Scrapie Protein Degradation by Cysteine Proteases in CD11c⁺ Dendritic Cells and GT1-1 Neuronal Cells

Katarina M. Luhr,¹*  Elin K. Nordström,¹  Peter Löw,¹  Hans-Gustaf Ljunggren,²  Albert Taraboulos,³ and Krister Kristensson¹

Department of Neuroscience, Karolinska Institutet, SE-171 77 Stockholm,¹ and Center for Infectious Medicine, Department of Medicine, Karolinska Institutet, Huddinge University Hospital, SE-141 86 Stockholm,² Sweden, and Department of Molecular Biology, Hebrew University Hadassah Medical School, 91120 Jerusalem, Israel³

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Dendritic cells (DC) of the CD11c⁺ myeloid phenotype have been implicated in the spread of scrapie in the host. Previously, we have shown that CD11c⁺ DC can cause a rapid degradation of protease-resistant prion proteins (PrPSc) in vitro, indicating a possible role of these cells in the clearance of PrPSc. To determine the mechanisms of PrPSc degradation, CD11c⁺ DC that had been exposed to PrPSc derived from a neuronal cell line (GT1-1) infected with scrapie (ScGT1-1) were treated with a battery of protease inhibitors. Following treatment with the cysteine protease inhibitors (2S,3S)-trans-epoxysuccinyl-1-leucylamido-3-methylbutanate (E-64c), its ethyl ester (E-64d), and leupeptin, the degradation of PrPSc was inhibited, while inhibitors of serine and aspartic and metalloproteases (aprotinin, pepstatin, and phosphoramidon) had no effect. An endogenous degradation of PrPSc in ScGT1-1 cells was revealed by inhibiting the expression of cellular PrP (PrPC) with RNA interference, and this degradation could also be inhibited by the cysteine protease inhibitors. Our data show that PrPSc is proteolytically cleaved preferentially by cysteine proteases in both CD11c⁺ DC and ScGT1-1 cells and that the degradation of PrPSc by proteases is different from that of PrPC. Interference by protease inhibitors with DC-induced processing of PrPSc has the potential to modify prion spread, clearance, and immunization in a host.

Prion diseases are neurodegenerative diseases that affect humans (e.g., Creutzfeldt-Jakob disease and variant Creutzfeldt-Jakob disease) as well as animals (e.g., scrapie, bovine spongiform encephalopathy, and chronic wasting disease). At the biochemical level, these diseases are characterized by the conversion of a normal cellular prion protein (PrPC) into an abnormal isoform that is enriched in β-structures and is partially resistant to proteinase K (PrPSc) (41, 42). Prion-affected tissues show accumulation of PrPSc, and this may be paralleled by neuronal vacuolization and nerve cell death. Although prion diseases are associated with an accumulation of PrPSc in the brain, indirect evidence has recently been obtained that PrPSc can be degraded within an infected cell. Such evidence derives from the treatment of scrapie-infected neuronal cells, the neuroblastoma N2a cell line, with antibodies against PrPC (19, 40). This treatment can cause clearance of PrPSc from the cultured cells, and it is suggested that this is caused by inhibition of the formation of new PrPSc concomitant with degradation of previously formed PrPSc. From in vitro and in vivo studies, there is also indirect evidence that macrophages may be involved in the degradation of PrPSc (2, 6).

We have previously described that CD11c⁺ dendritic cells (DC) can efficiently degrade PrPSc presented to them by scrapie-infected gonadotropin-releasing cells (GT1-1 cells) in vitro (29). This raises the questions of whether certain pro-

* Corresponding author. Mailing address: Department of Neuroscience, Retzius väg 5, Karolinska Institutet, SE-171 77 Stockholm, Sweden. Phone: 46-8-728 78 27. Fax: 46-8-325 325. E-mail: katarina.luhr@neuro.ki.se.

MATERIALS AND METHODS

GT1-1 cell culture and scrapie infection. GT1-1 cells, a subtype of immortalized mouse gonadotropin-releasing hormone neurons (36), were a generous gift from Pamela Mellon (University of California, San Francisco). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (4.5 g of glucose per liter) containing Glutamax I and supplemented with 5% heat-inactivated fetal bovine serum (FBS), 5% heat-inactivated horse serum (HS), and 50 U of penicillin-streptomycin per ml (all from Gibco-BRL, Paisley, United Kingdom). The GT1-1 cells were infected with mouse-adapted scrapie by incubation with a 0.1% homogenate of mouse brains infected with the Rocky Mountain Laboratory strain of scrapie (the homogenates were obtained as a generous gift from Stanley B. Prusiner, University of California, San Francisco) at 30°C. After 3 days, the medium was changed and the temperature was raised to 37°C. Western blotting confirmed the presence of protease-resistant PrPSc after six passages.

Differentiation and isolation of CD11c⁺ DC. Murine bone marrow-derived DC were obtained from the femur and tibia bone marrow of C57BL/6 mice (obtained from the Microbiology and Tumor Biology Center, Karolinska Institutet) essentially as described by Inaba et al. (24). Briefly, after removal of small pieces of bone and debris, the cells were pelleted and resuspended in DMEM (4.5 g of glucose/liter) with Glutamax I, 15% FBS, and 50 U of penicillin-streptomycin per ml to which 10 ng of recombinant murine granulocyte-macrophage colony-stimulating factor per ml and 10 ng of murine interleukin-4 (Peprotech, Rocky Hill, N.Y.) per ml were added. The cells were grown in 5% CO₂ at 37°C and replated after 7 days. To obtain pure DC, the cells from the bone marrow cultures were affinity purified with magnetic cell separation (MACS) CD11c⁺ magnetic micro beads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.
Protease inhibitors. The inhibitors used were leupeptin hydrochloride, (2S,3S)-trans-epoxysuccinyl-l-leucylamido-3-methylbutane (E-64c), (2S,3S)-trans-epoxysuccinyl-l-leucylamido-3-methylbutane ethyl ester (E-64d), aprotinin, and pepstatin A (all from Sigma-Aldrich Chemie, Steinheim, Germany; Sigma catalog numbers E-0514, E-6684, A-1153, and P-265, respectively) and phosphoramidon (Roche Diagnostics GmbH, Mannheim, Germany). Pepstatin A was diluted in 70% ethanol to a 1.6 mM stock solution and stored at −70°C. All other inhibitors were made up to a stock concentration of 15 mM and stored at −20°C. Leupeptin, aprotinin, and phosphoramidon were all diluted in phospho-
buffered saline (PBS). E-64c and E-64d were made up in 50% ethanol.

Treatment with protease inhibitors. Cultured ScGT1-1 cells were established by growing ScGT1-1 cells overnight in 10-mm tissue culture dishes (Corning Inc., Corning, N.Y.) to a concentration of 10⁶ cells/dish. CD11c+ sorted DC were then added at a concentration of 10⁶ cells/dish, giving an approximate DC:GT1-1 cell ratio of 1:1. The cells were incubated in AIM-V serum-free medium (Gibco-BRL) to avoid interference with serum proteases, supplemented with 10 ng of granulocyte-macrophage colony-stimu-
lating factor (PeproTech) per ml. To some of these cocultures and to some
scattered DC were then added at a concentration of 10⁶ cells/dish, as described below. Western blotting, as described below.

lysates were then mixed with loading buffer, boiled, and analyzed by Western
blotting (see above), and the pellets from the spun cell medium were added. The
2 min. Attaching DC were then lysed with the lysis buffer used for Western

C. After incubation, the samples were
as controls. At 0 and 48 h, the cell medium was removed and spun at 400
C for 2 min at 4°C. Then 50
L of homogenate was added to each dish of DC
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Exposure of homogenates of ScGT1-1 cells to living DC. ScGT1-1 cells were
harvested and diluted in sterile water at a concentration of 10⁶ cells/ml, frozen and
thawed twice, homogenized with a 27-gauge needle, and spun for 2 min at 400 × g to remove debris; 30 μl of homogenate was added to each dish of DC
cultured in AIM-V medium, 1.2 × 10⁶ cells/dish. After incubation for 12 h at
37°C, the medium was removed and spun for 2 min at 400 × g to sample
free-floating DC, which were then reintroduced to the dishes. Then 50 μM
leupeptin was added to some of the dishes at time zero, while others were kept
as controls. At 0 and 48 h, the cell medium was removed and spun at 400 × g for
2 min. Attaching DC were then lysed with the lysis buffer used for Western
blotting (see above), and the pellets from the spun cell medium were added. The
lysates were then mixed with loading buffer, boiled, and analyzed by Western
blotting, as described below.

Incubation of lysates at different pHs. ScGT1-1 cells and purified DC were
harvested, spun at 1,500 × g, diluted in PBS separately at a concentration of 2 × 10⁷
cells/ml, and then frozen at −70°C. The frozen cell homogenates were lysed in
lysis buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1% Triton X-100). The
ScGT1-1 cell lysates were divided into 12 aliquots, and to eight of these, lysates
d of DC (ScGT1-1/DC ratio, 1:2) were added. Leupeptin (15 μM) was added to
d to the ScGT1-1/DC lysates. Lysis buffer was added to adjust the volume in
the ScGT1-1 lysates without DC. The pHs of the lysates were adjusted to 5.5 or
7.8 by adding 10 μl of 0.1 M sodium acetate buffer (pH 5.5) or of 1 M Tris-HCl
buffer (pH 7.8), respectively, to a total of 15 μl of ScGT1-1/DC lysates. All the
samples were incubated for 1 h at 37°C. After incubation, the samples were
mixed on a rotating wheel and boiled.

Generation of siRNA. A 21-nucleotide short interfering RNA (siRNA) duplex
(sense, UUUAGGAGAGACCGAGCAAU) corresponding to positions 123 to
143 (GenBank accession number NM_011170.1) on the mRNA for PrP, was
designed as recommended (18), with uridine residues in the two-nucleotide
overhangs. DNA templates for the chosen siRNA, containing a region comple-
mentary to the T7 promoter primer (CCTGTCGC) at the 3′ end, were ordered from
Invitrogen (Paisley, United Kingdom). siRNA were synthesized by in vitro
transcription with the Silencer siRNA construction kit according to the manu-
facturer’s instructions (Ambion, Austin, Tex.). siRNAs were labeled with indo-
carcyanine (Cy3) with the Silencer siRNA labeling kit according to the manu-
facturer’s instructions (Ambion).

Transfection of cells with siRNA. ScGT1-1 cells were plated on 35-mm cell
culture dishes in regular cell culture medium (described above) without antibi-
cotics. 24 h after plating, cells were transfected with siRNA (final concentration, 20 nM; final volume, 1 ml) with Oligofectamine (Invitrogen) (3 μl of reagent/ml of medium) according to the manufacturer’s instructions. Transfection was carried out under serum-free conditions in OptiMEM I (Gibco-BRL) without antibiotics. At 4 h after trans-
fec tion, 500 μl of DMEM with 15% FBS and 15% HS was added to the cell
culture and used to replace the previous siRNA-containing medium. The
scattered cells were transfected with siRNA (final concentration, 20 nM; final volume, 1 ml).

The cells were analyzed by Western blotting and immunofluorescence with Fab
D13, obtained from Stanley B. Prusiner (32, 57), 1 to 10 days after transfection,
to determine the presence of PrPSc and PrPSc-. Cultures analyzed by Western
blotting were standardized to the same amount of protein before proteinase K
(PK) treatment. Transfection efficiency was evaluated by fluorescence micros-
copy. Cells transfected with Cy3-labeled siRNA were stained with Hoechst 33342
(Sigma) (final concentration, 5 μg/ml) in PBS for 10 min to visualize cell nuclei.
The cells were rinsed twice with PBS, fixed in 10% formalin (Merck KGaA,
Darmstadt, Germany) in PBS, rinsed again in PBS, and finally mounted in
glycerol (Merck).

Western immunoblotting. Before blotting, the protein contents of the lysates
were lysed with the Bradford protein assay (Bio-Rad) and spectrosco-
petry (Ultralumin Plus; Pharmacia LKB, Cambridge, United Kingdom) at 595 nm
according to the manufacturer’s instructions. The lysate was then split into two
aliquots. One aliquot was treated with 20 μg of PK (Boehringer, Mannheim,
Germany) per ml at 37°C for 40 min and then incubated with 3 mM phenyl-
methylsulfonyl fluoride (Sigma) to stop the reaction. The other aliquot was not
PK treated. The samples were boiled in sodium dodecyl sulfate (SDS) sample
buffer, loaded on NuPAGE 12% Bis-Tris gels with MOPS (morpholinepropane-
sulfonic acid)-SDS running buffer, and resolved at 200 V according to the
manufacturers’ instructions (Invitrogen). Proteins were transferred to Immob-
ilion-P38 transfer membranes (Millipore, Bedford, Mass.) at 35 V for 3 h
 blocked in 5% bovine serum albumin (BSA, Sigma), and incubated with recom-
binant Fab D13, 1 μg/ml, followed by the secondary goat anti-human F(ab′)2
peroxidase-conjugated antibody (Pierce, Rockford, Ill.) at 0.16 μg/ml. Detection
was performed by enhanced chemiluminescence (ECL Plus; Amersham Phar-
macia Biotech, Buckinghamshire, United Kingdom).

Immunofluorescence. The cells grown on the cell culture dishes were fixed in
10% formalin (Merck KGaA) for 30 min, permeabilized with 0.1% Triton X-100
(Sigma, St Louis, Mo.) in PBS for 5 min, and treated with 3 M guanidinium
thiocyanate (Merck-Schuchardt, Hohenbrunn, Germany) for detection of PrP
(53) for 5 min. After blocking with 5% BSA (Sigma) for 40 min, the cells
were incubated with the primary antibody (Fab D13 diluted in PBS containing 5%
BSA to 3.5 μg/ml) overnight at 4°C; followed by addition of Cy3-conjugated
donkey anti-rat immunoglobulin G (Jackson Immunoresearch, West Grove,
Pa.) at a concentration of 7.5 μg/ml. Double labeling with Fab D13 and a rat
monoclonal immunoglobulin G, LAMP-1 (ID48), 0.2 μg/ml or a goat polyclonal
transferrin receptor antibody (CDT1) (both obtained from Santa Cruz Biotech-
nology, Santa Cruz, Calif.), 4 μg/ml, was performed. Cy3-conjugated donkey
anti-rat immunoglobulin G (Jackson Immunoresearch), 15 μg/ml, and fluores-
cin isothiocyanate-conjugated donkey anti-goat immunoglobulin G, 30 μg/ml,
respectively, served as secondary antibodies. DC were visualized with an anti-
major histocompatibility complex (MHC) class II antibody purified from the
supernatant of the hybridoma M5/114.15.2 (BD Pharmingen), 10 μg/ml, with
Cy3-labeled donkey anti-goat immunoglobulin G, 15 μg/ml, as a secondary
antibody. The cells were rinsed in PBS with 1% NH₄Cl (Sigma) between each
treatment and mounted in glycerol with 2.5% diazabicyclooctane (Sigma).

RESULTS
Effects of protease inhibitors on degradation of PrPSc by
DC. In the first series of experiments, we determined whether
DC-mediated degradation of exogenous PrPSc could be
blocked by inhibition of proteases. For this experiment we used
leupeptin, which is a commonly used protease inhibitor. As
described previously (29), when purified CD11c+ DC were
added to ScGT1-1 cells and cocultivated for 48 h, the
latter were engulfed by the DC, and this was followed by a marked
reduction in the intensity of PK-resistant PrP compared to the

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unexposed ScGT1-1 cells (Fig. 1A). The amount of PK-resistant PrP was also decreased after 48 h in coculture compared to that at time zero (data not shown). When the cocultures of ScGT1-1 and DC were treated with leupeptin, degradation was impeded (Fig. 1A). In such cocultures, the accumulation of PrP-immunopositive material was seen in the cytoplasm of the DC after guanidine thiocyanate treatment (Fig. 1B).

To determine whether any family of proteases is particularly involved in PrPSc degradation, the cocultures of ScGT1-1 cells and DC were then treated with protease inhibitors that block the catalytic activities of cysteine (E-64c, E-64d, and leupeptin), aspartic (pepstatin), serine (leupeptin and aprotinin), and metalloproteases (phosphoramidon). E-64c, E-64d, and leupeptin prevented the decrease in the intensity of the PK-resistant bands (Fig. 1C). Aprotinin, pepstatin, and phosphoramidon had no effect in the DC-mediated degradation of exogenous PrPSc (Fig. 1C). These experiments show that the DC-mediated degradation of exogenous PrPSc can be impeded by some but not all protease inhibitors. To further show that the inhibitors had a direct effect on DC, homogenates of the ScGT1-1 cells were added to living DC. After 48 h of incubation most of the PK-resistant PrP had disappeared. This degradation could also be inhibited by leupeptin (Fig. 1D).

**Effect of pH on DC-mediated degradation of PK-resistant PrP in vitro.** To determine whether the PrPSc-degrading activity of the protease inhibitors is pH dependent, lysates of DC were coincubated with lysates of ScGT1-1 cells at different pHs with and without leupeptin. Since CD11c+ DC in culture do not express PrPC (29), lysates from these cells have no intrinsic PrP that could interfere with the detection of PrP derived from ScGT1-1 cells. During the incubation period, most of the full-length PrP was degraded, but bands corresponding to the size of the PK-resistant PrP remained in lysates of the ScGT1-1 cells (Fig. 2). The intensity of these bands decreased after coincubation with the DC lysates at pH 5.5, and this decreased intensity was inhibited by leupeptin. PrPSc was not degraded at pH 7.8, which implies that degrading proteases in DC require an acidic environment in the cell.

**Effect of RNA interference on PrP in ScGT1-1 cultures.** In order to verify that endogenous degradation of PrPSc occurs in ScGT1-1 cells, these cells were treated with an siRNA directed to the mRNA for PrPC (to stop PrP synthesis) and harvested daily for 10 days after siRNA transfection. Transfection efficiency was high, as evaluated by fluorescence microscopy showing that almost all cells contained Cy3-labeled siRNAs (Fig. 3A). Subsequently, treatment with siRNA caused a reduction in PrPC and clearance of PrP Sc, as shown by Western blotting (Fig. 3B). Immunofluorescence also showed reduced levels of PrPSc in siRNA-treated cells (Fig. 3D) compared to untreated controls (Fig. 3C). No reduction in PrP expression was seen in mock-transfected cells (data not shown). These data show that there is an endogenous clearance of PrPSc in infected cells, and this result tallies with a recently published observation on N2a and GT1-1 cells treated with chemically synthesized siRNAs (13).

**Effect of protease inhibitors and pentosan polysulfate on PrP in ScGT1-1 and GT1-1 cells.** In order to analyze whether the protease inhibitors can affect the level of PrPSc within ScGT1-1 cells, these cells were treated with the different protease inhibitors for 48 h. There were no overt cytopathic or cytostatic effects of the treatment. Lysates from cell cultures

![FIG. 1. Effect of protease inhibitors on DC-induced degradation of PrPSc derived from ScGT1-1 cells. (A) Immunoblot showing levels of PK-resistant PrP in ScGT1-1 cells, ScGT1-1 cells after exposure to DC, and DC combined with leupeptin treatment and (B) DC (green) after 24 h of coculture with ScGT1-1 cells in the presence of leupeptin. Note the presence of intracellular PrPSc (red). (C) Levels of PK-resistant PrP from ScGT1-1 cells exposed to DC for 48 h and after treatment of these cocultured cells with E-64c, E-64d, leupeptin, phosphoramidon, pepstatin, and aprotinin for 48 h. (D) Levels of PrPSc after incubation of ScGT1-1 homogenate (H) with DC in the absence and presence of leupeptin at 0 and 48 h. The duplicates represent different cultures.

![FIG. 2. Effect of pH on PrPSc degradation. Immunoblot showing levels of PrPSc after incubation of ScGT1-1 lysates at pH 5.5 and 7.8 with the addition of DC lysates and DC lysates combined with leupeptin for 1 h. Note the decrease in PrP intensity at pH 5.5 after incubation with DC lysates, which was not seen when leupeptin was added. The duplicates represent different samples.

![FIG. 3. Effect of RNA interference on PrP in ScGT1-1 cultures. In order to verify that endogenous degradation of PrPSc occurs in ScGT1-1 cells, these cells were treated with an siRNA directed to the mRNA for PrPC (to stop PrP synthesis) and harvested daily for 10 days after siRNA transfection. Transfection efficiency was high, as evaluated by fluorescence microscopy showing that almost all cells contained Cy3-labeled siRNAs (Fig. 3A). Subsequently, treatment with siRNA caused a reduction in PrPC and clearance of PrP Sc, as shown by Western blotting (Fig. 3B). Immunofluorescence also showed reduced levels of PrPSc in siRNA-treated cells (Fig. 3D) compared to untreated controls (Fig. 3C). No reduction in PrP expression was seen in mock-transfected cells (data not shown). These data show that there is an endogenous clearance of PrPSc in infected cells, and this result tallies with a recently published observation on N2a and GT1-1 cells treated with chemically synthesized siRNAs (13).
exposed to E-64c, E-64d, and leupeptin subjected to Western blotting showed an increased intensity of PK-resistant PrP (Fig. 4A). No increase in PrPSc was seen in ScGT1-1 cells treated with aprotinin, pepstatin, or phosphoramidon.

Immunolabeling of guanidine thiocyanate-treated cells with the anti-PrP antibody showed immunopositive punctate structures in more than 80% of the ScGT1-1 cells. These speckled accumulations occurred often as small, distinct clusters in the cytoplasm of the cells (Fig. 4B). After treatment with leupeptin and E-64d, the immunopositive punctate structures were more spread throughout the cytoplasm of the cells (Fig. 4C), which may reflect an increase in the number of these structures and/or increased accumulation of PrPSc within them during their transport in the cell. Previous ultrastructural studies show that scrapie accumulates in lysosomes, residual bodies, and numerous compartments not yet identified (35). In agreement with this result, we found a partial colocalization of D13-positive materials and LAMP-1, a lysosome-associated membrane protein, after treatment with E-64d (Fig. 5). No colocalization was apparent between D13 and the transferrin receptor, which labeled the plasma membranes (data not shown). However, a more detailed immunocytochemical analysis of the compartments for PrP accumulation after treatment with protease inhibitors requires ultrastructural studies.

To determine that the levels of PrPSc observed were not caused by increased formation of PrPSc, the cells were incubated with pentosan polysulfate, a polyanion known to inhibit scrapie formation (7, 9). When PrPSc formation was inhibited by this treatment, leupeptin and E-64d still enhanced the intensity of PrPSc (Fig. 6) indicating that no increased synthesis of PrPSc was induced by the protease inhibitors. In addition, to determine that the increase in PrPSc induced by E-64c, E-64d, and leupeptin did not reflect effects on PrPSc, uninfected cells were treated with the inhibitors. There was a tendency to an increase in the level of PrPSc after treatment with E-64c, E-64d, and leupeptin but, in contrast to the experiments with PrPSc, also after treatment with the other inhibitors (Fig. 7).

Taken together, these observations suggest that cysteine proteases are involved in the endogenous proteolytic cleavage of PrPSc in ScGT1-1 cells, similar to the degradation of exogenous PrPSc by DC.

DISCUSSION

The present study shows that degradation of PrPSc by CD11c+ DC and ScGT1-1 cells can be interfered with by protease inhibitors and that this degradation requires an acidic environment. It also shows that the effects of protease inhibitors on degradation of PrPSc and PrPSc differ.

We have recently shown that CD11c+ DC in culture can degrade exogenous PrPSc derived from phagocytosed ScGT1-1

FIG. 3. Effect of PrPSc RNA interference on the occurrence of PrPSc and PrPSc in ScGT1-1 cells. (A) ScGT1-1 cells were treated with Cy3-labeled siRNAs (red), showing transfection of the majority of the cells; cell nuclei are shown in blue. (B) Immunoblot showing reduction of PrPSc and loss of PK-resistant PrP 7 days after PrPSc siRNA treatment of ScGT1-1 cells. a, untreated cells; b, PrPSc siRNA-treated cells. −, non-PK-treated samples; +, PK-treated samples. (C and D) PrP immunofluorescence of untreated (C) and PrPSc siRNA-treated (D) ScGT1-1 cells. The cells in both panels C and D were treated with guanidine thiocyanate prior to immunostaining to expose PrPSc. Bars, 20 μm.

FIG. 4. Presence of PrPSc in ScGT1-1 cell cultures after treatment with protease inhibitors. (A) Immunoblot showing the presence of PK-resistant PrP in ScGT1-1 cell cultures untreated or treated with E-64c, E-64d, leupeptin, aprotinin, pepstatin, and phosphoramidon for 48 h. (B and C) PrP immunofluorescence with Fab D13 of (B) untreated and (C) leupeptin-treated ScGT1-1 cells. Cells were exposed to guanidine thiocyanate prior to immunostaining. Bars, 20 μm.
Here we demonstrate that this degradation can be inhibited by treatment with the two cysteine protease inhibitors, E-64c and E-64d, and with leupeptin. Leupeptin inhibits both cysteine and serine proteases (15, 43), but since the serine protease inhibitor aprotinin had no such effect, it is likely that the effect of leupeptin on PrPSc degradation reflects inhibition of cysteine protease activity. The observation that the RNA interference with PrP could clear PrPSc from the cultures demonstrates an endogenous turnover of PrPSc in ScGT1-1 cells, which is in agreement with recent observations of clearance of PrPSc from scrapie-infected N2a cells following treatment with PrP-binding antibodies (19, 40). The half-life of PrPSc is probably much longer than that of PrPC (3), which has a half-life estimated to 3 to 6 h in N2a cells (3, 8) and 1.5 to 2 h in primary splenocyte and cerebellar granule cell cultures (39).

Part of the PrPC bound to cell membranes may be cleaved by serum phospholipases and/or metalloenzymes and released from the cell surface, whereas the rest may be targeted for intracellular degradation (52, 55). In a study of human PrPC degradation with protease inhibitors on lysates from cerebral and cerebellar cortex, the metal-chelating agents EDTA and EGTA and inhibitors of cysteine proteases were effective in inhibiting PrPC degradation (26). In the present study, we found no signs of selective inhibition of PrPC degradation by cysteine proteases. These results therefore indicate that the cleavage sites available for proteolysis of PrPSc, the degradation of which was inhibited only by cysteine protease inhibitors, are more restricted than those available for proteolysis of PrPC.

Cysteine proteases represent a class of multifunctional proteolytic enzymes that can function both in lysosomal degradations (cathepsins) and in programmed cell death (caspases) (28). In the present study, we found that the cysteine proteases degraded PrPSc at an acidic pH, which indicates that the PrPSc-degrading activity occurs in endosomal and lysosomal compartments. Thus, the degradation of PrPSc and of PrPC seems to employ similar compartments in the cell (52) and of the N-terminal trimming of PrPSc by acid proteases into a 19-kDa (unglycosylated) fragment that occurs soon after its formation in scrapie-infected N2a and HaB cells (10, 51).

Ultrastructurally, PrPSc has been identified in vesicles and lysosomes in both N2a and hamster brain-derived cells (35, 53). The degradation of bona fide PrPSc, as observed in the present study, therefore seems to occur in compartments distinct from that of the misfolded prion-like PrP species that can accumulate in the cytosol upon treatment with proteasome inhibitors (30, 58) and cyclosporin (11). The mammalian cysteine proteases that are localized to the lysosomal compart-

FIG. 5. Double immunolabeling of (A) PrP and (B) LAMP-1 in ScGT1-1 cells treated with E-64d. Cells were exposed to guanidine thiocyanate prior to immunolabeling. A merged image of the red and green channels shows partial colocalization of PrP and LAMP-1 (C). A bright-field micrograph of the cells is also shown (D). Bar, 20 μm.

FIG. 6. Immunoblot showing levels of PK-resistant PrP in ScGT1-1 cells after treatment with pentosan polysulfate (PP), leupeptin, or E-64d or combinations of pentosan polysulfate and leupeptin or E-64d. The cultures were treated for 48 h, and the duplicates represent different cultures.

FIG. 7. Immunoblot showing the effects of protease inhibitors on PrPSc in uninfected GT1-1 cells after treatment with protease inhibitors. The cultures were treated for 48 h, and the duplicates represent different cultures.
ments are known as cathepsins, although not all cathepsins are cysteine proteases (for reviews, see references 28 and 34).

Since various cell types differ in their protease contents, the observation that cysteine proteases are selectively involved in PrPSc degradation may be relevant for understanding the susceptibility of particular cell types to prion infections. Thus, a cell’s proteolytic enzyme content could be one of the factors that determine the susceptibility of a cell to prion infection (25). It would therefore be of interest to identify the individual lysosomal cysteine protease(s) that is active in PrPSc degradation and to analyze whether cells with various susceptibilities to scrapie infection differ in their content of such enzymes. For instance, differences in catalytic properties may account for the observation of reduced levels of PrPSc in scrapie-infected N2a cells following treatment with leupeptin and E-64 (16) as opposed to the increased levels in GT1-1 cells. Recently, an upregulation of cathepsin B and cathepsin L activities was described in scrapie-infected N2a cells (59). Whether protease inhibitors can be used to improve the susceptibility of cells to prion infection or to stabilize an already established prion infection remains to be seen. One strategy could be to use cathepsin knockout mice (14, 20) and to study their susceptibility to prion infections.

CD11c+ DC have been implicated in facilitating the spread of scrapie to the nervous system from peripheral sites of inoculation (1). These observations may seem paradoxical in view of our finding that this type of DC in culture can efficiently degrade PrPSc. However, proteolytic processing of PrPSc in CD11c+ DC could conceivably generate fragments of PrPSc that are still infectious. The smallest identified infectious PrPSc molecule is a 106-amino-acid prion protein expressed in transgenic mice lacking wild-type PrPSc. This is a prion protein with two deletions, an N-terminal truncation and an internal deletion, designated a miniprion, or PrP106 (48). In addition, a subset of residues 89 to 140 spontaneously induce protease resistance in synthetic PrP (49). The minimum PrP peptide size required to induce infectivity in wild-type PrP is not yet known, but should such fragments resist an initial proteolytic cleavage of PrPSc by cysteine proteases, fragments that retain infectivity may be generated. Although it seems unlikely that such fragments could be transmitted to neighboring cells in context with major histocompatibility complex class II molecules, they might be exported to the cell surface by other mechanisms or be released into the environment by so-called exosomes (38, 54). Processing by DC could also increase the infectivity of ingested prions by inducing alterations beyond proteolysis. For instance, endosomal hydrolases could digest glycans such as the N-linked sugars that are attached to PrPSc. The influence of such chemical modifications on prions remains to be determined. Low pH within DC endosomes as well as the hydrolysis by lipases of membranes attached to PrPSc could also encourage conformational alterations of PrPSc or disperse it into smaller aggregates, with a concomitant increase in effective prion infectivity.

Although these cultured CD11c+ DC never showed levels of PrPSc comparable to those of GT1-1 cells, they could still express PrPSc at levels high enough to support replication of PrPSc in the in vivo situation (5, 21, 47), as has been suggested for the facilitation of prion spread by follicular DC (4, 33, 37). Prion spread to the peripheral nervous system may be facilitated by the fact that CD11c+ DC are in close contact with peripheral nerve fibers in the epithelium (17, 22), which is in contrast to follicular DC, which are of a different origin and have different functions than the CD11c+ DC (12). In fact, in his original study, Langerhans described connections of the new type of cells in dermal epithelium (the DC that were later given his name) to nerve fibers, and he believed that the cells were of neuronal origin (27).

The foregoing observation of efficient cysteine protease-dependent degradation of PrPSc by DC may relate to recent studies on the effect of prion peptide immunization on PK-resistant PrP (46). Immunization with peptides predicted to fit the major histocompatibility complex class II binding motif causes a marked reduction in the level of PK-resistant PrP in scrapie-infected tumors transplanted into mice without affecting PrPSc or tumor growth. One possible explanation for this is that degradation of PrPSc occurs in vivo and that this degradation can be increased by immunization. Immunization with recombinant PrP delays the onset of prion disease in mice (45), and stimulation of innate immunity prolongs the survival of scrapie-infected mice even as a postexposure treatment (31, 44, 50). Knowledge of the mechanisms involved in proteolytic cleavage of PrPSc serves as a foundation for therapies employing immune modulation of prion diseases or the design of specific protease activators for the degradation of PrPSc.

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REFERENCES


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