRG-β1. The broad ap-
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Small proportion of unprocessed membrane-anchored NRGs expose their extracellular domains on the cell surface (23). To investigate the effect of meltrin β on appearance of the extracellular domains on the cell surface, the cells expressing HA-NRG-β1 together with or without meltrin β were stained with anti-HA antibody under the nonpermeabilized condition. In this experiment, another type of expression plasmids were constructed in which wild-type or ΔMP meltrin β was expressed together with green fluorescent protein (GFP) by inserting internal ribosomal entry site sequence between cDNAs encoding these proteins. The result shown in Fig. 3D showed that efficient exposure of the N-terminal HA-tag of HA-NRG-β1 significantly decreased in the cells expressing wild-type meltrin β. Such an effect could not be seen in ΔMP meltrin β expressing cells. Thus, enhanced processing of membrane-

**Fig. 3.** Meltrin β participates in the processing of NRG-β1. A, diagram of the domain organization of NRG isoforms used in this study. Boxes represent the major structural motifs of NRGs: an immunoglobulin-like domain (Ig), a glycosylated spacer domain (Glyco.), an EGF-like domain (EGF), and a transmembrane domain (TM). Variation among these isoforms occurs in the C terminus of the EGF-like domain and in the juxta-}

The appearance of processed NRG-β1 band might represent the variety of multiple N-linked and O-linked glycosylation in its spacer region (26). We further investigated whether coexpression of meltrin β affects the release of mature NRG-β1. Overexpression of wild-type meltrin β considerably increased the release of mature NRG-β1 (Fig. 3B, lane 3). Western blotting of cell extracts using the anti-C terminus of NRGs antibody showed that overexpression of wild-type meltrin β increased the ratio of processed cytoplasmic tail of NRG-β1 (74 kDa, open triangle) and decreased the ratio of full-length NRG-β1 (120 kDa, filled triangle) (Fig. 3C, upper panel, lane 3). These results strongly suggest that meltrin β could potentiate the basal processing activity of NRG-β1.

To investigate whether the meltrin β protease activity is necessary for the processing of NRG-β1, several mutants of meltrin β were constructed. In E347Q and H346A,H350A meltrin β, glutamine, and alanine residues were substituted for the glutamic acid and histidine residues, respectively, which are essential for the metalloprotease activity. In ΔMP meltrin β, metalloprotease domain is completely deleted. Western blotting using anti-meltrin β antibody revealed two immunoreactive species with apparent molecular masses of 125 and 100 kDa in the cell expressing E347Q meltrin β as shown in the cell expressing wild-type meltrin β (Fig. 3C, lower panel, lanes 3 and 4). The 100-kDa form is considered to be generated by removal of the prodomain from the 125-kDa form, probably by a furin-like pro-protein convertase, which cleaves ADAMs at the sequence motif RXRR in a late Golgi compartment (27, 28). Western blotting of the cells expressing H346A,H350A meltrin β revealed mainly the 125-kDa unprocessed form (Fig. 3C, lower panel, lane 5).

Expression of E347Q meltrin β made no change in the basal processing of NRG-β1 (Fig. 3, B, lane 4, and C, upper panel, lane 4). This observation clearly demonstrates that protease activity of meltrin β is essential for the increase of NRG-β1 processing. On the other hand, expression of H346A,H350A meltrin β remarkably suppressed the release of mature NRG (Fig. 3B, lane 5). At the same time, expression of H346A,H350A meltrin β increased the ratio of the unprocessed form of NRG-β1 and decreased the ratio of its processed cytoplasmic tail in the cells (Fig. 3C, upper panel, lane 5). Expression of ΔMP meltrin β decreased the production of NRG-β1 by unknown reasons (data not shown). However, Western blotting of an increased amount of the extract revealed that expression of ΔMP meltrin β also increased the ratio of the unprocessed form of NRG-β1 and decreased the ratio of the processed form of NRG-β1 (Fig. 3C, upper panel, lane 6). Thus, expression of these mutants of meltrin β exert dominant negative effects on the basal processing of NRG-β1. Taken together, these results indicate that meltrin β participates in the processing of NRG-β1 through its metalloprotease activity.
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FIG. 4. Substrate specificity of meltrin β. A, plasmids encoding several isoforms of NRG were transfected to L929 cells together with plasmid encoding wild-type or H346A,H350A (mut) meltrin β. CM was subjected to Western blotting using anti-HA antibody as described in Fig. 3B. B, L929 cells were transfected with plasmids encoding NRG-α2 (upper panel) or NRG-β1 (lower panel) and meltrin β. Cells were labeled for 1 h with [35S]methionine/cysteine (PerkinElmer Life Sciences) and immediately frozen (0) or chased with cold media for 5 h (5). Cells were extracted, and NRGs were immunoprecipitated from extracts with anti-HA antibody. All samples were subjected to SDS-PAGE. Arrowheads indicate the migration of full-length NRGs.

Brefeldin A-sensitive and monensin-insensitive cleavage of NRG-β1 by meltrin β. Plasmids encoding NRG-β1 were transfected to L929 cells together with plasmid encoding wild-type or H346A,H350A (mut) meltrin β. Cells were labeled for 1 h and then chased with cold media for 5 h in the absence or presence of 10 µg/ml brefeldin A or 2 µg/ml monensin. Cells were extracted, and NRGs were immunoprecipitated from extracts with anti-HA antibody. All samples were subjected to SDS-PAGE and autoradiography. Arrowheads indicate the migration of full-length NRGs.

NRGs mediate a variety of biological functions including glial cell development, synaptogenesis, and cardiac development through the activation of the ErbB family of tyrosine kinase receptors (33). Most NRG isoforms encode membrane-anchored proteins that generate soluble ligands for the ErbB family by proteolytic cleavages. It is not yet clear, however, whether the functions of NRGs depend on actions of processed and released soluble NRGs or whether the transmembrane form is biologically active. Genetic disruption of only the intracellular domain of membrane-anchored NRG isoforms results in a similar phenotype of embryonic maldevelopment to that observed with disruption of the entire gene (34). Furthermore, deletion of the cytoplasmic tail of membrane-anchored NRGs completely abrogated the release of mature NRGs (34). These results strongly suggest that the proteolytic processings of membrane-anchored NRGs are critical regulatory mechanisms...
of NRG functions.

In the present study, we provided evidence that meltrin β participates in the processing of β-type NRGs. Initially, both meltrin β and NRG proteins were found to be expressed in dorsal root ganglia at the same stages during embryogenesis (Fig. 1). During neurogenic differentiation of P19 cells, the expression of meltrin β and NRGs mRNA was activated in a similar fashion (Fig. 2). Next, overexpression of wild-type meltrin β potentiated the release of mature soluble NRG-β1 (Fig. 3B, lane 3) with a concomitant decrease in the cell surface expression of extracellular domains of NRG-β1 (Fig. 3D). The protease activity of meltrin β is indispensable for the potentiation of NRG-β1 processing (Fig. 3, B and C, lane 4). Furthermore, expression of H346A,H350A or ΔMP meltrin β remarkably suppressed the release of soluble NRG-β1 (Fig. 3B, lanes 5 and 6) with a concomitant increase in the ratio of full-length NRG-β1 and a decrease in the ratio of processed forms of NRG-β1 in the cells (Fig. 3C, lanes 5 and 6). We further confirmed the enhanced processing of NRG-β1 with meltrin β protease by the pulse-chase experiment shown in Fig. 4B. These results clearly demonstrate that meltrin β has functional processing activity of NRG-β1 and that the protease activity of meltrin β is necessary for constitutive processing of NRG-β1. This is the first report on the function of meltrin β and, at the same time, the first report that indicates the involvement of ADAM metalloproteases in the proteolytic processing of membrane-anchored NRGs. It is considered that meltrin β plays a pivotal role in the development of several organs through the processing of NRGs.

As reported previously, NRG-α2 is the predominant isoform in mesenchymal cells, whereas NRG-β1 is the major neuronal isoform (35). The main cleavage sites in these NRG molecules are in exon-α and exon-β, respectively (28). While L299 cells possess endogenous proteolytic processing activities for both α- and β-type NRGs, both overexpression of wild-type and H346A,H350A meltrin β only affected the cleavage of β-type NRGs. It is plausible that α-type NRG is cleaved by a pro tease(s) other than meltrin β in L299 cells. Alternatively, L299 cells may lack some regulatory factors that cooperate with overexpressed meltrin β to cleave α-type NRG efficiently.

Meltrin β expressed in L299 cells was mainly localized in the Golgi apparatus (data not shown) although intracellular localization of meltrin β remains to be determined precisely. Examination of the effects of brefeldin A and monensin on the processing revealed that meltrin β participates in the intracellular processing of NRGs, probably in the Golgi apparatus or in monensin-insensitive secretory pathways. Recently, several reports demonstrated that some ADAMs are processed and activated in the trans-Golgi network (27, 28), and localized mainly in the Golgi apparatus (13, 27). Furthermore, Skovronsky et al. (36) have found activity of TACE and/or Kuzbanian in the trans-Golgi network. These observations and our results indicate that multiple ADAMs function in the trans-Golgi network as intracellular processing enzymes.

Expression of H346A,H350A or ΔMP meltrin β markedly suppressed the basal processing activity of NRG-β1 (Fig. 3). Genetic and biochemical characterization of other ADAM pro teases also indicated such dominant negative effects of protease-deficient mutants (11–14, 37). In preliminary experiments, we found that small proportion of meltrin β and NRGs expressed in L299 cells could be communoprecipitated (data not shown). H346A,H350A and ΔMP meltrin β might show dominant-negative effects through the interaction with NRGs, thereby blocking the interaction of endogenous proteases with NRGs. On the other hand, expression of E347Q meltrin β did not affect the basal processing activity (Fig. 3). As shown in Fig. 3C, the prodomain of E347Q meltrin β is removed precisely while those of H346A,H350A and ΔMP meltrin β are not removed. These meltrin β mutants might have different conformation from wild-type or E347Q meltrin β, and their conformational abnormality might affect endogenous meltrin β or similar proteases to act on NRG-β1. The identification of the domain of meltrin β required for the dominant negative effect on the processing will provide further insight into the mechanism by which meltrin β recognizes and processes NRG-β1.

Phorbol ester induces the processing of several membrane-anchored proteins through the activation of protein kinase C (PKC). As reported previously in other cell types, we found that phorbol ester induces the processing and release of mature soluble NRG-β1 in L299 cells (Ref. 23, and data not shown). This induced processing was not suppressed by expression of H346A,H350A mutant of meltrin β (data not shown). Our observation indicates that meltrin β accounts for the constitutive processing but not for the PKC-regulated processing of NRG-β1. Thus, distinct pathways for the processing of NRG-β1 are suggested: one pathway is dependent on meltrin β protease while, in the other PKC-regulated pathway(s), processing is carried out by other proteases. Several reports have demonstrated that TACE, Kuzbanian, and meltrin γ take part in PKC-regulated processing (7, 10, 12, 13). As shown in Fig. 4C, meltrin γ is not able to process NRG-β1 as a mature form. Further studies are warranted to determine whether or not other ADAMs such as TACE and Kuzbanian participate in the PKC-regulated processing of NRG-β1.

In summary, we showed that meltrin β and NRGs are simultaneously expressed in the nervous system during development and meltrin β participates in the proteolytic processing of β-type NRG isoforms which are involved in neurogenesis and synaptogenesis. During differentiation of P19 cells the activation of the meltrin β and NRG genes preceded that of glial fibrillary acidic protein (Fig. 2, data not shown), suggesting regulatory roles of meltrin β in glial cell differentiation through the release of mature NRGs. Further analysis including genetic disruption of meltrin β will be required to demonstrate the role of meltrin β in the development of the nervous system.

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