The C-terminal Products of Cellular Prion Protein Processing, C1 and C2, Exert Distinct Influence on p53-dependent Staurosporine-induced Caspase-3 Activation*

Claire Sunyach1, Moustapha Alfa Cisse, Cristine Alves da Costa, Bruno Vincent, and Frédéric Checler2

From the Institut de Pharmacologie Moleculaire et cellulaire du CNRS, UMR6097, Université Nice-Sophia-Antipolis, Equipe labellisée Fondation pour la Recherche Médicale, 660 route des Lucioles, Sophia-Antipolis, 06560 Valbonne, France

The cellular prion protein (PrPc) undergoes various endoproteolytic attacks within its N-terminal domain, leading to the production of C-terminal fragments (C) tethered to the plasma membrane and soluble N-terminal peptides (N). One of these cleavages occurs at position 110/111, thereby generating C1 and N1 products. We have reported that disintegrins ADAM-10, -9, and -17 participate either directly or indirectly to this proteolytic event. An alternative proteolytic event taking place around residue 90 yields C2 and N2 fragments. The putative function of these proteolytic fragments remained to be established. We have set up two novel human embryonic kidney 293 cell lines stably overexpressing either C1 or C2. We show that C1 potentiates staurosporine-induced caspase-3 activation through a p53-dependent mechanism. Thus, C1 positively controls p53 transcription and mRNA levels and increases p53-like immunoreactivity and activity. C1-induced caspase-3 activation remained unaffected by the blockade of endocytosis in HEK 293 cells and was abolished in p53-deficient fibroblasts. Conversely, overexpression of the C2 fragment did not significantly sensitize HEK 293 cells to apoptotic stimuli and did not modify p53 mRNA levels or activity. Therefore, the nature of the proteolytic cleavage taking place on PrPc yielded C-terminal catabolites with distinct function and could be seen as a switch mechanism controlling the function of the PrPc in cell survival.

The glycosylphosphatidylinositol-anchored cellular prion (PrPc)3 gained considerable attention with the emergence of bovine spongiform encephalopathy and its transmission to human beings. Tremendous efforts have been devoted to understand the mechanisms by which this normal form converts to the pathological form PrPSc (for recent reviews, see Refs. 1 and 2). Nevertheless, the physiological function of PrPc remains puzzling. Several reports suggested a putative role of PrPc in cell adhesion (3–5), neurite outgrowth (6), and synaptogenesis (7). The involvement of PrPc in cell viability is still under debate. Thus, a fair number of reports suggest that PrPc may have a role in the control of oxidative stress and cell survival (for review, see Ref. 8). It has been reported that PrPc protects against tumor necrosis factor α- (9) and Bax-induced cell death (10, 11), although this phenotype varies according to the cell type (12). Conversely, we recently established that PrPc overexpression led to an increased staurosporine-evoked cell death (13) in transfected or inducible cell lines and showed that this cellular response was associated with an activation of caspase-3 linked to a transcriptional and post-transcriptional control of the p53 transcription factor (14). Moreover, we and others have described that down-regulation of PrPc in cell lines (14, 15) or deletion in vivo decreased p53 expression (16, 14). This last observation would plead for a p53-dependent proapooptotic function of PrPc rather than a protective one given the pivotal role of this tumor suppressor in triggering cell death.

Shortly before or after its appearance at the cell surface, PrPc undergoes a variety of proteolytic processing events. In human brain, PrPc is mainly endoproteolyzed on the N-terminal side, at the 110/111 pepitidyl bond, to produce a 17-kDa C-terminal fragment C1 tethered to the plasma membrane (17, 18). A 9-kDa soluble N-terminal counterpart referred to as N1 is released in the extracellular medium (19). We have established in several cell systems that this cleavage is both constitutive and protein kinase C-regulated (19). We have demonstrated that three disintegrins contribute to this processing, either directly for ADAM (A Disintegrin And Metalloprotease) -10 and -17 (20) or indirectly for ADAM-9 (21). In agreement with these data, ADAM-10 appears as a strong candidate for this cleavage in vivo in human brain (22). In pathological situations, PrPc apparently undergoes an additional upstream cleavage, within the octapeptide repeats, around the 90/91 residues (18), yielding a 21-kDa C-terminal fragment referred to as C2 and its 7-kDa N2-corresponding N-terminal peptide. The nature of the proteases involved in the pathological cleavage remains to be established.

Very few works have examined the putative functions of C1 and C2, and particularly, their potential as a regulator of cell death, a function harbored by the parent protein. In other words, little is known concerning the fact that these cleavages could be seen as an activating process leading to biologically

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1 A recipient of a fellowship from the Fondation pour la Recherche Médicale.
2 To whom correspondence should be addressed: Tel.: 33-4-93-95-77-60; Fax: 33-4-93-95-77-08; E-mail: checler@ipmc.cnrs.fr.
3 The abbreviations used are: PrPc, cellular prion protein; XTT, sodium 3-(phenylaminocarbonyl)-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzene sulfonic acid; LDH, lactate dehydrogenase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling.
active fragments or as an inactivating mechanism aimed at abolishing PrP\textsuperscript{c}-mediated control of cell death. Whether C1 or C2 fragments harbor similar or distinct function also remained a matter of question. In this context, we have established two novel cell lines overexpressing either C1 or C2 fragments. We now show that C1 displays pro-apoptotic function and regulates p53 mRNA transcription and activity, whereas C2 remains biologically inert in this paradigm.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Materials**

The PRI-308 monoclonal antibody was raised against a synthetic peptide corresponding to the human 106–126 sequence. SAF32 and SAF61 monoclonal antibodies are directed against PrP\textsuperscript{79–92} residues and 142–160, respectively. All three antibodies were generously provided by Dr. J. Grassi (Commissariat à l’Énergie Atomique/Saclay, Gif sur Yvette, France). Anti-human p53 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-tubulin monoclonal antibody and staurosporine were purchased from Sigma (St. Quentin-Fallavier, France).

**cDNA Constructs**

The 3F4-tagged PrP\textsuperscript{c} coding vector (3F4MoPrP\textsuperscript{c}) drives the expression of mouse PrP\textsuperscript{c} in which 2 methionines in positions 109 and 112 of the human sequence replace lysine 108 and valine 111 in the mouse PrP\textsuperscript{c} cDNA, thereby allowing its detection by human-specific PRI-308). To obtain C1 and C2 coding vectors, 24–89 and 24–110 deletions were created in 3F4-tagged PrP\textsuperscript{c} cDNA using a QuikChange\textsuperscript{TM} site-directed mutagenesis kit according to the manufacturer’s instructions.

**Cell Systems and Transfections**

Human embryonic cells (HEK 293) stably expressing PrP\textsuperscript{c} or C2 fragments were established and maintained as described previously for mock-transfected and 3F4 MoPrP\textsuperscript{c}-expressing HEK293 cells (19). Murine TSM-1 neuronal cells (23) were transiently transfected using Lipofectamine 2000 reagent (Invitrogen, Cergy-Pontoise, France) according to the manufacturer’s instructions. p19\textsubscript{Arf}-deficient and p19\textsubscript{Arf}/p53 double knock-out fibroblasts kindly provided by Dr. M. Roussel (24) were transfected by means of the mouse embryonic fibroblasts Nucleofector\textsuperscript{TM} kit according to the manufacturer’s instructions (Amaza Biosystems, Koeln, Germany). In brief, 5 million cells were nucleofected (program A23) with 6 μg of various cDNA prediluted in 100 μl of mouse embryonic fibroblasts Nucleofector solution. The nucleofected samples were then transferred to Eppendorf tubes containing 500 μl of prewarmed culture medium, gently homogenized, and equally dispatched to 6-well dishes. Twenty-four hours after transfection cells were treated with either Me\textsubscript{2}SO (vehicle control) or 1 μM staurosporine for 2 h and recovered for further analysis.

**Western Blot Analysis**

Cells were homogenized in lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate, 5 mM EDTA) for PrP\textsuperscript{c} immunodetection. Samples of equal amounts of proteins were resolved on 12% SDS-PAGE and analyzed by standard immunoblotting techniques. For p53 detection, nuclear enriched fractions were prepared as detailed previously for cytochrome c translocation experiments (25). Equal amounts of proteins were separated on 8% gels and probed using a 1:10,000 dilution of anti-human p53 antibody. Blots were developed using ECL methods according to the manufacturer’s instructions (Roche Applied Science, Meylan, France). Chemiluminescence was recorded using a luminescence image analyzer LAS-3000 (Raytest, Courbevoie, France), and quantification of captured images was performed using the Aida Image Analyzer software (Raytest).

**Cycloheximide and N-Glycosidase F Treatment**

Cells were seeded in 6-well dishes and allowed to reach 80% confluency in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum. Culture medium was then replaced with 1 ml of Dulbecco’s modified Eagle’s medium containing cycloheximide (15 μg/ml) (Sigma). Treatment was then carried out for the appropriate time periods. When deglycosylation was required, 25 μg of proteins were recovered in denaturing buffer (25 mM Tris, pH 7.5, 0.5% SDS, 1% 2-mercaptoethanol) and boiled for 5 min. One percent Nonidet P-40 and 0.5 units of N-glycosidase F (Roche Applied Science) were added, and the samples were incubated for 2 h at 37 °C. The reaction was stopped by the addition of an equal volume of loading buffer (0.5 mM Tris, pH 6.8, glycerol 10%, SDS 1%, bromophenol blue 0.05%). Samples were then resolved on a 12% SDS-PAGE gel for Western blot analysis as described above. When specified, cells were treated with metalloprotease inhibitors a-phenanthroline (Sigma) or BB3103 (hydroxamic acid-based zinc metalloprotease inhibitor, kindly provided by British Biotech) as described previously (Vincent et al. (20)). Cells were then lysed, and protein samples were deglycosylated as detailed above.

**Cell Viability Assays**

**XTT Assay**—Cells were grown in 96-well plates in the presence or absence of staurosporine (2 μM, 16 h). XTT is metabolized by mitochondrial dehydrogenase to a water-soluble formazan salt only by metabolically active viable cells. Absorbance is measured at 452 nm as detailed previously (23).

**Lactate Dehydrogenase (LDH) Assay**—Cells were cultured in 6-well 35-mm plates. At confluency, they were treated with staurosporine (2 μM for 16 h). Forty μl of culture medium were analyzed for the release of LDH using CytoTox-ONE\textsuperscript{TM} homo-
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generative membrane integrity assay (Promega, Charbonnières, France) according to the manufacturer’s recommendations.

Caspase 3-like Activity Measurements

Cells were grown in 6-well plates and incubated with staurosporine (16 h with 2 μM) (Sigma) after they reached confluency. Samples were processed for caspase-3 like activity assay as described in detail elsewhere (23). Fluorometry was recorded at 360 and 460 nm (excitation and emission wavelengths) by means of a microtiter plate reader (Labsystems, Fisher Bioblock scientific, Illkirch, France). Caspase activity is calculated from the linear part of fluorometry recorded and expressed in units/hr/mg of proteins (established by the Bio-Rad procedure). One unit corresponds to 4 nmol of 4-methyl 7-amino-coumarin released. When blockade of internalization was required, cells were pretreated with 0.45 M sucrose for 30 min as detailed previously (26). Caspase-3 activity was then monitored as above.

Immunohistochemistry

Control (mock-transfected) and C1- and C2-expressing HEK 293 were cultured on glass coverslips in 35-mm dishes. Cells were fixed with 1.5% paraformaldehyde for 20 min and washed three times in phosphate-buffered saline, and the primary antibodies (SAF61 and SAF83) were applied for 2 h. After three washes with phosphate-buffered saline, cells were incubated with goat anti-mouse secondary antibody conjugated to Alexa Fluor-594 (Interchim, Montluçon, France) for 1 h. Hoechst (Interchim) was added to phosphate-buffered saline during the last three washes to stain the nuclei. Coverslips were then mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA), and staining was visualized with an Axiosplan 2 imaging microscope (Carl Zeiss, Sartrouville, France) with oil immersion objective x63 coupled to a cooled CCD camera (Raper Scientist, Tucson, AZ).

TUNEL Analysis

For each stably transfected cell line, the same number of treated cells were plated on glass coverslip. At confluency, they were treated with staurosporine (1 μM, 16 h) and processed for labeling. Samples previously fixed 20 min in paraformaldehyde, rinsed and permeabilized overnight in 70% ethanol, and then processed for the dUTP nick end-labeling in situ cell death detection kit, POD (Roche Applied Science) as stated by the supplier. For quantification, total cells were counterstained with erythrosine B. Images were captured on an Olympus BX41 microscope (Olympus, Rungis, France) using Olympus DP12 software. Cells undergoing cell death (diaminobenzidine-labeled nuclei) were counted on 10 independent optical fields for each experimental condition.

p53 Transcriptional Activity and Promoter Transactivation

p21(FAG-1)-luciferase and PG13-luciferase constructs (provided by Dr. B. Vogelstein, Baltimore, MD) that were used to measure p53 transcriptional activity have been extensively described (27, 28). Briefly, cells grown in 12-well plates were co-transfected with a 1:0.25 ratio of P21(FAG-1) or PG13-luciferase p53 gene reporter constructs and β-galactosidase expression vector (to normalize transfection efficiencies). Luciferase and β-galactosidase activities were measured 48 h after transfection according to well described procedures (25) using a luciferase assay system and β-galactosidase enzyme assay system (Promega, Charbonnières, France). The p53 promoter-luciferase construct (kindly provided by Dr. M. Oren, Rehovot, Israel), which has been described earlier (29), was used to determine p53 promoter transactivation as above.

Real-time Quantitative PCR

Total RNA from cells were isolated using the RNeasy kit (Promega, Charbonnières, France) following the instructions of the manufacturer. After DNase I treatment, 1 μg of total RNA were reverse-transcribed using oligo(dT) priming and avian myeloblastosis virus reverse transcriptase (Promega). Real-time PCR was performed in an ABI PRISM 5700 sequence detector system (Applied Biosystems, Courtaboeuf, France) using the SYBR Green detection protocol as outlined by the manufacturer. Human p53-specific primers were designed using the Primer Express software (Applied Biosystems) as follows: forward, 5’-GAA CCC TTG CTT GCA ATA GG-3’, and reverse, 5’-GTG TAG GTG CAA ATG CC-3’. Relative expression level of p53 gene is normalized for RNA concentrations with housekeeping gene (human GAPDH) using the following primers: forward, 5’-TGG CCT ACA CTG AGC ACC AG-3’, and reverse, 5’-CAG CGT CAA AGG TGG AGG AG-3’. mRNA values are expressed as arbitrary units; they represent means ± S.D. of triplicates and are representative of 3 independent experiments.

Statistical Analysis

Statistical analysis was performed with PRISM software (GraphPad Software, San Diego, CA) by using the Newman-Keuls multiple comparison tests for one-way analysis of variance.

RESULTS

Characterization of C1- and C2-expressing Cells—To elucidate the biological function of cellular prion protein N-terminal endoproteolytic events, we have established HEK 293 stable transfectants expressing C1 or C2 fragments. Immunological characterization of these cell lines was performed by means of three monoclonal antibodies directed against distinct epitopes spanning the complete PrPc sequence (Fig. 1B). As expected, SAF32 detects the C-terminal domain of PrPc, whereas it is complete in C2 (Fig. 1B, left panel). The epitope recognized by PRI-308 is truncated in C1, whereas it is complete in C2 (Fig. 1A). SAF61 recognizes the octapeptide repeat (amino acids 79–92), the hydrophobic region (amino acids 106–126), and the C-terminal domain (amino acids 142–160), respectively (Fig. 1A) (30). As expected, SAF32 detects overexpressed 3F4 MoPrPc together with the endogenous cellular PrPc but failed to label C1 and C2 (Fig. 1B, left panel). The epitope recognized by PRI-308 is truncated in C1, whereas it is complete in C2 (Fig. 1A). Accordingly, this antibody detects C2 and the full-length 3F4 MoPrPc but not C1 (Fig. 1B, central panel). As anticipated, SAF61, which is specific of the core region of the C-terminal domain of PrPc, reveals all three constructs (Fig. 1B, right panel). Shifts of the electrophoretic mobility observed between C1 and C2 lanes (Fig. 1B, SAF61) are in agreement with the expected molecular mass of C1 and C2 (i.e. 17 and 21 kDa, respectively). Both fragments retain intact glycosylation sites located in
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positions 180 and 186 in the C-terminal region of PrPc (31) explaining the complex pattern obtained. To clarify this pattern, we submitted the cell lysates to deglycosylation. N-glycosidase F treatment revealed one single 27-kDa band in 3F4MoPrPc overexpressing cells corresponding to PrP full-length (Fig. 2A). As expected, the heterogeneous pattern observed in C1 and C2 cells resolved into one single band of 17 and 21 kDa, respectively (Fig. 2A). In addition, immunohistochemical labeling of fixed cells overexpressing C1 and C2 using SAF61 showed that both fragments are correctly and similarly addressed to the plasma membrane (Fig. 2B).

C2 Is Not Proteolytically Converted into C1—To rule out a possible interference of C1 in putative C2-associated phenotype, we examined whether C2 could undergo proteolytic cleavages generating C1. To address this question, we first analyzed C1 and C2 metabolic stability after treatment with cycloheximide (to prevent de novo synthesis of C1 and C2 fragments) followed by deglycosylation. Fig. 3, A–C, indicate that C1 and C2 immunoreactivities time-dependently decreased with similar kinetic slopes (C1y = -3.3 x + 95.8; C2y = -3.1 x + 93.6). The C1 fragment results from the proteolytic attack of PrPc full-length protein in position 110/111 by disintegrins (ADAM-10 and -17). The C2 fragment, which contains this cleavage site, could theoretically produce C1. This does not appear to be the case. First, long time incubation of C2 does not lead to C1-like immunoreactivity even when C2 was totally degraded (Fig. 3B); second, the general inhibitor of metalloproteases, o-phenanthroline (Fig. 3D), as well as the specific inhibitor of ADAM-10 and -17, BB3103 (Fig. 3E), did not increase C2 immunoreactivity; third, transient overexpression of ADAM-10 or -17 neither accelerated C2 catabolism nor led to C1 production (data not shown). Overall, our data show that C2 does not produce C1, and therefore, that putative C2-mediated phenotype could not be indirectly due to C1.

C1 Expression Sensitizes HEK 293 Cells to Stauroporine-induced Toxicity—We previously described that overexpression of PrPc enhanced stauroporine sensitivity of several cell systems including HEK293 and TSM-1 cells (13, 14, 32) as shown by increased cytotoxic response. We therefore examined whether the overexpression of C1 or C2 could modulate cell survival after stauroporine treatment. Full-length PrPc (3F4MoPrP) and C1 fragment both increased stauroporine-induced cell toxicity measured by XTT (Fig. 4A) and increased LDH release (Fig. 4B). By contrast, C2 did not modulate cell viability in these conditions (Fig. 4, A and B). To examine whether apoptosis could account for the toxicity in stauroporine-treated C1 overexpressing cells, we further analyzed C1- and C2-overexpressing cells using the TUNEL labeling technique. Fig. 5, A and B, clearly show a 3-fold increase in the number of TUNEL-positive nuclei in 3F4MoPrPc- and C1-expressing cells, whereas there was no modification of the number of apoptotic nuclei in C2 cells fields (Fig. 5, A and B).

C1 but Not C2 Potentiates Stauroporine-induced Caspase-3 Activation in HEK 293 Cells and in TSM-1 Neurons—To further confirm that “classical” apoptotic mechanisms could account for the increase in TUNEL-positive cells, we examined the levels of caspase-3 activity in C1- and C2-expressing cells. Overexpression of PrPc increased stauroporine-stimulated caspase-3-like activity (13, 14, 32). Here again, the full-length PrPc and C1 fragments potentiated stauroporine-induced caspase-3 activation (Fig. 6A), whereas C2 remained biologically inert in this paradigm. Neither PrPc, C1, nor C2 could...
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**Blockade of Endocytosis Does Not Abolish C1-associated Proapoptotic Function**—We previously demonstrated that caspase-3 activation triggered by PrP<sup>C</sup> was dependent on clathrin-dependent endocytosis, suggesting that the PrP<sup>C</sup>-dependent apoptotic pathway was linked to PrP<sup>C</sup> internalization (26). One classical strategy to stop internalization is to submit cells to an hypertonic shock using 0.45 m sucrose. This treatment proved efficient to block both transferrin and PrP<sup>C</sup> entering intracellular compartments in HEK 293 cells (26) (Fig. 7). Cells overexpressing C1 or C2 were subjected to sucrose treatment followed by staurosporine stimulation of cell death. We show that unlike for PrP<sup>C</sup> (Fig. 7), C1-mediated caspase-3 activation is not prevented by blockade of endocytosis.

**C1 but Not C2 PrP<sup>C</sup> Modulate p53**—We previously demonstrated that endogenous PrP<sup>C</sup> regulated p53-dependent caspase-3-mediated cell death (14). Thus, overexpression of PrP<sup>C</sup> indeed increased p53 expression and activity, whereas PrP<sup>C</sup> deletion reduced overall cellular p53 activity and expression (14). We therefore examined p53 expression and activity together with p53-promoter transactivation in C1 and C2 stably expressing cell lines.

We first looked at p53 activity using a PG13-luciferase reporter construct. This construction is based on the genomic DNA consensus sequence recognized by p53 on the promoter of its target genes. Fig. 6A shows that p53 activity is drastically enhanced in 3F4MoPrP<sup>C</sup>- and C1-expressing cells, whereas no modifications could be measured in C2-expressing cells (Fig. 8A). The same results are obtained using the promoter of p21<sup>waf-1</sup>, a downstream effector of p53 involved in cell growth arrest (Fig. 8B). Expression of p53 was monitored in the mock-transfected and 3F4MoPrP<sup>C</sup>, C1, and C2 stable transfectants (Fig. 8C). The quantification of nuclear p53 illustrated in Fig. 6D clearly shows that immunoreactivity of p53 is increased in PrP<sup>C</sup> and C1 cells. To evaluate whether the impact of C1 and C2 overexpression on p53 transcription could explain our results on p53 activity and expression, we monitored human p53 promoter activity using a luciferase reporter construct (Fig. 8E) and performed real-time PCR analysis of p53 transcripts (Fig. 8F). p53 promoter transactivation together with mRNA levels were drastically enhanced in C1 cells, similarly to what was observed in full-length PrP<sup>C</sup> overexpressing cells. By contrast and in good agreement with the unmodified p53 activity (Fig. 8, A and B), C2-expressing HEK 293 cells did not show any significant modification of p53 level of transcription (Fig. 8, E and F).

modulate caspase-3 activity in basal conditions. Similar data were observed after transient transfection of C1 and C2 coding cDNAs in TSM-1 neurons (Fig. 6B), suggesting that the difference observed was not cell-specific. It should be underlined here that since C2 appeared biologically inert, this confirms first that C2 is not converted into C1 in our experimental conditions, and second, that endogenous PrP<sup>C</sup> does not interfere with the C1-associated pro-apoptotic phenotype.
C1-induced Caspase-3 Activation Is Fully p53-dependent—
We recently established that PrP<sup>c</sup>-mediated caspase-3 activation is fully dependent on p53 (14). We therefore examined whether C1-mediated caspase-3 activation was partly or totally dependent of p53. p53 can either induce cell apoptosis or regulate senescence and block cell cycle progression via the tumor suppressor p19<sub>Arf</sub>. In the absence of p19<sub>Arf</sub>, p53 can nevertheless still regulate apoptosis (24). Thus, p19<sub>Arf</sub>/H11002 and p19<sub>Arf</sub>/p53 null fibroblastic cell lines are two cell systems particularly relevant to explore p53-dependent cell death unrelated to cell cycle progression. We transiently transfected the cytomegalovirus-driven cDNAs coding for C1 and C2 in p19<sub>Arf</sub> and p19<sub>Arf</sub>/p53 null fibroblasts. Although highly expressed in p19<sub>Arf</sub>/p53 double knock-out cells (Fig. 9B), C1 was nevertheless unable to modify caspase-3 activation after staurosporine treatment (Fig. 9A). In sharp contrast, C1 was still able to significantly increase staurosporine-evoked caspase-3 activity in p19<sub>Arf</sub>—/— control cells (Fig. 9, A and B). This result indicates that C1-induced caspase-3 activation is fully dependent on p53. In agreement with the above data, we could not detect any modification of caspase-3 activity in both cell lines after transfection of C2 (Fig. 9, A and B).

DISCUSSION

PrP<sup>c</sup> undergoes several proteolytic cleavages. In normal conditions, one major cleavage takes place on the N-terminal side of the molecule, at the 110/111 peptidyl-bond, giving rise to the C-terminal glycolipid-anchored fragment C1 and its N-termi-
neurons (14). We therefore analyzed the p53-dependent apoptosis in these cells challenged with staurosporine and found that C1-expressing cells exhibit increased staurosporine susceptibility as was observed with the full-length PrPc (13, 14, 26), whereas C2-expressing cells behave like the control (mock-transfected) cells. The fact that C1 and PrPc holoprotein both trigger p53-dependent caspase-3 activation suggests that the cleavage generating C1 indeed corresponds to an activation process that could account for the proapoptotic function exhibited by PrPc.

We previously showed that point mutations of the N-terminal polybasic motif of PrPc, membrane sequestration of PrPc by specific antibodies, or hypertonic treatment all block clathrin-mediated endocytosis of PrPc and abolish p53-dependent caspase-3 activation linked to PrPc overexpression (Ref. 26 and the present report). Unlike PrPc, C1-mediated p53-dependent apoptosis could not be abolished by hypertonic sucrose treatment. Interestingly, several studies clearly showed that the N-terminal domain (25–90) of PrPc, which is absent in C1, was essential for endocytosis via clathrin-coated pits (34–36). These results argue in favor of two distinct mechanisms leading to PrPc- and C1-mediated caspase-3 activation. PrPc holoprotein relies on clathrin-mediated endocytosis, and thus, hence a putative transmembrane receptor, which suggests the involvement of a yet unidentified interacting protein to trigger p53 transactivation, whereas the mechanism by which C1 leads to increased p53 mRNA transcription likely involves distinct cell signaling. Thus, the C1 fragment either remains at the cell surface or, if internalized, enters the cell by a clathrin-independent pathway, therefore remaining insensitive to sucrose treatment. In this context, the localization of C1 in lipid raft domains of the

**FIGURE 7.** Blockade of endocytosis by sucrose treatment does not abrogate C1-mediated caspase-3 activation. Mock-transfected (Mock) cells and 3F4MoPrP, C1, and C2 stably expressing cell lines were incubated with 0.45 M sucrose for 30 min to prevent internalization and then treated with staurosporine (16 h, 2 μM) and analyzed for caspase-3 activity as described under "Experimental Procedures." Bars are the means ± S.E. of 4–6 independent experiments. *, p < 0.01; ns, nonstatistically significant; UA, arbitrary units.

**FIGURE 8.** C1 overexpression increases p53 activity, expression, and promoter transactivation in HEK293 cells. Mock-transfected (Mock) cells and 3F4MoPrP, C1, or C2 stable transfectants were monitored for p53 transcriptional activity using PG13 (A) and P21 waf-1 (B) luciferase reporter constructs as detailed under "Experimental Procedures." ns, nonstatistically significant. C, p53-like immunoreactivity in the nucleus was assessed by Western blotting in nuclear enriched fractions prepared as detailed under "Experimental Procedures." Tubulin (Tub) was used to control the total amount of protein in fractionated samples. M, mock-transfected cells. D, quantified p53 immunoreactivity in the indicated cell line. p53 mRNA transcription was assessed by the measurement of promoter transactivation using p53 promoter-luciferase construct (E) and real-time PCR analysis of p53 mRNA (F). Bars are the means ± S.E. of 4 independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**FIGURE 9.** Increased sensitivity to staurosporine triggered by C1 overexpression is p53-dependent. A, 3F4MoPrP, C1, or C2 constructs were transiently transfected in p19Arf null and p19Arf/p53-deficient fibroblasts. Twenty-four hours after transfection, cells were treated with staurosporine (1 μM, 2 h) and processed for caspase-3 activity measurement as described under "Experimental Procedures." Bars are the means of 4 independent determinations ± S.E. **, p < 0.01; ns, nonstatistically significant. B, representative PrP-like immunoreactivity probed with the indicated monoclonal antibody in each transfected cell line.
plasma membrane (36, 37) prompted us to investigate a signaling pathway involving fyn kinase (38). PP2, a selective inhibitor of the Src family kinases, did not affect PrP\(^\text{Sc}\)-induced staurosporine-evoked caspase-3 activation in TSM-1 neurons (13) or in HEK293 cells but decreased C1-mediated proapoptotic phenotype (data not shown), reinforcing the fact that PrP\(^\text{Sc}\) and C1 lead to caspase-3 activation through two distinct signaling pathways.

It is interesting to emphasize that the lack of the N-terminal part of PrP\(^\text{Sc}\) is not a structural feature that always yields proapoptotic fragments. Thus, unlike C1, C2 that is also truncated at its N terminus appears biologically inert on staurosporine-induced p53-dependent caspase-3 activation. In this context, it is interesting to note that functional studies performed on transgenic mice indicate that PrP\(^\text{Sc}\) lacking residues 33–92 (i.e. which resembles the C2 fragment) is biologically inert (39). Conversely, PrP\(^\text{Sc}\) proteins lacking 33–120 and 33–133 (i.e. devoid of the 90–110 domain, thereby mimicking the C1 structure) led to neuronal death in the granular layer of the cerebellum (39). It is tempting to speculate that this exacerbated cell death observed in vivo could account for C1-dependent caspase-3 activation.

We cannot completely rule out the possibility that the phenotype observed after expression of C1 is due to co-expression of this truncated form with endogenous PrP\(^\text{Sc}\). However, it is rather unlikely because C1 is present in our cells in vast excess in comparison with the endogenous holoprotein (Fig. 1B). Furthermore, endogenous PrP\(^\text{Sc}\) is obviously also present in C2-expressing cells but not sufficient to interfere with the lack of p53-dependent staurosporine-evoked caspase-3 activation associated with C2. Therefore, despite the presence of endogenous full-length PrP\(^\text{Sc}\), C1 and C2 overexpressing cells appear to be a suitable system to delineate the function of these two catabolites. Altogether, our results are the first demonstration that the function of PrP\(^\text{Sc}\) could be regulated by proteolytic events and that alternative cleavages could lead to various catabolites displaying distinct influence on the control of p53-dependent neuronal cell death.

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