Activity-dependent α-Cleavage of Nectin-1 Is Mediated by A Disintegrin and Metalloprotease 10 (ADAM10)*

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Received for publication, March 24, 2010, and in revised form, May 17, 2010 Published, JBC Papers in Press, May 25, 2010, DOI 10.1074/jbc.M110.126649

Nectin-1 is a Ca\(^{2+}\)-independent cell adhesion molecule, which, via the adaptor protein afadin, connects to the actin cytoskeleton in E-cadherin-based adherens junctions (1). Nectin-1 is required for development of ectodermal structures; mutations in the nectin-1 gene can cause cleft lip/palate ectodermal dysplasia and in severe cases mental retardation (2, 3). In the nervous system, nectin-1 is abundantly transcribed in all neuronal populations of cortex and hippocampus (4), but has perhaps been best characterized as a component of puncta adherentia junctions (PA); mechanical adhesive sites that connect pre- and postsynaptic membranes between hippocampal mossy fiber terminals and CA3 pyramidal dendrites (5). In the hippocampal stratum lucidum, the nectin-1/afadin adhesion system co-localizes with components of the cadherin/catenin system (5, 6) and with synaptic scaffolding molecules ZO-1 (7) and S-SCAM (8). Nectin-1 colocalizes with N-cadherin prior to synapse formation in cultured hippocampal neurons, and these adhesion complexes are later found at excitatory mature synapses on dendritic spines (9). Cultured neurons from nectin-1 KO mice show altered dendritic spine morphology as compared with wild type; hence nectin-1 is likely an important determinant of spine shape and structure (10).

Dendritic spine shape is continuously modulated by excitatory stimulation. This correlation has been suggested to be the cellular basis of memory formation. The spine head swells transiently in response to N-methyl-D-aspartic acid (NMDA) receptor stimulation and calmodulin activation, long-lasting spine enlargement on the other hand requires CaMKII, and is associated with an increase in AMPA receptor-mediated currents (11, 12). Correspondingly, low frequency stimulations used to induce hippocampal long-term depression (LTD) results in shrinkage and elimination of spines (13). The three-dimensional structure of a spine is determined by its underlying actin cytoskeleton, and the physical associations adhesion molecules form with surrounding matrix and neighboring cells (14). It has been suggested that neuronal cell adhesion molecules are actively involved in spine remodeling. In this context, it is of interest that several cell adhesion molecules, among them cadherin family members, undergo both proteolytic shedding of their extracellular NH\(_2\)-terminal domains and subsequent processing of the remaining COOH-terminal fragment (CTF) by γ-secretase (15–17). Nectin-1 undergoes ectodomain shedding upon treating with SF/HGF or TPA in MDCK cells (18) and in CHO cells (19), generating a large soluble fragment and small CTF. The shedding of nectin-1 also occurs at the synapses in mature hippocampal neurons when the actin cytoskeleton is disrupted (9). This shedding was inhibited by metalloprotease inhibitors in epithelial cells (18), suggesting that a metalloprotease may be involved. Ectodomain shedding and γ-secretase cleavage of synaptic cell adhesion molecules would comprise a rapid and elegant means by which neurons might remodel spine structure in response to synaptic transmission. Currently, it is not well understood how secretase cleavage of synaptic adhesion molecules is regulated by neuronal transmission. To

* This work was supported, in whole or in part, by National Institutes of Health Grant R01 AG027233.

† Supported through the Deutsche Forschungsgemeinschaft (SFB877) and the DeZentif-FPVI network of the European Community.

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explore this area, we have studied nectin-1 processing by α- and γ-secretase in cortical neurons and identified ADAM-10 as the metalloprotease responsible for α-secretase activity.

MATERIALS AND METHODS

Cell Lines, Antibodies, and Plasmids—Wild-type MEFs, ADAM10−/−, and ADAM17−/− were generated as described previously (20, 21). COS-7 and HEK293 cells were purchased from American Type Culture Collection. All cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% of fetal bovine serum with antibiotics (Invitrogen, Carlsbad, CA). Rabbit anti-nectin-1 antibody was prepared as described (9). Mouse anti-N-cadherin was from BD Transduction Laboratory, and mouse anti-HA and rabbit anti-actin antibodies were purchased from Sigma. Human nectin-1 (a gift from Dr. Patricia G Spear, Northwestern University) was Flag-tagged at the COOH terminus of ADAM10 to distinguish exogenous ADAM10 from endogenous ADAM10. Neuronal Cell Culture—Primary neurons were prepared from E15.5 embryos harvested from timed-pregnant C57Bl/6 mice or Sprague-Dawley E18 rat embryos (Taconic Labs, Germantown, NY). For biochemical experiments single cell suspensions were plated on PEI-coated 12-well tissue culture plates (3 × 104 cells per well) and maintained in Neurobasal Medium with B27 supplement with 1:1 media changes every 3–4 days. Ara-C was added at DIV 4 to prevent proliferation of non-neuronal cells. Signaling Experiments—Cortical neurons at DIV 10–14 were subjected to stimulation for 30 min, lysed in 1× SDS-PAGE loading buffer, boiled, and separated on 12% Tris-glycine gels. Reagents were added to the culture medium at final concentrations (in μM) of Sp-cAMP, 50; isoproterenol, 50; dopamine, 50; ATP, 50; NMDA, 100; glycine, 500. Inhibitors were added to the culture medium 30 min prior to NMDA stimulation at final concentrations (in μM) of: APV, 10–50; EGTA, 2000; KN93, 0.5; deltamethrin, 0.5; W7, 5; KT5720, 5; BIM, 1; PD98059, 50; D4476, 50; DMAT, 2.5; GM6001, 25; SB-3CT, 25; l-685,458, 1; myristoylated AIP, 0.05; HA 1004, 20, and PI3K Inhibitor, 2. Ionomycin Treatments—For the induction of endogenous nectin-1 or N-cadherin shedding, fibroblast cells were treated with different concentrations of ionomycin (IM, 1 and 5 μM, respectively) or vehicle control (DMSO) for different exposure times (from 5 to 15 min). To determine the involvement of MMP activity in IM-induced shedding of nectin-1 or N-cadherin, the MMP inhibitor GM6001 (12.5 μM) was pretreated for 6 h before the treatment of 5 μM IM for 5 min in fibroblasts. To block the γ-secretase dependent cleavage of nectin-1 or N-cadherin CTFs, cells were treated with 1 μM γ-secretase inhibitor for 1 h before the induction of shedding processes by 5 μM IM for 5 min. After drug treatment, cells were lysed in reducing sample buffer. Samples were loaded and fractionated on 12% SDS-PAGE gels and transferred onto nitrocellulose membrane. Anti-nectin-1 cytoplasmic Antibody (1:1000) and anti-N-cadherin antibody (1:2500) were used. Changes in the amount of protein were analyzed compared with F-actin control.

Transient Transfection of Cells—Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. For each experiment, 24 h after plating, COS-7 cells were cotransfected with 0.5 μg nectin-1 and various amount of ADAM10-HA (0, 0.5, 1, 2, 4 μg, respectively). The total amount of cDNAs for each sample was 4.5 μg by adding the proper amount of empty pVAX vector. After 1 h of incubation at 37 °C, the medium was changed with fresh DMEM. Cells were lysed in reducing sample buffer for Western blot analysis 48 h after transfection. Cell Lysis and Immunoblot Analysis—A crude synaptosomal membrane fraction was prepared from freshly dissected P1 ADAM10 conditional KO mouse brains as previously described (23). Cell homogenates for wild type, ADAM17−/−, and ADAM10−/− mouse embryonic fibroblasts were prepared by directly lysing in radioimmunoprecipitation assay buffer. After quantification by BCA protein assay kit, 100 μg of protein were loaded on 12% SDS-PAGE gels, blotted onto nitrocellulose membrane (Bio-Rad) and probed with antibodies as indicated in the figure legends. ShRNA Construct, Viral Production, and Viral Transduction—To construct the shRNA-expressing vector, the following sequences of the mouse shRNA used were as follows: shRNA#1 5′-GCAGATTTACTTATGGGAAT-3′; shRNA#2 5′-CTGTT-GCAGATCATTCAGTA-3′. The sequences were separated by an eight-nucleotide noncomplementary spacer (TCCTGAGA) from the corresponding reverse complement of the same 21-nucleotide sequence. These sequences were then annealed and cloned into the pSuperior vector (OligoEngine, Seattle, WA), which drives expression of the shRNA from an inducible H1 promoter. The H1 and shRNA sequence was then cloned into the pAd-Track CMV vector (the kind gift of Dr. Vogelstein, Johns Hopkins University School of Medicine, Baltimore, MD) (24). Transfer vectors were linearized with Pmnl, and the Pmnl-digested transfer vector and pAdeasy-1 DNA were co-transformed into BJ5183 competent bacterial cells. The transformation mix was resuspended in 500 μL of LB-broth and incubated for 45 min at 37 °C. Cells were plated onto LB/kanamycin plates. Colonies were picked and grown in LB broth. Minipreps were performed using the conventional alkaline lysis method. HEK-293A cells were plated on 6-cm2 tissue culture dishes and transfected using 4 μg of linearized plasmid DNA and 12 μL of Lipofectamine 2000 (Invitrogen) in 200 μL of Opti-MEM (Invitrogen). Virus was harvested and added to T-175 tissue culture flasks. Adenovirus expressing scramble shRNA was generated as previously described (25). High-titer viral stocks were obtained by CsCl gradient purification. At 5 DIV cultures were transduced with recombinant adenovirus. After 1 h of exposure to virus the neurons were washed with warmed HBSS and the reserved conditioned medium was returned to wells with the addition of one-half volume of feeding media. No toxicity was observed with virus transduction at 48 h (data not shown).

RESULTS

NMDA Receptor Activation Elicits Ectodomain Shedding of Nectin-1—Ectodomain shedding of nectin-1 can be induced in MDCK and CHO cells by activating PKC, but does not occur
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constitutively in these cells (18, 19). Because nectin-1 is present at excitatory synapses in vitro and in vivo, and localizes at puncta adherentia junctions (PAJs) between pre- and postsynaptic membranes (5, 9), we surmised that shedding of nectin-1 is a regulated event influencing synaptic adhesion under conditions of synaptic plasticity.

We examined ectodomain shedding of nectin-1 during synapse formation in primary cortical neurons harvested from 1 DIV to 17 DIV prepared from E15.5 mice cortex. On Western blots, nectin-1 appeared to be highly abundant in primary E15 neuronal cultures (Fig. 1A). Notably, nectin-1-derived CTFs were rarely detected in our cultures; hence constitutive shedding appears not to be a prominent feature of cortical synapse formation in vitro. We also examined N-cadherin, a Ca$^{2+}$-dependent cell-cell adhesion molecule, in these cultures. N-cadherin-derived CTFs were not detected in our culture (data not shown).

To identify physiological signaling pathways able to elicit nectin-1 shedding at neuronal synapses, we stimulated mature cortical neurons harvested from E15.5 mice with a number of neurotransmitters and analyzed the levels of nectin-1 derived CTFs resulting from each treatment (Fig. 1B). Of the agents tested in this screen, only NMDA elicited consistent and robust ectodomain shedding; we could easily detect the appearance of a 30-kDa nectin-1-derived CTF (named as an α-CTF) recognized by our polyclonal nectin-1 COOH-terminal antibody. When neurons were pretreated with the NMDA receptor antagonist α-2-amino-5-phosphovalerate (APV) prior to stimulation, ectodomain shedding was abolished when NMDA was added to the culture medium (Fig. 1C). Accumulation of 30-kDa bands was substantially reduced when neurons were pretreated with 1,10-phenanthroline, a metal ion chelator, which acts as a general matrix metalloproteinase inhibitor (Figs. 1C and 2A).

We also addressed whether excitatory neurotransmission mediated by AMPA or metabotropic glutamate receptors would also elicit nectin-1 ectodomain shedding. We found that this was not the case. Neurons that were stimulated with AMPA or ACPD, an agonist for metabotropic glutamate receptors, in parallel with NMDA at equimolar concentrations did not elicit nectin-1 shedding (Fig. 1B). Treatment with GABA also did not elicit the ectodomain shedding of nectin-1 (Fig. 1B). In conclusion, these results show that ectodomain shedding of nectin-1 in neuronal cells is selectively regulated by NMDA receptor activation and mediated by metalloproteases.

NMDA-induced Cleavage of Nectin-1 Is Mediated by α- and γ-Secretases—NMDA-mediated cleavage of nectin-1 in primary neurons results in the generation of two

FIGURE 1. NMDA receptor activation elicits nectin-1 ectodomain shedding in mouse cortical neurons. A, 50 μg of RIPA extracts harvested from E15.5 cortical cultures at DIV1–17 were separated on 12% Tris-glycine gels and analyzed by immunoblotting with nectin-1 and actin antibodies. Full-length nectin-1 accumulates in early culture time points; however, shedding of nectin-1 was undetectable throughout DIV1–DIV17. B, cortical neurons were stimulated for 30 min with agonists for AMPA, metabotropic glutamate, NMDA, and GABA receptors, lysed directly in SDS-PAGE sample buffer and analyzed by immunoblotting with nectin-1 and actin antibodies. Only NMDA elicited nectin-1 ectodomain shedding, indicated by the appearance of the 30 kDa α-CTF (lane 4). C, cortical neurons preincubated with 1,10-phenanthroline (Phe; 2 mg/ml) or 2-amino-5-phosphonovaleric acid (APV; 50 μM) as indicated were stimulated with NMDA (100 μM) for 30 min, lysed directly in SDS-PAGE sample buffer, and analyzed by immunoblotting with nectin-1 and actin antibodies. In the presence of APV, NMDA induced nectin-1 cleavage was inhibited (lane 3). The equal sample loading was shown by F-actin.

FIGURE 2. NMDA-mediated α- and γ-secretase cleavage of nectin-1 requires Ca$^{2+}$/calmodulin. A, mouse cortical neurons pretreated for 30 min with 1,10-phenanthroline, GM6001, SB-3CT and L-685,458 were stimulated with NMDA (100 μM) for 30 min, lysed directly in SDS-PAGE sample buffer, and analyzed by immunoblotting with nectin-1 and actin antibodies. Only NMDA elicited nectin-1 ectodomain shedding, indicated by the appearance of the 30 kDa α-CTF (lanes 4–5). In addition, γ-secretase inhibition caused accumulation of the 30-kDa α-CTF. B, cortical neurons pretreated for 30 min with APV, EGTA, and inhibitors for CAMKII (KN93), calcineurin (deltamethrin; DM), calmodulin (W7), PKA (KT5720), PKC (BIM), ERK1/2 (PD98059), casein kinase 1 (D4476), and casein kinase 2 were stimulated with NMDA (100 μM) for 30 min, lysed directly in SDS-PAGE sample buffer, and analyzed by immunoblotting with nectin-1 and actin antibodies. In the presence of APV (lane 3), EGTA (lane 4), and the calmodulin inhibitor W7 (lane 7) both α- and γ-secretase cleavage of nectin-1 was prevented. Not one of the kinase inhibitors applied prevented NMDA induced α- and γ-secretase cleavage of nectin-1. F-actin was used as a loading control for each experiment.
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FIGURE 3. MMP involvement in the cleavage of nectin-1 in fibroblast cells. After stimulation with IM (1 μM or 5 μM) or vehicle control (DMSO) for different time periods from 5 to 15 min, cells were lysed, and analyzed by Western blotting. A, enhanced calcium influx by IM rapidly accumulated nectin-1 (upper gel) and N-cadherin CTFs (lower gel), products of nectin-1 and N-cadherin shedding. The equal loading was shown by F-actin. B, blockade of IM-induced nectin-1 and N-cadherin shedding by treatment with GM6001, a MMPs inhibitor. The pretreatment of GM6001 (12.5 μM) for 6 h significantly blocked IM-induced nectin-1 (upper blot) and N-cadherin (lower blot) shedding. F-actin was used as a loading control for each experiment.

major CTFs when blots are exposed longer to the films; one prominent fragment migrating at ~30 kDa, and a less prominent at ~24 kDa (named as γ-CTF). The 30-kDa CTFs corresponded in size to nectin-1 CTFs generated after 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment of CHO and MDCK cells (18). The 24-kDa fragments corresponded in size to a smaller nectin-1 CTF, the generation of which was blocked by three different γ-secretase inhibitors (19). We asked if the nectin-1 fragments generated in response to NMDA receptor activation were likely to be products of α- and γ-secretase activities. To investigate if α-secretase was mediating neuronal nectin-1 cleavage we pretreated cortical neurons with 1,10-phenanthroline and GM6001, a potent, broad-spectrum hydroxamate inhibitor of MMPs and ADAMs (including TACE). We also pretreated neurons with SB-3CT, an inhibitor specific for MMP2 and MMP9, highly abundant matrix metalloproteinases present in the brain (Fig. 2A). Upon NMDA stimulation we found that generation of the 30-kDa nectin-1 CTF could be prevented in the presence of 1,10-phenanthroline and GM6001, but not with SB-3CT; hence, this cleavage event is likely mediated by α-secretase activity, but not by MMP2 and MMP9. In the presence of the γ-secretase inhibitor L685,458, the nectin-1 derived CTF migrating at 24 kDa was reduced, concomitantly with an increase in the 30-kDa α-CTF (Fig. 2A). These observations suggest that α- and γ-secretase sequentially process nectin-1 in response to NMDA receptor activation, and that cleavage of the nectin-1 α-CTF by γ-secretase is a prerequisite for continued α-secretase cleavage.

cleavage, hence influx of Ca2+ through the NMDA receptor and subsequent activation of calmodulin are necessary events for nectin-1 cleavage (Fig. 2B). Surprisingly, CaMKII activation was not necessary for NMDA-mediated nectin-1 processing; in the presence of the CaMKII antagonist KN93 nectin-1 cleavage proceeded at the same levels as in control cells. To further confirm that CaMKII is not involved in nectin-1 processing, we tested two additional CaMKII inhibitors (myristoylated AIP and HA 1004). Blocking CaMKII activity with these additional inhibitors had no effect on nectin-1 ectodomain shedding (data not shown). Inhibiting PKA, PKC, calcineurin, ERK1/2, and casein kinase 1/2 also had no effect on secretase-mediated nectin-1 cleavage (Fig. 2B). We also treated neurons with a PI3K inhibitor and found that the PI3K inhibitor did not block nectin-1 shedding elicited by NMDA receptor activation, indicating that the PI3K pathway is not associated with nectin-1 processing (data not shown). In conclusion, these experiments show that NMDA induced secretase cleavage of nectin-1 in neuronal cells requires Ca2+ and calmodulin, but not CaMKII activation.

Calcium Influx Induces the Ectodomain Shedding of Nectin-1 in MEF Cells—We treated wild-type MEF cells with ionomycin, an agent that promotes shedding of cadherins through stimulation of calcium influx (15, 16, 27). Treating cells with ionomycin (IM) for various time points triggered shedding of nectin-1, generating α-CTFs (Fig. 3A). IM treatment also increased γ-CTFs, suggesting that generation of α- and γ-CTFs is a sequential event. Interestingly the accumulation of both α- and γ-CTFs were diminished in longer IM treatment (Fig. 3), sug-
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FIGURE 4. ADAM10 as a major α-secretase in the ectodomain shedding of nectin-1. A, comparison of constitutive nectin-1 cleavage between wild-type, ADAM17−/− and ADAM10−/− fibroblasts. An equal amount of protein (100 μg) from three different types of cells was loaded for Western blot analysis. Compared with wild-type MEFs, the significant elevation of the full-length nectin-1 (100 kDa) and the reduction of CTF (30 kDa) produced by α-secretase-like activity were observed in ADAM10-deficient fibroblasts. B, relative ratio of total full-length nectin-1 expression in cell lines. The intensity of full-length nectin-1 was measured by densitometric analysis in three independent experiments. C, IM-induced the specific increase of nectin-1 α-CTFs (upper blot) processed by α-secretase-like activity in ADAM17-deficient MEFs cells. The pretreatment of γ-secretase inhibitor (1 μM) for 1 h resulted in further accumulation of CTF of nectin-1 after 5 μM IM treatment in ADAM17−/− cells. A similar effect is also detected for N-cadherin processing (lower blot). D, IM stimulation did not increase the nectin-1 α-CTFs or N-cadherin CTF1 in ADAM10−/− cells. F-actin was used as a loading control for each experiment.

suggesting that they are rapidly degraded. An additional 36-kDa band was detected in MEF cells, and this band was not affected by IM treatment indicating that the 36-kDa CTF may constitute a cleavage product of nectin-1 mediated by one or more unknown sheddases (Fig. 3A). These data indicate that the shedding of nectin-1 can occur in constitutive and regulated fashions by different sheddases. To confirm the activity of ionomycin, we also measured the ectodomain shedding of N-cadherin, which is known to undergo rapid shedding upon ionomycin treatment (15, 27). The rapid accumulation of 40-kDa N-cadherin CTF1 was observed (Fig. 3A). These results indicate that the activity-dependent shedding of nectin-1 and N-cadherin is regulated by similar mechanisms and possibly mediated by a common sheddase(s).

ADAM10 Is the Main α-Secretase Participating in Nectin-1 Stepping—Previous studies showed that metalloproteinase inhibitors block the cleavage of nectin-1 and N-cadherin, indicating that MMPs play an essential role in this shedding process of adhesion molecules (15, 18). In particular, ADAM10 has been shown to be responsible for ectodomain shedding of N-cadherin; however it is not clear whether this metalloproteinase is involved in the cleavage of nectin-1. To demonstrate the contribution of metalloproteinase activities to nectin-1 shedding, we pretreated cells with a broad-spectrum MMP inhibitor, GM6001, 6 h before ionomycin treatment in MEFs (Fig. 3B). Pretreatment of cells with GM6001 completely blocked the generation of the 30-kDa CTF in ionomycin-treated cells. Thus, as in primary neurons, calcium influx in fibroblasts promotes metalloproteinases that are responsible for the inducible cleavage of nectin-1. This effect was also observed in N-cadherin shedding as previously reported (Fig. 3B) (15).

Recently it has been reported that nectin-4 undergoes a proteolytic cleavage event mediated by ADAM17 (28). N-cadherin and E-cadherin, which colocalize with nectin-1 at synapses and adherens junctions respectively, undergo ectodomain shedding mediated mainly by ADAM10 (15, 16). These observations suggested the possibility that nectin-1 undergoes proteolytic events mediated by similar enzymes. To address whether ADAM10 or ADAM17 are involved in nectin-1 processing, we examined expression levels of endogenous nectin-1 in ADAM10- or ADAM17-deficient cell lines. When we compared the expression of full-length nectin-1 in the different cell types, both ADAM10- and ADAM17-deficient cells have greater levels of full-length nectin-1 (Fig. 4A). Densitometric analysis showed that the levels were greater in ADAM10−/− cells than those of both wild type and ADAM17−/− cells. The α-CTFs were detected in both wild type and ADAM17−/− cells, whereas the α-CTF was undetected in ADAM10−/− cells (Fig. 4A). These cell line analyses suggest that ADAM10 is responsible for the regulated cleavage of nectin-1 and also for the constitutive cleavage of nectin-1. Interestingly two additional bands at 18 and 14 kDa were detected in both wild-type and ADAM17−/− cells, indicating that there are further downstream cleavage events present. In ADAM10−/− cells, instead of these additional bands, a single distinct band at 17 kDa was detected, demonstrating that downstream cleavage events were altered in ADAM10−/− cells. We did not pursue these downstream events in this study.

Next we examined the contribution of ADAM10 and ADAM17 to the cleavage of nectin-1 and N-cadherin enhanced by ionomycin treatment in the presence or absence of γ-secretase inhibitor. Consistent with the difference in constitutive levels of nectin-1 shedding, more significant cleavage of nectin-1 mediated by α-secretase occurred in IM-stimulated ADAM17-deficient cells than ADAM10 knockouts (Fig. 4, C and D). The addition of a γ-secretase inhibitor to prevent further rapid processing of the α-secretase cleavage product revealed an increase of nectin-1 α-CTF in ADAM17-deficient cells (Fig. 4C) but not in ADAM10−/− cells (Fig. 4D). This result clearly demonstrates the generation of α-CTF is dependent on ADAM10 activity. To confirm the activity of ionomycin, the ectodomain shedding of N-cadherin was measured in both cell lines. The rapid accumulation of 40 kDa N-cadherin CTF1 was measured by densitometric analysis in the different cell types, both ADAM10- and ADAM17-deficient cells have greater levels of full-length nectin-1 (Fig. 4A). Densitometric analysis showed that the levels were greater in ADAM10−/− cells than those of both wild type and ADAM17−/− cells. The α-CTFs were detected in both wild type and ADAM17−/− cells, whereas the α-CTF was undetected in ADAM10−/− cells (Fig. 4A). These cell line analyses suggest that ADAM10 is responsible for the regulated cleavage of nectin-1 and also for the constitutive cleavage of nectin-1. Interestingly two additional bands at 18 and 14 kDa were detected in both wild-type and ADAM17−/− cells, indicating that there are further downstream cleavage events present. In ADAM10−/− cells, instead of these additional bands, a single distinct band at 17 kDa was detected, demonstrating that downstream cleavage events were altered in ADAM10−/− cells. We did not pursue these downstream events in this study.
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FIGURE 5. The selective accumulation of nectin-1 α-CTF by ADAM10. A, COS-7 cells were cotransfected with nectin-1 (0.5 μg) and various amounts of ADAM10-HA (0.5, 1, 2, 4 μg). ADAM10 expression significantly increased nectin-1 α-CTFs in a dose-dependent manner. Longer exposure exhibited that ADAM10 expression also increased γ-CTFs. B, relative ratio of nectin-1 α- and γ-CTFs in COS-7 cells. The intensity of CTFs was measured by densitometric analysis in three independent experiments.

FIGURE 6. ADAM10-mediated nectin-1 shedding in neurons and the brain. A, COS-7 cells were co-transfected with ADAM10 cDNA and vector alone (lane 2), or vector encoding shRNA to a nonspecific sequence (Scramble, lane 3) or to ADAM10 sequences (lanes 4 and 5). Western blot analysis demonstrates the substantial knockdown of ADAM10 by both shRNAs specific to ADAM10 sequences. B, MEF cells were infected with recombinant adenovirus expressing GFP or coexpressing ADAM10 shRNA and GFP. Cells were lysed in reducing sample buffer 48 h after infection. Membranes were probed with nectin-1, ADAM10, and GFP antibodies. C, densitometric analysis of FL nectin-1 and α-CTF in B. D, rat cortical neurons at 5 DIV were infected with adenovirus expressing scramble shRNA or ADAM10 specific shRNA at two different MOIs for 48 h. Neurons were stimulated with 100 μM NMDA for 15 min and cells were lysed in reducing sample buffer. The accumulation of CTFs was analyzed by immunoblots. E, brain homogenates were prepared from P1 Flox/Flox mice and ADAM10 conditional knock-out mice. Sixty micrograms of brain homogenate was separated on 4–12% gradient gels and analyzed by immunoblotting with nectin-1 and ADAM10 antibodies. The nectin-1 α-CTF level was decreased by 60% in ADAM10 knock-out mice compared with non-infected cells and cells transduced with scrambled shRNA whereas non-infected cells and cells transduced with scrambled shRNA have high levels of ADAM10 (Fig. 6B). The nectin-1 α-CTF level was increased in cells transduced with ADAM10 shRNA.

Overexpression of ADAM10 Significantly Increases the α-CTF—To further characterize the involvement of ADAM10 in nectin-1 cleavage process, various concentrations of ADAM10 were overexpressed with a constant amount of nectin-1 in COS-7 cells and changes in nectin-1 CTFs were analyzed by Western blotting. In order to distinguish exogenous ADAM10 from endogenous ADAM10, we inserted an HA tag at the COOH terminus of ADAM10. The α-CTF was significantly increased in ADAM10 expressing cells in a dose-dependent manner (Fig. 5A). The densitometry analysis further demonstrated the increase in α-CTF (Fig. 5B). In addition, γ-CTF, a product of intramembranous cleavage event by γ-secretase was increased in the same manner (Fig. 5, A and B). Taken together, these data demonstrate that ADAM10 plays a major role in the α-secretase-mediated cleavage of nectin-1.

ADAM10 Is Responsible for Nectin-1 Cleavage in Cortical Neurons and the Brain—Nectin-1 is highly expressed in neurons and plays a role in synaptogenesis (5, 9). To test whether ADAM10 was the metalloprotease responsible for nectin-1 shedding in neurons (Figs. 1 and 2), we utilized virus-vector based delivery of shRNA. To determine whether ADAM10 directed shRNA reduced overexpressed ADAM10 in a cell line, we co-transfected COS-7 cells with ADAM10 and two shRNA constructs. Both shRNA constructs efficiently reduced ADAM10 in COS-7 cells (Fig. 6A). We generated recombinant adenovirus expressing shRNA #1 and examined whether transduced shRNA reduced the endogenous levels of ADAM10 in MEF cells. After 48 h, both pro- and mature forms of ADAM10 were markedly reduced in MEF cells transduced with shRNA whereas non-infected cells and cells transduced with scrambled shRNA have full-length nectin-1 and ADAM10 antibodies.

observed in ADAM17−/− (Fig. 4C) but not in ADAM10−/− cells (Fig. 4D) as previously reported (15, 27).

To examine whether ADAM10 is responsible for the generation of 30-kDa α-CTF in neurons, we infected cortical neurons with adenovirus expressing ADAM10 shRNA #1. The cells were stimulated with NMDA, and the cell lysates were analyzed for the level of 30-kDa α-CTF. Nectin-1 α-CTF was greatly increased in control neurons after NMDA stimulation (Fig. 6D). A similar increase was seen in neurons expressing the scrambled shRNA control, whereas no increase was detected in
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neurons transduced with ADAM10 shRNA (Fig. 6D). These data indicate that ADAM10 is a major α-secretase for nectin-1 processing in neurons.

Finally, to test whether ADAM10 affected nectin-1 cleavage in vivo, we examined the nectin-1 ectodomain shedding in P1 brain homogenates prepared from ADAM10 conditional knock-out mice. In these mice both pro- and mature ADAM10 were substantially reduced as previously reported (Fig. 6E) (23). The reduction of the 30-kDa α-CTF was observed in these mice brains compared with that of the Flox mice, indicating that nectin-1 processing is regulated by ADAM10 in the brain.

DISCUSSION

In this study, we have investigated α- and γ-secretase-mediated processing of nectin-1 in primary cortical neurons and MEF cell lines. In cortical neurons, nectin-1 undergoes ectodomain shedding mediated by both α- and γ-secretase in response to the activation of NMDA receptors. However, stimulation of AMPA or metabotropic glutamate receptors did not elicit the ectodomain shedding of nectin-1. α- and γ-secretase cleavage of nectin-1 required influx of Ca2+ through the NMDA receptor, and activation of calmodulin, but was not dependent on CamKII kinase activation. These observations suggest that α- and γ-secretase processing of nectin-1 is a Ca2+/calmodulin-regulated event that occurs under conditions of activity-dependent synaptic plasticity. Secretase cleavage of synaptic adhesion molecules such as nectin-1 may allow the activated synapse to rapidly undergo both the increase and decrease in spine size observed during induction of LTP and LTD.

Our experiments using different ADAM-deficient MEF cells, co-expression and shRNA studies demonstrated that ADAM10 is critically involved in the both constitutive and regulated cleavage of nectin-1 in fibroblast cells. Our shRNA approach demonstrates that ADAM10 represents the major nectin-1 sheddase in neurons. Activation of ADAM10 via calcium influx may also provide an important mechanism for modulating dynamic processes in synaptic plasticity, tissue regeneration after injury, and tissue remodeling. Ca2+ influx activates ADAM10 by triggering the dissociation of calmodulin from pro-ADAM10 (29). Therefore, NMDA-stimulated Ca2+ influx might be the cause of enhanced ADAM10 activity, leading to increased nectin-1 shedding.

The ectodomain shedding and γ-secretase cleavage of nectin-1 may play several biological functions. First, the shedding directly regulates the cell-cell dissociation and synaptic plasticity at the synapse. Nectin plays an essential role in the organization of the junctional complex comprised of E-cadherin-based AJs and claudin-based TJs in epithelial cells and fibroblast cells (30). E-cadherin also undergoes ectodomain shedding mediated by ADAM10 (16). Treating MDCK cells with TPA or SF/HGF induces shedding of cell adhesion molecules including nectin-1, resulting in cell-cell dissociation (18). These data suggest that cell-cell disassociation requires shedding of both cell adhesion molecules as mediated by ADAM10.

In neurons, shedding of cell adhesion molecules may regulate neurite outgrowth, synapse formation, and remodeling. Nectin-1 also colocalizes with N-cadherin prior to synapse formation and is highly enriched at the tips of large growth cones in the early cultured hippocampal neurons (9), suggesting that shedding of these molecules plays an important role in neurite outgrowth. In the stratum lucidum, the nectin-1/afadin adhesion system co-localizes with components of the cadherin/catenin system (5, 6) and these adhesion complexes are found at excitatory mature synapses on dendritic spines in cultured neurons (9, 31). Ectodomain shedding and γ-secretase cleavage of synaptic cell adhesion molecules including nectin and N-cadherin (32) would comprise a rapid and elegant means by which neurons might remodel spine structure in response to synaptic transmission. These structural modifications of synapses likely underlie the plasticity implicated in learning and memory.

Second, the released soluble ectodomain of nectin-1 may act as a signaling molecule. It is known that soluble extracellular domains of cell adhesion molecules play roles in various physiological functions. For instance, the soluble extracellular domain of L1 released from cultured neurons promotes neurite outgrowth and influences neuronal differentiation (33–36), whereas neurons from transgenic mice over-expressing the soluble NCAM extracellular region in the developing brain exhibit a lack of NCAM-dependent neurite branching and outgrowth (37). The soluble ectodomain of nectin-1 may elicit biological responses by binding to either nectin-1 or 3 through homo- or hetero-trans-dimerization or perhaps to an unknown ligand. A fusion protein composed of the ectodomain of nectin-1 and the Fc portion of IgG trans-interacts with cellular nectin-1 and nectin-3, and induces filopodia and lamellipodia by the activation of Rap1, Cdc42, and Rac small G-proteins through the activation of c-Src (38–40). Nectin-1 has three isoforms: α, β, and γ. The β form is missing a conserved PDZ binding motif of four amino acid residues at the COOH terminus, whereas the γ form is a secreted protein, which lacks the transmembrane region. The biological role of nectin-1 γ is still unknown. It will be interesting to determine what physiological roles the soluble ectodomain of nectin-1 and nectin-1 γ play, and whether they have distinctive roles, in vivo.

Third, the nectin-1 intracellular domain released by γ-secretase activity may act either as a transcriptional stimulator or repressor. Sequence analysis indicates that the intracellular domain of nectin-1 contains a presumptive nuclear localization signal (RRRH) right after the transmembrane domain. This suggests that the nectin intracellular domain may translocate into the nucleus and participate in gene transactivation. On the other hand, analogous to intracellular domains of N-cadherin, it may associate with cytoplasmic proteins such as CBP and act as a repressor (41).

Fourth, shedding of nectin-1 may regulate the subcellular localization of afadin between the plasma membrane and the nucleus. Nectin interacts with afadin, an actin binding protein. Afadin has four splicing variants: the longest one is named l-afadin and the shortest one is named s-afadin (42, 43). S-afadin lacks the actin binding domain and the third proline-rich domain (42, 44). Interestingly s-afadin can shuttle between the plasma membrane and nucleus (45), suggesting that s-afadin is involved in gene transactivation. This finding is very similar to N-cadherin and β-catenin system. Shedding of N-cadherin, mediated by ADAM10, enhances β-catenin nuclear signaling (15). It is possible that shedding of nectin-1 releases afadin from
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Acknowledgment—We thank Dr. William G. Rebeck for comments on the manuscript.

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12. Acknowledgment—We thank Dr. William G. Rebeck for comments on the manuscript.

peripheral membranes and causes translocation of s-afadin into the nucleus.

Nectins are composed of four members. Nectin-1, -2, and -3 are ubiquitously expressed in many cell types whereas the expression of nectin-4 is restricted to embryos (46). Nectin-4 is also undergo multiple cleavage events, forming several CTFs in epithelial and fibroblast cells (data not shown). However, it is not known whether nectin-2 and nectin-3 are cleaved by similar sheddases. The ectodomain shedding of the nectin family may provide a major process regulating various biological aspects such as cell differentiation, proliferation, migration, and survival.

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