Presenilin-dependent Intramembrane Proteolysis of CD44 Leads to the Liberation of Its Intracellular Domain and the Secretion of an Aβ-like Peptide*

Sven Lammich‡, Masayasu Okochi‡, Masatoshi Takeda§, Christoph Kaether‡, Anja Capell¶, Ann-Katrin Zimmer‡, Dieter Edbauer‡, Jochen Walter‡, Harald Steiner‡, and Christian Haass†

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Alzheimer's disease (AD)-associated γ-secretase is a presenilin (PS)-dependent proteolytic activity involved in the intramembranous cleavage of the β-amyloid precursor protein, Notch, LDL receptor-related protein, E-cadherin, and ErbB-4. This cut produces the corresponding intracellular domains (ICD), which are required for nuclear signaling of Notch and probably ErbB-4, the β-amyloid precursor protein, E-cadherin, and the LDL receptor-related protein as well. We have now investigated CD44, a cell surface adhesion molecule, which also undergoes an intramembranous cleavage to liberate its ICD. We demonstrate that this cleavage requires a PS-dependent γ-secretase activity. A loss-of-function PS1 mutation, a PS1/PS2 knockout, as well as two independent and highly specific γ-secretase inhibitors, abolish this cleavage. Surprisingly, small peptides similar to the amyloid β-peptide (Aβ) are generated by an additional cut in the middle of the transmembrane region of CD44. Like Aβ, these CD44 β-peptides are generated in a PS-dependent manner. These findings therefore suggest a dual intramembranous cleavage mechanism mediated by PS proteins. The dual cleavage mechanism is required for nuclear signaling as well as removal of remaining transmembrane domains, a general function of PS in membrane protein metabolism.

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† To whom correspondence may be addressed. Tel.: 49-89-5996-480; Fax: 49-89-5996-415; E-mail: hsteiner@pbm.med.uni-muenchen.de (to C. H.)

‡ From the ‡Adolf-Butenandt-Institute, Department of Biochemistry, Laboratory for Alzheimer's and Parkinson's Disease Research, Schillerstr. 44; Ludwig-Maximilians-University, 80338 Munich, Germany

§ Department of Post-Genomics and Diseases, Division of Psychiatry and Behavioral Proteomics, Osaka University Graduate School of Medicine, 565-0871 Osaka, Japan

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braneous proteolysis leading to ICD liberation and the production of βA-like peptides (32), a hypothesis that may exclude a direct function of PSs in γ-secretase processing.

Identification of PS substrates is of pivotal importance for the development of safe γ-secretase inhibitors. In that regard it has been shown that γ-secretase inhibitors not only block βA generation but also severely interfere with T lymphocyte differentiation in cultured cells (39, 40) and lateral inhibition in zebrafish (Danio rerio) (41). Very recently CD44, a cell surface adhesion protein, has been shown to undergo an intramembraneous cleavage, which results in the generation of CD44-ICD (42). CD44-ICD is targeted to the nucleus where it regulates genes containing TPA-responsive elements (42). Based on the inhibition of this cleavage by MG132, an unspecific proteasome inhibitor (43), which also blocks β- and γ-secretase (44), it has been hypothesized that CD44-ICD generation could be mediated by γ-secretase (42). However, as described above at least three different intramembraneous cleavage mechanisms are currently known and evidence for a PS-dependent CD44-ICD generation is missing. We therefore investigated if inactivation of PS function through highly selective inhibitors, a complete knockout of PS genes, or a dominant negative PS1 mutation affects CD44-ICD production. Upon demonstration of a PS-dependent CD44-ICD generation, we further found that a second PS-dependent γ-secretase cut located in the middle of the TMD liberates a small and hydrophobic peptide similar to βA. Therefore our data suggest a novel function of PSs in TMD removal. Moreover, our findings demonstrate very similar mechanisms of βAPP and CD44 γ-secretase processing and therefore further support a direct role of PSs in intramembranous proteolysis.

EXPERIMENTAL PROCEDURES

Cell Lines, Cell Culture, and Transfections—Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 200 μg/ml zeocin to select for PS1 expression. Immortalized mouse embryonic fibroblast cells derived from PS1 C1 (Roche Diagnostics, Mannheim, Germany) or LipofectAMINE (Invitrogen) for mouse embryonic fibroblasts according to the supplier’s instructions.

cDNA Constructs—A full-length CD44 construct with a C-terminal Myc epitope tag was generated by PCR using a human brain cDNA library (Clontech) and the following primers: CD44FL forward, cgcagcttcgagctgaagctttgagtggtc and CD44FL reverse, cgctegctggaCAAGCATCCTTCTCTACTAGAGTTTGTGGCCTACACTTCC. Capital letters denote the C-terminal Myc epitope tag. The PCR product was subcloned into the HindIII/XhoI sites of pcDNA3.1/Hygro (+) (Invitrogen). The CD44AE and CD44E-FLAG fragments were generated by PCR using the following primers: CD44AE forward, cgcaagtcctgaagcctttctgctccattgaagccttgcttctgactgagcgtc and CD44E-FLAG forward, cgcaagtcctgaagcctttctgctccattgaagccttgcttctgactgagcgatc. Capital letters denote the N-terminal Flag-epitope tag. The resulting cDNA constructs were sequenced for verification.

Antibodies—The monoclonal antibody 9E10 to the Myc epitope was obtained from the hydridoma bank. Anti-Flag-M2-agarose, anti-Myc-agarose and the monoclonal Flag-M2 antibody were from Sigma.

Analysis of CD44 Expression—HEK293 cells or HEK293 cells stably expressing wild type PS1 or PS1 D385N were transiently transfected with CD44 cDNA encoding CD44FL. 24 h after transfection, cell lysates were prepared and analyzed for CD44FL by immunoblotting with antibody 9E10. Detection was performed using enhanced chemiluminescence (ECL, Amersham Biosciences).

CD44-ICD and CD44-β Generation—HEK293 cells stably expressing wild type PS1 or PS1 D385N were grown on poly-l-lysine coated 6-cm dishes. 6 h after transfection, CD44FL forward, cgcagcttcgagctgaagctttgagtggtc and CD44FL reverse, cgctegctggaCAAGCATCCTTCTCTACTAGAGTTTGTGGCCTACACTTCC. Capital letters denote the N-terminal Flag-epitope tag. The resulting cDNA constructs were sequenced for verification.

RESULTS

The cell surface protein CD44 was previously shown to undergo ectodomain shedding in various cancer cell lines to produce a membrane-anchored C-terminal fragment (CTF) (45). Very recently it was demonstrated that this CTF could further be proteolytically cleaved within the transmembrane region to release a chimeric substrate for PS-mediated proteolysis (46).

PS1-dependent CD44-ICD Generation—HEK293 cells express endogenous PS-dependent γ-secretase activity and have been widely used to investigate PS-dependent processing of βAPP (15, 46), LRP (35), ErbB-4 (38), E-cadherin (36), and Notch (13). Therefore we transiently transfected HEK293 cells or HEK293 cells stably expressing either wild type PS1 or the biologically inactive mutant PS1 D385N (12) with a CDNA construct encoding C-terminal Myc-tagged full-length CD44 (CD44FL) (Fig. 1B). CD44FL is expressed as a 90–100 kDa protein in transfected cells (Fig. 2). Additionally, we observed a robust accumulation of a protein species of ~25 kDa in cells expressing the non-functional dominant negative mutant PS1 D385N (Fig. 2). This cut is reminiscent to the PS1/γ-secretase-dependent generation of AICD (Fig. 1A). We therefore investigated whether intramembranous proteolytic processing of CD44 is dependent on PS activity.

CD44-CTF in the presence of the non-functional PS1 D385N mutation also results in a concomitant decrease of CD44-ICD. Therefore
HEK293 cells stably expressing wild type PS1 or PS1 D385N were transiently transfected with CD44 FL. Cell lysates were prepared and CD44 FL was analyzed by immunoblotting with antibody 9E10. Note the robust accumulation of CD44 CT-FL upon the expression of the non-functional PS1 D385N variant.

HEK293 cells stably expressing wild type PS1 or PS1 D385N were transiently transfected with CD44 ΔE (Fig. 1B), which mimics the product of the ectodomain-shedded CD44 and is reminiscent to the constitutively active Notch ΔE frequently used to monitor PS-dependent Notch endoproteolysis (47). Consistent with Okamoto et al. (42) we obtained proteins with a molecular mass of 25–30 kDa upon expression of CD44 ΔE.

These fragments accumulated to high levels either in the presence of the non-functional PS1 D385N mutant or in wild type PS1-expressing cells treated with specific γ-secretase inhibitors (48, 49) (Fig. 3A, upper panels). This observation is reminiscent of the accumulation of APP-CTFs upon γ-secretase inhibitor treatment (31). Consistent with previous results (42), immunoprecipitation of cell lysates with anti-Myc-agarose resulted in the detection of CD44 ΔE in cells treated with γ-secretase inhibitors. Asterisk indicates that this band results from CD44 ΔE overexposure. B, in vitro generation of CD44 ICD. Membrane preparations were incubated at 4 or 37 °C for 2 h in the presence or absence of 5 μM L-685,458. The reaction mixtures were separated by ultracentrifugation into pellet (P100) and soluble fraction (S100). The S100 fraction was further analyzed for CD44 ICD by immunoblotting with antibody 9E10.

These results were confirmed by a previously established in vitro assay for PS-dependent AICD generation (31). Membranes were prepared from HEK293 cells transiently expressing CD44 ΔE and incubated either at 4 or 37 °C. As shown in Fig. 3B incubation of membranes at 37 °C led to the production of CD44 ICD. When L-685,458 was added to inhibit PS-dependent γ-secretase activity no CD44 ICD was observed (Fig. 3B). Moreover, CD44 ICD was also not produced when membranes isolated from HEK293 cells expressing PS1 D385N were used in the in vitro assay (Fig. 3B). The lack of CD44 ICD production upon interference with PS activity thus unequivocally proves
Fig. 4. Identification of secreted CD44 β-peptides. HEK293 cells were transiently transfected with CD44ΔE or CD44ΔE-FLAG and incubated for 15 h with 1 μM DAPT, 5 μM L-685,458, or Me₂SO as vehicle. Upper panels, equal amounts of proteins were directly subjected to SDS-PAGE, blotted, and probed with 9E10. Note the robust accumulation of CD44ΔE and CD44ΔE-FLAG in cells expressing PS1 ΔD385N and in cells treated with γ-secretase inhibitors. Lower panels, conditioned media were immunoprecipitated with anti-FLAG-M2-agarose, subjected to SDS-PAGE, and immuno-blotted using FLAG-M2 antibody.

FIG. 5. Lack of CD44-ICD or CD44-β generation in the absence of PS. PS1ΔD385N mouse embryonic fibroblasts were transiently transfected with CD44ΔE-FLAG. As expected, CD44ΔE-FLAG and CD44-β are generated in PS1ΔΔ/PS2ΔΔ cells whereas ablation of PS1 and PS2 results in a robust accumulation of CD44ΔE-CTF (Fig. 5). PSs are essential for the generation of CD44-ICD and of CD44-β, since both cleavage products could not be observed in cells derived from PS1ΔΔ/PS2ΔΔ mice (Fig. 5). Moreover, the generation of both CD44-ICD and CD44-β in PS1ΔΔ/PS2ΔΔ cells is blocked in the presence of the γ-secretase inhibitor L-685,458 (49) (Fig. 5). Taken together the above findings provide unequivocal evidence for a PS-dependent generation of CD44-ICD and CD44-β.

In order to determine the cleavage site, which results in the release of CD44-β we performed MALDI-TOF mass spectrometry using immunoprecipitated CD44-β. This revealed a major peptide species with a molecular mass of 4385 Da (Fig. 6A). Computer-based analysis revealed that the proteolytic cleavage, which results in the secretion of this peptide occurs between Ala278 and Leu279 (Fig. 6B) in cells expressing endogenous or ectopic wild type PS1. Similar to the heterogeneous γ-secretase generated Aβ additional peptides were observed (Fig. 6A and B). All peptides were generated by a PS-dependent activity, since the corresponding peaks were not observed upon expression of PS1 ΔD385N (Fig. 6A, inset). Thus, like βAPP (31–34), CD44 is cleaved at two topologically different sites within the TMD (Fig. 7). Both cleavages are dependent on PS function, since the γ-secretase inhibitors DAPT and L-685,458, and the biologically inactive PS1 ΔD385N mutant inhibit formation of CD44-β and of CD44-ICD. This suggests a dual cleavage mechanism directly mediated by a PS1-dependent γ-secretase activity.

Overall, our data demonstrate that intramembranous processing of CD44 occurs by a PS-dependent γ-secretase activity at two topologically different sites (summarized in Fig. 7). One cleavage (close to the cytoplasmic border) liberates CD44-ICD for putative nuclear signaling, the other cut (in the middle of the TMD predominantly between Ala278 and Leu279) splits the TMD and results in the secretion of a peptide similar to Aβ.

Cleavage close to the cytosolic border of the TMD results in the generation of CD44-ICD. This cut is remarkably similar to AICD and NICD generation, since it not only occurs at a topologically similar site but it is also fully PS-dependent. This is demonstrated by the inhibition of CD44-ICD generation through DAPT, L-685,458, and by a dominant negative mutation of PS1. Moreover no CD44-ICD could be detected in PS1/2 double knockout cells.

DAPT and L-685,458 specifically inhibit the γ-secretase in cultured cells and in vivo (48, 49). In transgenic mice, which develop amyloid plaques due to the overexpression of mutant βAPP, DAPT significantly reduces the Aβ burden (48). In addition PS-dependent Notch endoproteolysis is also blocked by γ-secretase inhibitors in cultured cells (29, 50, 51). Moreover, DAPT causes a severe Notch phenotype in zebrafish.
A sites located in the middle of the transmembrane for the generation of AICD or CD44-ICD. Arrowheads evidently is a prerequisite for the subsequent shedding. Moreover, they all undergo ectodomain shedding, which apparently is a prerequisite for the subsequent γ-secretase activity. Arrows indicate the cleavage site liberating AICD or CD44-ICD. Arrowheads indicate the cleavage sites located in the middle of the transmembrane for the generation of ABα and ABβ or for the novel cleavage site for CD44.

Intramembranous cleavage leading to ICD generation seems to take place close to the cytoplasmic domain. PS-dependent ICD generation occurs constitutively immediately after ectodomain shedding. This raises the question how nuclear signaling of the different ICDs is regulated. Up- or down-regulation of PS genes has so far not been reported. Moreover, artificial overexpression of PSs by transfection does not lead to substantially higher PS levels since only endogenous PSs are replaced by the overexpressed variant (56). This may also be the case for other components of the PS/γ-secretase complex such as nicastrin (22–24). It appears therefore that the activity of ICDs like that of conventional transcription factors may be regulated by proteolytic degradation. In fact, NICD (57, 58) and LRP-ICD (35) have been shown to be degraded by the proteasome whereas AICD is predominantly removed by a metalloprotease activity, most likely IDE (59).

In addition to ICD generation we also found a PS-dependent production of Aβ-like CD44 peptides, which we called CD44-β. MALDI-TOF MS revealed that these peptides are generated by cleavage within the middle of the TMD. This cut is apparently homologous to the cleavages, which lead to the secretion of the two major Aβ species (Fig. 7). Our findings therefore reveal a remarkably similar intramembraneous cleavage mechanism of βAPP and CD44. Moreover, we have recently identified a secreted Notch peptide, which also resembles Aβ and CD44-β (60). Therefore we identified a dual intramembranous cut mediated by a PS-dependent γ-secretase activity. Apparently one cut allows the generation of ICDs, which may be involved in nuclear signaling. The other cut, which occurs within the middle of the TMD allows the efficient removal of the remaining membrane associated stub. Thus we propose that PS-mediated endoproteolysis is required at least in some cases for nuclear signaling but also for the general clearance of TMDs. This is supported by the finding that even artificial TMDs may allow γ-secretase processing in cultured cells (61) and in Drosophila (62).
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