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Role of ADAMs in the Ectodomain Shedding and Conformational Conversion of the Prion Protein*

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The cellular prion protein (PrP^C) is essential for the pathogenesis and transmission of prion diseases. PrP^C is bound to the plasma membrane via a glycosylphosphatidylinositol anchor, although a secreted, soluble form has also been identified. Previously we reported that PrP^C is subject to ectodomain shedding from the membrane by zinc metalloproteinases with a similar inhibition profile to those involved in shedding the amyloid precursor protein. Here we have used gain-of-function (overexpression) and loss-of-function (small interfering RNA knockdown) experiments in cells to identify the ADAMs (a disintegrin and metalloproteinases) involved in the ectodomain shedding of PrP^C. These experiments revealed that ADAM9 and ADAM10, but not ADAM17, are involved in the shedding of PrP^C and that ADAM9 exerts its effect on PrP^C shedding via ADAM10. Using dominant negative, catalytically inactive mutants, we show that the catalytic activity of ADAM9 is required for its effect on ADAM10. Mass spectrometric analysis revealed that ADAM10, but not ADAM9, cleaved PrP between Gly^{228} and Arg^{229}, three residues away from the site of glycosylphosphatidylinositol anchor attachment. The shedding of another membrane protein, the amyloid precursor protein β-secretase BACE1, by ADAM9 is also mediated via ADAM10. Furthermore, we show that pharmacological inhibition of PrP^C shedding or activation of both PrP^C and PrP^{Sc} shedding by ADAM10 overexpression in scrapie-infected neuroblastoma N2a cells does not alter the formation of proteinase K-resistant PrP^{Sc}. Collectively, these data indicate that although PrP^C can be shed through the action of ADAM family members, modulation of PrP^C or PrP^{Sc} ectodomain shedding does not regulate prion conversion.

The prion protein (PrP)[3] is the causative agent of the transmissible spongiform encephalopathies such as Creutzfeldt-Jakob disease in humans, scrapie in sheep, bovine spongiform encephalopathy in cattle, and chronic wasting disease in deer and elk (1). In these diseases the cellular form of PrP (PrP^C) undergoes a conformational conversion to the infectious form PrP^{Sc} that is characterized biochemically by its resistance to digestion with proteinase K (PK) (2). Mature PrP^C is anchored to the extracellular surface of the cell membrane through a glycosylphosphatidylinositol (GPI) anchor and, like most GPI-anchored proteins, is clustered into cholesterol-rich, detergent-resistant membrane rafts (reviewed in Ref. 3). Although the precise subcellular site of conversion remains undefined, conformational conversion of PrP^C to PrP^{Sc} is believed to occur either at the cell surface or within the endocytic pathway (4–6).

A number of studies indicate that modulation of PrP^C levels at the cell surface may represent a possible future disease intervention strategy. For example, the retention of PrP^C at the cell surface and concomitant prevention of its endocytosis through the use of PrP antibodies inhibited PrP^{Sc} formation (7). Furthermore, the sterol-binding polyene antibiotic filipin reduced endocytosis, and induced cellular release, of PrP^C with a concomitant reduction in PrP^{Sc} accumulation (8). More recently, it has been shown that modulation of cell surface PrP^C levels by the novel sorting nexin SNX33 can interfere with PrP^{Sc} formation in cultured cells (9). Nonetheless, the natural processes regulating PrP^C levels at the cell surface remain poorly defined. One such mechanism of regulation is via shedding of the bulk of the ectodomain of PrP^C either through cleavage of the polypeptide close to the GPI anchor or within the GPI anchor itself. Indeed, it has long been established that PrP^C can be shed into the medium of cultured cells and is present as a soluble form *in vivo* in human cerebrospinal fluid (10, 11).

Numerous cell surface proteins can be proteolytically shed by the action of a group of zinc metalloproteinases known collectively as secretases or sheddases (reviewed in Refs. 12, 13). Whereas most proteolytically shed proteins are derived from transmembrane polypeptide-anchored substrates, several GPI-anchored proteins, including the folate receptor (14), the ecto-ADP-ribosyltransferase ART2.2 (15), and a GPI-anchored construct of angiotensin-converting enzyme (16) are shed by the action of metalloproteinases. We have previously shown that PrP^C can also be proteolytically shed from the cell surface through the action of one or more zinc metalloproteinases with similar properties to those of the α-secretases responsible for the shedding of the amyloid precursor protein (APP) of Alzheimer disease (17). This α-secretase-mediated ectodomain shed-

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[3] The abbreviations used are: PrP, prion protein; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; BACE1, β-site APP-cleaving enzyme-1; GPI, glycosylphosphatidylinositol; HEK, human embryonic kidney; MS, mass spectrometry; PK, proteinase K; PrP^C, cellular form of PrP; PrP^{Sc}, proteinase-resistant form of PrP; rprP, recombinant PrP; sAPPα, soluble form of APP derived from α-secretase cleavage; sBACE1, shed form of BACE1; SNX, sorting nexin; sPrP, shed form of PrP; RT, reverse transcription; siRNA, small interfering RNA; dn, dominant negative.

ding of APP from the cell surface is carried out by at least three members of the a disintegrin and metalloproteinase (ADAM) family, namely ADAM9, -10, and -17 (reviewed in Ref. 18). In addition to cleavage by ADAMs, APP is also cleaved by the β-secretase, BACE1 (β-site APP-cleaving enzyme) and the γ-secretase complex to release the neurotoxic amyloid-β peptide (19). BACE1 itself is also subject to ectodomain shedding by as yet unidentified members of the ADAM family (20).

The similarities between the ectodomain shedding of APP and PrP^C, in particular the similar profile of inhibition by a range of hydroxamate-based zinc metalloproteinase inhibitors (17), led us to investigate whether the same members of the ADAM family were also involved in the shedding of PrP^C. It should be noted that this ectodomain shedding of PrP^C by cleavage of the polypeptide chain near to the site (Ser^{231}) of GPI anchor addition in the C terminus of the protein is distinct from the so-called α-cleavage between residues 111 and 112 in the middle of the protein (21, 22). This latter "endoproteolytic" cleavage of PrP^C is reported to be carried out by members of the ADAM family (23, 24).

To investigate the role of ADAMs in the ectodomain shedding of PrP^C, we used loss-of-function and gain-of-function experiments in cultured cells in which candidate PrP sheddases were either knocked down by siRNA or overexpressed. We have also further characterized the shedding of BACE1 by comparison to the shedding of APP and PrP^C. In addition, we have explored whether proteolytic shedding of PrP^C is of importance in regulating its conversion into PrP^{Sc}.

EXPERIMENTAL PROCEDURES

Antibodies—Antibody 3F4 (Cambridge Bioscience Ltd., Cambridge, UK) recognizes the 3F4 epitope tag (corresponding to amino acid residues 109–112 of human PrP) at residues 108–111 of murine PrP. Antibody SAF-32 (Cayman Chemical, Ann Arbor, MI) recognizes the octapeptide repeats in the N-terminal half of PrP. The epitope for antibody 6D11 (Eurogentec Ltd., Southampton, UK) falls within amino acids 93–109 of PrP. Antibody 6H4 (Prionics AG, Schlieren, Switzerland) recognizes the amino acid sequence DYEDRYYRE (human PrP: amino acids 144–152). Antibody 6E10 (Cambridge Bioscience Ltd., Cambridge, UK) recognizes amino acid residues 1–17 of the human amyloid-β sequence within APP. Antibody Ab54 was kindly provided by Dr. I. Hussain (GlaxoSmithKline, Harlow, UK) and recognizes the C terminus of APP. Anti-ADAM9, anti-ADAM10, and anti-ADAM17 antibodies were from Merck. The anti-actin antibody (AC15) and the anti-BACE1 antibody (EE17) were from Sigma.

Recombinant Proteins—Recombinant murine PrP (residues 23–231) was from Allprion AG (Schlieren, Switzerland). Recombinant human ADAM9 and ADAM10 were purchased from R & D Systems (Abingdon, UK).

Cell Culture—Human embryonic kidney (HEK), mouse neuroblastoma N2a, and scrapie-infected N2a cells (ScN2a) (25) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (50 units ml^{-1}), streptomycin (50 mg ml^{-1}), and 2 mM glutamate (all from Invitrogen). Cells were maintained at 37 °C in 5% CO_2 in air. GW0264 (provided by Dr. I. Hussain, GlaxoSmithKline, Harlow, UK) was used at a concentration of 10 μM. For all HEK cell incubations, the medium was changed at confluence to Opti-MEM (Invitrogen), and the cells were incubated for 5 h with the indicated compounds. For N2a and ScN2a cell incubations, the medium was changed at confluence to Opti-MEM, and the cells were incubated for 24 h with the indicated compounds. The medium was then harvested and concentrated 50-fold using Vivaspin 6 concentrators (Sartorius, Epsom, UK). For analysis of cell-associated proteins, cells were washed with phosphate-buffered saline (20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.15 M NaCl, pH 7.4) and scraped from the flasks into phosphate-buffered saline. Following centrifugation at 500 × *g* for 5 min, the pelleted cells were lysed in 0.1 M Tris-HCl, 0.15 M NaCl, 1% (v/v) Triton X-100, 0.1% (v/v) Nonidet P-40, pH 7.4.

Stable cDNA Transfections—cDNA encoding either murine PrP (containing a 3F4 tag at amino acid residues 108–111) (26) in the mammalian expression vector pIRES*neo* (Invitrogen) or human BACE1 in the vector pIRES*hyg* were introduced into HEK cells by electroporation. Recombinant cells were selected using 500 μg ml^{-1} gentamycin sulfate (G418; Sigma) or hygromycin, respectively.

Transient cDNA and siRNA Transfections—HEK or ScN2a cells (at 80% confluence) were transiently transfected with 8 μg of pcDNA3.1b containing the cDNA encoding either murine ADAM9, bovine ADAM10, or murine ADAM17 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were used 36 h post-transfection. For siRNA treatments, HEK cells were grown to 60% confluence in antibiotic-free growth medium and transfected with siRNA duplexes (Eurogentec Ltd., Southampton, UK) using DharmaFECT-1 (Dharmacon) according to the manufacturer's instructions. Cells were transfected with 100 nM duplexes targeted to ADAM9 (sense sequences: duplex 1, 5′-GUGCACAGCUAGUUCUAAAdTdT-3′; duplex 2, 5′-GGAGGAAACUGCCUUCUUAdTdT-3′; or duplex 3 5′-GAGGAUUGCUGCAUUUAGAdTdT-3′); or ADAM10 (sense sequences: 5′-GCCAAGUUCUUGAGAAGAAdTdT-3′, 5′-GAUAUCCAGUCAUGUUAAAdTdT-3′, and 5′-CUGGAAUUAUUACUGUUCAdTdT-3′). Control cells were subjected to transfection with control Smartpool siRNA (Dharmacon). Cells were used 48 h post-transfection.

Generation of Catalytically Inactive ADAM9 and ADAM10—Catalytically inactive dominant negative (dn) mutants of murine ADAM9 and bovine ADAM10 were generated by site-directed mutagenesis using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic primer sequences were as follows: dnAD9 E348A 5′-CATCCATTGTTGCTCATGCATTGGGGCATAACCTTGG-3′ and dnAD10 E384A 5′- CTCACATTACGTTTGCTCATGCAGTTGGACATAACTTTGGATC-3′. Mutants were verified by DNA sequencing (The Sequencing Service, Dundee, UK).

Protein Assay and Enzyme Treatments—Protein was quantified using bicinchoninic acid (27) with bovine serum albumin as standard. Peptide:*N*-glycosidase F deglycosylation was performed by adding 40 μl of 5× peptide:*N*-glycosidase F buffer (30 mM Na_2HPO_4/NaH_2PO_4, pH 7.2, 20 mM EDTA) to 200 μg of cell lysate along with 5 μl of 10% (w/v) SDS and 5 μl of β-mercaptoethanol. The samples were boiled for 5 min and

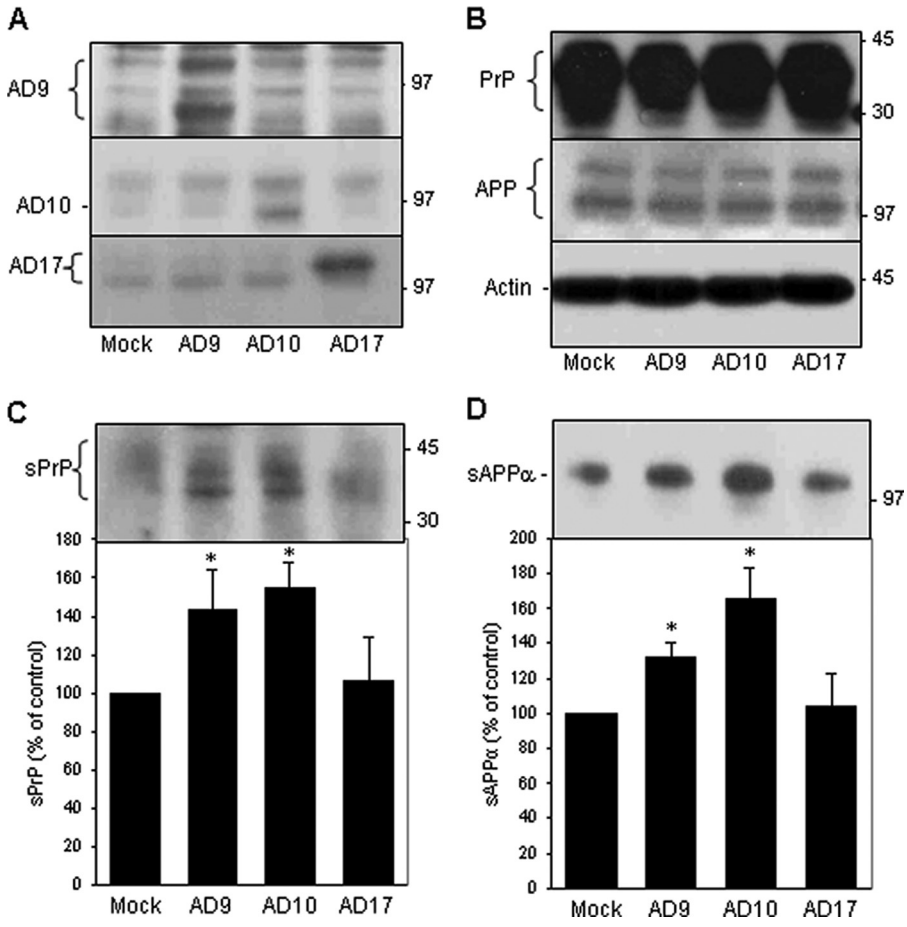

FIGURE 1. **Shedding of PrP^C is enhanced by ADAM9 and ADAM10.** HEK cells stably expressing PrP^C were transiently transfected with either empty vector (*Mock*) or the cDNAs encoding ADAM9 (*AD9*), ADAM10 (*AD10*), or ADAM17 (*AD17*). Transfected cells were incubated for 5 h in serum-free medium before harvesting the conditioned medium and preparing cell lysates. *A,* immunodetection of ADAMs in cell lysates. *B,* immunodetection of PrP^C (antibody 3F4), APP (antibody Ab54), and actin in cell lysates. *C,* immunodetection of sPrP in conditioned medium (antibody 3F4). *D,* immunodetection of sAPPα in conditioned media (antibody 6E10). *C* and *D*, multiple immunoblots were quantified by densitometric analysis. Results are the mean ± S.D. ($n = 3$). *, significant at $p \leq 0.05$.

then 20% (v/v) Triton X-100 (5 μl) was added along with 1 unit of peptide:*N*-glycosidase F. Samples were then deglycosylated for 16 h at 37 °C. For PK digestion, 200 μg of cell lysate protein in a volume of 200 μl was incubated for 30 min at 37 °C with 5 μg of PK. The reaction was stopped by the addition of phenylmethanesulfonyl fluoride to a final concentration of 3 mM. Protein was precipitated by the addition of 800 μl of ice-cold methanol, incubation overnight at 4 °C, pelleted by centrifugation, and then resuspended in electrophoresis sample buffer prior to SDS-PAGE.

SDS-PAGE and Immunoelectrophoretic Blot Analysis—Samples were mixed with an equal volume of reducing electrophoresis sample buffer and boiled for 3 min. Proteins were resolved by SDS-PAGE using 7–17% polyacrylamide gradient gels and transferred to Immobilon P polyvinylidene difluoride membranes as described previously (28). Antibodies were used at the following dilutions: 3F4 at 1:4000; SAF-32 at 1:3000; 6D11 at 1:10,000; 6H4 at 1:20,000; 6E10 at 1:2500; Ab54 at 1:10,000; anti-ADAM9, -10, and -17 at 1:2000; EE17 at 1:2500; and the anti-actin antibody at 1:5000. Bound antibody was detected using peroxidase-conjugated secondary antibodies in conjunction with the enhanced chemiluminescence detection method (Amersham Biosciences).

Quantitative RT-PCR Analysis—To monitor endogenous ADAM9 expression, RNA from cell samples was extracted using TRIzol (Invitrogen). Samples containing 1 μg of RNA were then subjected to total cDNA strand synthesis followed by ADAM9 or β-actin amplification using Titanium one-step RT-PCR kit (Clontech). The ADAM9 primer sequences were as follows: 5′-GCTAGTTGGACTGGAGATTTGG-3′ and 5′-TTATTACCACAGGAGGGAGCAC-3′ giving a PCR product of 486 bp. The actin primers were those provided with the Titanium kit. RT-PCR products were resolved on an agarose gel containing ethidium bromide.

Mass Spectrometry—To assess cleavage of PrP by ADAM9 and ADAM10, recombinant PrP was diluted to a final concentration of 10 μM in 25 mM Tris-HCl, pH 9.0, 2.5 μM $ZnCl_2$, 0.005% (v/v) Brij-35. Samples were incubated in the presence or absence of 200 ng of recombinant ADAM9 or ADAM10 for 3 h at 37 °C. Samples were then drop dialyzed into 1 M ammonium acetate, and subsequently, 5 μl of sample was diluted to 1:1 (v/v) methanol, 0.1% aqueous formic acid and analyzed by Z-spray nanoelectrospray ionization MS using a quadrupole-ion mobility spectrometry-orthogonal time-of-flight MS (Synapt HDMS, Waters). The MS was operated in positive TOF mode using a capillary voltage of 1.0 kV, cone voltage of 50 V, nanoelectrospray nitrogen gas pressure of 0.1 bar, and backing pressure of 2.01 mbar. The source and desolvation temperatures were set at 80 and 150 °C, respectively. Nitrogen was used as buffer gas, at a pressure of 7.87×10^{-3} mbar in the trap and transfer regions and 3.1×10^{-4} mbar in the ion mobility spectrometry cell. Mass calibration was performed by a separate injection of sodium iodide at a concentration of 2 μg/μl. Data processing was performed using the MassLynx version 4.1 suite of software supplied with the mass spectrometer.

ADAM Activity Assay—The activity of recombinant ADAM9 and ADAM10 was assessed using a fluorogenic peptide substrate Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2 (R & D Systems). The peptide substrate was diluted to a final concentration of 10 μM in 25 mM Tris-HCl, pH 9.0, 2.5 μM $ZnCl_2$, 0.005% (v/v) Brij-35 and incubated, where indicated, with 200 pg of ADAM9 or ADAM10, and the inhibitor

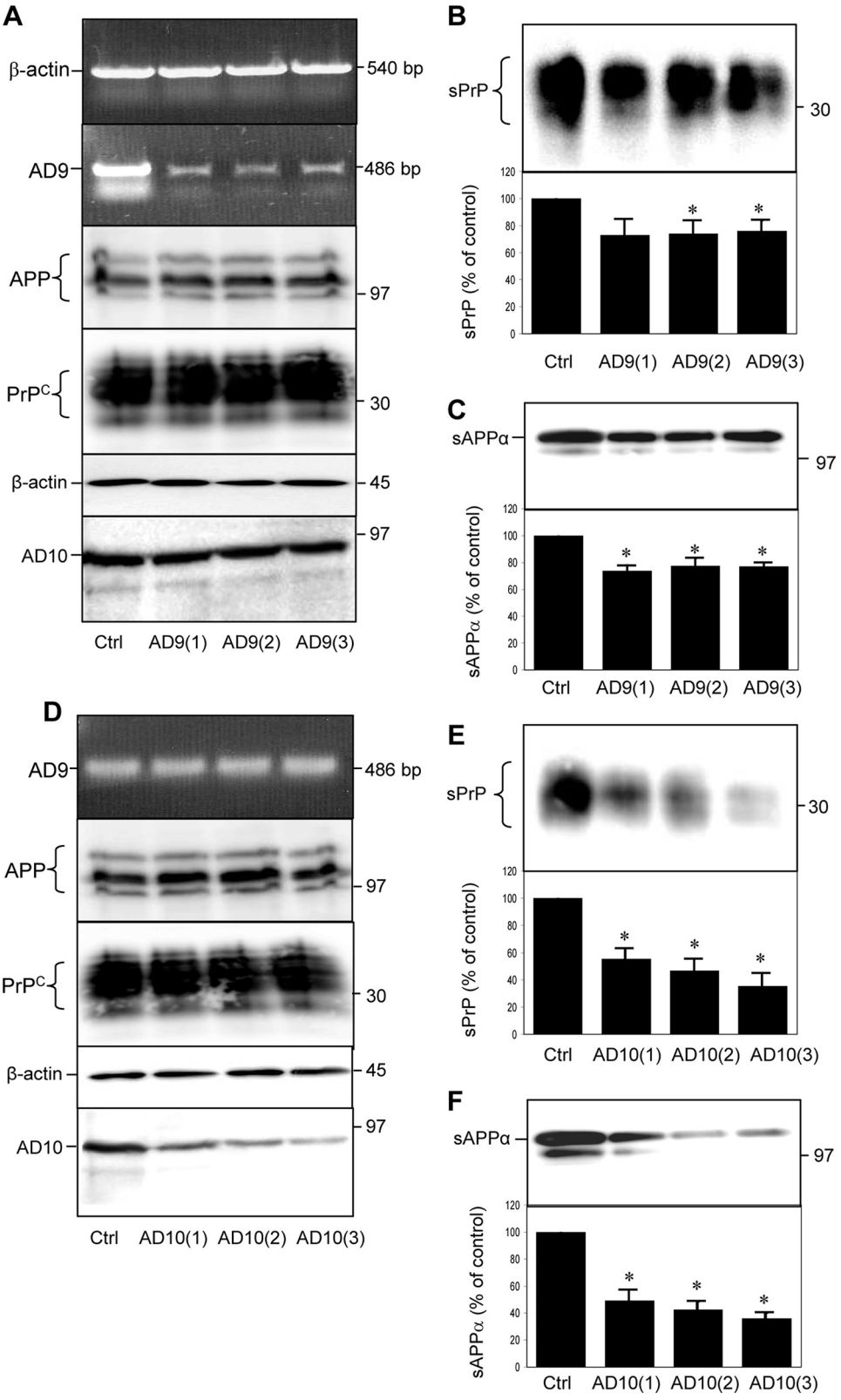

FIGURE 2. **Knockdown of ADAM9 and ADAM10 by siRNA reduces the shedding of PrP^C.** HEK cells stably expressing PrP^C were transiently transfected with one of three different siRNA duplexes to either ADAM9 (*AD9*) (*A–C*) or ADAM10 (*AD10*) (*D–F*) as detailed under "Experimental Procedures." Transfected cells were incubated for 5 h in serum-free medium before harvesting the conditioned medium and preparing cell lysates. *A* and *D*, immunodetection of ADAM10, PrP^C, APP, and actin in cell lysates. ADAM9 was detected by semi-quantitative RT-PCR. Immunodetection of sPrP (*B* and *E*) and sAPPα (*C* and *F*) in conditioned medium using antibodies 3F4 and 6E10, respectively, is shown. Multiple immunoblots were quantified by densitometric analysis. Results are the mean ± S.D. ($n = 3$). *, significant at $p \leq 0.05$. *Ctrl*, control.

GW4023 (final concentration 10 μM). Peptide cleavage was monitored using an OptiPlate fluorescence plate reader (PerkinElmer Life Sciences) with excitation at 310 nm and emission at 400 nm.

Statistical Analysis—Statistical analysis was performed using unpaired *t* tests for pairwise comparison in all instances. Significance was assigned when $p \leq 0.05$.

RESULTS

PrP^C Is Shed by ADAM9 and ADAM10—To determine whether the same ADAM family members responsible for shedding APP could also shed PrP^C, we transiently transfected cDNAs encoding ADAM 9, ADAM10, or ADAM17 into HEK cells stably expressing PrP^C (Fig. 1). Immunoblot analysis of ADAM9 in cell lysates (Fig. 1*A*) revealed a number of nonspecific bands along with an increased intensity of two immunoreactive bands at 110 and 84 kDa representing the prodomain containing and mature forms of ADAM9, respectively, consistent with previous studies (24, 29). The anti-ADAM10 antibody detected a nonspecific band at 105 kDa, which was also present in the mock-transfected cell lysates (Fig. 1*A*), along with a specific band at 90 kDa that corresponds to the prodomain containing form of ADAM10 in agreement with previous reports (24, 30). The prodomain containing form of ADAM17 was detected as a 110-kDa immunoreactive band consistent with a previous report (30).

The levels of cell-associated PrP^C and APP were not significantly altered by co-expression of any of the ADAMs (Fig. 1*B*). However, co-expression of ADAM9 and ADAM10 significantly enhanced the release of shed PrP (sPrP) into conditioned medium (1.44- and 1.55-fold, respectively) (Fig. 1*C*). Similarly, ADAM9 and ADAM10 significantly enhanced the shedding of sAPPα into the conditioned medium by 1.32- and 1.65-fold, respectively (Fig. 1*D*). Expression of ADAM17 had no significant effect on the shedding of sPrP or of sAPPα (Fig. 1,

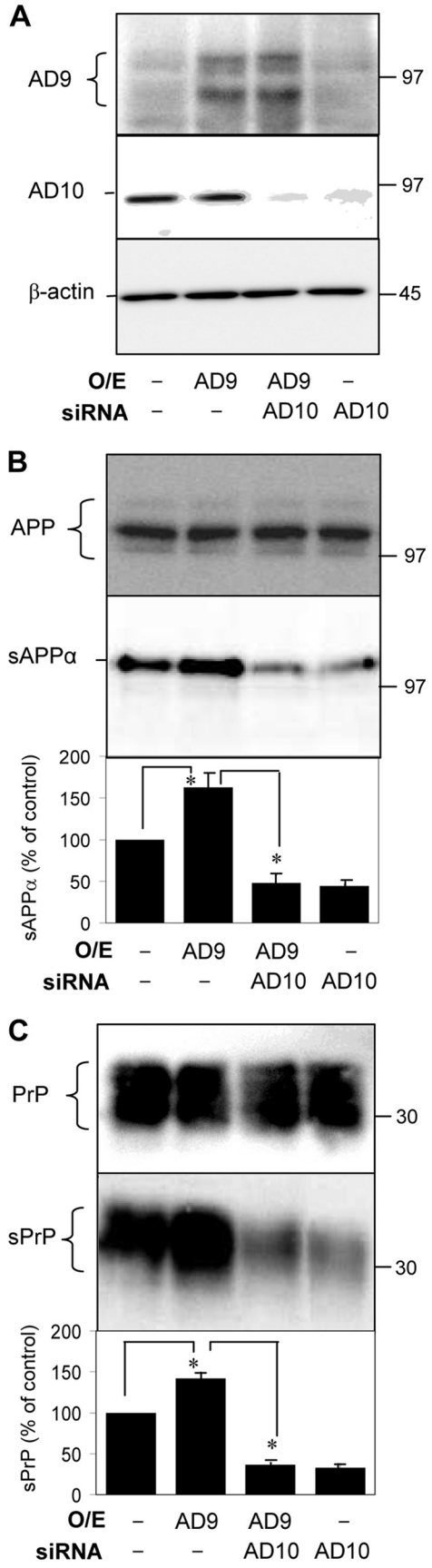

FIGURE 3. **Knockdown of ADAM10 by siRNA inhibits the increased shedding of PrP^C by ADAM9.** HEK cells stably expressing PrP^C were transiently transfected with or without ADAM10 (*AD10*) siRNA duplexes as detailed under "Experimental Procedures." After 18 h cells were transfected with or without the cDNA encoding ADAM9 (*AD9*). After a further 25 h, cells were incubated for 5 h in serum-free medium before harvesting the conditioned medium and preparing cell lysates. *A,* immunodetection of ADAM9, ADAM10, PrP^C, APP, and actin in cell lysates. Immunodetection of sPrP (*B*) and sAPPα (*C*) in conditioned medium using antibodies 3F4 and 6E10, respectively, is shown. Multiple immunoblots were quantified by densitometric analysis. Results are the mean ± S.D. ($n = 3$). *, significant at $p \leq 0.05$. *O/E*, overexpressing.

C and *D*), even though this construct of ADAM17 has been shown to increase the shedding of another membrane protein, angiotensin-converting enzyme-2 (31). These data indicate that ADAM9 and ADAM10, but not ADAM17, are capable of shedding the ectodomain of PrP^C.

To confirm that ADAM9 and ADAM10 were responsible for the shedding of PrP^C, siRNAs were employed to knock down the expression of endogenous ADAM9 and ADAM10 in the HEK cells. Three individual duplexes were targeted to both ADAM9 and ADAM10, with a commercially available control Smartpool siRNA reagent used as a control. As the anti-ADAM9 antibody did not detect endogenous ADAM9 in the HEK cells because of the high amount of nonspecific bands recognized (Fig. 1*A*), its knockdown was confirmed by RT-PCR. The siRNA duplexes against ADAM9 reduced its expression by between 78.7 and 89.2% depending on the duplex used, but it had no effect on the expression of ADAM10 (Fig. 2*A*). The siRNA duplexes against ADAM10 reduced its expression by between 66.5 and 79.3%, but it had no effect on ADAM9 expression (Fig. 2*D*). Neither knockdown of ADAM9 nor of ADAM10 altered the amount of PrP^C or APP in the cell lysate (Fig. 2, *A* and *D*). However, knockdown of ADAM9 reduced the amount of sPrP in the conditioned medium by 21.0, 26.2, and 24.1% for duplexes 1, 2, and 3, respectively (Fig. 2*B*). Knockdown of ADAM10 by the ADAM10-specific duplexes 1–3 reduced sPrP shedding by 44.6, 53.5, and 64.5%, respectively (Fig. 2*E*). Similar reductions in the shedding of sAPPα by knockdown of ADAM9 and ADAM10 were also observed (Fig. 2, *C* and *F*). These data confirm that endogenous ADAM9 and ADAM10 are involved in the shedding of PrP^C.

Shedding of PrP^C by ADAM9 Is Mediated via ADAM10—It has been reported that ADAM9 mediates its effect on the endoproteolytic α-cleavage of PrP^C and the α-secretase cleavage of APP indirectly, through modulating ADAM10 activity (24). To determine whether this was also the situation for the involvement of ADAM9 in the shedding of PrP^C, we expressed ADAM9 in the presence of siRNA knockdown of ADAM10 (Fig. 3*A*). As before, expression of ADAM9 led to an increase in the shedding of both sPrP and sAPPα, and siRNA knockdown of ADAM10 reduced the shedding of both proteins (Fig. 3, *B* and *C*). In the presence of the siRNA knockdown of ADAM10, expression of ADAM9 did not lead to an increase in the shedding of either sPrP or sAPPα above that seen in the presence of the ADAM10 knockdown alone (Fig. 3, *B* and *C*). These data show that the shedding of PrP^C, similarly to the shedding of sAPPα, by ADAM9 is dependent on the presence of ADAM10.

Modulation of ADAM10 by ADAM9 Requires the Proteolytic Activity of ADAM9—To further investigate the mechanism by which ADAM10 activity is modulated by ADAM9, the require-

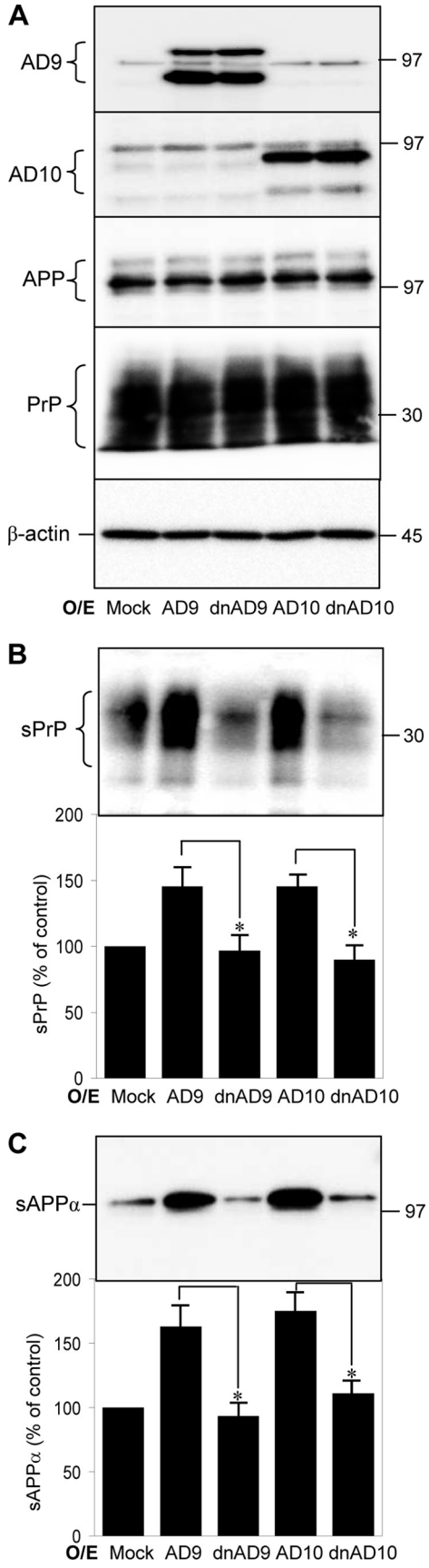

FIGURE 4. **Proteolytic activities of both ADAM9 and ADAM10 are required for the shedding of PrP^C.** HEK cells stably expressing PrP^C were transiently transfected with either empty vector (*Mock*) or the cDNAs encoding ADAM9 (*AD9*), dnADAM9 (*dnAD9*), ADAM10, or dnADAM10 (*dnAD10*). Transfected cells were incubated for 5 h in serum-free medium after which the conditioned medium was harvested and cell lysates prepared. *A,* immunodetection of ADAMs, PrP^C, APP, and actin in cell lysates. *B,* immunodetection of sPrP in conditioned medium. *C,* immunodetection of sAPPα in conditioned medium. *B* and *C,* multiple immunoblots were quantified by densitometric analysis. Results are the mean ± S.D. ($n = 3$). *, significant at $p \leq 0.05$. *O/E*, overexpressing.

ment for the proteolytic activity of ADAM9 was assessed. Catalytically inactive dn mutants of ADAM9 and ADAM10 were generated by mutating the conserved catalytic Glu residue in the zinc-binding His-Glu-Xaa-Xaa-His motif of the enzymes to Ala (32, 33). The mutants were then transfected into HEK cells, and the effect of their expression on the shedding of sPrP and sAPPα was assessed, relative to wild-type ADAM9 and ADAM10 transfectants (Fig. 4). Both mutants were expressed to similar levels as the corresponding wild-type proteins (Fig. 4*A*). However, neither dnADAM9 nor dnADAM10 led to an increase in the detection of either sPrP or sAPPα in the conditioned medium (Fig. 4, *B* and *C*). These data indicate an absolute requirement for the proteolytic activity of ADAM9 in its modulation of ADAM10 activity and for the catalytic activity of ADAM10 in shedding PrP^C.

ADAM10, but Not ADAM9, Cleaves Recombinant PrP—To further understand the cleavage of PrP by the ADAMs family members, recombinant PrP was incubated with recombinant ADAM9 or ADAM10, and the samples were analyzed by nanoelectrospray ionization mass spectrometry. The mass spectrum from rPrP yielded a charge state distribution of ions corresponding to a single species of 23,161 Da (Fig. 5*A*). Similarly, in the sample digested with ADAM9, only peaks corresponding to intact rPrP were observed (Fig. 5*A*). However, in the ADAM10-digested sample, in addition to peaks corresponding to a molecular mass of 23,161 Da, a second species of 22,675 Da was observed (Fig. 5*A*). No species of this molecular weight was observed on the spectrum of recombinant ADAM10 alone (data not shown), indicating that the 22,675 Da species was generated by the ADAM10-mediated cleavage of rPrP. Analysis of the N- and C-terminal sequences of rPrP indicated that the 22,675 Da species was the result of a 4-amino acid C-terminal truncation of rPrP, with cleavage occurring at the Gly^{228}–Arg^{229} peptide bond in the C-terminal rPrP sequence QAYY-DGRRSS (it should be noted that rPrP has an additional Ser residue at the C terminus as compared with mature PrP^C). Thus, this lower molecular weight species corresponds to cleavage of PrP^C between Gly^{228} and Arg^{229}, three residues N-terminal to the site of GPI anchor addition at Ser^{231} (Fig. 5*B*). To confirm that the recombinant ADAM9 was catalytically active, the enzyme was assayed with a quenched fluorescent peptide substrate. Both recombinant ADAM9 and ADAM10 cleaved the peptide substrate (Fig. 5*C*). These data indicate that ADAM10, but not ADAM9, can directly cleave PrP close to the site of GPI anchor attachment, strengthening the hypothesis that the ADAM9-mediated shedding of PrP^C occurs though modulation of ADAM10 activity.

Shedding of BACE1 by ADAM9 Is Also Mediated via ADAM10—Shed forms of the Alzheimer disease β-secretase BACE1 have been identified in both cultured cells and human

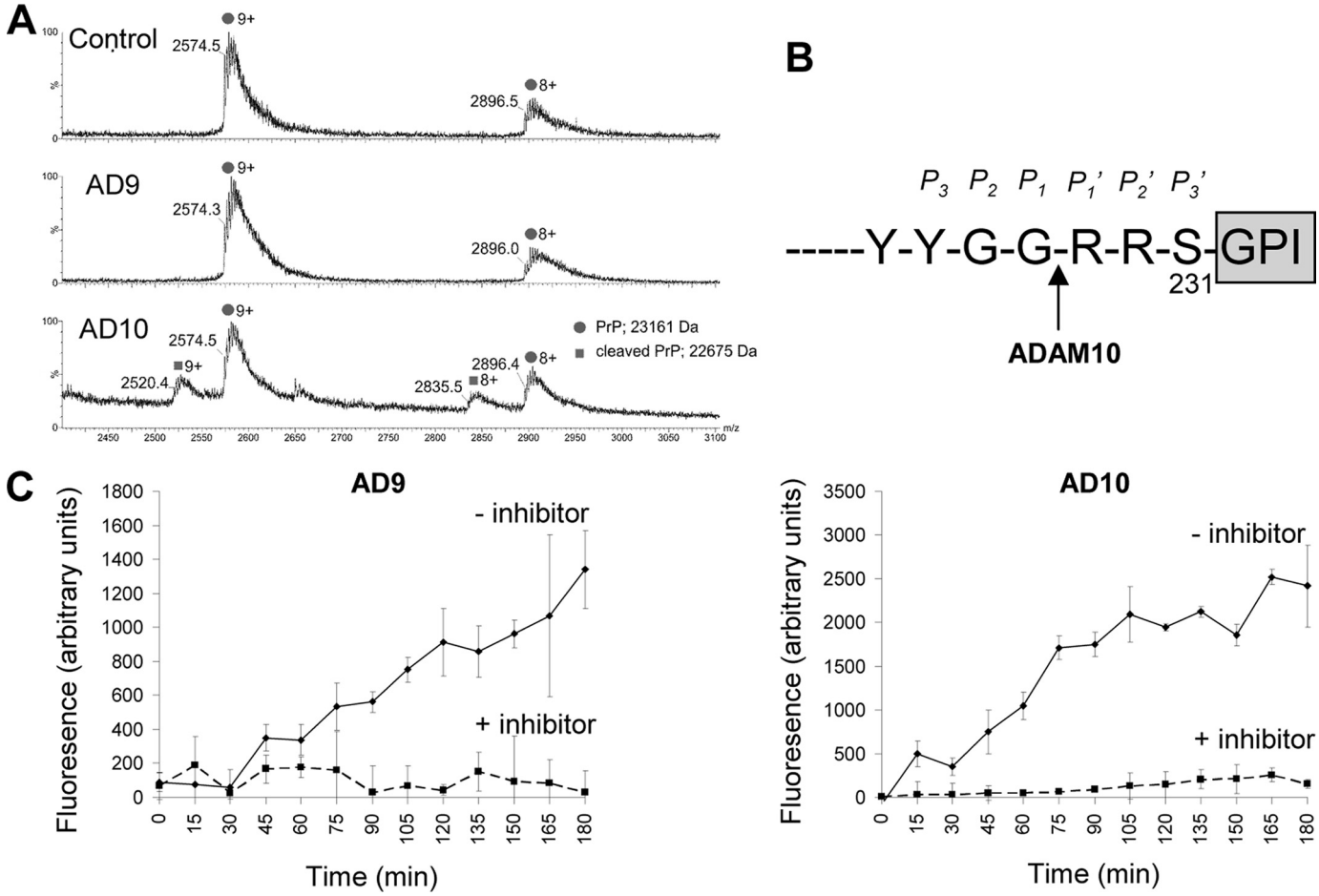

FIGURE 5. **ADAM10, but not ADAM9, cleaves recombinant PrP.** *A,* recombinant PrP (final concentration 10 μM) was incubated with 200 ng of recombinant ADAM9 or ADAM10 for 3 h at 37 °C. Samples were then prepared for analysis by nanoelectrospray mass spectrometry as described under "Experimental Procedures." Nanoelectrospray mass spectra of rPrP only, rPrP with ADAM9(*AD9*), and rPrP with ADAM10 (*AD10*) showing the 9+ and 8+ charge state envelope are shown. PrP (●) is present in all samples, and the proteolytically cleaved product (■) is present in the ADAM10-treated sample only. The *m/z* ratios for the $[M + nH]^{n+}$ signals are labeled, where *n* is the charge state; adjacent signals correspond to sodium substitution adducts of the protonated signal. *B,* C-terminal sequence of PrP^C showing the site of GPI anchor addition and the determined cleavage site by ADAM10. *C,* activity of recombinant ADAM9 and ADAM10 was assessed by measuring the cleavage of a fluorogenic peptide substrate Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2 for 3 h at 37 °C in the presence or absence of the inhibitor GW4023. Values are mean ± S.D. ($n = 3$). *Mca*, 7,4-methoxycoumarin-4-acetyl; Dpa, N^{β}-(2,4-dinitrophenyl)-L-2,3-diaminopropionic acid.

cerebrospinal fluid (20, 34, 35). Based on the observation that BACE1 shedding was sensitive to specific zinc metalloproteinase inhibitors, it was concluded that ADAM10 was a candidate BACE1 sheddase (20). To confirm that ADAM10 was involved in the shedding of BACE1 and to explore the role of ADAM9 in the shedding of this protein, HEK cells expressing BACE1 were transfected with the cDNAs encoding either ADAM9 or ADAM10. The level of cell-associated BACE1 was not significantly altered by co-expression of either of the ADAMs (Fig. 6, *A* and *B*). However, expression of ADAM9 or ADAM10 enhanced the release of shed BACE1 (sBACE1) into the conditioned medium (2.3- and 7.8-fold, respectively) (Fig. 6, *A* and *B*). To determine whether, like PrP^C and APP, ADAM9 was shedding BACE1 via modulation of ADAM10, HEK cells were transfected with ADAM9 cDNA in the presence of siRNA knockdown of ADAM10 (Fig. 6*C*). The increase in the shedding of sBACE1 observed upon expression of ADAM9 was inhibited to control levels in the presence of the ADAM10 siRNA (Fig. 6*D*). These data indicate that both ADAM9 and ADAM10 are capable of shedding BACE1 and that the ADAM9-mediated shedding of BACE1 requires the presence of ADAM10, as seen for the shedding of PrP^C and APP.

ADAM9, -10, and -17 Are Not Responsible for the Cell-associated Endoproteolytic α-Cleavage of PrP^C—In addition to the juxtamembrane cleavage of PrP^C, which occurs during ectodomain shedding, the protein is also subject to endoproteolytic α-cleavage between residues His^{111} and Met^{112} to yield N- and C-terminal fragments (termed N1 and C1, respectively) (reviewed in Ref. 22). Previously it has been reported that ADAM9 and -10 are involved in the constitutive endoproteolytic cleavage of PrP^C (23, 24). To investigate this in our system, lysates from cells expressing either ADAM9, ADAM10, or ADAM17 were subjected to peptide: *N*-glycosidase F deglycosylation, to permit resolution of full-length PrP^C from its C1 fragment. Expression of any of the three ADAMs did not significantly affect the amount of C1 fragment in the cell lysates (Fig. 7*A*). Furthermore, siRNA depletion of either ADAM9 or ADAM10 did not alter the amount of C1 fragment relative to full-length PrP^C (Fig. 7*B*). Collectively, these data indicate that ADAM9, -10, and -17

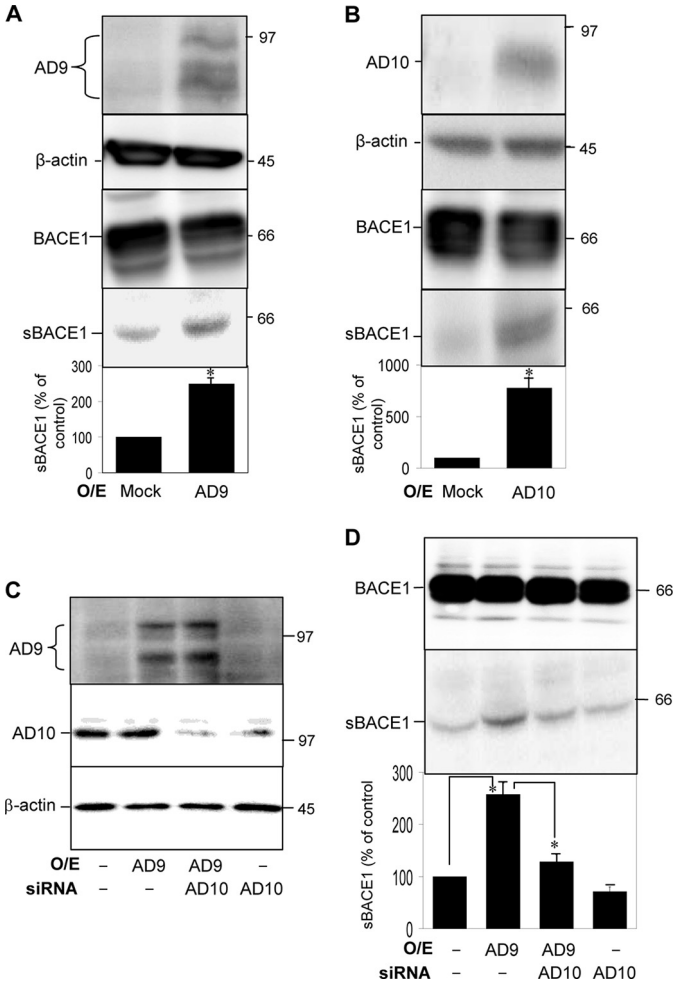

FIGURE 6. **ADAM9 and -10 are involved in the shedding of BACE1.** HEK cells stably expressing BACE1 were transiently transfected with either empty vector (*Mock*) or the cDNAs encoding either ADAM9 (*AD9*) or ADAM10 (*AD10*). Transfected cells were incubated for 5 h in serum-free medium before harvesting the conditioned medium and preparing cell lysates. Immunodetection of overexpressed ADAM9 (*A*) or ADAM10 (*B*) along with BACE1 and actin in cell lysates and sBACE1 in conditioned medium is shown. HEK cells stably expressing BACE1 were transiently transfected with or without ADAM10 siRNA duplexes as detailed under "Experimental Procedures." After 18 h cells were transfected with or without the cDNA encoding ADAM9. After a further 25 h, cells were incubated for 5 h in serum-free medium before harvesting the conditioned medium and preparing cell lysates. *C,* immunodetection of ADAM9, ADAM10, and actin in cell lysates. *D,* immunodetection of BACE1 in lysates and conditioned medium. Multiple immunoblots were quantified by densitometric analysis. Results are the mean ± S.D. ($n = 3$). *, significant at $p \leq 0.05$. *O/E*, overexpressing.

are not responsible for the cell-associated constitutive endoproteolytic α-cleavage of PrP^C.

Modulation of ADAM-mediated PrP^C Shedding Does Not Alter PrP^{Sc} Formation—There is accumulating evidence indicating that the level of PrP^C at the cell surface or the amount of PrP^C that is subsequently endocytosed is a strong determinant in the formation of PrP^{Sc} (7–9, 36). Molecular mechanisms capable of regulating the amount of PrP^C at the cell surface might therefore be of critical importance in the control of PrP^{Sc} propagation. Consequently, we sought to determine whether modulation of ADAM-mediated cell surface PrP^C shedding would alter PrP^{Sc} formation using two approaches. First, scrapie-infected ScN2a mouse neuroblastoma cells (25) were incubated with the hydroxamate-based inhibitor GW0264, a potent inhibitor of ADAM10 that has >100-fold reduced potency toward ADAM17 (20). Shedding of sPrP from the ScN2a cells was inhibited by 77.3% following treatment with GW0264 (Fig. 8*A*). To detect PrP^{Sc}, cell lysates were digested with PK prior to SDS-PAGE and subsequent immunoblotting using antibody 6D11 (Fig. 8*B*). Incubation of the cells for 96 h with GW0264 had no effect on the amount of PK-resistant PrP^{Sc}, whereas incubation with Congo red, which is known to prevent conversion of PrP^C to PrP^{Sc} (37), resulted in a 76.1 ± 10.7% reduction of PK-resistant PrP^{Sc} over the same time period (Fig. 8*B*). To confirm that the shedding of PrP^C had been inhibited in the ScN2a cells over this extended time period, conditioned medium samples from each day of incubation were analyzed for sPrP (Fig. 8*C*). These data indicate that inhibition of PrP^C shedding does not alter PrP^{Sc} formation.

In the second approach, ScN2a cells were transiently transfected with the cDNA encoding ADAM10 and then left for 96 h to assess whether shedding of PrP was increased in this cell line and whether this increased shedding could modulate prion conversion. When cells were transfected with the cDNA encoding ADAM10, there was a significant increase in both expression of ADAM10 (272.6 ± 18.5%) and in the shedding of total sPrP (Fig. 9*A*). When medium samples were treated with PK prior to analysis, the amount of PK-resistant PrP^{Sc} shed into the medium was also increased in cells transfected with ADAM10 (Fig. 9*A*). However, when cell lysate samples were digested with PK to assess the effect of ADAM10 overexpression on the total cell load of PK-resistant PrP^{Sc}, no change was observed in cells overexpressing ADAM10 (Fig. 9, *B* and *C*). Again, Congo red was used as a positive control to show that a 79.3 ± 6.0% reduction in PK-resistant PrP^{Sc} could be achieved over the time course of this experiment (Fig. 9, *B* and *C*). These data indicate that promoting the shedding of both PrP^C and PrP^{Sc} by ADAM10 does not modulate cell-associated PrP^{Sc} formation.

DISCUSSION

Whereas shed forms of PrP have long been identified both *in vitro* and *in vivo*, it was thought that this shedding was the result of the action of a phospholipase on the GPI anchor of PrP^C (11, 38). Proteolysis as a mechanism of PrP^C ectodomain shedding was first hypothesized with the observation that the mobility of secreted PrP^C was not altered after treatment with aqueous hydrofluoric acid as would be expected had the protein been shed by the action of a phospholipase (10). Confirming that PrP^C can be proteolytically shed, we have previously shown that a range of hydroxamate-based zinc metalloprotease inhibitors have an identical inhibitory profile in relation to PrP^C shedding and the shedding of APP (17), strongly suggesting that the two proteins are shed by the same protease(s). Here we have extended that study and directly assessed the roles of ADAM9, -10, and -17 in PrP^C shedding by direct comparison to APP shedding. We show that overexpression of either ADAM9 or ADAM10, but not of ADAM17, enhances the shedding of PrP^C. Using siRNA-mediated depletion of endogenous ADAM9 and -10, we were able to confirm that these two proteases were responsible for the shedding of PrP^C at least in cell culture models.

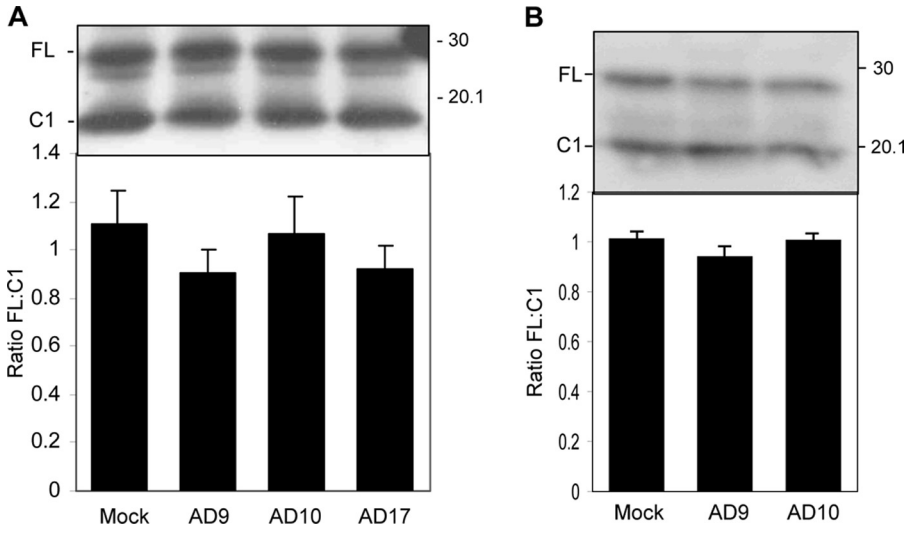

FIGURE 7. **ADAM9, -10, and -17 are not involved in the endoproteolytic α-cleavage of PrP^C.** HEK cells stably expressing PrP^C were transfected with either empty vector (*Mock*) or the cDNAs encoding ADAM9 (*AD9*), ADAM10 (*AD10*), or ADAM17 (*AD17*) (*A*) or siRNA targeted to either ADAM9 or ADAM10 (*B*). Cell lysates were prepared and deglycosylated as described under "Experimental Procedures." Full-length (*FL*) PrP^C and its C1 fragment in cell lysates were immunodetected using antibody 6H4. Multiple immunoblots were quantified by densitometric analysis. Results are the mean ± S.D. ($n = 3$).

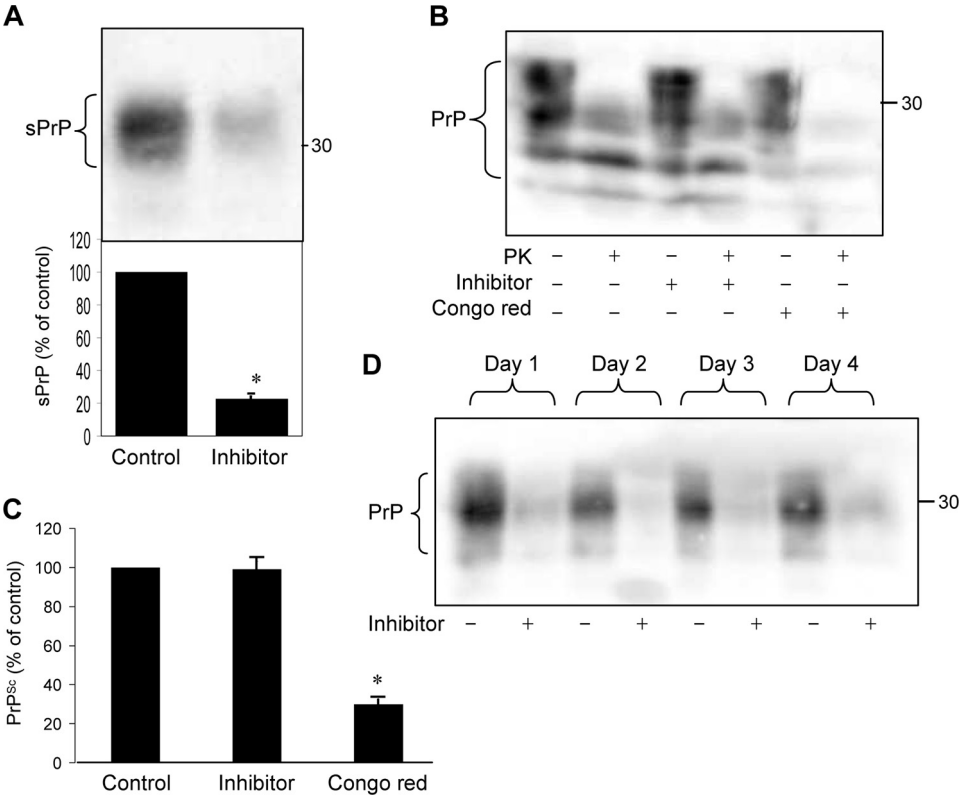

FIGURE 8. **Inhibition of PrP^C shedding does not alter PrP^{Sc} formation.** *A,* ScN2a cells were incubated for 12 h in the absence or presence of GW0264 (10 μM). sPrP in conditioned medium was detected using antibody 6D11. *B,* ScN2a cells were incubated in the presence or absence of GW0264 (10 μM) for 96 h (inhibitor was replaced every 12 h). As a positive control for anti-PrP^{Sc} activity, cells were treated with 1 μg/ml Congo red for 96 h (with media replaced every 12 h). Cells were harvested, lysed, and digested with PK as described under "Experimental Procedures." Immunoblotting for PrP was performed using antibody 6D11. *C,* densitometric analysis of PK-resistant PrP^{Sc} levels for each treatment, relative to those of mock-treated cells, from multiple blots from three independent experiments is shown. *D,* to confirm that shedding had been inhibited in ScN2a cells treated with GW0264 for 4 days, medium samples from each day of incubation were pooled and then concentrated. Equal volumes of concentrated medium were resolved by SDS-PAGE and immunoblotted using 6D11. *, significant at $p \leq 0.05$.

Koike *et al.* (39) showed that co-expression of ADAM9 with APP in COS cells enhanced production of soluble APP and made the logical conclusion that the catalytic activity of ADAM9 was directly responsible for APP shedding. However, it has since been demonstrated that ADAM9 enhances APP shedding in an indirect fashion via stimulation of ADAM10 activity (24). Using the novel approach of overexpression of ADAM9 in the presence of siRNA knockdown of ADAM10, we were able to confirm that ADAM9 exerts its effect on APP shedding by modulation of ADAM10. Furthermore, we were able to demonstrate that this phenomenon is not restricted to the ADAM9-mediated shedding of APP, as the shedding of both PrP^C and BACE1 by ADAM9 was also dependent on ADAM10. In addition, we have shown that the proteolytic activity of ADAM9 is critical for its ability to modulate ADAM10, as a catalytically inactive mutant of ADAM9 did not enhance the shedding of PrP^C, and that ADAM9 on its own was unable to cleave recombinant PrP. These results raise the question as to whether ADAM9 directly cleaves and sheds other membrane proteins or always acts via ADAM10. In the light of this, the role of ADAM9 in the shedding of other "substrates," *e.g.* Kit ligand, p75 neurotrophin receptor, Delta-like ligand-1, and pro-epidermal growth factor (40), may need to be revisited.

Recently, both ADAM9 and ADAM15 have been shown to proteolytically cleave ADAM10 releasing the ectodomain into the extracellular medium (41). The resulting membrane-bound stub was then subject to cleavage by γ-secretase, and the intracellular domain translocated to the nucleus, providing evidence that ADAM10 itself undergoes regulated intramembrane proteolysis (41). It is unclear whether the soluble ectodomain of ADAM10 is capable of cleaving membrane-bound substrates, and although it was catalytically active against

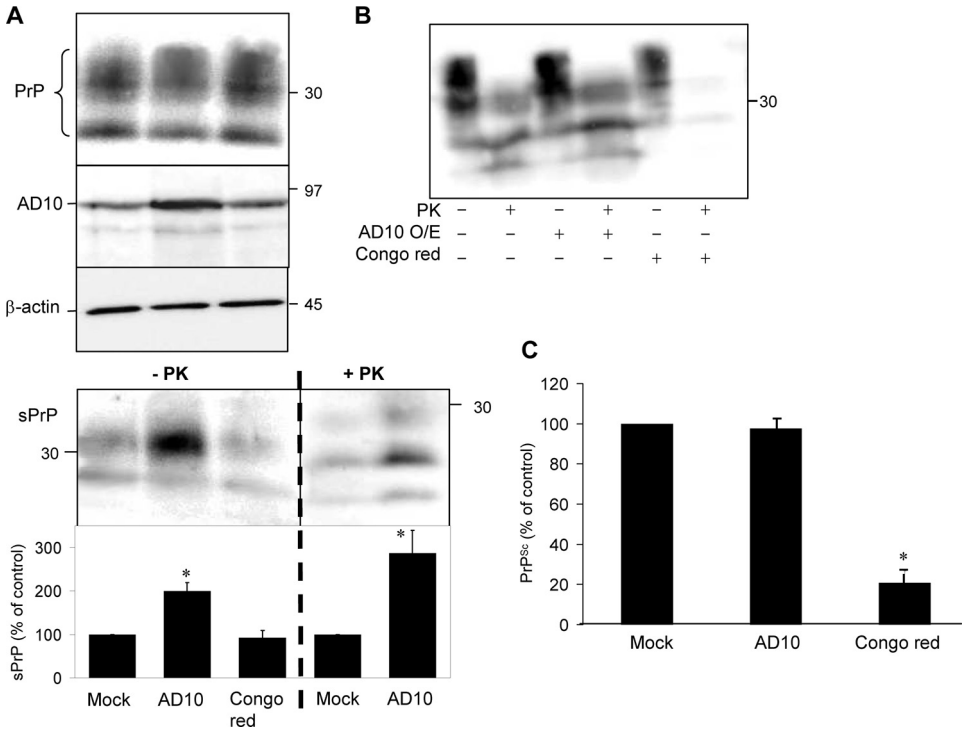

FIGURE 9. **ADAM10 overexpression increases the shedding of PrP^C and PrP^{Sc} but does not alter prion conversion.** ScN2a cells were transiently transfected with empty vector (*Mock*) or the cDNA encoding ADAM10 (*AD10*) or treated with 1 μg/ml Congo red. The cells were then left for 96 h. *A,* 12 h prior to the end of the experiment, the medium was replaced with serum-free medium before harvesting the conditioned medium and preparing cell lysates. Immunodetection of PrP, ADAM10, and actin in cell lysates is shown. Medium samples were concentrated and then treated with PK, where indicated. Equal volumes of concentrated medium were resolved by SDS-PAGE and immunoblotted using 6D11 for sPrP. *B,* cells were harvested, lysed, and digested with PK as described under "Experimental Procedures." PK-resistant PrP^{Sc} was detected using antibody 6D11. *C,* densitometric analysis of PK-resistant PrP^{Sc} levels of ADAM10-transfected and Congo red-treated cells, relative to those of mock-treated cells. Data from multiple blots from three independent experiments are shown. *, significant at $p \leq 0.05$.

synthetic peptide substrates (41, 42), it was incapable of shedding APP (42).

Shedding of the ectodomain of PrP^C by ADAM10 would require cleavage of the polypeptide chain close to the site of the GPI anchor attachment. Mass spectrometric analysis showed that this is indeed the case, with recombinant ADAM10 cleaving PrP three residues upstream of the Ser residue to which the GPI anchor is attached (43). Cleavage at this site is consistent with known ADAM10 cleavage sites in other substrates, in particular that basic residues are preferred at the P_1' and P_2' sites and Ser is strongly preferred at P_3'. Interestingly, the analysis of C-terminal peptides derived from endoproteinase Lys-C digests of hamster PrP^{Sc} revealed that although the majority of the molecules were GPI anchored, ~15% had a C-terminal peptide that ended with Gly^{228} (43), consistent with cleavage at this site by ADAM10. Recently, an anchorless fragment of PrP^{Sc}, which lacks a few amino acids at the very end of the C terminus together with the GPI anchor, was detected in the brains of patients with both sporadic and variant Creutzfeldt-Jakob disease (44). In light of the data presented in this study it is possible that these C-terminally truncated forms of PrP^{Sc} identified *in vivo* are a result of the action of ADAM10 in shedding PrP^{Sc}. Cleavage at the Gly^{228}–Arg^{229} bond is also consistent with our previous data that sPrP was recognized by the R1 antibody (17) whose epitope is within residues 220–231 (45). Recently, a novel 9-kDa C-terminal fragment of PrP^C, termed C3, has been reported (46). This C3 fragment does not appear to correspond to the C-terminal fragment following ectodomain shedding by ADAM10, as its generation was inhibited by E64, and it was recognized by the antibody R1, suggesting that C3 is cleaved upstream of residue 220 by a cysteine protease.

In addition to the proteolysis of PrP^C within its juxtamembrane region, we also examined the role of ADAMs in the endoproteolytic α-cleavage of PrP^C between amino acid residues His^{111} and Met^{112}. We show that both overexpression of ADAM9, -10, and -17 and depletion of ADAM9 and -10 did not alter levels of the PrP^C proteolytic fragment C1 in HEK cell lysates. These results appear to contradict previous reports that demonstrated an increase in N1 secretion from HEK cells overexpressing the same ADAMs (23, 24). However, these previous studies examined only the secretion of a minor pool of N1 into conditioned medium and did not examine the effect of the ADAMs on the cell-associated production of proteolytic PrP^C fragments. Recently, we have reported that the endoproteolytic α-cleavage of PrP^C occurs predominantly in a late compartment of the secretory pathway, prior to full-length PrP^C reaching the cell surface (47). In this study we have examined cell-associated levels of C1, as opposed to secreted N1, and we show that ADAM9, -10, and -17 overexpression, and ADAM9 and -10 depletion, did not affect the production of the former fragment. These data indicate that alternative unidentified proteinases are responsible for the bulk cell-associated endoproteolytic α-cleavage of PrP^C.

For the first time, we have examined the effect of modulating proteolytic ectodomain shedding of PrP^C on the production of PrP^{Sc} in scrapie-infected N2a cells. As PrP^C transport to the cell surface and/or its subsequent endocytosis appears to be required for *de novo* PrP^{Sc} production (7, 8), it might be expected that the inhibition of PrP^C shedding and the subsequent accumulation of the protein at the cell surface would promote PrP^{Sc} formation. Conversely, one might expect that enhanced shedding of PrP^C would reduce the amount of PrP^C substrate at the cell surface available for prion conversion. However, although) PrP^C shedding was clearly inhibited by GW0264 treatment and PrP^C and PrP^{Sc} shedding were increased by ADAM10 overexpression, we observed no concomitant alteration in the accumulation of proteinase K-resistant PrP^{Sc} in the ScN2a cells. These data imply that alterations to the basal shedding of PrP^C and PrP^{Sc} do not influence PrP^{Sc} formation, at least in this cell system. One possible

explanation for this lack of effect of altering the proteolytic shedding of either PrP^C or PrP^{Sc} on the conversion process is because the shedding by ADAM10 affects only a minority of the membrane-bound protein, whereas cleavage by phospholipase C or exposure to a PrP^C-specific antibody (48) affects a larger proportion of the membrane-bound protein, thus impacting on the conversion process.

From the observation that SNX33 overexpression increased the shedding of PrP^C and reduced PrP^{Sc} conversion, it was concluded that modulation of PrP^C shedding may represent a novel therapeutic approach in the treatment of prion diseases (9). However, this explanation may be oversimplified as these authors also demonstrated that SNX33 overexpression impaired the endocytic trafficking of PrP^C. As the endocytic pathway is a suggested site of prion conversion (4, 49, 50), it may be through blocking this that SNX33 affects the formation of PrP^{Sc}. By modulation of one of the proteinases, ADAM10, directly responsible for PrP^C and PrP^{Sc} shedding, our data argue that subtle modulation of PrP shedding is unlikely to offer a new avenue for therapeutic strategies for the treatment of prion diseases. Nevertheless, the precise dissection of the cell biology of PrP^C by studies such as these provides a realistic avenue toward the development of new treatments for prion diseases by increasing our understanding of the cellular processes governing prion conversion.

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