Prion peptide-mediated cellular prion protein overexpression and neuronal cell death can be blocked by aspirin treatment

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Received November 22, 2010; Accepted January 25, 2011

DOI: 10.3892/ijmm.2011.626

Abstract. Prion diseases are infectious neurodegenerative disorders characterized by the conversion of the cellular prion protein (PrPc) to the misfolded isoform (PrPsc). Prion peptide PrP 106-126 [PrP (106-126)] shares many physiological properties with PrPsc; it is neurotoxic *in vitro* and *in vivo*. PrP (106-126) induces neurotoxicity by the overexpression of PrPc and activation of the mitogen-activated protein (ERK1/2). Aspirin, an anti-inflammatory drug, is a known ERK inhibitor and prevents neurodegenerative disorders including prion diseases. The influence of aspirin treatment on prion protein-mediated neurotoxicity and expression of PrPc were the focus of this study. Cell viability and apoptosis were assessed by crystal violet staining and the TUNEL and DNA fragmentation assays. Apoptosis-associated protein expression of PrPc, p-53, p-ERK1/2, p-p38, Bcl-2 and cleaved-caspase-3 was examined by Western blotting and immunocytochemistry. Aspirin treatment inhibited PrP (106-126)-induced neuronal cell death in SH-SY5Y neuroblastoma cells. In addition, the PrP (106-126)-mediated increase of p-p38, p53, cleaved-caspase-3 and decrease of Bcl-2 expressions were blocked by aspirin and the ERK inhibitor, PR98059. Furthermore, we showed that the PrP (106-126)-mediated increase of PrPc and p-ERK1/2 were inhibited by PD98059 and aspirin. Taken together, these results demonstrate that ERK1/2 is a key modulator of the protective effect of aspirin on PrP-106-126-mediated cellular prion protein overexpression and neurotoxicity and also suggest that aspirin may prevent neuron cell damages caused by the prion peptide.

Introduction

Prion diseases or transmissible spongiform encephalopathies (TSE) are a family of infectious neurodegenerative disorders characterized by CNS spongiosis, vacuolation, neuronal loss, and microglial activation (1). The main abnormality associated with the pathogenesis of prion disease is the formation of scrapie prion proteins (PrPsc), which are proteinase K-resistant misfolded proteins that are modified forms of the normal cellular prion protein (PrPc) (2,3).

The production of PrPsc and the progression of prion diseases are dependent upon the presence of PrPc (4,5). Several studies on conditional prion protein gene knock-out mice, have shown that depletion of PrPc protein in neurons could prevent the disease (4,6). These findings emphasize the importance of therapeutic strategies targeting the PrPc protein. Some reports have shown that the PrPsc and the prion protein peptide induced neuronal oxidative injury and increased PrPc protein levels. In addition, PrPc knock-out cells prevented prion-mediated oxidative injury (4,7). In addition, recent studies have shown that glimepiride prevented prion-mediated neurotoxicity by reduction of PrPc expression (8). Thus, any treatment that affects the amount of PrPC expression may also affect prion-mediated neurotoxicity.

The prion protein peptide 106-126 [PrP (106-126)] contains the amino acid residues 106-126 of the cellular prion protein and resides near the N-terminus of PrPsc (9,10). It possesses many characteristics of the entire PrPsc protein (11). PrP (106-126) is frequently used to study PrPsc pathogenesis, as well as aggregation of endogenous PrPc to an amyloidogenic form that shares several characteristics with PrPsc (12,13). Recent studies have linked the increased phosphorylation of the ERK1/2 pathway to the neurotoxic effects of PrPsc and PrP (106-126) (14,15). In a model of prion disease, overstimulation of the ERK1/2 pathway was correlated with oxidative injury of neuronal and non-neuronal cells by reactive oxygen species (ROS) generation (15,16).

Aspirin is a known anti-inflammatory drug with a broad spectrum of pharmacological activity and multiple sites of action (17). Recent findings suggest that aspirin exerts direct neuroprotective effects, including NF-κB inhibition (18-20). Similarly, one study showed that aspirin prevented prion peptide-induced neurotoxicity by inhibition of NF-κB (18). Substantial evidence has indicated that ERK1/2 is also one of the targets of aspirin in neurodegenerative disease (21). However, the effect of aspirin-mediated ERK1/2 inhibition on prion peptide-induced neurotoxicity has not been evaluated.

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Key words: prion peptide (106-126), oxidative stress, neuronal cell death, ERK1/2 phosphorylation, aspirin

Thus, the influence of aspirin-mediated ERK1/2 inhibition on the PrP (106-126)-mediated overexpression of PrPc protein and neuronal cell damage were investigated. The results show that aspirin treatment decreases PrP (106-126)-mediated PrPc over-expression and prevents neuronal cell death by inhibition of ERK signaling. The results of the present study support the view that regulation of ERK1/2 is a viable therapeutic strategy for neurodegenerative diseases including prion disease.

Materials and methods

Cell culture. Human neuroblastoma cell lines (SH-SY5Y) were obtained from the American Type Culture collection (ATCC, Rockville, MD, USA). The cells were cultured in Minimum Essential Medium (Hyclone Laboratories, Logan, UT, USA) that contained 10% fetal bovine serum (FBS) (Invitrogen-Gibco, Grand Island, NY, USA) and gentamycin (0.1 mg/ml) in a humidified incubator maintained at 37˚C and 5% CO_2.

PrP (106-126) treatment. Synthetic PrP (106-126) (sequence, Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly) was synthesized from Peptron (Seoul, Korea). The peptides were dissolved in sterile DMSO at a concentration of 12.5 mM and stored at -80˚C.

Crystal violet staining. Cell viability was evaluated by crystal violet staining, as previously described (22). Briefly, the cells were stained with staining solution (0.5% crystal violet in 30% ethanol and 3% formaldehyde) for 10 min at room temperature (RT), and washed four times with water. The stained cells were lysed with 1% sodium dodecyl sulfate (SDS) solution, and the absorbance was measured at 550 nm. Cell viability was calculated based on the relative dye intensity compared to controls.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. TUNEL analysis was performed to measure the degree of cellular apoptosis using an *in situ* Apo-BrdU DNA fragmentation assay kit (BioVision, San Francisco, CA, USA) following the manufacturer's instructions.

DNA fragmentation assay. Cell pellets were suspended in 0.5 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 10% (v/v) NP-40, 20 mM EDTA and 0.5% (w/v) SDS, and digested with 500 μg/ml proteinase K for 4 h at 65˚C. After digestion, the DNA was sequentially extracted with phenol/chloroform (1:1, v/v), and precipitated with ethanol at -20˚C overnight. Purified DNA was electrophoresed on a 1.5% agarose gel and visualized with ethidium bromide staining and ultraviolet transillumination.

Immunofluorescence. SH-SY5Y cells were cultured on glass coverslips in a 24-well plate. Cells were washed with phosphate-buffered saline (PBS) and fixed with cold acetone for 90 sec. Cells were then washed with PBS again, blocked with 5% FBS in Tris-buffered saline with Tween (TBST), and incubated with cleaved caspase-3 monoclonal antibodies (2 μg/ml) for 48 h at room temperature (20˚C). Unbound antibody was removed by an additional PBS wash, and cells were incubated with labeled anti-rabbit Alexa Fluor 546 (for cleaved caspase-3) IgG antibody (4 μg/ml) for 2 h at room temperature. Finally, cells were mounted with DakoCytomation fluorescent medium and visualized via fluorescence microscopy.

Western blot analysis. SH-SY5Y cells were lysed in lysis buffer (25 mM HEPES, pH 7.4; 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM DTT, and protease inhibitor mixture). The proteins were electrophoretically resolved on a 10-15% SDS gel, and immunoblotting was performed as previously described. Equal amounts of lysate protein were resolved on a 10-15% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. Immunoreactivity was detected through sequential incubation with horseradish peroxidase-conjugated secondary antibodies and ECL reagents. The antibodies used for immunoblotting were p53 (Santa Cruz Biotechnology), Bcl-2 (Santa Cruz Biotechnology), phosphorylated-p38 (p-p38) (Cell Signaling Technology) phosphorylated-ERK (p-ERK) (Cell Signaling Technology), PrPc (Millipore) and β-actin (Santa Cruz Biotechnology).

Results

PrP (106-126)-induced apoptosis is inhibited by aspirin treatment in SH-SY5Y neuroblastoma cells. The influence of aspirin on PrP (106-126)-induced apoptosis in SH-SY5Y cells was determined by crystal violet staining. The SH-SY5Y cells were exposed to aspirin alone or in combination with PrP (106-126). The cells were responsive to PrP (106-126) treatment, and aspirin had no effect on cell viability. However, aspirin treatment inhibited PrP (106-126)-induced neuronal cell death (Fig. 1B). These results were confirmed by morphological examination of the cell population (Fig. 1A). Consistent with these results, the DNA fragmentation assay and TUNEL assay also showed that aspirin treatment completely inhibited PrP (106-126)-induced apoptosis (Fig. 1C and D).

Next, we examined whether aspirin had an effect on the protein levels of p53, p38, Bcl-2 and cleaved caspase-3. The SH-SY5Y cells were pretreated with 0, 2.5, 5 or 10 μM aspirin, and then exposed to PrP (106-126). The prior peptide-treated cells showed increased p53 and p-p38 protein levels and decreased Bcl-2 protein levels. By contrast, aspirin treatment was associated with decreased p53 and p-p38 protein levels and increased Bcl-2 protein levels in neuronal cells exposed to PrP (106-126) (Fig. 2A), in a dose-dependent manner. Immunofluorescent analysis using an antibody against cleaved caspase-3 detected the cleaved product in SH-SY5Y cells pretreated with aspirin and exposed to PrP (106-126) (Fig. 2B). Collectively, these results are consistent with the idea that aspirin treatment decreases PrP (106-126)-mediated apoptosis.

Aspirin treatment prevents PrP (106-126)-induced apoptosis by regulation of the ERK1/2 signaling pathway in SH-SY5Y cells. The phosphorylation of ERK1/2 has been shown to be responsible for the neurotoxicity associated with prion infections (14,15). Aspirin-induced PrP (106-126) resistance was studied by assessing the ERK1/2 activity after PrP (106-126) treatment. As shown in Fig. 3, p-ERK1/2 protein levels were increased after PrP (106-126) treatment, whereas they were inhibited by aspirin or PD98059 (ERK1/2 inhibitor) treatment.

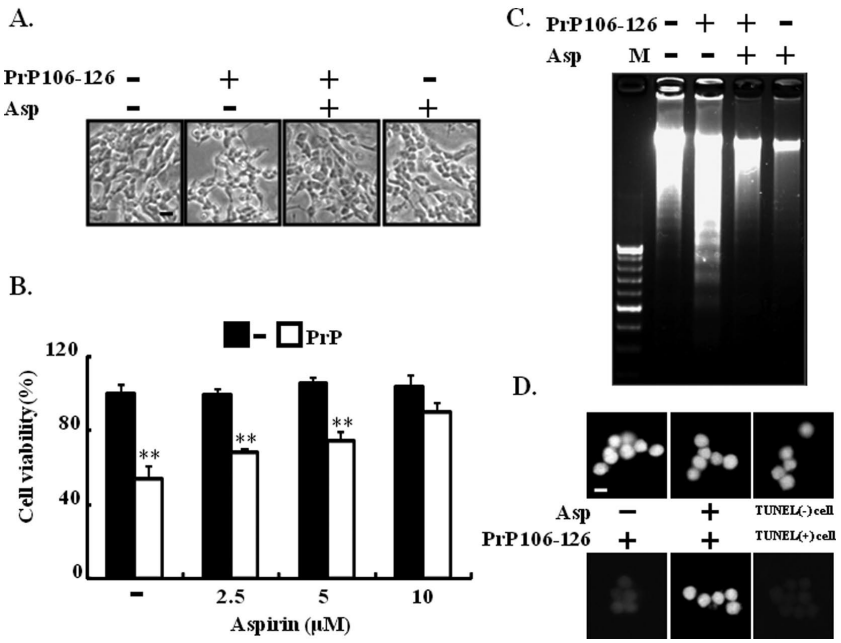

Figure 1. Aspirin protects PrP (106-126)-induced cell death in SH-SY5Y. (A) SH-SY5Y cells were pretreated with 0, 2.5, 5, or 10 μM aspirin for 6 h, and then exposed to 200 μM PrP (106-126) for 42 h. The treated cells were photographed by light microscopy (x200). Scale bar, 100 μm (B) Cell viability was measured by crystal violet staining. Viability of the control cells was set at 100%, and viability relative to the control was measured. The bar graph denotes the mean ± SEM. (n=3). **P<0.05. (C) Analysis of genomic DNA isolated from SH-SY5Y cells that were pretreated with 10 μM aspirin for 6 h and then treated with 200 μM PrP (106-126) for 42 h. Marker, 100 bp DNA ladder. (D) Representative immunofluorescence images of TUNEL-positive SH-SY5Y cells at 42 h after exposure to 200 μM of PrP (106-126) in the absence or presence of 10 μM aspirin. The cells were counterstained with propidium iodide to show all cell nuclei. Magnification x400; scale bar, 10 μm.

Since the neurotoxicity of PrPsc and PrP (106-126) has been linked to the overexpression of the PrPc protein (7), we investigated whether aspirin affects the levels of PrPc proteins. The SH-SY5Y cells were pretreated with various concentrations of aspirin, and then exposed to PrP (106-126). The PrPc protein levels were increased with PrP (106-126) treatment, whereas they were inhibited by aspirin treatment (Fig. 3A). Consistent with these results, the PrP (106-126)-induced overexpression of PrPc protein levels was inhibited by PD98059 treatment (Fig. 3B).

To determine whether aspirin had a neuroprotective effect associated with ERK1/2 inactivation in PrP (106-126)-induced neuronal cell death, SH-SY5Y cells were pretreated with aspirin or PD98059, and then exposed to PrP (106-126). The cells were responsive to PrP (106-126) treatment, while aspirin and PD98059 had no effect on cell viability. However, aspirin and PD98059 treatment inhibited PrP (106-126)-induced neuronal cell death (Fig. 4A). These results were confirmed by morphological examination of the cell population (Fig. 4B). Consistent with these findings, the DNA fragmentation assay and the TUNEL assay showed that aspirin or PD98059 treatment completely inhibited PrP (106-126)-induced apoptosis (Fig. 4C and 4D). Collectively, these results are consistent with the notion that the aspirin-mediated protection from PrP (106-126)-induced apoptosis occurs via regulation of ERK1/2-dependent PrPc expression.

Discussion

The purpose of this study was to investigate the influence of aspirin treatment on the PrP (106-126)-induced PrPc overstimulation and neuronal cell death. The results of this study suggest the potential of aspirin for therapeutic intervention strategies in neurodegenerative diseases, including prion disease. The PrP (106-126) peptide served as a suitable model for PrPsc neurotoxicity; this is because it possesses many properties of the PrPsc protein (9-11,16,23). PrPsc protein and PrP (106-126) have been shown to induce neuronal cell death by increasing the production of prostaglandin E2 (PGE2) (8,24,25). Some studies have reported that PGE2 production is regulated by p53, p-p38, and ERK1/2 activation (26,27). Consistent with this, PrP (106-126) treatment of SH-SY5Y cells increased p53, p-p38, cleaved caspase-3 and p-ERK protein levels and decreased Bcl-2 protein levels (Figs. 2 and 3A).

A few studies have demonstrated that aspirin treatment influences the progression of prion disease (18,28). Bate *et al* observed that non-steroidal anti-inflammatory drugs (NSAIDs) protected against prion-induced neurotoxicity by inhibition of PGE2 production (28). Consistent with this, PrP (106-126)-mediated elevation of p53, p-p38, cleaved caspase-3 and p-ERK protein levels were decreased by aspirin treatment (Figs. 2 and 3A). Taken together, this data suggest that aspirin prevented prion peptide-induced neuronal cell death by the inhibition of p53, p-p38 and p-ERK protein levels.

One report has shown that PrPc overstimulation is a key factor of PrPsc or prion peptide-mediated neurotoxicity (7). Pietri *et al* suggested that PrP (106-126)-induced neurotoxicity is caused by overstimulation of PrPc-associated signaling pathways, including oxidative stress conditions (7). Using Western blot analysis, we found that aspirin inhibited PrP (106-126)-mediated over-stimulation of PrPc protein levels (Fig. 3A) and blocked neuronal cell death (Fig. 1A).

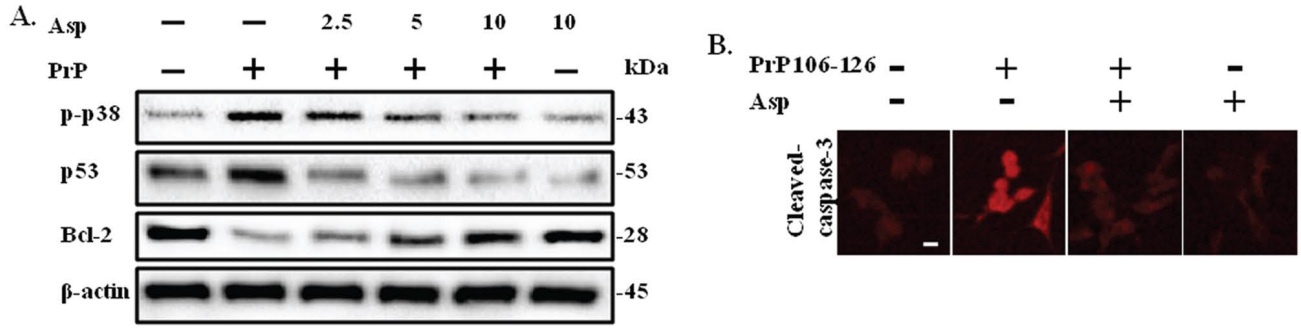

Figure 2. Aspirin regulates the expression levels of apoptosis-related proteins in PrP (106-126)-treated SH-SY5Y cells. (A) SH-SY5Y cells were pretreated with 0, 2.5, 5 or 10 μM aspirin for 6 h, and then exposed to 200 μM PrP (106-126) for 24 h. The treated cells were assessed for p-p38, p53 and Bcl-2 protein levels by Western blot analysis. Results were normalized to β-actin. (B) SH-SY5Y cells were pretreated with aspirin (10 μM) for 6 h, and then exposed to 200 μM PrP (106-126) for 24 h. The treated cells were immunostained with cleaved caspase-3 (red), as described in Materials and methods, and observed with a fluorescence microscope. Magnification x400; scale bar, 10 μm.

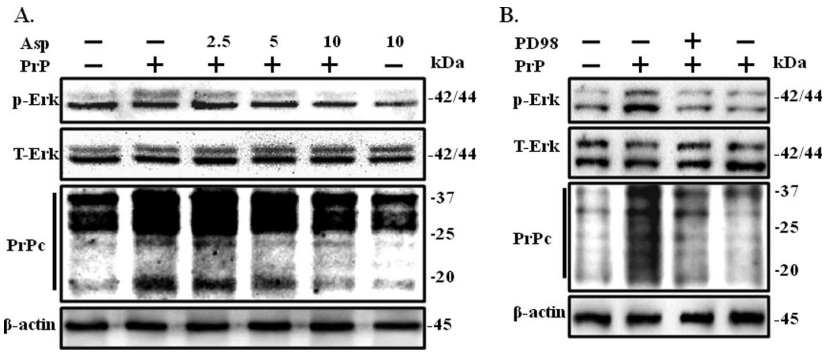

Figure 3. Aspirin inhibits the protein expression of PrPc protein by inhibition of ERK phosphorylation. (A) SH-SY5Y cells were pretreated with a range of aspirin concentrations (0, 2.5, 5 or 10 μM) for 6 h, and then exposed to 200 μM PrP (106-126) for 24 h. The treated cells were assessed for p-ERK, total-ERK (T-ERK) and PrPc levels by Western blot analysis. Results were normalized to β-actin (B) SH-SY5Y cells were pre-treated with 10 μM aspirin (6 h) or 10 μM PD98059, and then exposed to 200 μM PrP (106-126) for 24 h. Western blot analysis of p-ERK, T-ERK and PrPc levels in SH-SY5Y cells. Results were normalized to β-actin.

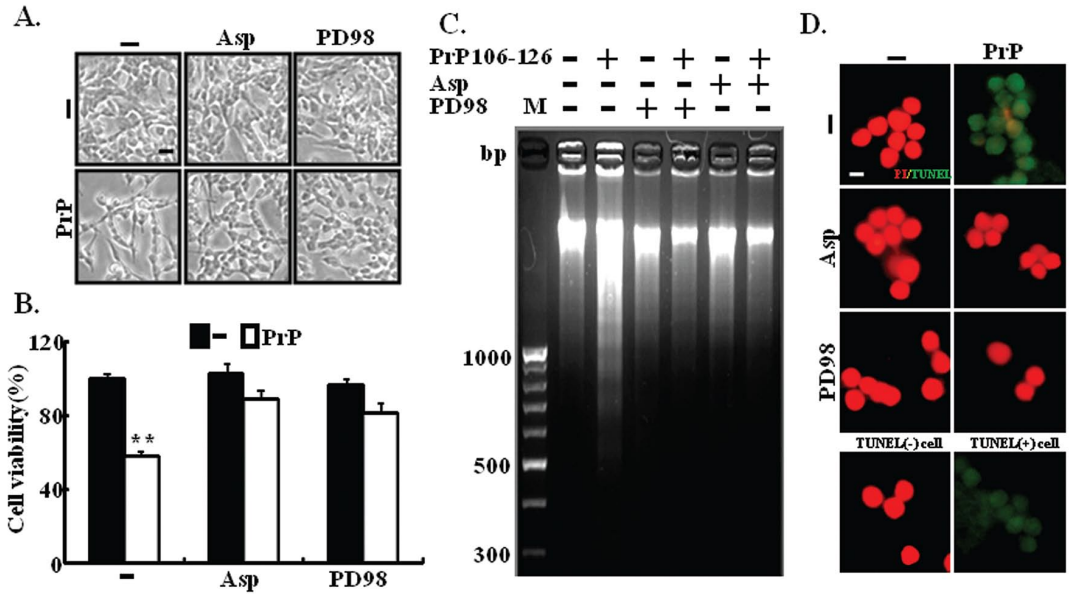

Figure 4. Aspirin protects PrP (106-126)-induced neuronal cell death by inhibition of ERK activation. (A) SH-SY5Y cells were pretreated with aspirin (10 μM) or PD98059 (10 μM) for 6 h before PrP (106-126) (200 μM) treatment for 36 h. The treated cells were photographed by light microscopy (x200). Scale bar, 100 μm. (B) Cell viability was measured by crystal violet staining. Viability of control cells was set at 100%, and viability relative to the control was measured. The bar graph denotes the mean ± SEM (n=3). **P<0.05. (C) Analysis of genomic DNA isolated from SH-SY5Y cells treated as described in (A). Marker, 100 bp DNA ladder. (D) Representative immunofluorescence images of TUNEL-positive (green) SH-SY5Y cells at 42 h after exposure to 200 μM of PrP (106-126) in the absence or presence of 10 μM aspirin (6 h) or 10 μM PD98059 (6 h). The cells were counterstained with propidium iodide (red) to show all cell nuclei. Magnification x400; scale bar, 10 μm.

Aspirin is a known anti-inflammatory drug and ERK inhibitor (17,21). Thus, we investigated the effect of aspirin-mediated ERK1/2 inhibition on prion peptide-induced PrPc expression. The results of this study showed that PrP (106-126)-induced PrPc overexpression was inhibited by treatment with aspirin and the ERK inhibitor (PD98059) (Fig. 3A, C and D). In addition, treatment of PD98059 or aspirin protected the cells from PrP (106-126)-induced neurotoxicity (Fig. 4).

Thus, the observations noted in this study support the notion that aspirin treatment protects cells from prion peptide-mediated apoptosis by regulation of ERK1/2-mediated PrPc expression. In addition, these results suggest that ERK1/2 inhibitors may be clinically useful for the treatment of neurodegenerative diseases, including prion disease.

Acknowledgements

This study was supported by a National Research Foundation of Korea grant funded by the Korean Government (2009-0076873) and the Regional Research Universities Program and the Center for Healthcare Technology Development.

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