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Mechanism and significance of specific proteolytic cleavage of Reelin

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ABSTRACT

Reelin is a secreted glycoprotein essential for normal brain development and function. In the extracellular milieu, Reelin is subject to specific cleavage at two (N-t and C-t) sites. The N-t cleavage of Reelin is implicated in psychiatric and Alzheimer's diseases, but the molecular mechanism and physiological significance of this cleavage are not completely understood. Particularly, whether the N-t cleavage affects the signaling activity of Reelin remains controversial.

Here, we show that the protease in charge of the N-t cleavage of Reelin requires the activity of certain proprotein convertase family for maturation and has strong affinity for heparin. By taking advantage of these observations, we for the first time succeeded in obtaining "Uncleaved" and "Completely Cleaved" Reelin proteins. The N-t cleavage splits Reelin into two distinct fragments and virtually abolishes its signaling activity. These findings provide an important biochemical basis for the function of Reelin proteolysis in brain development and function.

Reelin is a large secreted glycoprotein that plays essential roles in brain development and function [1–3]. Reelin interacts with very low-density lipoprotein receptor and apolipoprotein E receptor 2, leading to tyrosine phosphorylation of the intracellular protein Dab1 [4–6]. Further downstream signaling components are not well established.

The N-terminal region of Reelin is required for full signaling activity [7,8]. In the central region of Reelin, there are eight Reelin Repeats (RR), each of which is composed of 350–390 amino acids [1,9]. The fifth and sixth RR (RR5 and RR6) consist minimal receptor-binding unit [10]. The C-terminal region is highly basic and participates in the activation of downstream signaling [11]. Reelin is specifically cleaved at two sites located between RR2 and RR3 (N-t site) and between RR6 and RR7 (C-t site), respectively (Fig. 1A) [12–14]. The identity of protease(s) in charge of these cleavages remains unknown, except that it is a metalloprotease(s) [12,14]. The amount of the N-terminal fragment generated by the N-t cleavage (from the N-terminus to RR2; hereafter called NR2, Fig. 1A) is decreased in bipolar and depressed patients [15], and increased in Alzheimer's disease patients [16,17], suggesting the linkage between Reelin cleavage and brain malfunctions. However, neither regulatory mechanism of the N-t cleavage nor its physiological significance is understood thus far.

What is the relationship between the cleavage of Reelin and its signaling activity? By using artificial Reelin fragments, Jossin et al. [13] suggested that the cleavage of Reelin did not affect its signaling activity. Other groups took similar approaches but reached the different conclusion: the N-t cleavage of Reelin greatly reduces its signaling activity [8,10]. One of the problems in these studies was the contamination of cleaved products generated by basal processing activity in "full-length" Reelin sample, which made quantitative comparison difficult. The other problem was using artificially truncated fragments because they may not reflect the structure and function of the naturally occurring fragments. It should also be pointed out that the exact cleavage sites are unknown and all the groups chose different sites for preparation of truncated mutants.

In this study, we found that Furin Inhibitor I (FI-1; decanoyl-RVKR-CMK) strongly inhibits the cleavage of Reelin and that the protease in charge of the N-t cleavage has strong affinity for heparin. By combining these observations and samples, we provide the proof that the cleavage of Reelin indeed affects its signaling activity.

Materials and methods

Animals. Jcl:ICR mice were obtained from Charles River Japan (Yokohama, Japan). All of the experimental procedures used here

Abbreviations: ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin motifs; FI-1, Furin Inhibitor I; FI-2, Furin Inhibitor II; PC, proprotein convertase; RR, Reelin repeat

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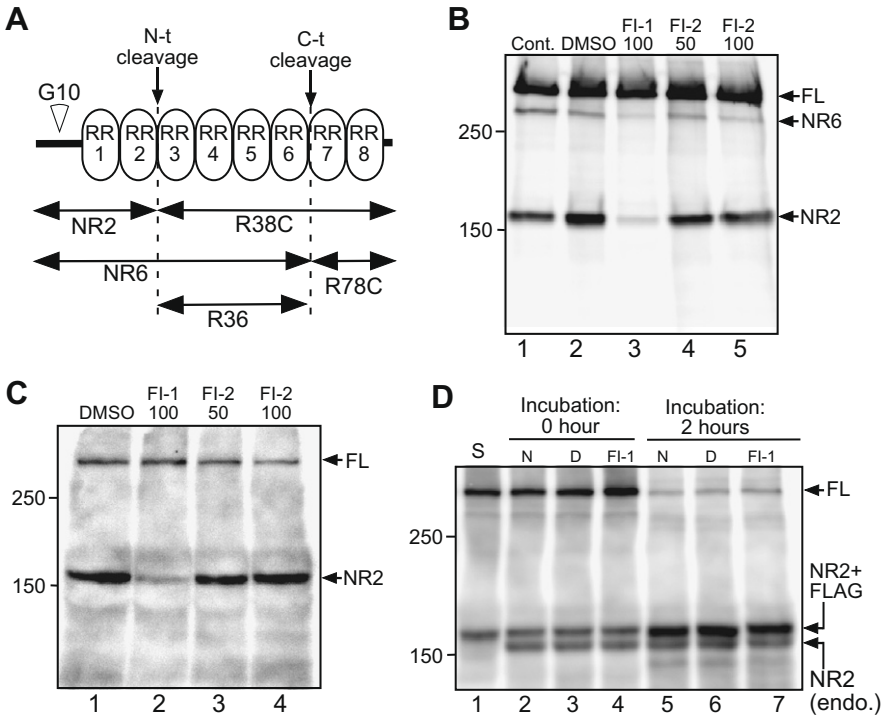

Fig. 1. Furin Inhibitor I, but not Furin Inhibitor II, inhibits the maturation of the protease(s) in charge of the N-t cleavage of Reelin. (A) Schematic representation of Reelin protein. The epitope of G10 antibody is shown. The N-t and C-t cleavage sites are located after RR2 and RR6, respectively. RR; Reelin repeat. (B) HEK293T cells were transfected with Reelin expression vector and cultured in the presence of none (lane 1), 2% DMSO (vehicle, lane 2), 100 μM Furin Inhibitor I (FI-1, lane 3), 50 μM Furin Inhibitor II (FI-2, lane 4), or 100 μM FI-2 (lane 5) for 48 h. The culture supernatant was then collected and analyzed by western blotting with G10. FL indicates the position of full-length Reelin. (C) Primary cortical neurons were cultured in the presence of 2% DMSO (vehicle, lane 1), 100 μM FI-1 (lane 2), 50 μM FI-2 (lane 3) or 100 μM FI-2 (lane 4) for 3 days. The culture supernatant was then collected and analyzed by western blotting with G10. (D) FI-1 does not directly inhibit the N-t cleavage. Reelin protein with insertion of FLAG-tag between R2 and R3 (Reelin-RR2/RR3-FLAG) was prepared from transfected HEK293T cells (S; substrate, lane 1). This protein was mixed with the culture supernatant of primary-cultured cortical neurons in the presence of none (N; lanes 2 and 5), 2% DMSO (D; lanes 3 and 6), or 100 μM FI-1 (lanes 4 and 7). The samples were immediately mixed with SDS–PAGE sample buffer (lanes 2–4) or incubated at 37 °C for 2 h and mixed with SDS–PAGE sample buffer (lanes 5–7). (B–D) Positions of the molecular weight markers (kDa) are shown on the left.

were approved by the Animal Care and Use Committee of Nagoya City University and were performed according to the guidelines of the Science Council of Japan.

Materials. FI-1 and Furin Inhibitor II (FI-2; hexa-D-arginine) were purchased from Merck (Whitehouse Station, NJ). Anti-Reelin antibodies G10 and E5 were purchased from Millipore (Temecula, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-Reelin antibody CR50 was described previously [18]. Anti-phosphotyrosine 4G10, anti-Dab1, and anti-Myc were purchased from Upstate Biotechnology (Lake Placid, NY), Millipore, and Sigma (St. Louis, MO), respectively.

Expression vectors. The plasmid pCrl [19] was used for Reelin expression and as the template for PCR amplification. Expression vector for Reelin with insertion of FLAG-tag between RR2 and RR3 (Reelin-R2/R3-FLAG) was constructed in pcDNA3 by utilizing PCR. The sequences of all of the fragments amplified by PCR were verified by DNA sequencing. Further details of the methods and the maps of the plasmids will be supplied on request.

Cell culture and transfection. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Equitech-Bio, Kerrville, TX) and antibiotics. Transfection was performed using Lipofectamine 2000 (Invitrogen), in accordance with the manufacturer's instructions. Preparation of primary cortical neurons and Dab1 phosphorylation assay were performed as described previously [11].

SDS–PAGE and western blotting. Samples were prepared in SDS–PAGE sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.05% bromophenol blue), separated by SDS–PAGE, and transferred on to a polyvinylidene difluoride membrane, as described previously [11]. Blotted membranes were analyzed with the indicated primary antibodies and the appropriate HRP-conjugated secondary antibodies using chemiluminescence reagents (Immobilon Western HRP Substrate (Millipore)) and LAS-3000 image-capturing system (Fuji, Tokyo, Japan).

Heparin Sepharose chromatography. Primary cortical neurons were cultured for 4 days and the culture supernatant was collected. The culture supernatant was concentrated using Amicon Ultra 50 (Millipore), diluted with buffer A (10 mM Tris pH 7.5), and applied to HiTrap Heparin column chromatography equipped in AKTA system (GE Healthcare, Piscataway, NJ). The flow rate was 1 ml/minute. The column was washed with 100 mM NaCl in buffer A and elution was performed with linear gradient (100 mM to 1 M) of NaCl.

Immunoprecipitation. Reelin protein was incubated with 5 μg/ml anti-Reelin antibody CR50 or anti-Myc (negative control), and Protein G Sepharose (GE Healthcare) for 2 h at 4 °C. The beads were collected by centrifugation, washed with 20 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20 for 4 times, and dissolved in SDS–PAGE sample buffer.

Preparation of "Uncleaved" and "Completely Cleaved" Reelin. HEK293T cells were transfected with the expression vector for Reelin and cultured for 2 days in the serum-free media in the presence of 100 μM FI-1. The culture supernatant was incubated with control buffer or protease-containing fraction from Heparin Sepahrose column chromatography for overnight at 37 °C to obtain "Uncleaved" and "Completely Cleaved" Reelin, respectively.

Results and discussion

Furin Inhibitor I, but not Furin inhibitor II, inhibits the N-t cleavage of Reelin

To look for a reagent that affects the proteolytic cleavage of Reelin, HEK293T cells were transfected with the expression vector for Reelin and cultured in the presence of various reagents. The culture supernatant was then collected and analyzed by western blotting using anti-Reelin antibody G10 that recognizes its N-terminal region (Fig. 1A) [20]. In the absence of a specific reagent or in the presence of dimethylsulfoxide (DMSO, vehicle) only, NR6 and NR2 fragments (approximately 330 kDa and 160 kDa, respectively) were detected (Fig. 1B, lanes 1 and 2, respectively). Thus, HEK293T cells secrete the protease(s) that catalyzes the N-t and C-t cleavage, as previously shown [5,6,13]. Among many reagents tested, FI-1 strongly inhibited the N-t and C-t cleavage of Reelin (Fig. 1B, lane 3). The inhibitory effect of FI-1 on the N-t cleavage was observed when its concentration was lowered to 3 μM (data not shown). Furin belongs to proprotein convertase (PC) family proteases that include at least 8 members [21,22]. As PC family proteases mediate the maturation of prohormones and extracellular enzymes [21], it was suggested that a certain member of PC family proteases are required for the maturation of the protease(s) in charge of the N-t cleavage on HEK293T cells. Interestingly, FI-2, another inhibitor of Furin, has no effect on the Reelin even at 100 μM (Fig. 1B, lane 5). We next asked if FI-1 could inhibit the N-t cleavage in primary-cultured cortical neurons that endogenously secrete Reelin and the protease(s) in charge of the N-t cleavage [12]. As shown in Fig. 1C, FI-1 (lane 2), but not FI-2 (lanes 3 and 4), decreased the generation of NR2 fragment. Therefore, the N-t cleavage in neuronal systems is mediated by the protease(s) whose maturation is inhibited by FI-1, but not by FI-2.

We investigated if FI-1 directly inhibits the N-t cleavage by incubating recombinant Reelin protein with the culture supernatant of primary-cultured cortical neurons in a cell-free system. To discriminate the NR2 fragment generated by the N-t cleavage *in vitro* from the endogenous one, Reelin protein with insertion of FLAG-tag between RR2 and RR3 (Reelin-R2/R3-FLAG) was used as the substrate (Fig. 1D, lane 1). This protein was subject to the N-t cleavage and generated NR2 fragment plus FLAG-tag, whose molecular weight was slightly bigger than the endogenous NR2 (Fig. 1D). After incubation of Reelin-R2/R3-FLAG with the culture supernatant of primary-cultured cortical neurons for 2 h, the amount of full-length Reelin-R2/R3-FLAG decreased and that of NR2 fragment with FLAG tag increased (Fig. 1D, lane 5). Addition of FI-1 to this reaction did not affect the cleavage amount (Fig. 1D, lane 7). Therefore, the protease(s) inhibited by FI-1 is necessary for maturation of the protease(s) in charge of N-t cleavage, but once it is maturated it is not inhibited by FI-1.

Furin belongs to proprotein convertase (PC) family proteases that include at least 8 members [21,22]. PC family proteases are mainly localized in trans-Golgi network, endosomes, and secretory granules, and are required for the maturation of many prohormones and extracellular enzymes [21]. The results above suggested that the metalloprotease(s) in charge of the N-t cleavage undergoes maturation step catalyzed by the PC family member(s). Among the PC family members, proprotein convertase 2 (PC2) is inhibited by FI-1 [23], but not by FI-2 [24]. Therefore, PC2 may be a good candidate for maturation of the protease(s) in charge of the N-t cleavage. PC2 is expressed mainly in neuronal systems [22], further supporting its potential role in Reelin signaling. On the other hand, Furin, the founding member of PC family, would not be involved in the maturation of the protease(s) in charge of the N-t cleavage since Furin is strongly inhibited by FI-2 [24]. The sensitivity of other PC family proteases against FI-1 and FI-2 has not been systematically investigated.

The protease in charge of N-t cleavage of Reelin has strong affinity for heparin

We next tried to purify the protease(s) in charge of the N-t cleavage from the supernatant of primary-cultured cortical neurons by column chromatography. In all chromatographies tested, the N-t cleavage activity was eluted in a single peak (data now shown), strongly suggesting that the N-t cleavage was catalyzed by a single protease. Unfortunately, we found that the protease was quite unstable after partial purification and have not succeeded in purification or identification of the protease thus far. We, however, found that this protease has strong affinity for heparin. The culture supernatant of primary cortical neurons were desalted and applied to Heparin Sepharose column chromatography. Most of the proteins did not bind to Heparin Sepharose and were recovered in the flow-through fraction (Fig. 2, upper panel). The N-t cleavage activity was, however, not detected in the flow-through fraction (data not shown) and eluted by approximately 300–400 mM NaCl (Fig. 2, lanes 2–5). This result indicates that the protease in charge of the N-t cleavage has strong affinity for heparin.

By testing many specific inhibitors against protease families, Lambert de Rouvroit et al. [12] suggested that the protease that cleaves Reelin might belong to a disintegrin and metalloproteinase (ADAM) family. Particularly, ADAMTS (ADAM with thrombospondin motifs) family proteases are secreted metalloproteases that are known to degrade many extracellular proteins [25]. Interestingly, most of them require PC family for maturation [25], and some of them (including ADAMTS-1 [26] and ADAMTS-5 [27]) have strong affinity for heparin. These are good candidates of the protease in charge of the N-t cleavage of Reelin.

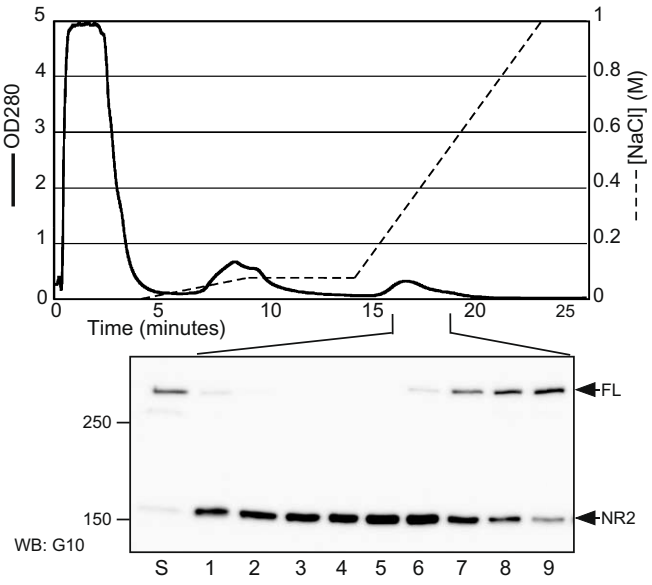

Fig. 2. The protease in charge of the N-t cleavage has strong affinity for heparin. Proteins secreted by primary-cultured cortical neurons were separated by HiTrap Heparin Sepharose column chromatography. In the top panel, protein profile (OD280, bold line) and NaCl concentration ([NaCl], broken line) are shown. The eluted fractions (labeled with the numbers) were used for the N-t cleavage assay using recombinant Reelin (S in the lower panel) as substrate. Positions of the molecular weight markers (kDa) are shown on the left of the lower panel.

The N-t cleavage splits Reelin protein into two distinct fragments

The N-terminal region of Reelin mediates multimerization that is required for effective activation of downstream signaling [7,8]. The N-t cleavage of Reelin potentially separates the NR2 fragment from the receptor-binding region (RR56) and decreases its signaling activity (Fig. 3A, scenario 1). The other possibility is that the NR2 and R38C fragments remain in a same complex after the N-t cleavage either by disulfide bonds or by non-covalent interaction (Fig. 3A, scenario 2). To know which scenario is correct, we performed immunoprecipitation study. We first prepared "Uncleaved" Reelin by adding FI-1 to Reelin-expressing HEK293T cells (Fig. 3B, lane 1). Then we treated this sample with the partially purified protease from Heparin Sepharose column chromatography (Fig. 2) and obtained "Completely Cleaved" Reelin (Fig. 3B, lane 2). These samples were incubated with CR50 antibody (that recognizes the N-terminal region [7,18]) or anti-Myc (negative control). The immunocomplex was precipitated with Protein G Sepharose and analyzed by western blotting using E5 antibody that recognizes RR8. The "Uncleaved" Reelin was completely precipitated by CR50 (Fig. 3C, lane 9) and did not remain in the unbound fraction (Fig. 3C, lane 5). On the other hand, the R38C fragment from "Completely Cleaved" Reelin was not detected in the bound fraction (Fig. 3C, lane 10) and remained in the unbound fraction (Fig. 3C, lane 6). Control experiments using anti-Myc antibody guaranteed the specificity of this system (Fig. 3C, lanes 3, 4, 7, 8). It was thus concluded that the N-t cleavage splits Reelin protein into two distinct fragments, namely NR2 and R38C.

The N-t cleavage abolishes the signaling activity of Reelin

Whether the N-t cleavage affects the signaling activity of Reelin is the most important question. We thus incubated primary-cultured cortical neurons with "Uncleaved" or "Completely Cleaved" Reelin as prepared above, and measured their ability to induce Dab1 phosphorylation (Fig. 3D). It was revealed that "Completely Cleaved" Reelin had little signaling activity (Fig. 3D, lanes 2–4, performed in triplicate) while "Uncleaved" Reelin induced robust Dab1 phosphorylation (Fig. 3D, lanes 5–7, performed in triplicate). We previously showed that the recombinant fragment consisting

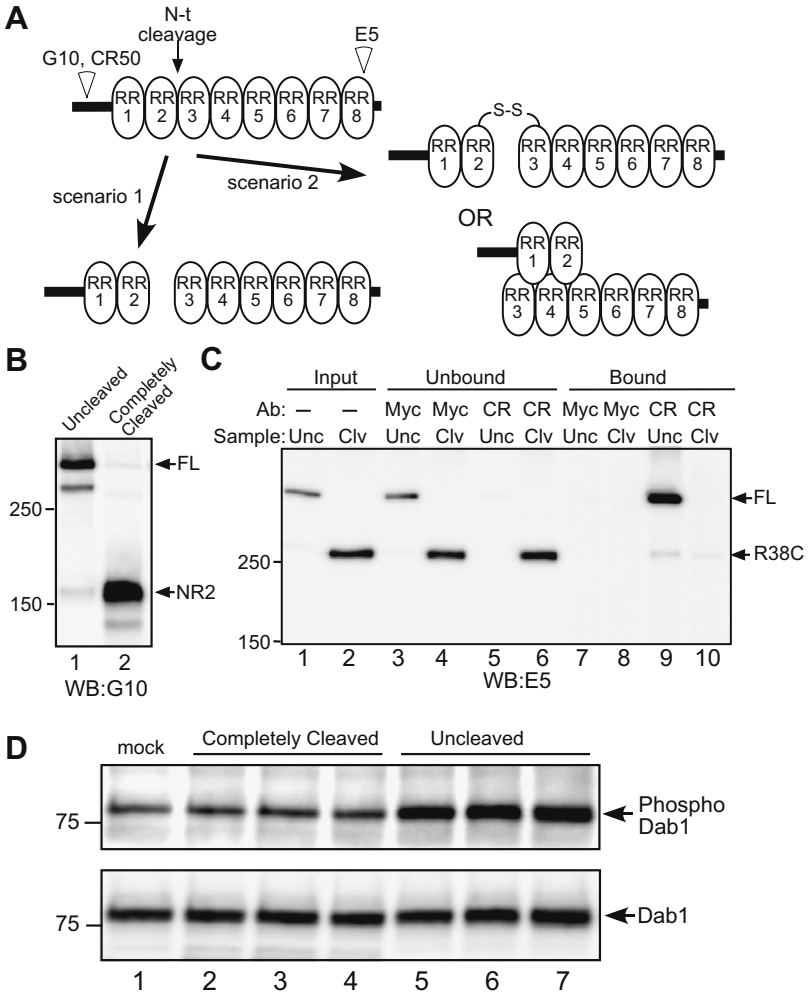

Fig. 3. The N-t cleavage splits Reelin into distinct fragments and abolishes its signaling activity. (A) Schematic drawing of two scenarios regarding Reelin protein after the N-t cleavage. In scenario 1, the N-t cleavage splits Reelin into two fragments. In scenario 2, NR2 and R38C fragments remain in the same complex after the N-t cleavage either by disulfide bonds (top) or by non-covalent interaction (bottom). (B) Preparation of "Uncleaved" and "Completely Cleaved" Reelin. "Uncleaved" Reelin was obtained from Reelin-expressing HEK293T cells cultured in the presence of 100 μM FI-1. "Completely Cleaved" Reelin was obtained by treating "Uncleaved" Reelin with the partially purified protease in charge of the N-t cleavage from Heparin column chromatography. (C) The N-t cleavage splits Reelin into distinct fragments. "Uncleaved" (Unc) or "Completely Cleaved" (Clv) Reelin were incubated with anti-Myc (Myc, negative control) or CR50 (CR). The immunocomplex was precipitated with Protein G Sepharose, and analyzed by western blotting with anti-RR8 antibody E5. (D) "Completely Cleaved" Reelin does not effectively induce Dab1 phosphorylation. Primary-cultured cortical neurons were incubated with serum-free culture supernatant containing "Completely Cleaved" or "Uncleaved" Reelin for 20 min at 37 °C. The whole cell lysate was prepared and analyzed with western blotting with anti-phosphotyrosine (top panel) or with anti-Dab1 (bottom panel). In B–D, positions of the molecular weight markers (kDa) are shown on the left.

of RR5 and RR6 induced Dab1 phosphorylation, but its ability was nearly 100 times lower than that of wild-type Reelin [10]. We thus presume that the N-t cleavage lowers the signaling ability of full-length (Uncleaved) Reelin by approximately 100-fold. We do not exclude the possibility that a certain molecule interacts the Reelin fragments and increases its signaling activity *in vivo*.

Conclusion

In this study, we found that the protease in charge of the N-t cleavage of Reelin is synthesized as proprotein and maturated by means of PC family (but not by Furin). The protease has strong affinity for heparin, which may be important for its localization in the extracellular matrix. Although the identity of the protease remains to be determined, the characteristics provided by this study will help understanding the role of Reelin and its specific proteolysis. Moreover, for the first time, the signaling activity of the full-length Reelin with little contamination of cleaved fragments was quantitatively compared with the naturally occurring Reelin fragment. The result clearly indicated that the N-t cleavage virtually abolishes the signaling activity. Next challenges include the identification of the protease in charge of Reelin cleavage and its loss-of-function experiments, which will clarify the physiological role of the cleavage *in vivo*, particularly in the pathogenesis of psychiatric and neurodegenerative diseases.

Acknowledgments

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