ADAM10, the Rate-limiting Protease of Regulated Intramembrane Proteolysis of Notch and Other Proteins, Is Processed by ADAMS-9, ADAMS-15, and the γ-Secretase

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ADAM10 is involved in the proteolytic processing and shedding of proteins such as the amyloid precursor protein (APP), cadherins, and the Notch receptors, thereby initiating the regulated intramembrane proteolysis (RIP) of these proteins. Here, we demonstrate that the sheddase ADAM10 is also subject to RIP. We identify ADAM9 and -15 as the proteases responsible for releasing the ADAM10 ectodomain, and Presenilin/γ-Secretase as the protease responsible for the release of the ADAM10 intracellular domain (ICD). This domain then translocates to the nucleus and localizes to nuclear speckles, thought to be involved in gene regulation. Thus, ADAM10 performs a dual role in cells, as a metalloprotease when it is membrane-bound, and as a potential signaling protein once cleaved by ADAM9/15 and the γ-Secretase.

ADAMs8 (A disintegrin and metalloprotease) are type 1 transmembrane proteins related to snake venom integrin ligands and metalloproteases. All 38 different family members feature a common modular ectodomain structure (1–4) (Fig. 1A). In addition to the membrane-bound, full-length prototype, soluble ADAM variants have also been identified, consisting of only the ectodomain or fragments thereof that are released into the intercellular space. Such variants are generated by partial gene duplication (ADAM9) (5), alternative splicing (ADAM12) (6, 7), or proteolysis (ADAMs 8, 13, and 19) (8–10). ADAMs can be grouped either by their tissue distribution and/or functional properties. One major group (ADAMs 2, 3, 5, 6, 16, 18, 20, 21, 24, 25, 26, 29, and 30) is expressed exclusively in the male gonad, with an emerging role in sperm maturation. A second group (ADAMs 2, 7, 11, 18, 22, 23, and 29) is characterized by an inactive protease domain, and they seem to be mainly important for cell adhesion and fusion. A large third group of ADAMs displays a broad expression profile and has demonstrated (ADAMs 8, 9, 10, 12, 17, 19, and 28) or predicted (ADAMs 15, 20, 21, 30, and 33) proteolytic activity. These proteases play a major role in the ectodomain shedding of proteins involved in paracrine signaling, cell adhesion, and intracellular signaling (reviewed in Refs. 11 and 12). The site specificity of the cleavage of these substrates is rather relaxed, and apparently different family members can mutually compensate for each other. This has been illustrated particularly well for the amyloid precursor protein (APP) (13–17).

ADAM10 is one of the proteolytically active ADAM members (15, 18–21). The list of ADAM10 substrates is still growing, confirming the central role of ADAM10 in many important biological processes, such as cell migration and axonal navigation (robo receptors and ephrins (22, 23), cell adhesion (cadherins (19, 21), CD44 and L1 (24)), and regulation of immune reactions, and control of apoptosis (FasL) (25). Importantly, genetic ablation of ADAM10 in vertebrates (15) and invertebrates (26–29) mainly results in loss of Notch phenotypes, indicating the crucial role for this protease in the Notch signaling pathway (30, 31). Finally, ADAM10 is emerging as a major player in human disease. It is up-regulated in several tumors (32), and it is also considered to be protective in Alzheimer disease as it is one of the major α-secretases, cleaving APP within the amyloid-β (Aβ) peptide sequence, which thus pre-
cludes amyloid plaque formation (13, 18, 20, 33). Interestingly, two other ADAMs (9 and 17) have also demonstrated α-secretase activity in vitro (13, 14, 16, 18). Thus, stimulating α-secretase cleavage is an interesting therapeutic option for Alzheimer disease (20).

A fascinating aspect of ADAM10-mediated proteolysis is the initiation of regulated intramembrane proteolysis (RIP), which is characterized by two consecutive cleavage steps. First, the ectodomain is shed to generate a soluble ectodomain (11, 34). Then, the remaining transmembrane C-terminal fragment (CTFs) becomes a substrate for intramembrane cleaving proteases such as Presenilin/γ-Secretase (35). The fragments generated by this cleavage are released both externally and internally from the membrane and are, in many instances, involved in cell signaling pathways. Notch signaling has been particularly well investigated and it is well known that the Notch intracellular domain, upon release by Presenilin/γ-Secretase, translocates to the cell nucleus and regulates transcription of a series of Notch target genes in so-called transcription factories (36–41).

Presenilin (PS), an aspartyl protease, is the catalytic subunit of the γ-Secretase complex (42, 43). Two different genes encode PS1 and PS2, respectively, which each integrate into different γ-Secretase complexes (44). Although many γ-Secretase substrates have been discovered (reviewed by Kopan (35)), the extent to which the released intracellular domain fragments are important for signaling is not completely clear as most of the work is based on in vitro experiments. Thus, the possibility exists that, in many cases, the γ-Secretase could act as an “intramembrane proteasome,” removing residual transmembrane protein fragments that were generated by for instance, ectodomain shedding mediated by ADAM members, to avoid creating a bottleneck in the plasma membrane (35).

Here we demonstrate the surprising finding that ADAM10, apart from its central role in protein shedding and the initiation of regulated intramembrane proteolysis of several substrates, is itself subject to a similar proteolytic cascade. This suggests that ADAM10 has, in addition to its important function as a membrane-tethered sheddase, also the potential to be a signal transducing protein itself.

**EXPERIMENTAL PROCEDURES**

Animals, Cell Cultures, and Tissues—Mice and derived cell lines and the technique used for their derivation and maintenance were as published (17, 45, 46). Primary murine glial and cortical neuronal cultures were established from brains of embryonic day 14.5 mice, as described previously (47).

cDNA Constructs—Full-length murine ADAM9 and ADAM15 as well as ADAM9EA and ADAM15EA catalytically inactive mutant constructs were kind gifts from C. P. Blobel (48, 49). ADAM10 cDNA (complete cds of GenBank™ sequence AF011379) was obtained by PCR from a murine 129/SvJ cDNA library and was recloned into a modified PSG5 expression vector (Stratagene). A VP16-Gal4 sequence (50) was subcloned into mADAM10 cDNA after introduction of an Hpal restriction site in the ADAM10 C terminus via site-directed mutagenesis (Stratagene) at positions G745V,H746N. A mADAM10 construct lacking the ectodomain (containing a signal peptide sequence (amino acids 1–19) joined to amino acids 669–749) was FLAG-tagged (CTTGTCACTCGTCGTC–CTTGTAGTC) before the stop codon at the C terminus. The PCR product was ligated into a pcDNA3.1 vector (ADAM10E-flag). All constructs were sequenced and contained no errors. For COS and HEK293 cell transfections we used FuGENE 6 (Roche) or Genejuice (Merck Biosciences), according to the manufacturer’s protocol.

Sample Preparation—Cell extracts were obtained as described before (15). Phenanthrolines was added to the protease inhibitor mixture to prevent autolysis during the extraction procedure, as previously described for TACE (51). The γ-Secretase inhibitor X (carbamic acid tert-butyl ester L-685,458, Calbiochem) was used at 0.1 μM in medium with 2% fetal calf serum, unless otherwise specified (52). Membrane extracts and whole protein extracts were prepared as described previously (53).

Shedding Assay—After 24 h of serum starvation (53) culture medium was replaced with fresh serum-free medium containing one of the following protease inhibitors (Calbiochem): TAPI-1 (25 μM), TAPI-2 (25 μM), GM6001 (50 μM), or the appropriate vehicle control. Following 24 h incubation cell viability was checked, cell extracts were obtained, and cell culture supernatants were concentrated ×20 by ultrafiltration (Centricron-10/Millipore).

α-Secretase Fluorescence Resonance Energy Transfer Assay—Cell extracts and concentrated supernatant of ADAM10−/− and WT MEFs, after overnight conditioning in serum-free medium, were incubated with a fluorogenic substrate peptide mimicking the APP α-secretase cleavage site as indicated by the manufacturer (R&D Systems). Fluorogenic emission was measured by Victor2 (PerkinElmer Life Sciences) at 495 nm.

Subcellular Fractionation—Postnuclear supernatants were prepared using a sucrose step gradient protocol (adapted from Fleischer and Kervina (54)). Pooled cells from five 10-cm culture dishes, after 1.5 h treatment with 20 ng/ml leptomycin B (Sigma) (52), were harvested and homogenized in ice-cold buffer (20 mM Hepes-NaOH, pH 7.4, 5 mM MgCl2, 0.25 M sucrose with 0.2 M dithiothreitol, protease inhibitors, without EDTA) using a glass Dounce homogenizer (type S). Cell disruption and integrity of nuclei were checked. SHM2.1 (20 mM Hepes-NaOH, pH 7.4, 5 mM MgCl2, 2.1 M sucrose) was added to the homogenate to obtain a final sucrose concentration of 1.5 M and after centrifugation at 29,000 × g (TST41), the pellet was resuspended in SHM 0.25 (20 mM Hepes-NaOH, pH 7.4, 5 mM MgCl2, 0.25 M sucrose). Fractions were collected and analyzed by Western blotting.

SDS-PAGE proteins were separated and transferred as described before (15). Primary antibodies (overnight at 4 °C) and horseradish peroxidase-tagged (Dako) secondary antibodies (1 h at room temperature) were applied. ADAM10 was detected using the polyclonal antiserum (B42.1), generated against the 17 C-terminal amino acid residues (15). N-terminal-specific antibody MAB946 (R&D Systems) only detected ADAM10 when sample buffer contained 1 μM N-ethylmaleimide (Pierce) instead of β-mercaptoethanol (55). APP fragments,
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**A**

MAB946  
MAB946  
Pro  
Protease  
Dis  
Cys  
TM  
Cyto  
C

sADAM10:  
ADAM10 FL:  
−55 kDa  
−10 kDa  
Pro ADAM10:  
−85 kDa

**B**

MEF ADAM10

| Mr(K) | CE | SN |
|-------|----|----|
| 64    | ADAM10+ | ADAM10− |
| 62    | ADAM10 FL | ADAM10 |
| 38    | ADAM10 CTF | ADAM10 |

**C**

% α-secretase activity

SN

**D**

MAB946  
Pro ADAM10  
ADAM10 FL  
ADAM10 CTF

**E**

Spleen  
Liver  
Lung  
Heart  
Kidney  
Brain  
Cerebellum

FIGURE 1. Ectodomain shedding of ADAM10 in MEFs. A, model of the domain organization of ADAM10, which consists of a pro-domain (Pro) that is proteolytically removed in the trans-Golgi network by pro-protein convertases, a zinc-binding metalloprotease (Protease) domain, a disintegrin domain (Dis), which binds to integrin cell adhesion molecules, a cystein-rich domain (Cys), which can interact with cell surface proteoglycans and in some cases also contains a fusion peptide sequence, a variable stalk region, a transmembrane (TM) domain, and a cytoplasmic (Cyto) domain. Epitopes for C (B42.1) and N terminus (MAB946) specific antibodies are indicated. Ectodomain shedding (arrow) leaves a ∼10-kDa membrane-anchored CTF and releases a ∼55-kDa soluble ectodomain (sADAM10). B, Western blot analysis of total wild-type (+/+ ) MEF cell extracts (CE) (50 μg/lane) shows a Pro-ADAM10 (∼85 kDa) and a mature ADAM10 (ADAM10 FL, ∼65 kDa) after prodomain removal. A third 10-kDa band is detectable (ADAM10 CTF) with B42.1, the C-terminal ADAM10 antibody, but not by MAB946, the N-terminal antibody. Additional bands (asterisk) are observed in wild-type cells, but not in ADAM10-deficient cells. These bands may represent ADAM10 splice variants (see www.genecards.org/cgi-bin/carddisp.pl?gene=ADAM10) or degradation products. A secreted ADAM10 ectodomain (sADAM10) is observed in the culture supernatant (SN) (30 μg/lane), detected by MAB946, but not B42.1. C, SN from WT MEFs was able to cleave a synthetic peptide containing the APP α-secretase cleavage site in a fluorescence resonance energy transfer assay. In MEFs lacking ADAM10 we observed a strong reduction in this cleavage compared with WT. D, ADAM10 CTes are detected by Western blot analysis in cell lysate samples from both neuronal and glial cell cultures (30 μg/lane). E, Western blot of total tissue extracts of E16.5 CD1 mouse embryos (50 μg/lane). ADAM10 CTes were detected to a various extent in all tested organs.
**RESULTS**

**The ADAM10 Ectodomain Is Shed from Fibroblasts in Vitro**—In Western blots of whole cell homogenates, ADAM10 appears as a doublet band of ~85 and 65 kDa, corresponding to the unprocessed pro-form and the mature enzyme, respectively (58). In addition a band at ~10 kDa is observed that reacts exclusively with C terminus-specific antibodies (ADAM10 CTF, Fig. 1, A and B). It is noteworthy that in some experiments the ADAM10 CTF appears as a doublet band (e.g. Fig. 2B, fourth panel). In the culture supernatant samples of the cells, we also observed a soluble protein at ~55 kDa that was immunoreactive with antibodies against the ADAM10 N terminus but not C terminus (soluble = sADAM10, Fig. 1B). These bands were undetectable in cell extracts and supernatants from ADAM10−/− MEFs (Fig. 1B). Thus, ADAM10 is apparently processed by an unknown protease generating a membrane-bound C-terminal fragment and a secreted, soluble ectodomain. We checked whether the ADAM10 ectodomain shed in the medium retained its proteolytic activity. The supernatant of wild-type MEFs cleaves a synthetic peptide containing the α-secretase cleavage site of APP in a fluorescence resonance energy transfer assay. This activity is strongly reduced in supernatant of MEFs lacking ADAM10 (Fig. 1C). In separate experiments we could demonstrate that removal of ADAM10 from the supernatant by immunoprecipitation also reduces significantly proteolytic activity (data not shown).

ADAM10 CTFs were also observed in cell lysates of cultured neurons and astrocytes (Fig. 1D) and in vivo in brain, liver, lung, heart, and kidney tissue from both embryo (Fig. 1E) and adult mice (data not shown). As shown in Fig. 1E, considerable differences in ADAM10 processing are observed in different tissues. In particular the heart (which is strongly affected by ADAM10 deficiency, see Ref. 15) displays an abundant accumulation of the ADAM10 CTF.

**ADAM10 Shedding Depends on ADAMs 9 and 15**—To identify the proteases responsible for ADAM10 shedding, we screened wild-type MEF cultures with a panel of inhibitors against all major classes of proteases, but only the metalloprotease inhibitors GM6001, TAPI1, and TAPI2 reduced ADAM10 CTF and sADAM10 accumulation in MEFs, suggesting that ADAM10 sheddase(s) belong(s) to the metalloprotease family (Fig. 2A). Members of the ADAM family are known to be important ectodomain shedding metalloproteases. So far, only 12 of the 38 ADAMs have demonstrated (ADAMs 8, 9, 10, 12, 17, 19, and 28) or predicted (ADAMs 15, 20, 21, 30, and 33) active MP domains. Consequently, we investigated ADAM10 shedding in MEF cell lines deficient in expression of ADAMs 9, 15, and 19 and cell lines deficient for both ADAMs 9 and 15. We found a significant, albeit somewhat variable, reduction in ADAM10 shedding in ADAM9-deficient MEF lines (Fig. 2B, fourth and seventh panels, lanes 4–6), whereas shedding was virtually abolished in a cell line lacking both ADAMs 9 and 15 (Fig. 2B, fourth and seventh panels, lanes 7–9). No difference in ADAM10 shedding was observed in ADAM19−/− MEFs (data not shown). We confirmed that ADAM10 is a novel substrate for ADAM9 and ADAM15 by overexpression experiments in COS cells. Only low amounts of endogenous ADAM10 holoprotein and no CTFs could be detected in untransfected cells (Fig. 2C, first and third panels, lanes 1 and 2). However, upon transient overexpression of ADAM10 an intense doublet band of ADAM10 FL as well as ADAM10 CTF and the soluble ADAM10 ectodomain were observed (Fig. 2C, first and third panels, lanes 5–7). When increasing amounts of ADAM9 (0.1 μg in Fig. 2C, second panel, lanes 14–15 versus 1 μg in lanes 8–10) were co-overexpressed with ADAM10, more ADAM10 fragments were generated (Fig. 2C, third and sixth panels, lanes 14–15 and 8–10, respectively), whereas this effect was undetectable following transfection with similar concentrations of the catalytically inactive ADAM9EA mutant (Fig. 2C, lanes 16–17 and 11–13). A similar increase in sADAM10 was shown when ADAM15, but not ADAM15EA, was overexpressed in COS cells, demonstrating the ability of ADAM15 to cleave ADAM10 as well. Interestingly, accumulation of the cell-bound ADAM10 CTF was not observed following cleavage by ADAM15 (supplemental Fig. S1), whereas it is a consistent feature of ADAM9-mediated cleavage. Although the precise mechanism is unclear, the observed absence of the ADAM10 CTF following cleavage by ADAM15 suggests the ADAM10 CTF is rapidly degraded after proteolytic processing by ADAM15.

Finally, we sought to determine whether ADAM9 and -15 are involved in ADAM10 proteolytic processing in vivo. Thus, we examined ADAM10 CTF generation in ADAM9- and ADAM9/15-deficient mouse brain samples from different postnatal ages (Fig. 2D). We observed a decrease in ADAM10 CTF generation in the ADAM9- and ADAM9/15-deficient mouse brain samples relative to WT control samples, demonstrating in vivo correlation of the in vitro cell culture studies conducted in MEF and COS cell lines. Interestingly, the reduction in ADAM10 CTF generation was less prominent in tissues such as the liver and lung, which suggests the presence of additional tissue-specific ADAM10 sheddases. Apparently, the deficiency of ADAM10 shedding did not result in the accumulation of full-length ADAM10, indicating a tight control of ADAM10 holoprotein levels in the cell.

**ADAM10 CTFs Are Cleaved by γ-Secratease to Release a Free Intracellular Domain**—In many instances, the CTF of type I integral membrane proteins generated by ectodomain shedding are subsequently cleaved by γ-Secratease, a protein complex that contains presenilin as the catalytic subunit. Therefore, we determined whether the ADAM10 CTFs generated by ADAM9 and -15 are also substrates for γ-Secratease (Fig. 3A). Inhibiting γ-Secratease activity with the γ-Secratease inhibitor X resulted in an accumulation of ADAM10 CTFs in wild-type cells (Fig. 3B). In accordance with this finding, there was a clear accumulation of ADAM10 CTF fragments in P51 and P51/P52 double deficient MEFs (Fig. 3C, second and fourth or sixth

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9 T. Tousseyn, E. Jorissen, D. Hartmann, and B. De Strooper, unpublished observations.
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**A**

![Image](image-url)  
**FIGURE 2. ADAM10 ectodomain shedding is mediated by ADAMs 9 and 15.** A, Western blots of total MEF cell extracts (CE) (50 μg/lane) and culture supernatants (SN) (30 μg/lane). Metalloprotease inhibitors TAPI-1 (25 μM), TAPI-2 (25 μM), and GM6001 (50 μM) significantly reduce ADAM10 processing.  

**B**

![Image](image-url)  
**FIGURE 2B.** Western blots of total MEF cell extracts and culture supernatants (lanes 1–9). Metalloprotease inhibitors TAPI-1 (25 μM), TAPI-2 (25 μM), and GM6001 (50 μM) significantly reduce ADAM10 processing.  

**C**

![Image](image-url)  
**FIGURE 2C.** Western blots of COS cell extracts and supernatants (lanes 1–9). Metalloprotease inhibitors TAPI-1 (25 μM), TAPI-2 (25 μM), and GM6001 (50 μM) significantly reduce ADAM10 processing.  

**D**

![Image](image-url)  
**FIGURE 2D.** Western blots of brain extracts from 4-week-old (4wk) and 1-year-old (1y) mice. Metalloprotease inhibitors TAPI-1 (25 μM), TAPI-2 (25 μM), and GM6001 (50 μM) significantly reduce ADAM10 processing.

Interestingly, the pattern of ADAM10 CTF accumulation in these cell lines is very similar to the CTF accumulation observed with other γ-Secretase substrates, such as APP and Notch, in agreement with the conclusion that the ADAM10 CTF is a valid substrate for γ-Secretase. Additional studies utilizing genetic rescue experiments with wild-type human PS1, but not a PS1 active site mutant, confirmed that ADAM10 CTF is an authentic γ-Secretase substrate (Fig. 3C, seventh to ninth lanes).

We confirmed the role of the γ-Secretase in ADAM10 CTF turnover in vivo, by determining ADAM10 CTF generation in various tissues from PS1- and PS2-deficient mice. ADAM10 CTF accumulation was established in brain, lung, liver, heart, and kidney from E16.5 PS1+/− and PS2−/− mice. ADAM10 CTF accumulation was prominent in the brain and lung of PS1-deficient mice, whereas ADAM10 CTF accumulation was the highest in the liver of PS2−/− mice, exceeding the deficit in ADAM10 CTF processing observed in PS1+/− mice (Fig. 3D).

Intriguingly, the liver displays the highest levels of PS2 expression in wild-type animals (59). Thus, these data strongly suggest the ADAM10 CTF is a substrate for both PS1- and PS2-related γ-Secretases.

To confirm the release of ADAM10 intracellular domains (ICD) from ADAM10 CTFs we introduced a VP16-GAL4 transcriptional activation domain. Overexpression of ADAM9 resulted in a 10-fold activation of the luciferase reporter (Fig. 3E; white bar in ADAM10–GAL), which was reduced by the γ-Secretase inhibitor X (InhX) to the level of dimethyl sulfoxide controls (Fig. 3E; red bar in ADAM10–GAL). Moreover, cotransfection with ADAM9 resulted in a ~100-fold
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A schematic representation of an ADAM10 CTF generated by ADAM9 or -15 processing. γ-Secretase cleavage of this CTF releases a soluble ICD from the membrane into the cytoplasm. Loss of γ-Secretase activity results in accumulation of the ADAM10 CTF. Western blots of total cell extracts from MEFs (50 μg/lane) probed with the indicated antibodies. Cells were treated with the γ-Secretase inhibitor InhX (0.1 μM). ADAM10 CTFs and reporter activation as a result of the increased generation of the ADAM10 CTF available for processing by the γ-Secretase, effects that were entirely blocked by the γ-Secretase inhibitor X (Fig. 3F, yellow and blue bars, respectively). Thus, these results are consistent with reporter activation initiated by ectodomain cleavage of ADAM10 via ADAM9 and consecutive RIP of the generated ADAM10 CTF by the γ-Secretase resulting in the release of the ADAM10 ICD.

RIP of ADAM10 Leads to an Intracellular Accumulation of Its ICD—Regulated intramembrane proteolysis of several proteins such as the Notch receptor is followed by nuclear translocation of the intracellular domain. Endogenous nuclear ADAM10 immunoreactivity was below detection limits in purified nuclear fractions from MEF cells (Fig. 4, first lane), probably reflecting the low nuclear concentrations of the ADAM10 ICD, similar to the Notch receptor ICD (60). However, addition of the nuclear export inhibitor leptomycin B allowed specific detection of a ∼4–5-kDa band in nuclear fractions that cross-reacted with ADAM10 C-terminal-specific antibodies (Fig. 4, second lane). This band was not observed in nuclei purified from ADAM10-deficient cells (Fig. 4, third lane) or from cells deficient in ADAM9 and -15 or the γ-Secretase components PS1/2 (Fig. 4, fourth and fifth lanes, respectively). Interestingly, reintroduction of hPS1 into PS1/2-deficient cells rescued the appearance of the ADAM10 ICD (Fig. 4, sixth lane).

We then confirmed the nuclear localization of the ADAM10 ICD by transfecting HEK293 cells with an ADAM10-GAL4 short fragment (Fig. 5A), which is a membrane-anchored fragment of ADAM10 that requires γ-Secretase processing to release the ICD, but does not require the rate-limiting ectodomain shedding step by ADAM9 or -15. A similar construct has been used previously to study intramembrane proteolysis of Notch. We found that about 30% of the cells transfected with this construct (displaying strong cell surface immunofluorescence when stained with FLAG antibodies), exhibited nuclear immunoreactivity (Fig. 5, B, C, and F, white bar). When inhibitor X was used, this percentage dropped to 5% of the FLAG-positive cells, confirming that the γ-Secretase was necessary to release the ADAM10 ICD (Fig. 5, D–F, black bar). More specifically, APP CTFs, known γ-Secretase substrates, accumulate in the presence of InhX. C, Western blots of PS1+/+ (second lane) and PS1/2−/− MEFs (fourth and sixth lanes) show ADAM10 CTF accumulation. Stable transfection of wild-type human PS1 (hPS1) into PS1/2−/− MEFs (seventh lane), but not the catalytically inactive PS1 D257A or D385A mutants, can rescue generation of the ADAM10 CTF (eighth and ninth lanes). D, Western blots of mouse tissue extracts and quantitative analysis of three experiments. ADAM10 CTF accumulation occurs in the PS1-deficient brain and lung and PS2-deficient liver. p values are indicated with asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.001). E, schematic representation of the luciferase reporter construct. A VP16 (activation domain)-GAL4 (binding domain) fusion sequence was cloned to the ADAM10 ICD (sixth lane). F, luciferase assay on COS cells transfected with either empty control vector (ø) or with ADAM10-GAL4 alone resulted in activation of luciferase expression (white bar). This effect was inhibited by the γ-Secretase inhibitor X at 0.1 μM (InhX, red bar). Cotransfection with ADAM9 (yellow bar) strongly stimulated luciferase expression, an effect that could be inhibited by InhX (blue bar), demonstrating that the execution of signaling depends on the combined action of ADAM9 and the γ-Secretase. Transfection of GAL4-VP16 fusion protein (p-GAL) directly drives luciferase expression strongly and is independent of γ-Secretase processing (red bar, absence of InhX).
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ADAM10 ICD immunoreactivity was visible in multiple intense spots in the nucleus (Fig. 5, B and C), independent of nucleoli, nuclear envelope indentations, and heterochromatin accumulations, as is shown by the respective B23, lamin B, and bromodeoxyuridine immunostainings (Fig. 6, D–L). A fraction of the ADAM10 ICD speckle-like structures appear to be closely associated with two known nuclear speckle subtypes, Cajal bodies and PML bodies, as identified by immunoreactivity for p80/coilin and PML, respectively (Fig. 6, M–R), but not with sc-35 speckles (Fig. 6, A–C). Notably, both Cajal bodies and PML bodies have been shown to localize to actively transcribed gene loci, indirectly supporting a role for ADAM10 in gene transcription control (61, 62).

DISCUSSION

ADAM10 functions as a membrane-tethered protease, initiating the RIP of proteins such as the Cadherins, the Notch receptor and its ligands, and cytokine receptors. We demonstrate here that ADAM10 is not only executor of, but also subject to, regulated intramembrane proteolysis, being shed by related metalloproteases and subsequently cleaved by γ-Secretase.

We identified two ADAM proteases, i.e., ADAM9 and ADAM15, as sheddases responsible for ectodomain cleavage of ADAM10. To date, ADAM9 has been reported to cleave heparin binding-epidermal growth factor and APP in vitro; however, ADAM9 knock-out mice display no deficiency in shedding of these proteins (17). In view of studies that suggest that members of the ADAM family can compensate for the absence of one or more family members (13–17), it is usually very difficult to precisely define the function of individual ADAMs, such as ADAM9. Nevertheless, given the reduced accumulation of the ADAM10 CTF in vivo in brains of ADAM9- and ADAM9/15-deficient mice and the in vitro evidence in MEF and COS cell lines, ADAM10 appears to be an authentic ADAM9 substrate. We demonstrate here that ADAM15, using the same criteria, is also a putative ADAM10 sheddase. Although ADAM9 and -15 seem to be important ADAM10 sheddases, tissue blots from compound ADAM9/15 knock-out animals, suggest that at least one other unidentified protease can cleave ADAM10 in its ectodomain in certain tissues. Importantly, ADAM10 is the first identified substrate for ADAM15, which has until now mainly been studied in the context of cell-cell interaction and cell migration, functions that are mediated by its disintegrin and cysteine-rich domains (57, 63, 64).

ADAM10 shedding results in the release of a proteolytically active

FIGURE 5. The ADAM10 ICD translocates to the nucleus in HEK293 cells transfected with ADAM10ΔE-FLAG. A, a schematic representation of the ADAM10ΔE-FLAG construct with release of ADAM10-ICD-FLAG to the nucleus following γ-Secretase cleavage. B–D, immunofluorescence labeling of transfected HEK cells using anti-FLAG primary and green fluorescent secondary antibodies (Alexa 488-conjugated). FLAG immunoreactivity (green) was observed in the nuclei of about 30% of transfected cells (B and C). Notice the multipunctate staining pattern resembling that of nuclear speckles (C). Cross-section through a speckle at the position of the white line, shown in the inset in C, confirms the intranuclear presence of speckles. Less than 5% of transfected cells show nuclear staining in the presence of the γ-Secretase inhibitor (InhX, 0.1 μM) (D and E). Hoechst nuclear counterstain is in blue. Bar, 10 μm. F, the number of speckled nuclei in the presence or absence of InhX (white bar, dimethyl sulfoxide (DMSO) control; black bar, + InhX) is represented by a bar graph relative to the number of transfected cells (means of three experiments). ***p value <0.001.
the ADAM10 CTF is a substrate for γ-Secretase. PS2 deficiency in fibroblasts alone had quantitatively little or no effect, in agreement with the relatively minor contribution of PS2 to γ-Secretase activity in fibroblasts (56, 65). ADAM10 CTF processing was reconstituted by expressing wild-type human PS1 in PS1/PS2 double-deficient MEFs, but not by expressing a catalytically inactive mutant hPS1, in which the catalytic aspartyl residues have been replaced by alanine (46). Treatment with γ-Secretase inhibitor X resulted in ADAM10-CTF accumulation. Finally, the accumulation of ADAM10-CTFs in brain, liver, and lung tissue from PS1<sup>−/−</sup> and PS2<sup>−/−</sup> mice in vivo, firmly establishes ADAM10 as an authentic γ-Secretase substrate.

To address the physiological significance of the sequential ADAM10 proteolytic processing, one option could obviously be that the intramembrane processing merely serves to remove the transmembrane fragments of ADAM10 after shedding of the soluble ectodomain. However, several indirect arguments suggest the possibility that the intracellular domain of ADAM10 exerts a nuclear function. First, we could demonstrate nuclear enrichment of the ADAM10 ICD following treatment with the nuclear export blocker leptomycin B. We could further demonstrate enhanced nuclear staining when we transfected HEK293 cells with a FLAG-tagged ADAM10 construct with a shortened ectodomain (ADAM10ΔE-FLAG), which bypasses the rate-limiting ectodomain shedding step by ADAM9 or -15 (66). Under these conditions, an intense multipunctate nuclear immunoreactivity for ADAM10 could be observed. Nuclear transport of the ADAM10 ICD was reduced following treatment with γ-Secretase inhibitors, providing additional evidence for RIP of ADAM10.

Finally we demonstrate that within the nucleus, the ADAM10 ICD localizes to a nuclear speckle-like compartment, similar to what has been described for Notch- and APP-ICDs (67). Speckles are interchromatin bodies that concentrate proteins involved in mRNA production, splicing, and maturation. Furthermore, a fraction of these ADAM10 ICD-containing speckle-like structures appears to be closely associated with two known nuclear speckle subtypes, Cajal bodies and PML bodies. Both Cajal bodies and PML bodies are associated with actively transcribed gene loci involved in cell survival control (61, 62), providing further circumstantial evidence for a nuclear function of ADAM10. Recently, translocation and colocalization of an ~60-kDa enzymatically active form of ADAM10 with the androgen receptor in the nuclear fraction of a prostate cancer was observed (68), opening the debate as to how these observations are interrelated with our observations of the 4-kDa ADAM10 nuclear fragment.

We conclude that regulated intramembrane proteolysis of ADAM10 and the nuclear accumulation of ADAM10 ICDs suggests a dual function for ADAM10 as a "disintegrin protease and signaling receptor," which is an intriguing idea as it would suggest that proteases can also function as signaling molecules. Recently, other membrane-tethered proteases such as MT1-MMP (69) and BACE1 (70) have been reported to undergo ectodomain shedding, and it may be speculated
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that other membrane proteases may have such dual functions as well.

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