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L-2,5-Dihydrophenylalanine, an Inducer of Cathepsin-dependent Apoptosis in Human Promyelocytic Leukemia Cells (HL-60)

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L-2,5-Dihydrophenylalanine (DHPA), a phenylalanine analogue, induced apoptosis in human promyelocytic leukemia cells (HL-60). This apoptosis was demonstrated by morphological changes of the cells, such as fragmentation of nuclei and chromatin condensation, and by some evidence found in biochemical analysis, such as DNA ladder and activation of caspase 3. The DHPA-induced apoptosis was prevented by a pan-caspase inhibitor, Z-VAD-fmk, and a cysteine protease inhibitor, E-64d, which inhibits calpains and cathepsin B and L. A calpain inhibitor, Z-LL-H, did not affect this apoptosis. A cathepsin B specific inhibitor, CA074-Me, prevented only chromatin condensation. However, E-64d and a cathepsin L specific inhibitor, Z-FY(*t*-Bu)-dmk, protected the cells from both chromatin condensation and oligonucleosomal DNA fragmentation. As proceeding to the apoptotic process, the activities of both cathepsin B and L increased gradually. These results indicated that DHPA was an inducer of cathepsin-dependent apoptosis in HL-60 cells.

L-2,5-Dihydrophenylalanine (DHPA) is a phenylalanine analogue, which was first isolated from *Streptomyces* sp.[1] as an antimicrobial metabolite. It has been reported to exhibit a growth inhibitory effect on various microorganisms including bacteria and fungi[1,2]. The mode of action was thought to be dependent on the metabolically antagonistic activity against phenylalanine[1,2]. DHPA is also able to be metabolized as a substrate for several enzymes, such as tryptophan hydroxylase, tryptophan 2,3-dioxygenase, indoleamine 2,3-dioxygenase, phenylalanine hydroxylase and phenylalanine ammonia-lyase[3~6]. And DHPA exhibited a cytotoxic effect on rat and the effect was reversed by the excess of phenylalanine[7].

We found that DHPA showed a growth inhibitory effect on human promyelocytic leukemia cells (HL-60). Moreover, DHPA induced apoptosis judging from morphological changes of the cells, such as fragmentation of nuclei and chromatin condensation. Although activation of various proteases, such as caspases and cathepsins, is well known to be involved in the process of apoptotic proteolysis, the physiological role of cathepsins compared with that of caspases has been poorly understood in apoptosis. Cathepsins are lysosomal proteases and have various isotypes containing cathepsin A to Z, legumain and napsin. Cathepsin B, F, H, L and X are cysteine proteases existing in almost all tissues[8]. Cathepsin C, K, S and legumain are cysteine proteases only expressed in the specialized tissue[8,9]. Cathepsin D, E and napsin are aspartic proteases[10]. Cathepsin A is a serine protease[11]. Cathepsin B contributes to apoptosis induced by serum deprivation[12] or some stimuli, TNF-α, bile salt and atractyloside[13~15]. Cathepsin B increases the release of cytochrome *c* from mitochondria[13]. On the other hand, cathepsin L induces apoptosis independently on the release of cytochrome *c*[16]. Cathepsin L has been reported to exhibit a processing activity of procaspases[17]. To reveal the role of the proteases in the DHPA-induced apoptosis, effects of protease inhibitors on the apoptosis were examined. In this report, we describe that DHPA is an inducer of cathepsin-dependent apoptosis.

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Materials and Methods

Isolation and Purification of DHPA

A strain of *Streptomyces* sp. producing DHPA was isolated from soil sample. DHPA was purified from the culture broth and the chemical structure was confirmed by the method described previously[1,3,18]. It was dissolved in sterile distilled water to make a stock solution, from which appropriate concentrations were prepared with cultured medium for the following tests.

Cell Culture

The HL-60 cell line was obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium (containing Penicillin-Streptomycin solution) supplemented with 10% FBS at 37°C in humidified air containing 5% CO_2 unless stated otherwise. Cells were sub-cultured twice a week, and only those in the exponential growth period were used in the following experiments.

Cell Proliferation Assay

Cell proliferation was examined by the Alamar Blue assay as described previously[19]. Briefly, cells were cultured in quadruplicate at 10^6 cells/ml in 96-well flat-bottomed tissue culture plates with each concentration of drugs for 24 hours. After addition of Alamar Blue solution, incubation was continued for another 24 hours. Fluorescence was detected with a Millipore Cytofluor 2300 (excitation wavelength, 530 nm; emission wavelength, 590 nm)

Fluorescent Staining of Nuclei

Cells were cultured at 10^6 cells/ml in 48-well flat-bottomed tissue culture plates with or without DHPA for 24 hours. After cultivation, cells were collected and washed with phosphate-buffered saline (PBS). Cells were fixed with 1% glutaldehyde in PBS for 1 hour and were washed with PBS twice. The fixed cells were stained with 1 mM Hoechst 33258 in PBS for 5 minutes. The stained cells were observed under an Olympus fluorescent microscope (excitation wavelength, 365 nm; emission wavelength, 420 nm). The number of cells having normal and apoptotic nuclei in randomly selected areas was counted. Apoptotic rate was represented as a percentage of apoptotic cells in total those.

Analysis of Oligonucleosomal DNA Fragmentation

Oligonucleosomal DNA fragmentation was analyzed by a slightly modified agarose gel electrophoresis method described previously[20]. Cells were cultured at 10^6 cells/ml in 48-well flat-bottomed tissue culture plates with or without drugs for 24 hours. The cells were digested in 50 mM Tris-HCl buffer (pH 7.8) containing 10 mM EDTA, 0.5% SDS, and 100 μg/ml of proteinase K at 37°C for 12 hours. The DNA was extracted with phenol/chloroform (1:1, v/v), precipitated with 0.5 M NaCl/ethanol (1:1, v/v), and then electrophoresed on 2% agarose gel. DNA fragments were stained with ethidium bromide and visualized under UV light.

Caspase 3 Assay

Caspase 3 activity was measured by the fluorometric assay, in which a fluorogenic synthetic peptide Ac-DEVD-MCA was used as a substrate. Cells were cultured at 10^6 cells/ml in 48-well flat-bottomed tissue culture plates with or without drugs for 24 hours. After cultivation, cells were collected and washed with PBS. The pellets were resuspended in 20 mM Hepes-KOH buffer (pH 6.8) with 250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, and 1 mM dithiothreitol on ice for 10 minutes. The cells were lysed with the addition of 10 μM digitonin for another 10 minutes. Cell lysates were incubated with 14 μM Ac-DEVD-MCA at 37°C for 1 hour. Fluorescence was measured with a Millipore Cytofluor 2300 (excitation wavelength, 380 nm; emission wavelength, 460 nm). One unit of the enzyme activity was defined as the cleavage of 1 pmol of the substrate per one minute.

Cathepsin Assay

Cathepsin was assayed with a slightly modified fluorometric assay described previously[21,22]. Fluorogenic synthetic peptides, Z-Arg-Arg-MCA and Z-Phe-Arg-MCA were used as substrates for cathepsin B and L, respectively. Cells were cultured at 10^6 cells/ml in 48-well flat-bottomed tissue culture plates with or without drugs for 24 hours. After cultivation, cells were collected and washed with PBS. The pellets were kept in 20 mM Hepes-KOH buffer (pH 6.8) with 1% Triton X-100 on ice for 10 minutes. And then the cells were lysed with the addition of 10 μM digitonin for another 10 minutes. Cell lysates were incubated with each substrate at 37°C for 30 minutes in 50 mM phosphate buffer (pH 6.0) containing 200 mM NaCl, 2.5 mM dithiothreitol, and 5 mM EDTA for cathepsin B, and in the same buffer except pH 5.5 and a further addition of 4 M urea for cathepsin L. Fluorescence was measured with a Millipore Cytofluor 2300 (excitation wavelength, 380 nm; emission wavelength, 460 nm). One unit of the enzyme activity was defined as the cleavage of 1 pmol of each substrate per one minute.

Chemicals

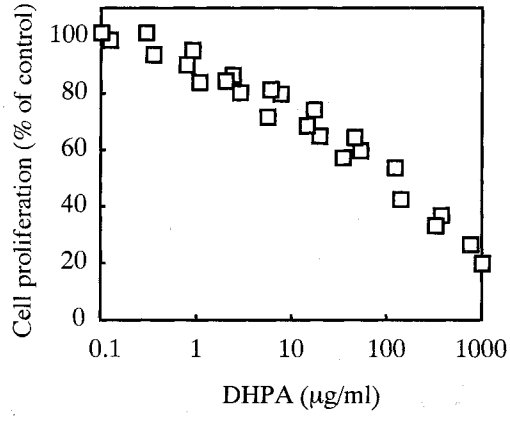
A cathepsin L inhibitor, Z-FY(*t*-Bu)-dmk, was obtained from Calbiochem (San Diego, U.S.A.). Other protease inhibitors and substrates were products of Peptide Institute (Osaka, Japan). Alamar Blue was purchased from Wako (Osaka, Japan). All other chemicals including amino acids and inhibitors of protein synthesis were obtained from Sigma-Aldrich (St. Louis, U.S.A.).

Results and Discussion

Growth Inhibitory Effect of DHPA on HL-60 Cells

Growth inhibitory effect of DHPA on HL-60 cells was evaluated using Alamar Blue assay (Fig. 1.). Alamar Blue is a non-cytotoxic reagent, which yields a fluorescent product after reduction. This chemical is an indicator of redox state in cells. Therefore, this is also used to assess cell proliferation[19]. DHPA showed a growth inhibitory effect on HL-60 cells dose-dependently as shown in Fig. 1. DHPA at 1000 μg/ml inhibited cell proliferation to 20% after 24-hour incubation. DHPA affects activity of phenylalanine hydroxylase[5]. The enzyme catalyzes hydroxylation of phenylalanine to tyrosine and is indispensable for tyrosine biosynthesis in mammalian cells. The lack of this enzyme causes accumulation of phenylalanine and insufficiency of tyrsoine, resulting in mental retardation and other abnormalities[23,24]. Because of this, either phenylalanine-free diet or supplement of tyrosine is needed for the therapy[23,24]. In HL-60 cells, phenylalanine and tyrosine showed no growth inhibitory effects at 1000 μg/ml. In addition, the growth inhibitory effect of DHPA was not cancelled by the supplement of 1000 μg/ml of tyrosine (date not shown). However, the growth inhibition was significantly decreased by the addition of 200 μg/ml of phenylalanine (date not shown). Therefore, these data indicated that the growth inhibition by DHPA did not depend on the metabolic block in tyrosine biosynthesis, but might depend on some antagonistic activity against phenylalanine.

Fig. 1. Growth inhibitory effect of DHPA on HL-60 cells.

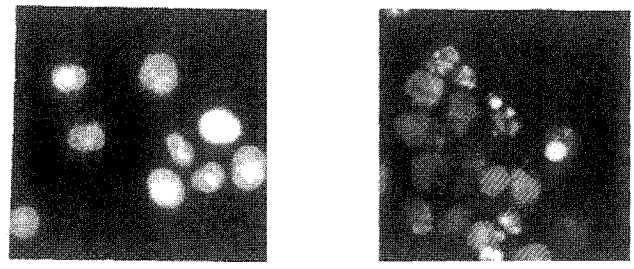


HL-60 cells were cultured at 10^6 cells/ml (□) in RPMI-1640 medium containing 10% FBS with various concentrations of DHPA under humidified air containing 5% CO_2 at 37°C for 1 day. The cell proliferation was estimated by Alamar Blue assay. Values are means of independent 3 observations.

Induction of Apoptosis of HL-60 Cells by DHPA

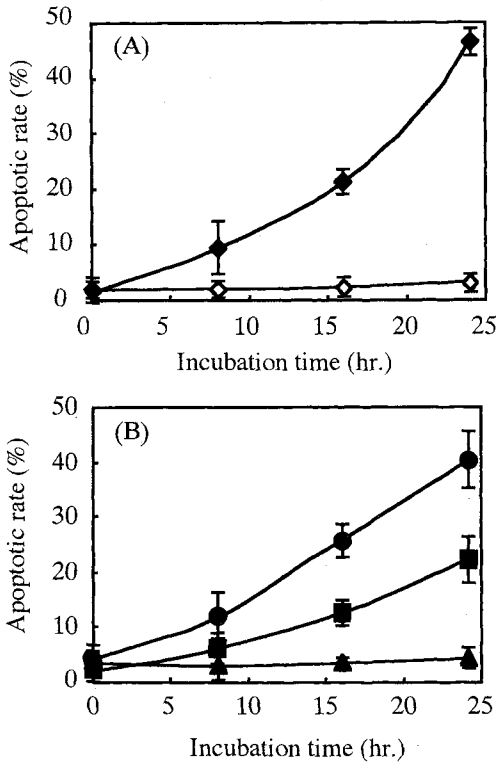
Fluorescent staining of nuclei in HL-60 cells treated with or without DHPA are shown in Fig. 2. In treated cells, the fragmentation of nuclei and chromatin condensation were observed, which were two pieces of evidence of typical apoptotic cells. Ratio of apoptotic cells was estimated by counting cells having apoptotic nuclei stained with Hoechst 33258 dye (Fig. 3. A). Caspase 3 is a cysteine protease, which is activated in caspase cascade in apoptotic process. The activity of caspase 3 was maintained at 80 unit/mg protein in DHPA-untreated cells throughout incubation. On the other hand, the activity increased up to 1771 unit/mg protein in cells treated with DHPA at 1000 μg/ml after 24-hour incubation. Oligonucleosomal DNA fragments were detected in the DNA fraction extracted from the treated cells (Fig. 4.). These data supported that DHPA was an inducer of apoptosis in HL-60 cells. Phenylalanine and tyrosine did not induce apoptosis even at the same

Fig. 2. Fluorescent micrographs of HL-60 cells treated with DHPA.



HL-60 cells were incubated without (Control) or with (Treated cells) 1000 μg/ml of DHPA for 12 hours. The cells were fixed and then stained with Hoechst 33258 dye.

Fig. 3. Induction of apoptosis by DHPA in HL-60 cells (A) and effects of protease inhibitors on the apoptotic rate (B).

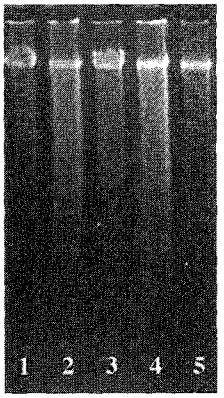


(A), HL-60 cells were incubated without (◇) or with (◆) 1000 μg/ml of DHPA.

(B), HL-60 cells were preincubated with 20 μM protease inhibitors; pan-caspase inhibitor, Z-VAD-fmk (▲), calpain inhibitor, Z-LL-H (●), cysteine protease inhibitor, E-64d, (■). After preincubation, HL-60 cells were incubated with 1000 μg/ml of DHPA. Apoptotic rate was quantified by counting cells having apoptotic nuclei stained with Hoechst 33258 dye.

Values are means±S.D. of independent 3 observations.

Fig. 4. Oligonucleosomal DNA fragmentation caused by DHPA and effects of various protease inhibitors on the fragmentation.



HL-60 cells were preincubated without (lane 1 and 2) or with 20 μM protease inhibitors; pan-caspase inhibitor, Z-VAD-fmk (lane 3), calpain inhibitor, Z-LL-H (lane 4), cysteine protease inhibitor, E-64d (lane 5). After preincubation, HL-60 cells were incubated without (lane 1) or with (lane 2~5) 1000 μg/ml of DHPA for 24 hours. DNA was extracted and then electrophoresed on agarose gel.

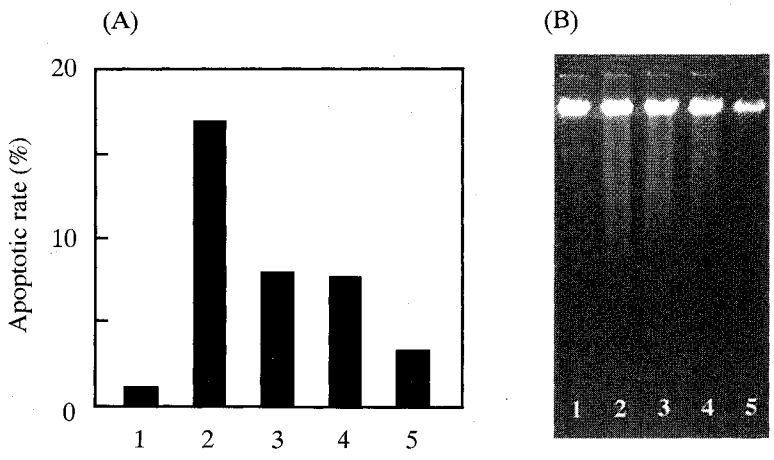
concentration. The DHPA-induced apoptosis was abolished by addition of 500 μg/ml of phenylalanine. This abolition might be due to the same reason as that for the growth inhibition induced by DHPA.

After 24-hour incubation, apoptotic cell population increased up to 48% in cells treated with DHPA at 1000 μg/ml, whereas in untreated cells the population remained less than 1%. Relatively high concentrations of DHPA are required for the induction of a significant apoptosis. It is thought to simply depend on the culture medium, which contains phenylalanine and other aromatic amino acids enough to antagonize apoptotic effects of DHPA at low concentrations. Cycloheximide, anisomycin, puromycin and ochratoxin A, which are inhibitors of protein synthesis[25~28], induced apoptosis completely within 24 hours in HL-60 cells (data not shown). Thus DHPA-induced apoptosis proceeded more slowly than that induced by the above apoptosis inducers initiated by the inhibition of protein synthesis. Slow proceeding apoptosis has been reported in apoptosis induced by radiation, divalent cation, retinoid, DNA-damaging agent, cell cycle inhibitor and topoisomerase inhibitor[29~34]. And this apoptosis accompanies with kinetically slow process such as moderate activation of caspases and chromatin condensation[29]. These slow apoptotic pathway is initiated by damage of nuclear DNA and related to protein synthesis[29]. Cell cycle arrest is also necessary for the slow apoptosis[32,33]. Studies on the initiation mechanism of DHPA-induced apoptosis are now in progress.

Prevention of DHPA-induced Apoptosis by Caspase Inhibitor and Cysteine Protease Inhibitor

Effects of protease inhibitors on DHPA-induced apoptosis are shown in Fig.3.B. Several protease inhibitors tested were pretreated for 10~60 minutes prior to addition

Fig. 5. Effects of cathepsin inhibitors on the apoptotic nuclei (A) and the oligonucleosomal DNA fragmentation (B) in DHPA-induced apoptosis.



HL-60 cells were preincubated without (1 and 2) or with 20 μM cathepsin inhibitors; cathepsin B inhibitor, CA074-Me (3), cathepsin L inhibitor, Z-FY(*t*-Bu)-dmk (4), nonspecific cathepsin inhibitor, E-64d (5). After preincubation, HL-60 cells were incubated without (1) or with (2~5) 1000 μg/ml of DHPA for 12 hours.

(A), Apoptotic rate was quantified by counting cells having apoptotic nuclei stained with Hoechst 33258 dye.

(B), DNA was extracted and then electrophoresed on agarose gel.

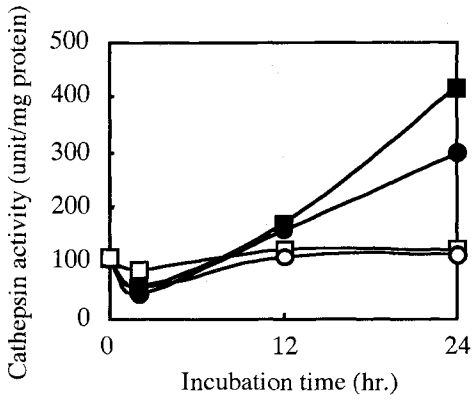
of DHPA. Apoptotic cells treated with each protease inhibitor were quantified as described above. All protease inhibitors themselves tested in Fig. 3. B did not affect cell growth and not induce apoptosis in HL-60 cells (data not shown). A pan-caspase inhibitor, Z-VAD-fmk, prevented the DHPA-induced apoptosis completely. Z-VAD-fmk inactivates irreversibly various caspases, such as caspase 1, 3, 4 and 7[35]. And a cysteine protease inhibitor, E-64d, which inhibits calpains and cathepsin B and L[36], reduced the apoptotic rate to 48% of that induced by DHPA. However, a calpain inhibitor, Z-LL-H[37], did not affect the DHPA-induced apoptosis. DNA fragmentation was analyzed by agarose gel electrophoresis (Fig. 4.). Z-VAD-fmk and E-64d prevented the DNA fragmentation induced by DHPA. On the other hand, Z-LL-H did not restrict the apoptotic DNA fragmentation. These results indicated that DHPA induced apoptosis *via* activation of caspases and cysteine proteases like cathepsins except calpains.

Prevention of DHPA-induced Apoptosis by Cathepsin Inhibitors

The quantification of apoptotic cells (Fig. 5. A) and analysis of oligonucleosomal DNA fragmentation (Fig. 5. B) were examined in the cells treated with cathepsin inhibitors in DHPA-induced apoptosis. The exposure of cathepsin inhibitors to the cells was performed for 1 hour prior to addition of DHPA. Three cathepsin inhibitors themselves did not affect cell proliferation and not induce apoptosis against HL-60 cells. A cathepsin B specific inhibitor, CA074-Me[38], reduced only chromatin condensation, which is one morphological aspect of apoptosis, induced by DHPA. A cathepsin L specific inhibitor, Z-FY(*t*-Bu)-dmk[39], and the non-specific cathepsin inhibitor, E-64d, decreased both chromatin condensation and DNA fragmentation induced by DHPA. These results suggested that cathepsin B and L were involved in the DHPA-induced apoptosis and at least activation of cathepsin B was needed for the exercitation of chromatin condensation in HL-60 cells.

Cathepsin B accelerates the release of cytochrome *c* from mitochondria during TNF-α induced apoptosis[13]. This release causes the activation of caspase 9[40]. On the other hand, cathepsin L induces apoptosis independently on the release of cytochrome *c*[16]. Cathepsin L has been reported to process procaspases directly to release activated forms[17]. In fact, addition of Z-FY(*t*-Bu)-dmk reduced the activity of caspase 3 to the control level, at which observed in DHPA-untreated cells (data not shown). This reduction indicated that the activation of cathepsin L was associated with that

Fig. 6. Elevation of cathepsin B and L activities in DHPA-induced apoptosis.



HL-60 cells were incubated with (closed) or without (open) 1000 μg/ml of DHPA for 24 hours. Cathepsin B (round) and L (square) activities were measured as described in materials and methods.

of caspase 3 in DHPA-induced apoptotic cascade.

Elevation of Cathepsin Activity in DHPA-induced Apoptosis

Cathepsins was assayed in the cells treated with or without DHPA (Fig. 6.). The activities of cathepsin B and L after 2-hour incubation slightly fell down. These responses are thought to be due to extracellular environmental changes, such as refreshment of medium. In DHPA-untreated cells, the activities of both cathepsin B and L were kept at low level throughout incubation. On the other hand, in DHPA-treated cells, activation of both cathepsin B and L were started after 2-hour incubation and the activities were gradually increasing up within 24-hour incubation. After 24-hour incubation, the activities of cathepsin B and L increased by 3 times and 4 times, respectively, being compared with untreated control cells. This activation was coordinated with the increase of apoptotic rate as shown in Fig.3.A. In HL-60 cells, cathepsin B expression is regulated by the differentiating agents, phorbol 12-myristate-13-acetate, calcitriol and retinoids[41]. Especially retinoids confer slow apoptosis[31]. DHPA-induced apoptosis seemed to be similar to retinoids-induced one. Although retinoids differentiates promyelocytes into granulocytes in HL-60[42], DHPA did not (data not shown).

During TNF-α-mediated hepatocyte apoptosis, caspase 8 stimulates the release of cathepsin B from lysosomes, resulting in the release of cytochrome *c* from mitochondria[13]. We could not identify factors or receptors associated with the activation of cathepsin B and L in DHPA-induced apoptosis.

To summarize these results and discussion, DHPA induced slow apoptosis in HL-60 cells. And the apoptosis was dependent on the activation of lysosomal protease cacthepsin B and L. Although the mechanism of cathepsin activation has been poorly understood, the restriction of phenylalanine by DHPA may be involved in the pathway. Therefore, DHPA is a novel type of apoptosis inducer accompanying with the activation of cathepsin B and L, and will be a useful tool for the investigation concerning activation mechanism of cathepsins.

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