The cellular prion protein (PrP\textsuperscript{c}) is physiologically cleaved in the middle of its 106–126 amino acid neurotoxic region at the 110/111 \( \downarrow \) 112 peptide bond, yielding an N-terminal fragment referred to as N1. We recently demonstrated that two disintegrins, namely ADAM10 and ADAM17 (TACE, tumor necrosis factor alpha converting enzyme) participated in both constitutive and protein kinase C-regulated generation of N1, respectively. These proteolytic events were strikingly reminiscent of those involved in the so-called “\( \alpha \)-secretase pathway” that leads to the production of secreted sAPP\( \alpha \) from \( \beta \)APP. We show here, by transient and stable transfection analyses, that ADAM9 also participates in the constitutive secretion of N1 in HEK293 cells, TSM neurons, and mouse fibroblasts. Decreasing endogenous ADAM9 expression by an antisense approach drastically reduces both N1 and sAPP\( \alpha \) recoveries. However, we established that ADAM9 was unable to increase N1 and sAPP\( \alpha \) productions after transient transfection in fibroblasts depleted of ADAM10. Accordingly, ADAM9 is unable to cleave a fluorimetric substrate of membrane-bound \( \alpha \)-secretase activity in ADAM10\( ^{–/–} \) fibroblasts. However, we established that co-expression of ADAM9 and ADAM10 in ADAM10-deficient fibroblasts leads to enhanced membrane-bound and released fluorimetric substrate hydrolyzing activity when compared with that observed after ADAM10 cDNA transfection alone in ADAM10\( ^{–/–} \) cells. Interestingly, we demonstrate that shedding ADAM10 displays the ability to cleave endogenous PrP\( \textsuperscript{c} \) in fibroblasts. Altogether, these data provide evidence that ADAM9 is an important regulator of the physiological processing of PrP\( \textsuperscript{c} \) and \( \beta \)APP but that this enzyme acts indirectly, likely by contributing to the shedding of ADAM10. ADAM9 could therefore represent, besides ADAM10, another potential therapeutic target to enhance the breakdown of the 106–126 and \( \beta \)\( \alpha \) toxic domains of the prion and \( \beta \)APP proteins.

Spongiform encephalopathies are fatal neurodegenerative diseases involving a highly protease-resistant protein referred to as prion scrapie (PrP\textsuperscript{sc})\( ^{1, 2} \). Human prion diseases include, among others, Creutzfeldt-Jakob disease, familial transmissible spongiform encephalopathies, new variant of Creutzfeldt-Jakob disease, as well as a few cases of iatrogenic transmission (3–5). PrP\textsuperscript{sc} corresponds to the abnormal conformation of an ubiquitous glycosylphosphatidylinositol-anchored protein called cellular prion (PrP\textsuperscript{c}) that is mainly synthesized in the central nervous system (6, 7). The mechanisms by which PrP\textsuperscript{c} is converted into PrP\textsuperscript{sc} are not fully understood. However, it appears clearly that inoculation of PrP\textsuperscript{sc} triggers pathology and ultimately cell death in mice only when PrP\textsuperscript{c} is present in the host animal. Thus, PrP\textsuperscript{sc} null mice resist infection and toxicity exhibited by scrapie-enriched inoculates (8–10). On the other hand, it appears that depletion of PrP\textsuperscript{c} is relatively innocuous (11, 12). Therefore, all strategies aimed at lowering endogenous PrP\textsuperscript{c} are potential therapeutic approaches.

Classical means to regulate endogenous concentrations of proteins are either clearance/uptake or proteolytic catabolism. PrP\textsuperscript{c} apparently undergoes distinct proteolytic events in normal and Creutzfeldt-Jakob-affected brains. In normal conditions, PrP\textsuperscript{c} is endoproteolyzed mainly at the 110/111 \( \downarrow \) 112 peptide bond (13), leading to a secreted product referred to as N1. This cleavage is likely of importance because it occurs within the 106–126 domain of PrP\textsuperscript{c} that is thought to convey intrinsic toxicity (14–16). Therefore, this cleavage could be seen as a physiological means to deplete cells of endogenous PrP\textsuperscript{c} and its 106–126 associated toxicity. Interestingly, in the pathology, an additional major breakdown occurs upstream, at the 90 \( \downarrow \) 91 peptide bond (13). Such “pathological” cleavage preserves the toxic potential of 106–126 that remains intact.

We recently established that N1, the physiological cleavage product of PrP\textsuperscript{c}, could be generated in cells upon constitutive and protein kinase C-mediated pathways (17). We identified ADAM10 and ADAM17, two members of the disintegrin and metalloprotease family (18), as the main enzymes responsible for constitutive and regulated production of N1, respectively (19). The nature of the disintegrins involved in N1 formation together with the PKC regulation of this cleavage were strikingly reminiscent of those taking place on \( \beta \)APP that ultimately leads to sAPP\( \alpha \) secretion (20).

Among the wide family of ADAM proteases, ADAM9 appeared as a likely candidate, particularly because this protease was suggested to contribute to sAPP\( \alpha \) formation (21, 22). Therefore, the close parallel between the \( \alpha \)-secreases-generating sAPP\( \alpha \) and N1 led us to postulate that ADAM9 could also contribute to N1 formation. Here we show that overexpression of ADAM9 in various cell systems including HEK293 cells, TSM neurons, and fibroblasts, all led to increased N1 production. Conversely, down-regulation of ADAM9 by an antisense approach drastically reduces N1 recovery. We also show that ADAM9-associated converting enzyme; TAPI, TACE inhibitor; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
increase of N1 was abolished by ADAM10 deficiency and that ADAM9 contributed to the shedding of a catalytically active form of ADAM10. Altogether, our study indicates that ADAM9 indirectly contributes to the N1 production by acting upstream to ADAM10, likely by enhancing its shedding.

**MATERIALS AND METHODS**

**Antibodies and Pharmacological Agents**—SAF32 is a monoclonal antibody raised against the 79–92 residues of PrP<sup>c</sup> and has been characterized previously (23). The polyclonal antibody directed against mouse ADAM9 was kindly provided by Dr. C. Blobel. Anti-murine ADAM10 were from Euromedex. 207 antibody was from Cephalon (West Chester, PA). 22C11 was purchased from Roche Applied Science (Ingelheim, France). o-Phenanthroline, pepstatin, E64, and AEBSF were purchased from Sigma (St. Quentin-Fallavier, France). BB3103 (hydroxamic acid-based zinc metalloprotease inhibitor) was kindly provided by TACE inhibitor mixture (Sigma). The lysate and media were supplemented with a protease inhibitor mixture (Sigma). 25 µg of proteins were separated by SDS-polyacrylamide gel electrophoresis on an 8% Tris/glycine gel. Proteins were transferred onto nitrocellulose membranes (2 h, 100 V) and incubated overnight at 4 °C with anti-ADAM9 or ADAM10 antibodies (1/1000 dilution in PBS/Tween 0.05%/milk 5%) overnight at 4 °C. PrP<sup>c</sup> was revealed by enhanced chemiluminescence as described (17).

**Cell Cultures and Transient Transfections**—βAPP and 3F4-MoPrP<sup>c</sup>-expressing HEK293 cells were obtained once with phosphate-buffered saline (PBS) and resuspended in 500 µl of lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 5 mM EDTA) supplemented with a protease inhibitor mixture (Sigma). 25 µg of proteins were separated by SDS-polyacrylamide gel electrophoresis on an 8% Tris/glycine gel. Proteins were transferred onto nitrocellulose membranes (2 h, 100 V) and incubated overnight at 4 °C with anti-ADAM9 or ADAM10 antibodies (1/1000 dilution in PBS/Tween 0.05%/milk 5%). Bound antibodies were detected using a goat anti-rabbit peroxidase-conjugated antibody (1/5000 dilution) (Beckman Coulter), and immunological complexes were revealed with enhanced chemiluminescence as described (17).

**Stable Transfections**—HEK293 cells overexpressing mouse ADAM9 or mouse ADAM9-antisense were obtained after transfection of 2 µg of the corresponding cDNAs with DAC30 reagent (Eurogentec). Positive clones were identified by Western blot analysis with anti-mouse ADAM9-specific polyclonal antibody. Cells were maintained at 37 °C in 5% CO<sub>2</sub> in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% fetal calf serum containing penicillin (100 units/ml<sup>−1</sup>), streptomycin (50 mg/ml<sup>−1</sup>), and Genetnic (0.5 mg/ml<sup>−1</sup>).
ADAM9 Contributes to PrPc Processing

FIGURE 2. Effect of stable transfection of ADAM9 cDNA on N1 recovery in HEK293 cells. HEK293 were stably transfected with empty pcDNA3 (mock-transfected, M) or with ADAM9 (A9) cDNA, and clones were screened for their ADAM9 content by Western blot analysis as described under “Materials and Methods.” (A) Note that anti-murine A9 does not label endogenous human A9 and ProA9 present in control HEK293 cells. Overexpression of ADAM9 in clone #8 increases N1 production as assessed by combined immunoprecipitation/Western blot with SAF32 as described under “Materials and Methods” [(B), PrP–] and ADAM9-like immunoreactivities in cell homogenates were examined by Western blot as described in the Methods. Bars correspond to the densitometric analysis of N1 (C) or PrP (D) and are expressed as the percent of control N1 secretion or PrP expression in mock-transfected cells. Values are the means ± S.E. of seven independent experiments. *, p < 0.0005.

FIGURE 4. N1 and sAPPα secretions are lowered in HEK293 cells displaying reduced ADAM9 expression. HEK293 cells were stably transfected with antisense pcDNA3 bearing ADAM9 then clones with reduced ADAM9-like immunoreactivity (As) were selected by Western blot analysis as described under “Materials and Methods” (note that anti-human A9 antibodies do not label ProA9) (A). Secretion media were analyzed for their N1 (B) or sAPPα (D) contents as described under “Materials and Methods.” Cell homogenates were prepared and analyzed for their PrP and βAPP as described under “Materials and Methods.” Bars correspond to the densitometric analysis of N1 (C) or sAPPα (E) recovery and are expressed as the percent of control N1/sAPPα secreted by mock-transfected cells. Values are the means ± S.E. of five independent experiments. *, p < 0.0005.

FIGURE 5. ADAM9 overexpression increases N1 recovery in neurons and fibroblasts. ADAM9 was transiently transfected in neocortical neurons (TSM1 cell line) or in fibroblasts (B). N1 recovered in media was immunoprecipitated and monitored by Western blot analysis as described under “Materials and Methods.” Cell lysates were examined for their ADAM9, PrPc, and tubulin immunoreactivities by Western blot as described under “Materials and Methods.” Immunoprecipitation and Western Blot Analysis of Endogenous sAPPα in HEK293-antisense-ADAM9—Cells were allowed to secrete for 2 h in 1 ml of serum-depleted DMEM, then media were collected as above. The medium was supplemented with RIPA and incubated overnight with a 1000-fold dilution of polyclonal antibody 207 and protein A-Sepharose beads (Amersham Biosciences). After centrifugation, pellets were washed once with 500 μl of RIPA buffer, once with 500 μl PBS and subjected to SDS-polyacrylamide gel electrophoresis on a 8% Tris/glycine gel. Proteins were transferred onto nitrocellulose membranes (2 h, 100 V) and incubated overnight at 4 °C with the monoclonal antibody WO2. Immunological complexes

ImmunoResearch) and revealed by electrochemiluminescence as described (17).

Immunoprecipitation and Western Blot Analysis of Endogenous sAPPα in HEK293-antisense-ADAM9—Cells were allowed to secrete for 2 h in 1 ml of serum-depleted DMEM, then media were collected as above. The medium was supplemented with RIPA and incubated overnight with a 1000-fold dilution of polyclonal antibody 207 and protein A-Sepharose beads (Amersham Biosciences). After centrifugation, pellets were washed once with 500 μl of RIPA buffer, once with 500 μl PBS and subjected to SDS-polyacrylamide gel electrophoresis on a 8% Tris/glycine gel. Proteins were transferred onto nitrocellulose membranes (2 h, 100 V) and incubated overnight at 4 °C with the monoclonal antibody WO2. Immunological complexes

Beads were washed twice with 500 μl of RIPA buffer, once with 500 μl of PBS, and subjected to SDS-polyacrylamide gel electrophoresis on a 16.5% Tris/Tricine gel. Proteins were transferred onto polyvinylidene difluoride membrane (Amersham Biosciences) (45 min, 100 V) and incubated overnight at 4 °C with the monoclonal antibody SAF32 (dilution 1/2000). Immunological complexes were detected with a goat antimouse peroxidase-conjugated antibody (dilution 1/2000) (Jackson

40626 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 280 • NUMBER 49 • DECEMBER 9, 2005
were detected as above. BAPP immunoreactivity was monitored in cell lysates with 22C11 (1/2000) and SAF32 (1/2000) antibodies, respectively.

**N1 Production by Shedded ADAM10**—Wild-type fibroblasts cultured in 100-mm dishes were washed once with PBS and incubated for 8 h at 37 °C in 5 ml of serum-depleted DMEM. Media were collected and depleted from N1 fragment by incubation overnight with a 500-fold dilution of monoclonal antibody SAF32 and protein A-Sepharose beads. After centrifugation, supernatants were collected and referred to as depleted conditioned medium. Then A10−/− mouse embryonic fibroblast cells grown in 35-mm dishes were washed once with PBS and incubated for 8 h with 1 ml of DMEM, with or without the disintegrin inhibitor batimastat. N1 recovery was analyzed by combined immunoprecipitation/Western blot as described above.

**Western Blot Analysis of BAPP and sAPPa in ADAM10−/− Fibroblasts and in BAPP-expressing HEK293 Cells**—Cells cultured in 35-mm dishes were washed once with PBS and incubated for 2 h at 37 °C in the absence (control) or in the presence of various pharmacological agents in 1 ml of serum-depleted DMEM. Media were collected, and cells were resuspended in 500 μl of lysis buffer. Both medium and lysates were complemented with a protease inhibitor mixture (Sigma). Twenty μl of media (sAPPa) or 25 μg of proteins from cell lysates (BAPP) were subjected to SDS-PAGE on a 8% Tris/glycine gel, transferred on nitrocellulose (2 h, 100 V), and incubated overnight at 4 °C with monoclonal antibody 22C11 (1/2000). Immunological complexes were detected with the ECL method using the goat anti-mouse peroxidase-conjugated antibody (1/5000 dilution).

**Disintegrin Fluorimetric Assay on Intact Cells**—Wild-type and ADAM10-deficient fibroblasts were cultured in 6-well plates and transiently transfected with empty vector or pcDNA3 encoding mouse ADAM9 or/and ADAM10 as described above. Forty-eight hours after transfection, the medium was replaced by 1.5 ml of phosphate-buffered saline containing the α-secretase fluorimetric substrate (7-methoxy-coumarin-4-yl)acetyl-P-L-A-Q-A-V-V-N-3-(2,4-dinitrophenyl)-i,i-i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,i,
ADAM9 Contributes to PrP<sub>c</sub> Processing

**Statistical Analysis**—Statistical analysis was performed with the Prism software (Graphpad software, San Diego, CA) using the unpaired t test for pairwise comparisons.

**RESULTS**

**Transient and Stable Transfections of ADAM9 cDNA Increases N1 and sAPP<sub>x</sub> Recoveries in HEK293 Cells**—We previously established that ADAM10 and ADAM17 contributed to the production of a 11/12-Da PrP<sup>c</sup>-related fragment referred to as N1 in HEK293 cells (19). We examined whether transient transfection of murine ADAM9 cDNA could influence the recovery of N1. Western blot analysis of ADAM9 expression revealed two immunoreactive bands at 110 and 84 kDa (Fig. 1A) in agreement with a previous study (28) indicating that ADAM9 is first synthesized as a precursor, which is later processed (removal of inhibitory prodomain) by a proprotein convertase in the secretory pathway to yield the 84-kDa active form. Transfection of murine ADAM9 cDNA increases N1 recovery (Fig. 1, A and C) without significantly affecting PrP<sup>c</sup>-like immunoreactivity (Fig. 1A). Interestingly, ADAM9 transient transfection also drastically increases sAPP<sub>x</sub> recovery in βAPP-expressing HEK293 cells (Fig. 1, B and D), thereby confirming that in HEK293 cells, ADAM9 could participate to the α-secretase-mediated cleavage of BAPP as it did in COS cells and human glioblastoma A172 cells (21, 29).

To confirm this finding, we stably transfected ADAM9 cDNA in HEK293 cells. A series of positive clones exhibited the expected expression pattern, i.e. the high molecular weight precursor of ADAM9 and mature ADAM9 (Fig. 2A). Clone #8 was selected for further experiments. Stably transfected cells secrete drastically higher amounts of N1 than mock-transfected HEK293 cells (Fig. 2, B and C), while PrP<sup>c</sup>-like immunoreactivity was not affected (Fig. 2, B and D).

N1 production in stably transfected ADAM9-expressing cells was significantly inhibited by the zinc-metalloprotease inhibitor o-phenanthroline, while AEBSF (a serine-protease inhibitor) and pepstatin (acidic protease inhibitor) were unable to affect N1 recovery (Fig. 3). Further analysis of the effect of disintegrin inhibitors indicated that BB3103 and, to a lesser extent, TAPI both reduced significantly ADAM9-induced increase in N1 production (Fig. 3, A and B). Thus, in the presence of BB3103, ADAM9-associated increase of N1 production was fully abolished and N1 recovery returned to control secretion obtained with mock-transfected cells (Fig. 3, A and B). The rather selective pharmacological inhibitory spectrum of BB3103 toward disintegrins together with the extent of inhibition of N1 production triggered by this inhibitor suggest that PrP<sup>c</sup> undergoes little if any proteolysis at the 110–111 kDa site by proteases other than disintegrins.

**Statistical Analysis**—We previously established that ADAM9 and ADAM17 contributed to the production of a 11/12-Da PrP<sup>c</sup>-related fragment referred to as N1 in HEK293 cells (19). We examined whether transient transfection of murine ADAM9 cDNA could influence the recovery of N1. Western blot analysis of ADAM9 expression revealed two immunoreactive bands at 110 and 84 kDa (Fig. 1A) in agreement with a previous study (28) indicating that ADAM9 is first synthesized as a precursor, which is later processed (removal of inhibitory prodomain) by a proprotein convertase in the secretory pathway to yield the 84-kDa active form. Transfection of murine ADAM9 cDNA increases N1 recovery (Fig. 1, A and C) without significantly affecting PrP<sup>c</sup>-like immunoreactivity (Fig. 1A). Interestingly, ADAM9 transient transfection also drastically increases sAPP<sub>x</sub> recovery in βAPP-expressing HEK293 cells (Fig. 1, B and D), thereby confirming that in HEK293 cells, ADAM9 could participate to the α-secretase-mediated cleavage of BAPP as it did in COS cells and human glioblastoma A172 cells (21, 29).

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N1 and sAPP<sub>x</sub> Secretion Are Reduced by ADAM9 Antisense Strategy—To establish the contribution of endogenous ADAM9 to N1 formation, we have established HEK293 cells stably overexpressing ADAM9 antisense cDNA. Among the various clones displaying reduced ADAM9 expression, the one selected shows a 54% reduction in mature ADAM9-like immunoreactivity (Fig. 4A). Lowering ADAM9 expression led to a statistically significant reduction of N1 (Fig. 4, B and C) and sAPP<sub>x</sub> (Fig. 4, D and E) recoveries without altering endogenous PrP<sup>c</sup>-like (B)- and βAPP (D)-like immunoreactivities. Altogether, these data indicate that, as documented above for overexpressed ADAM9, endogenous ADAM9 contributed to N1 formation and to the α-secretase pathway yielding sAPP<sub>x</sub> in HEK293 cells.

**Transient Transfection of ADAM9 cDNA Increases N1 and sAPP<sub>x</sub> Recoveries in TSM1 Neurons and Fibroblasts**—We examined whether the control of N1 production by ADAM9 was cell-specific. TSM1 is a neuronal cell line of neocortical origin (30) in which N1 production had been documented (17). As expected, N1 was readily detectable in mock-transfected TSM1 neurons, the production of which was enhanced by ADAM9 transient expression (Fig. 5A). Similar experiments carried out with immortalized fibroblasts led to similar N1 increased recovery (Fig. 5B) indicating that ADAM9-associated increase of N1 was not cell-specific. It should be noted that in both cell types, ADAM9 transfection led to increased expressions of both pro-ADAM9 and ADAM9, indicating that in neurons and fibroblasts, ADAM9 was properly processed and, according to increased N1 production, fully catalytically functional.

**Transient Transfection of ADAM9 cDNA Increases N1 and sAPP<sub>x</sub> Recoveries in Wild-type but Not in ADAM10<sup>−/−</sup> Fibroblasts**—Previous works demonstrated that the disintegrin metalloprotease ADAM10 had an α-secretase activity responsible for processing the amyloid precursor protein (31) and PrP<sup>c</sup> (19). Our study now clearly shows that ADAM9 contributes to the proteolytic processing of PrP<sup>c</sup> besides that of βAPP suggested previously (21). However, our data did not establish whether catalysis triggered by ADAM9 was direct or indirect and one could not preclude a possible functional cross talk between disintegrins for PrP<sup>c</sup> and βAPP proteolysis. Thus, ADAM9 and ADAM10 could independently participate in both PrP<sup>c</sup> or βAPP processing or, alternatively, one of these enzymes could be rate-limiting for full functionality of the other. To answer this question, we took advantage of recently established ADAM10<sup>−/−</sup> fibroblasts cell line (26) to assess whether the deletion of this enzyme could impair ADAM9-associated N1 and sAPP<sub>x</sub> productions. In wild-type fibroblasts, transient transfection of ADAM9 cDNA led to increased recoveries of released (but not intracellular) N1 (Fig. 6, A and B) and sAPP<sub>x</sub> (Fig. 7A) and full expression and maturation of ADAM9 (Fig. 6, C and D). In ADAM10-deficient fibroblasts, N1 (Fig. 6, A and B) and sAPP<sub>x</sub> (Fig. 7A) recoveries were drastically reduced in control conditions and were not enhanced by ADAM9 cDNA transient transfection, although ADAM9 remained fully expressed and maturated (Figs. 6, C and E, and 7B). Therefore, our data indicate that ADAM9 likely indirectly contributed, in an ADAM10-dependent manner, to N1 and sAPP<sub>x</sub> production.

**ADAM9 Does Not Affect ADAM10 Expression**—We designed several experiments to delineate the cross-talk between ADAM9 and ADAM10. First, we examined whether exogenous ADAM9 could affect endogenous ADAM10 expression. Co-expression of ADAM9 and
ADAM10 in ADAM10-deficient fibroblasts did not change ADAM10-like immunoreactivity observed after ADAM10 cDNA transfection, only (Fig. 8, A and B). Thus both mature and immature forms of ADAM10 remained unchanged. ADAM9 cDNA transfection did not modify the mRNA levels of endogenous ADAM10 in wild-type fibroblasts (data not shown). Interestingly, the use of the disintegrin inhibitor TAPI confirmed that the expression and ratio of mature and immature forms of ADAM10 remained unchanged in single and double transfection experiments (Fig. 8, A and B).

It should be noted that disintegrins activation mechanism is a well known maturation process consisting in the removal of a prosegment that could be either self-catalyzed or could necessitate the involvement of an additional prohormone convertase activity as was demonstrated for ADAM15 (32), ADAM9 (28), and ADAM17 (33). ADAM10 is also processed by the PC7 prohormone convertase that releases its prodomain (25). To further rule out the possibility of a modulation of ADAM10 maturation by ADAM9, we took advantage of a series of ADAM10 constructs recently designed to demonstrate the involvement of PC7 in the maturation of ADAM10. Thus, the 160 construct corresponds to an ADAM10 species rendered resistant to PC7 proteolysis by introduction of a NAQA sequence replacing the RKKR targeted by PC7 (25). Furthermore, we used a control ADAM10 construct (125) corresponding to the enzyme deleted of its prodomain (25). Therefore, these constructs allowed us to examine whether ADAM9 could substitute for PC7 to process ADAM10 at another site. Fig. 9A shows that as expected, ADAM10 but not ADAM9, increased N1 recovery after transfection in ADAM10-deficient fibroblasts. Interestingly, ADAM10-125 and ADAM10-160 alone or in combination with ADAM9 did not alter N1 production while co-expression of ADAM9 with wild-type ADAM10 led to increased N1 production (Fig. 9, A and B). These data clearly demonstrate the inability of ADAM9 to act as a maturation enzyme for ADAM10 when PC7-mediated cleavage is abolished. Finally, we established, by co-immunoprecipitation experiments, that ADAM9 and ADAM10 did not physically interact and that ADAM9 overexpression did not alter the intracellular distribution of endogenous ADAM10 (data not shown). Altogether, the above data indicate that the indirect ADAM10-dependent ADAM9-associated processing of cellular prion could not be explained by a modulation of ADAM10 transcription or maturation by ADAM9.

**ADAM9 Contributes to PrPc Processing**

**FIGURE 9. ADAM9 does not affect ADAM10 maturation. ADAM10-deficient (A10-/−) fibroblasts were transiently transfected with either pcDNA3 (Cl) or indicated cDNA (A and B). Forty-eight hours after transfection, cells were allowed to release N1, which was monitored as described under “Materials and Methods.” All ADAM9- and ADAM10-related expressions were monitored by Western blot as described under “Materials and Methods.”**

**FIGURE 10. ADAM9 influences membrane-bound and secreted α-secretase activity in an ADAM10-dependent manner. Kinetic analysis of the hydrolysis of the α-secretase quenched fluorimetric substrate by intact cells in ADAM10−/− fibroblasts transiently transfected with the indicated cDNA constructs allowed us to examine whether ADAM9 could substitute for ADAM10-125 and ADAM10-160 alone or in combination with ADAM9 did not alter N1 production while co-expression of ADAM9 with wild-type ADAM10 led to increased N1 production (Fig. 9, A and B). These data clearly demonstrate the inability of ADAM9 to act as a maturation enzyme for ADAM10 when PC7-mediated cleavage is abolished. Finally, we established, by co-immunoprecipitation experiments, that ADAM9 and ADAM10 did not physically interact and that ADAM9 overexpression did not alter the intracellular distribution of endogenous ADAM10 (data not shown). Altogether, the above data indicate that the indirect ADAM10-dependent ADAM9-associated processing of cellular prion could not be explained by a modulation of ADAM10 transcription or maturation by ADAM9.

ADAM9 Expression Increases the Levels of ADAM10-dependent Membrane-bound and Released α-Secretase-like Activity—We therefore examined whether ADAM9 could influence ADAM10 activity downstream to its membrane-bound localization. We took advantage of the availability of an α-secretase fluorimetric substrate to measure α-secretase activity in its accurate membrane-bound configuration. Thus, since the fluorimetric substrate is non-permeant, any activity detected on intact fibroblasts would be due to protease(s) with catalytic sites facing the extracellular space or present in the culture medium. This configuration is the one expected for genuine ectopeptidasic enzymes such as disintegrins. Clearly, overexpression of ADAM9 in ADAM10-deficient fibroblasts did not modify the α-secretase activity present at the membrane (Fig. 10A). This observation fits perfectly with the inability of ADAM9 to process βAPP and PrP in ADAM10−/− fibroblasts (see Figs. 6 and 7). Conversely, ADAM10 cDNA transfection
ADAM9 Contributes to PrPc Processing

led to increased detection of α-secretase activity at the membrane (Fig. 10A). Interestingly, ADAM9/ADAM10 co-transfection further increased α-secretase activity, indicating that while inactive in absence of ADAM10, ADAM9 functionally interacts with ADAM10.

To examine whether ADAM9 could participate to the shedding of ADAM10, we measured α-secretase activity released from ADAM10−/− fibroblasts. Independent transfection of ADAM9 or ADAM10 cDNA did not modify basal activity detectable in control conditions (Fig. 10B). However, the co-expression of ADAM9 and ADAM10 significantly enhanced the level of α-secretase-like activity released from ADAM10−/− cells (Fig. 10B). As ADAM9 is unable to cleave the substrate (see Fig. 10A), it could be concluded that the observable activity likely corresponded to shedded ADAM10.

Shedded ADAM10 Generates N1 from ADAM10−/− Fibroblasts—To examine directly whether ADAM10 could cleave endogenous PrPc and generate N1, we conditioned medium from wild-type fibroblasts, which we then depleted of its endogenous N1 (Fig. 11, A and B). This source of enzyme displays high batimastat-sensitive fluorometric substrate-hydrolyzing α-secretase-like activity (data not shown). This activity was able to generate N1 from ADAM10−/− fibroblasts, the formation of which was fully prevented by ADAM inhibitor (Fig. 11, C and D). Therefore, shedded ADAM10 displays the ability to directly cleave PrPc and, thereby, contributes to N1 formation.

DISCUSSION

One of the most puzzling discoveries in recent neurobiology was the demonstration that a new type of neurodegenerative diseases, transmissible spongiform encephalopathies, could be caused by an infectious proteinaceous agent genetically called prion (1). Interest in this type of pathology had been boosted by the observation that, besides well known cases reported for ovines (called scrapie) and later for bovines (bovine spongiform encephalopathies), some cases referred to as Creutzfeldt-Jakob disease and, more recently, new variants of Creutzfeldt-Jakob Disease, could be found in human beings (3–5), revealing a new problem of public health. The “infectious” agent (PrPSc or PrPSc+) was found to occur as an “abnormal” conformer of a ubiquitous protein called PrPc and differs from the latter by its lower susceptibility to proteolysis (6, 7). Of most interest was the demonstration that the most infectious innoculates of PrPSc remained innocuous in absence of endogenous PrPc. Thus, PrPSc null mice resist toxicity and infection by scrapie homogenates (8–10). Therefore, strategies aimed at lowering or depleting endogenous PrPc has the potential of slowing down or preventing disease transmission. Antibody sequestration/depletion of PrPc or, alternatively, enhancement of its proteolytic attack are therefore empirical therapeutic strategies to lower endogenous PrPc. Of importance is the observation that mice depleted of their endogenous PrPc are healthy, fertile, and viable (11).

Very few studies have documented the fate of PrPc in vivo. Chen et al. (13) first described a “normal” cleavage taking place at the 110–111/112 peptide bond (leading to the formation of a fragment referred to as N1) and reported on an additional breakdown specifically occurring upstream at the 90/91 peptide bond in pathological cases. However, the nature of the proteases involved in these cleavages remained unknown.

We previously demonstrated that PrPc undergoes cleavages by two proteases belonging to the ADAM (a disintegrin and metalloprotease) family. Indeed, we showed that ADAM10 and ADAM17 participated to the constitutive and protein-kinase C-regulated production of N1 (17, 19). Interestingly, there exists a close parallel between PrPc and βAPP metabolism, and ADAM10 and ADAM17 are some of the common denominators that can be clearly identified between prion and Alzheimer disease (34). However, one group previously documented the fact that βAPP could also undergo proteolysis by ADAM9 (21, 22). We therefore examined whether ADAM9 could also participate to PrPc physiological proteolysis. Indeed, overexpression of ADAM9 in several cell systems including HEK293 cells, TSM neurons, and fibroblasts drastically increases N1 recovery, while conversely, the down-regulation of endogenous ADAM9 reduces N1 formation. Therefore, ADAM9 contributes to both PrPc and βAPP proteolysis.

Is ADAM9 acting directly on PrPc and βAPP or upstream of another protease? Our study clearly suggests that ADAM9 acts upstream of ADAM10 that would be the end point of a catalytic cascade ultimately leading to N1 formation. Thus, ADAM9 expression increases N1 in wild-type fibroblasts but not in fibroblasts devoid of ADAM10. However, ADAM10 deficiency does not affect ADAM9 maturation, and therefore, the inability of ADAM9 to increase N1 in ADAM10−/− fibroblasts was due not to the impairment of ADAM10-mediated ADAM9 maturation process. On the other hand, ADAM9 does not affect ADAM10 mRNA levels or maturation. Furthermore, we established that in ADAM10−/− fibroblasts, ADAM9 did not hydrolyze a fluorometric substrate used as a reporter of α-secretase activity, in agreement with the inability to modulate N1 production. However, co-expression of both ADAM9 and ADAM10 clearly potentiated the cleavage of the
fluorimetric substrate observed after the expression of ADAM10 only. This potentiation was illustrated by increases of membrane-bound and released ADAM10-dependent α-secretase activity. Therefore, it seems reasonable to postulate that ADAM9 would act upstream of ADAM10, which would likely be the genuine PrP\(\text{Sc}\) and βAPP-cleaving enzyme, by increasing its shedding. Interestingly, a recent abstract (35) suggested that ADAM9 could shed ADAM10, thereby releasing a soluble ectodomain that remained catalytically active and could serve as a source of circulating protease.

As stated above, depletion of endogenous PrP\(\text{Sc}\) could be a means to prevent scrapie-associated pathologies. Therefore, ADAM9 could be seen as a therapeutic target and its activation or overexpression as a potential strategy. The feasibility of such strategy has been nicely documented in transgenic mice. Thus, overexpression of ADAM10 in an Alzheimer disease mouse model prevents amyloid plaques and reduced cognitive deficits (36). This potentiation was illustrated by increases of membrane-bound and released ADAM10-dependent APP-secretase activity. Therefore, it seems that the ADAM9/10-secretase activity, by modifying the N-terminus of the 106–126 domain, may delay the pathological stigmata but also abolishes the potential 106–126-associated toxicity of PrP\(\text{Sc}\). Further study is needed to examine whether ADAM9 or ADAM10 transgenic mice inoculated with the infectious agent could resist infection.

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