Cellular prion protein (PrP\textsuperscript{c}) undergoes a proteolytic attack at the 110/111,112 peptide bond, whereas the PrP isoform (PrP\textsuperscript{res}) that accumulates in the brain tissue in Creutzfeldt-Jakob disease reveals an alternate cleavage site at about residue 90. Interestingly, the normal processing of PrP occurs inside the 106–126 amino acid region thought to be responsible for the neurotoxicity of the pathogenic prions, whereas PrP\textsuperscript{res} cleavage preserves this potentially toxic domain. Therefore, any molecular mechanisms leading to enhanced cleavage at the 110/111,112 peptide bond could be of potential interest. We set up TSM1 neurons and HEK293 stable transfectants overexpressing the wild-type or 3F4-tagged murine PrP\textsuperscript{c}, respectively. Both mock-transfected and PrP\textsuperscript{c}-expressing cell lines produced an 11–12-kDa PrP fragment (referred to as N1), the immunological characterization of which strongly suggests that it corresponds to the N-terminal PrP\textsuperscript{c} fragment derived from normal processing. We have established that the recovery of secreted N1 is increased by the protein kinase C agonists PDBu and PMA in a time- and dose-dependent manner in both cell lines. In contrast, secretion of N1 remains unaffected by the inactive PDBu analog oPDD and by the protein kinase A effectors dibutyryl cAMP and forskolin. Overall, our data indicate that the normal processing of PrP\textsuperscript{c} is up-regulated by protein kinase C but not protein kinase A in human cells and murine neurons.

The group of spongiform encephalopathies covers a series of transmissible neurodegenerative diseases that includes among others human pathologies such as Creutzfeldt-Jakob disease and Gerstmann-Sträussler-Scheinker syndrome (1–3). The infectious agent thought to be likely responsible for these lethal diseases is closely associated with a host protein particle called prion (4), a 33–35 kDa polypeptide displaying a widespread distribution in mammals (5). Several lines of evidence indicate that the prion pathology is mainly underlined by the physical catabolic “depletion” of the potentially pathogenic 106–126 PrP domain. Whether it is a maturation process leading to the production of biologically active fragments or a proteolytic cleavage responsible for the final catabolic clearance of PrP\textsuperscript{c} is still questionable. However, it is striking that the “normal” cleaving enzyme responsible for PrP\textsuperscript{c} breakdown targets a PrP domain that appears as the potential “toxic” core of the protein. Thus, several studies have indicated that synthetic peptides corresponding to the 106–126 sequence of PrP could trigger toxic cellular responses (10–12). Along with this observation is the fact that the cleavage appears shifted through the N-terminal domain of the PrP in Creutzfeldt-Jakob brains around amino acid residue 90, thereby leaving intact the 106–126 sequence (8, 9). The identification of the 110/111,112-cleaving enzyme and a better understanding of putative mechanisms by which such a cleavage is regulated is of great interest.

We have set up two distinct cell lines from human embryonic kidney (HEK293) or mice neuronal (TSM1) origins that overexpress murine PrP\textsuperscript{c}. Both cell lines are shown here to secrete a fragment that biochemically and immunologically behaves as the N-terminal product (referred to as N1 below) of normal processing. We show that this cleavage is up-regulated by effectors of the protein kinase C pathway but not by protein kinase A agonists. Taken together, our study provides the first evidence of a protein kinase C-mediated regulation of normal PrP\textsuperscript{c} cleavage and thus a potential means to exacerbate the catabolic “depletion” of the potentially pathogenic 106–126 PrP domain.

MATERIALS AND METHODS

Antibodies—Monoclonal antibodies Pri-308 and Pri-917 have been raised against synthetic peptides corresponding to the 106–126 and 214–230 human PrP sequence, respectively (13). Western blot analyses indicated that Pri-917 recognizes human and mouse PrP whereas Pri-308 reacts more specifically with human PrP and shows a lower binding affinity to mouse PrP (13). Monoclonal antibodies SAF-32, SAF-70, and SAF-84 have been raised against scrapie-associated fibrils (SAFs) from infected hamster brain. They have been shown to bind linear peptides corresponding to human residues 79–92, 142–160, and 126–164 respectively. Western blot analyses revealed that SAF-32 and SAF-70 recognize PrP from most mammalian species, whereas SAF-84 does not react with human PrP (13). Monoclonal antibody 8G8 is directed against human recombinant PrP (14, 15) and binds the 95–110 sequence of human PrP with a significant cross-reactivity with mouse PrP. All monoclonal antibodies were used as dilutions of crude ascitic fluids. The 3F4 monoclonal antibody (kindly provided by Dr. Rakačak) was shown to react with human PrP with a significant cross-reactivity with mouse PrP.
to be specific for methionine 109 and 112 of human PrP (corresponding to the 108 and 111 positions of the mouse PrP sequence (16)).

Cell Culture and Stable Transfections in HEK293 and Neuronal TSM1 Cells—HEK293 cells overexpressing the 3F4MoPrP were obtained after transfection with DAC30 reagent (Eurogentec) of 2 μg of empty pDNA3 vector (mock) or pDNA3 containing cDNA encoding mouse PrP bearing the human 3F4 epitope (17). MoPrP was stably transfected in neuronal TSM1 cells by means of the Superfect reagent (Qiagen). Transfectants were screened by Western blot analysis with the SAF32 antibody. Cells were subsequently maintained at 37 °C in 5% CO2 in DMEM medium supplemented with 10% fetal calf serum containing penicillin (100 units/ml), streptomycin (50 mg/ml) and gentamicin (1 mg/ml).

Cell Treatments, Immunoprecipitations, and Detection of the N1 fragment—Cells cultured in 35-mm dishes were washed twice with PBS and incubated for 8 h at 37 °C in the absence (control) or in the presence of various pharmacological agents in 1 ml of serum-depleted DMEM. Medium was collected, and cells were resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 5 mM EDTA). Both medium and lysates were complemented with a protease inhibitor mixture (Sigma) and incubated overnight with a 500-fold dilution of monoclonal antibodies and protein A-Sepharose beads (Zymed Laboratories Inc.). After centrifugation, beads were washed twice with radioimmune precipitation buffer, once with PBS, and then resuspended in loading buffer and heated at 95 °C for 5 min. Proteins were separated on 16.5% Tris/Glycine gel, transferred onto nitrocellulose membrane (45 min, 100 V), and probed for 45 min with skim milk (5% in PBS) and incubated with various monoclonal antibodies (dilution 1:2000 in PBS, Tween (0.05%), skim milk (5%) overnight at 4 °C. Bound antibodies were detected using a sheep anti-mouse peroxidase-conjugated secondary antibody (dilution 1:5000) (Amersham Pharmacia Biotech), and immunological complexes were detected with the ECL method as described previously (18).

Methanol precipitation was performed by freezing secretion medium at −20 °C for 2 h, and then proteins were precipitated with 5 volumes of methanol. Samples were spun for 20 min at 5000 × rpm and then pellets were dried, resuspended in Laemml buffer, and electrophoresed as described above.

Western Blot Analysis of PrP—Cells grown in 35-mm dishes were washed twice with PBS and resuspended in lysis buffer (50 μl) as described above. Lysates were complemented with a protease inhibitor mixture and 25 μg of protein were subjected to SDS-PAGE on a 12% Tris/Glycine gel. Proteins were transferred onto nitrocellulose membrane (2 h, 100 V) and incubated with SAF32 antibody (dilution 1:2000 in PBS, Tween (0.05%), milk (5%)) overnight at 4 °C. PrP was detected as described above.

Statistical Analysis—Statistical analyses were performed with Prism software (Graphpad Software, San Diego, CA) using the Newman-Keuls multiple comparison test for one-way analysis of variance and the unpaired Student’s t test for pair-wise comparisons. All results were expressed as mean ± S.E., and p < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The aim of the present study was to set up stably transfected cell lines overexpressing the wild-type (MoPrP) or 3F4-tagged humanized (3F4MoPrP) mouse PrP. As is often the case when studying neurodegenerative diseases with a cell biology approach, the choice of cell line is of great importance. One of the parent cell lines chosen here was HEK293 because these cells have been widely used as a relevant cell model from human origin to study degenerative diseases such as Alzheimer’s disease (for a review see Ref. 19). TSM1 neurons were also used because these murine cells from neocortical origin display the unique feature to correspond to stably transfected neurons (20) whereas very often “neuron-like or neuron-derived” cell lines are used as a central nervous system cell model.

TSM1 neurons have been successfully used in our laboratory in the course of studying Alzheimer’s and Parkinson’s disease to examine the regulation of amyloid precursor protein (β-APP) maturation (21, 22) and more recently to establish stable transfectants overexpressing wild-type and mutant α-synucleins (23). Fig. 1 shows that analysis of geneticin-resistant clones resulting from MoPrP (Fig. 1A) or 3F4MoPrP cDNA transfection (Fig. 1B) reveals cell lines overexpressing PrP-like immunoreactivity, whose molecular weight and electrophoretic pattern correspond to previously described differently glycosylated PrP isoforms (see Figs. 3C, 4C, and 5B), the electrophoretic patterns of which were similar to those observed in transfected cells, indicating that the overexpressed protein appears to be properly glycosylated/ folded in the two cell systems.

MoPrP TSM1 neurons secrete in a time-dependent manner (Fig. 2B) an 11–12-kDa PrP-related fragment (N1) obtained after methanol precipitation or after immunoprecipitation with the SAF32 monoclonal antibody (Fig. 2A). The same N1 fragment was recovered from the secreted fraction of mock-transfected TSM1 neurons (Fig. 3A), indicating that N1 also results from the physiological cleavage of endogenous PrP in TSM1 neurons. Interestingly, both mock and 3F4MoPrP HEK293 cells secrete an identical 11–12-kDa SAF32-immunoprecipitable fragment (Fig. 3A, right lanes) that was particularly abundant in the PrP-transfected HEK293 cell line (see 3F4 in Fig. 3A). It should be noted that an identical 11.5-kDa N-terminal product was previously detected in the conditioned medium of N2a mouse neuroblastoma cells overexpressing chicken PrP (24).

To determine whether N1 could be also detected in cell lysates, we have shown (Fig. 3B) that mock and transfected cell lines all produce an intracellular N1. Further experiments are in progress to establish whether the cell lysate counterpart of N1 results from intracellular cleavage of PrP occurring during routing to the plasma membrane or whether membrane-bound PrP undergoes cleavage in early endosomes compartments soon after its documented internalization (25).

Chen et al. (8) suggests a physiological cleavage of PrP leading to the detection of a C-terminal fragment of about 18.5 kDa referred to as C1 that remained attached to the membrane by its phosphatidylglycerol–inositol anchor. This C1 product likely resulted from normal physiological processing of PrP as it was isolated from normal human brain and seemed to derive from an intrachain PrP attack occurring between the residues 110 and 112. The immunological characterization of the N1-secreted product detected in the present study indicates that it likely corresponds to the N-terminal counterpart of C1. Thus, by means of a series of monoclonals (13–15) interacting...
Regulated Cleavage of Cellular Prion Protein

FIG. 2. Kinetics of secretion of N1 fragment by MoPrPc-transfected TSM1 neurons. A, 1 ml of serum-free DMEM was conditioned for the times indicated (1, 4, 8, or 14 h) with MoPrPc-overexpressing TSM1 neurons then medium was either methanol-precipitated or immunoprecipitated with the SAF32 antibody (dilution 1:500) as described under “Materials and Methods.” Proteins were analyzed by 16.5% Tris/Tricine SDS-PAGE followed by Western blotting with SAF32 (dilution 1:2000) and ECL. B, densitometric analyses of gels of secreted N1 fragment are expressed as arbitrary units and data points are the mean values of two distinct experiments.

FIG. 3. Detection of secreted and intracellular N1 fragment in mock- and PrPc-transfected TSM1 neurons and HEK293 cells. After incubation for 8 h with 1 ml of serum-free DMEM, medium (A) or cell lysates (B) from the indicated mock or transfected cells were immunoprecipitated with SAF32 antibody and protein A-Sepharose as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.” The immunoprecipitated proteins were separated by SDS-PAGE on 16.5% Tris/Tricine gels, and Western blots were performed with SAF32 as described under “Materials and Methods.”

with various epitopes located along the PrP sequence (Fig. 4A), we have clearly shown that all monoclonals recognizing C-terminal epitopes, i.e. SAF70, SAF84, and Pri-917 do not label the N1 product (Fig. 4B) whereas they label intracellular PrPc (not shown). Interestingly, the 8G8 antibody that interacts with a sequence located at the extreme C terminus of a fragment theoretically derived from the PrP cleavage at the 110/111 bond (see Fig. 4A) still labels the N1 fragment (Fig. 4B). By contrast, Pri-308 that recognizes a sequence encompassing the theoretical cleavage site does not label N1. Of most interest was the use of 3F4, a well characterized monoclonal antibody recognizing the two methionine residues located at positions 109 and 112 of the human PrP sequence (lacking in the mouse sequence, see ref. 16). As expected, 3F4 antibody did not recognize intracellular mouse PrPc whereas intense labeling of 3F4-tagged MoPrPc was observed by Western blot (Fig. 4, upper right). However, the N1 fragment secreted by human cells was not immunoprecipitated by 3F4 antibody (Fig. 4C, lower right, lanes 3 and 4) indicating that the cleavage giving rise to N1 disrupt the 3F4-recognized epitope. Altogether, the immunological characterization of N1 strongly suggests that the N1 product is derived from a proteolytic attack occurring between the 108 and 111 residues.

N1 secretion by PrPc-expressing HEK293 and TSM1 cells can be drastically up-regulated by the protein kinase C effectors PDBu (Fig. 5A) and PMA (Fig. 6), but not by the PDBu-inactive analog aFDD (Fig. 5A). Interestingly, the phorbol esters also potentiate the recovery of N1 in the secreted fraction of mock-transfected cells (Fig. 5B), indicating that the protein kinase C control of N1 secretion also takes place during the course of endogenous PrPc processing. N1 recovery was also potentiated by the phosphatase okadaic acid (Fig. 6). To assess whether N1 secretion is selectively controlled by protein kinase C or more generally affected by various kinases, we examined the putative effect of protein kinase A effectors. Clearly, forskolin and 8-bromo-cyclic AMP (Fig. 6) did not modify the N1 recovery from mock- or PrP-transfected TSM1 neurons (Fig. 6A) and HEK293 cells (Fig. 6B). As expected, the inactive forskolin analog, dideoxyforskolin did not modify N1 secretion (Fig. 6).

It is important to note that PDBu treatment does not affect the expression of intracellular PrPc, whatever the cell type examined (Fig. 5). This indicates that the protein kinase C target does not correspond to phorbol esters-responsive-tran-
scriptional elements but more likely to downstream cellular intermediate(s) along the pathways that ultimately lead to PrPc incorporation in the plasma membranes. The possibility that this kinase directly targets PrPc, thereby modulating its susceptibility to proteolysis is supported by the recent demonstration that recombinant bovine PrP was indeed substrate of protein kinase C but not of protein kinase A (26). Alternatively, protein kinase C could also affect all the cellular intermediates involved in N1 formation including proteins participating to address or route processes or proteolytic activities themselves.

It is interesting to note that the β-amyloid precursor protein that appears to play a key role in Alzheimer's disease, is also physiologically maturated by a protein kinase C-controlled mechanism (for a review see Ref. 19). This regulated physiological cleavage leading to a secreted product (sAPPα) occurs inside the sequence of the Aβ peptide, the production of which appears to be central to the pathology (27) and disrupts its formation (28, 29). Therefore, the normal breakdown of βAPP can be seen as a means to deplete the cells from endogenous toxic Aβ species. In this context, it is interesting to note that the production of N1 also implies a cleavage inside the 106–126 PrPc sequence that is thought to be highly neurotoxic (10–12). Therefore, mechanisms leading to N1 production can also theoretically be seen as a way to deplete cells from PrP species bearing the 106–126 toxic domain. Supporting the view that catabolism of PrPc is central to prion pathologies is the fact that in the brains affected by Creutzfeld-Jakob disease, the cleavage of PrPc occurs around the amino acid 90, thereby leaving...
intact the potential of toxicity displayed by the 106–126 domain. It is therefore of great interest to demonstrate that the protein kinase C-mediated pathways could up-regulate N1 production, i.e. normal PrP metabolism. Whether protein kinase C can be seen as a potential target for therapeutic intervention aimed at stopping or slowing down the evolution of prion-related neurodegenerative disease remains to be established.

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