Proteolytic cleavage of HsRad51 during apoptosis

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Received 12 February 1998; revised version received 7 April 1998

Abstract The Rad51 gene of *Saccharomyces cerevisiae* is required for genetic recombination and recombinational repair of DNA strand breaks. In higher eukaryotes Rad51 is essential for embryonic development, and is involved in cell proliferation and DNA repair. Here we show that human Rad51 (HsRad51) is proteolytically cleaved during apoptosis in two T-lymphocyte cell lines, Jurkat and PFI-285. Apoptosis was induced by camptothecin or anti-Fas monoclonal antibody (anti-Fas mAb). HsRad51 was cleaved with similar kinetics as human poly(ADP-ribose) polymerase (HsPARP) after treatment with either agent. The time course of cleavage coincided with internucleosomal DNA fragmentation. The HsRad51 fragments observed in apoptotic cells were identical to those generated from in vitro translated (IVT) HsRad51 exposed to activated Jurkat S-100 extract in a cell-free system. In each case, cleavage of HsRad51 was abolished by acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO). However, cleavage of IVT HsRad51 could not be demonstrated using purified caspase-2, -3 or -6 to -10, and the identity of the responsible protease thus remains to be determined. In summary, we have shown that HsRad51 belongs to a group of repair proteins, including PARP and DNA-dependent protein kinase, which are specifically cleaved during the execution phase of apoptosis.

Key words: Human Rad51; Apoptosis; Caspase; Acetyl-Asp-Glu-Val-Asp-aldehyde; DNA repair; T-cell

1. Introduction

Apoptosis is a distinct form of programmed cell death mediated by different stimuli, including DNA damaging agents and Fas/Fas ligand interaction [1]. It is characterized by several features, two of these being plasma membrane blebbing and internucleosomal degradation of chromosomal DNA. Specific cysteine proteases of the interleukin-1β-converting enzyme (ICE) family initiate the execution stage of apoptosis [2]. These proteases, commonly called caspases [3], recognize a sequence of five amino acids: P_4–P_1 followed by P′, and specifically cleave substrates C-terminal to an aspartic acid residue at the P_1 site [4]. The cleavage can lead to activation or inactivation of substrates, which include cytoskeletal components and proteins involved in signal transduction, RNA processing and DNA repair [5,6]. Many of these proteins are likely to be involved in the morphological and biochemical changes that accompany apoptosis, although the exact functional roles of the cleavages are not clear. Recently the involvement of a caspase in the activation of the DNase activity causing DNA fragmentation in nuclei has been observed [7,8]. Poly(ADP-ribose) polymerase (PARP) and the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{cs}), both involved in DNA damage sensing and repair [9,10], are inactivated by caspase cleavage [11–16]. The significance of PARP cleavage is indicated by a recent study showing that PARP knock-out mice display enhanced susceptibility to DNA fragmentation [17]. Further identification of the targets of the caspases is important for the understanding of the apoptotic process.

Like PARP and DNA-dependent protein kinase (DNA-PK), the Rad51 protein binds to DNA and plays a role in repair. *Saccharomyces cerevisiae* Rad51 is involved in recombination and repair of DNA strand breaks [18,19]. Mutations in the gene sensitize yeast cells to ionizing radiation and abolish recombination [18,19]. Overall homology between the human Rad51 (HsRad51) and *S. cerevisiae* Rad51 proteins is 68% [20,21], and both proteins catalyze ATP- dependent DNA strand exchange reactions in vitro [22–25]. Recently a direct role for HsRad51 in recombination in human immortalized cells was suggested [26]. Several reports have indicated an involvement of mammalian Rad51 in repair; nuclear foci of Rad51 protein are formed in human fibroblasts after DNA damage [27], antisense inhibition of mouse Rad51 enhances radiosensitivity [28] and Rad51 homozygous mutant mouse embryos are hypersensitive to ionizing radiation [29]. Mammalian Rad51 may also have an essential role in replication, since targeted disruption of the murine Rad51 gene leads to embryonic lethality [30], and depletion of Rad51 in chicken B lymphocytes is accompanied by chromosome aberrations and cell death [31].

Here we demonstrate that HsRad51, like PARP and DNA-PK_{cs}, is proteolytically cleaved during apoptosis in two T-cell lines and in vitro. The cleavage is Ac-DEVD-CHO inhibitable, but the caspase/protease directly responsible for the cleavage remains to be determined.

2. Materials and methods

2.1. Cells and culture conditions

PFI-285, a transformed T-lymphocyte cell line with a nearly normal

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Abbreviations: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; Ac-YVAD-CHO, acetyl-Tyr-Val-Ala-Asp-aldehyde; DNA-PK, DNA-dependent protein kinase; DNA-PK_{cs}, catalytic subunit of the DNA-dependent protein kinase; DTT, dithiothreitol; HsPARP, human PARP; HsRad51, human Rad51; ICE, interleukin-1β-converting enzyme; IVT, in vitro translated; mAb, monoclonal antibody; NM, nutrient medium; PARP, poly(ADP-ribose) polymerase; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPCK, tosyl-L-phenylalanine chloromethyl ketone

PII S0014-5793(98)00433-5

karyotype [32], kindly provided by Dr. Anne-Lise Børresen, Norsk Radium Hospital, Oslo, was cultured as previously described [32]. Jurkat, a T-lymphocyte cell line, was cultured in nutrient medium (NM), which is RPMI 1640 purchased from Gibco (USA) containing 10% newborn calf serum, 10 μg/ml streptomycin, 150 IU/ml benzylpenicillin and 20 mM L-glutamine.

2.2. Reagents

Tosyl-L-phenylalanine chloromethyl ketone (TPCK) was purchased from Calbiochem (USA), acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) and acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO) from Neosystem Laboratoire (France). Camptothecin, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, pepstatin and dithiothreitol (DTT) were obtained from Sigma (USA) and anti-human Fas monoclonal antibody (IgM CH-11 clone) from Medical and Biological Laboratories, Nagoya (Japan). Cytochrome *c* was obtained from Calbiochem, and dATP from Pharmacia (Sweden).

2.3. Western blotting

Western blotting was performed as previously described [33]. 50 μg of protein per lane was resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Primary antibodies

HsRad51 polyclonal antibody was prepared as described previously [33]. PARP monoclonal antibody C-2-10 was generously provided by Dr. Guy Poirier, Centre Hospitalier de L'Université Laval, Canada.

2.5. Internucleosomal DNA fragmentation

Cells (1×10^6) were collected by centrifugation at $1000\times g$ for 5 min. Pellets were resuspended in 300 μl cold lysis buffer (5 mM Tris, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) and 300 μl polyethylene glycol (PEG) solution (5% PEG, 2 M NaCl). Samples were left on ice for 10 min, and supernatants were cleared by centrifugation at $12\,000\times g$ for 10 min. 1 ml ice-cold 99% ethanol and 30 μl 5 M NaCl was added to the supernatants. Samples were stored at −20°C overnight, and centrifuged at $12\,000\times g$ for 15 min the following day. Pellets were lyophilized and dissolved in 10 μl 1×TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). 10 μl RNase stock (500 U/ml RNase T1, 5 mg/ml RNase A) was added, and samples were incubated for 1 h at 37°C. 2 μl proteinase K (25 mg/ml) was added, followed by incubation for 1 h at 37°C. 8 μl loading buffer (356 mM Tris, 356 mM boric acid, 0.8 mM EDTA, 40% sucrose, 0.05% bromophenol blue, 0.5% SDS) was added, and DNA fragments were separated on an 1.8% agarose gel in 0.5×TBE buffer (44.5 mM Tris, 44.5 mM boric acid, 0.1 mM EDTA). The gel was stained for 30 min in 0.5 μg/μl ethidium bromide. DNA was visualized with UV (305 nm) light.

2.6. Generation and activation of Jurkat S-100 extracts

Human Jurkat cells were grown as described [34]. The cells (2 liters at 0.8×10^6/ml) were harvested by centrifugation. After one wash in ice-cold PBS the cells were resuspended in ice-cold buffer A (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). After incubation on ice for 15 min, the cells were lysed by douncing 20 times in a 7 ml Kontes douncer with the B pestle. Nuclei were centrifuged at $1000\times g$ for 10 min at 4°C. The supernatant was centrifuged further at $100\,000\times g$ yielding Jurkat S-100. Activation was achieved by incubating 100 μM dATP and 0.2 μg cytochrome *c* for every 25 μg S-100 in 20 μl at 30°C for 1 h. The resulting activated S-100 was tested for acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin (Ac-DEVD-AMC) cleaving activity prior to use with in vitro translated substrates. Ac-DEVD-AMC was synthesized as described [34].

2.7. Cleavage of in vitro translated HsRad51 and HsPARP

^{35}S-radiolabelled, in vitro translated (IVT) HsRad51 or HsPARP was generated using a TNT-linked transcription/translation kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, human Rad51 cDNA in pGEM5Z from Promega (USA) or human PARP cDNA in pBluescript from Stratagene (USA) were incubated with T7 polymerase, 40 μCi of [^{35}S]methionine, and TNT kit components. IVT HsRad51 or IVT HsPARP (2% of total TNT reaction) were incubated with activated Jurkat S-100 extract for 5 h at 30°C. Reactions were boiled in Laemmli buffer, resolved by either 14% or 10% SDS-PAGE, and the protein bands were visualized with a phosphoimager.

3. Results

In contrast to the induction of Rad51 expression by DNA damaging agents in yeast [35–37], no increase in HsRad51 protein levels could be detected in human peripheral blood lymphocytes after treatment with DNA damaging agents. Instead, a decrease in the HsRad51 protein level was observed after treatment with high doses of ionizing irradiation, methyl methanesulfonate or nitrogen mustard (data not shown). To examine a possible connection between the decrease of HsRad51 expression and apoptosis, further studies were carried out in Jurkat cells. Apoptosis was induced by treatment with the topoisomerase I inhibitor camptothecin or anti-Fas mAb. Cell extracts were prepared at indicated time points, and analyzed by Western blotting (Fig. 1A). The full-length HsRad51 has a predicted molecular weight of 37 kDa, but migrates at 41 kDa. In Jurkat cells treated with 13 μM camptothecin, a 33 kDa band appeared 3 h after treatment. Similar kinetics were observed for cleavage of the 116 kDa HsPARP protein into a 89 kDa product (Fig. 1A). The same pattern was seen in Jurkat cells treated with 37 ng/ml anti-Fas mAb (Fig. 1B). The time course of cleavage coincided with internucleosomal DNA fragmentation (Fig. 1C,D). This suggests that the 33 kDa fragment is an apoptosis-related cleavage product of HsRad51.

To confirm this conclusion, a cell-free system was used. IVT HsRad51 or HsPARP were added to activated Jurkat S-100 extract. The samples were incubated at 30°C for 5 h and resolved by 14% SDS-PAGE. A HsRad51 cleavage product of 33 kDa, similar in size to the 33 kDa fragment in Jurkat

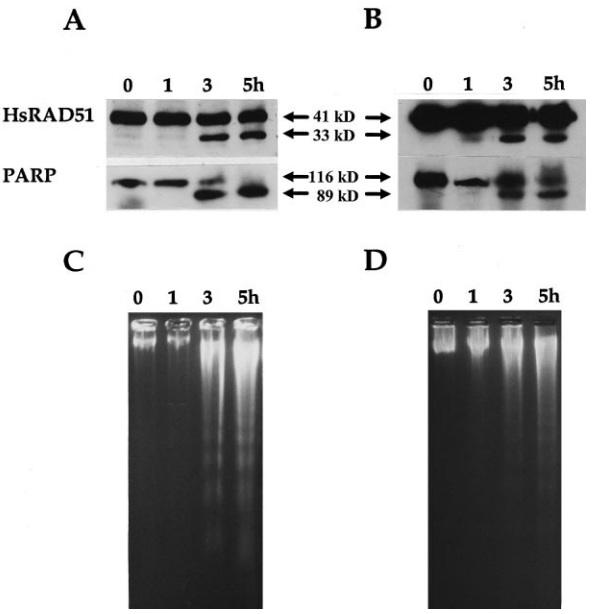

Fig. 1. Cleavage of HsRad51 and HsPARP in relation to DNA fragmentation in Jurkat cells. Western blot analysis of HsRad51 and HsPARP expression up to 5 h after treatment with (A) 13 μM camptothecin or (B) 37 ng/ml anti-Fas mAb. The membrane previously probed with HsRad51 antibody was stripped and re-probed with HsPARP antibody. Internucleosomal DNA fragmentation in Jurkat cells up to 5 h after treatment with (C) 13 μM camptothecin or (D) 37 ng/ml anti-Fas mAb.

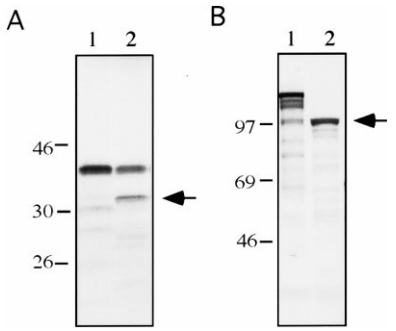

Fig. 2. Cleavage of in vitro translated ^{35}S-radiolabelled HsRad51 and HsPARP by apoptotic Jurkat S-100 extract. IVT HsRad51 (A) or IVT HsPARP (B) was incubated with: lane 1, control S-100 extract; lane 2, S-100 extract which had been activated by cytochrome *c* and dATP. Following incubation for 5 h, samples were resolved by (A) 14% or (B) 10% SDS-PAGE. Arrows indicate cleavage products in lanes A2 and B2.

cells, was observed (Fig. 2A). Cleavage of IVT HsPARP to generate an 89 kDa fragment was also observed under the same conditions (Fig. 2B). These results confirm that the 33 kDa band previously observed by immunoblotting is indeed derived from the HsRad51 protein, and not caused by cross-reaction of the antibody with another protein.

Studies with inhibitors were performed in two T-cell lines to assess which group of proteases was responsible for the observed HsRad51 cleavage. PFI-285 cells were pre-treated with the inhibitors for 15 min followed by exposure to 13 μM camptothecin. Cells were harvested 5 h after treatment, and proteins were analyzed by Western blotting (Fig. 3A). 200 μM of TPCK, an inhibitor of serine proteases [38], completely abrogated the cleavage (lane 3) observed in the sample treated with camptothecin without inhibitor (lane 2). 100 μM of the caspase-1-like protease inhibitor Ac-YVAD-CHO [11] did not significantly inhibit the cleavage (lane 4), while an equimolar concentration of the broader caspase-3-like inhibitor Ac-DEVD-CHO [11] completely abolished the cleavage (lane 5). The inhibition study was repeated in Jurkat cells using the same concentration of TPCK, but five times lower concentrations of the caspase inhibitors. Pre-treatment with 200 μM

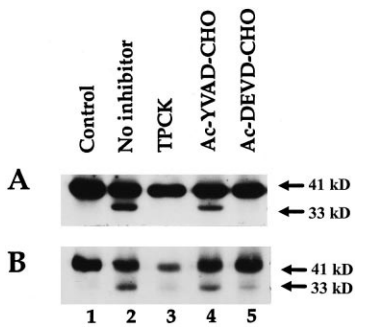

Fig. 3. Western blot analysis of HsRad51 in Jurkat and PFI-285 cells treated with camptothecin and protease inhibitors. A: PFI-285 cells treated with 1.0 μM camptothecin for 5 h. Lane 1, untreated control; lanes 2–5, camptothecin-treated cells preincubated for 15 min with: lane 2, no inhibitor; lane 3, 200 μM TPCK; lane 4, 100 μM Ac-YVAD-CHO; lane 5, 100 μM Ac-DEVD-CHO. B: Jurkat cells treated with 13 μM camptothecin for 5 h. Lane 1, untreated control; lanes 2–5, camptothecin-treated cells preincubated for 15 min with: lane 2, no inhibitor; lane 3, 200 μM TPCK; lane 4, 20 μM Ac-YVAD-CHO; lane 5, 20 μM Ac-DEVD-CHO.

TPCK or 20 μM Ac-YVAD-CHO did not have any effect, while 20 μM Ac-DEVD-CHO significantly inhibited the cleavage (Fig. 3B). Thus, the inhibition of HsRad51 cleavage by TPCK or Ac-DEVD-CHO was reproducible in two different T-cell lines.

The inhibition of HsRad51 cleavage by Ac-DEVD-CHO was confirmed in the cell-free assay described above. Ac-DEVD-CHO (200 nM) was added to the activated S-100 cell extract prior to the addition of IVT HsRad51. Cleavage of IVT HsRad51 was completely inhibited by Ac-DEVD-CHO (Fig. 4A). Inhibition of cleavage of HsPARP under the same conditions was also observed (Fig. 4B). Sensitivity to this concentration of the inhibitor suggests the involvement of a caspase-3- rather than caspase-1-like protease in HsRad51 cleavage. This finding is consistent with the above in vivo data.

An attempt was made to determine which caspase was responsible for the cleavage. IVT HsRad51 was exposed to recombinant, purified caspase-2, -3, -6, -7, -8, -9 or -10 for 1 h at 30°C. While HsPARP, which was used as a control, was cleaved by the caspases, no cleavage of HsRad51 was observed (data not shown).

4. Discussion

We have observed proteolytic cleavage of HsRad51 protein in Jurkat and PFI-285 cells treated with camptothecin or anti-Fas mAb. The cleavage coincided with internucleosomal DNA fragmentation, which indicates an association with apoptosis. HsRad51 was cleaved with similar kinetics to PARP, a protein implicated in the detection of DNA strand breaks [10]. Part of the HsRad51 protein in the cells and in the in vitro system remained uncleaved. This might be explained by the spatial distribution of the protein [27,39], or by interactions between HsRad51 and other proteins [40–47], which may protect the cleavage site.

Pre-incubation with the serine protease inhibitor TPCK abrogated the cleavage in cells treated with 13 μM camptothecin. This indicates that serine proteases may be involved in the apoptotic pathway leading to HsRad51 cleavage, an interpretation supported by a recent demonstration of a TPCK-sensitive step in the activation of caspase-3 [48]. On the other

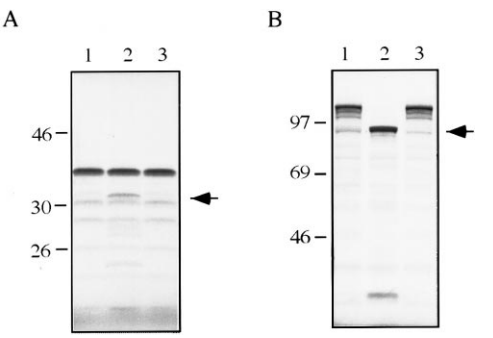

Fig. 4. Inhibition of cleavage of in vitro translated ^{35}S-radiolabelled HsRad51 and HsPARP. IVT HsRad51 (A) or IVT HsPARP (B) was incubated with: lane 1, control S-100 extract; lane 2, S-100 extract which had been activated by cytochrome *c* and dATP; lane 3, activated S-100 to which 200 nM Ac-DEVD-CHO was added just prior to incubation with IVT products. Following incubation for 5 h, samples were resolved by (A) 14% or (B) 10% SDS-PAGE. Arrows indicate cleavage products in lanes A2 and B2.

hand, 200 μM TPCK has been shown to induce necrosis in Jurkat cells and abolish the features of apoptosis [49]. Therefore, we cannot rule out the possibility that the inhibitory effect of TPCK on HsRad51 cleavage may be due to induction of necrosis instead of apoptosis.

Ac-YVAD-CHO, an effective inhibitor of proteases with the highest homology to caspase-1 [50], did not significantly inhibit the cleavage of HsRad51 in cells. Ac-DEVD-CHO, originally designed as an inhibitor of caspase-3 [11], abolished the cleavage of HsRad51 in cells and in vitro. In an earlier study 50 μM Ac-DEVD-CHO did not substantially prevent anti-Fas mAb induced cell death and DNA laddering in Jurkat cells [49]. Thus, the observed inhibitory effect of 20 μM Ac-DEVD-CHO on HsRad51 cleavage in Jurkat cells is not likely to be caused by an abrogation of apoptosis. However, neither purified caspase-3 nor caspase-2, -6, -7, -8, -9 or -10 was able to cleave IVT HsRad51. Thus HsRad51 cleavage is sensitive to Ac-DEVD-CHO inhibition, but not executed directly by a known Ac-DEVD-CHO-inhibitable caspase such as caspase-7 or -3. One explanation for this observation could be that an Ac-DEVD-CHO inhibitable caspase activates a downstream protease for HsRad51 cleavage. Alternatively an Ac-DEVD-CHO inhibitable caspase other than the ones tested could be directly responsible for HsRad51 cleavage. Multiple active forms of unidentified caspases have been discovered in HL-60 cells following induction of apoptosis with etoposide [51]. Thus it is possible that HsRad51 is cleaved by either a caspase which is unknown to date or by a non-caspase protease.

There is no DEVD site in the HsRad51 amino acid sequence that would generate a 33 kDa fragment following cleavage. However, DEVD-based compounds are broad inhibitors which cannot be assumed to selectively inhibit caspase-3 [6], especially when used at high concentrations [50]. Thus an Ac-DEVD-CHO inhibitable caspase could act directly on one of a few putative HsRad51 cleavage sites containing a P_1 Asp and an amino acid other than Asp in the P_4 position, e.g. AQVD-G, amino acids 271–275. Cleavage here would give a product of approximately 30 kDa. In vitro mutagenesis of the putative cleavage sites in the HsRad51 protein may help to elucidate the involvement of specific caspases.

What is the functional importance of the HsRad51 cleavage? Rad51 is involved in recombinational repair of DNA strand breaks in yeast [18,19], and several recent reports indicate that the Rad51 homologues in higher eukaryotes may have a similar role [26–28,52,53]. An association with DNA replication is suggested by increased Rad51 expression during S/G_2 phase [33], and formation of Rad51 positive nuclear foci during the S phase in mammalian cells [39]. In *S. cerevisiae*, repair using intrachromosomal recombination between sister chromatids in G_2 is preferred to interchromosomal recombination between homologous chromatids [54]. Similarly, interchromosomal recombination is suppressed in human cells [55]. It is tempting to speculate that HsRad51 might exert functions in the repair of DNA strand breaks by catalyzing intrachromosomal recombination between sister chromatids during the S/G_2 phase. Such an activity might obstruct the apoptotic process, and this could be prevented by inactivating HsRad51. Since HsRad51 is essential for cell survival, cleavage of the protein may also be of importance for the irreversibility of apoptosis [29,30].

In conclusion, we have shown that HsRad51, like PARP and DNA-PK_{cs}, is cleaved during the execution phase of apoptosis. The cleavage is Ac-DEVD-CHO inhibitable, but the protease responsible for the cleavage remains to be determined.

Acknowledgements: We thank Dr. Guy Poirier for providing the PARP monoclonal antibody; Fiona Benson for the gift of HsRad51 polyclonal antibody and purified HsRad51; Dr. Anne-Lise Børresen for providing the PFI-285 cell line; Dr. Andrew Slater and Professor Bo Lambert for valuable discussions. This work was supported by Karolinska Institutet, King Gustaf V:s Jubilee Fund, Swedish Radiation Protection Institute, Swedish Cancer Society, Alex and Eva Wallström foundation, Foundation Lars Hiertas Minne and Swedish Board for Animal Protection.

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