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Induction and intracellular localization of a 72-kDa heat shock protein in rat gastric mucosa after water-immersion stress

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Abstract: We investigated the expression and changes in the intracellular localization of a 72-kDa heat shock protein (HSP72) in rat gastric pyloric and fundic mucosa before and after water-immersion stress. Severe mucosal damage was found in the fundic mucosal area of the stomach after this stress. However, no mucosal lesion developed in the pyloric mucosal area. HSP72 in both the soluble and insoluble fractions of the pyloric and the fundic mucosal areas was significantly increased after water-immersion stress, peaking 6 h after the initiation of the stress. The increase in HSP72 was more significant in the pyloric mucosal area than in the fundic mucosal area under both normal and stress conditions. The increase of HSP72 in the pyloric mucosal cells occurred prior to the formation of the mucosal lesions, whereas the increase of HSP72 in the fundic mucosal cells was observed after ulcer formation. An immunohistochemical study showed that HSP72 was constitutively expressed in the cytoplasm of the gastric mucosal cells, and that the intranuclear induction of HSP72 was remarkably intense in the pyloric mucosal cells, especially in the proliferative zone, compared with the fundic mucosal cells. Our results may suggest that HSP72 has an important cytoprotective function in gastric mucosal cells and that there is a "biophysical" difference between pyloric and the fundic mucosal cells.

Key words: 72-kDa heat shock protein (HSP72), rat, gastric mucosa, gastric ulcer, cytoprotection

Introduction

Many stresses, including elevated temperature or exposure to toxins or heavy metals, activate the stereotyped response of heat shock genes, the heat shock response, in cultured cells.[1–3] The products of several highly conserved heat shock genes (heat shock proteins) protect the cells against subsequent stresses in vitro.[4] Heat shock proteins (HSPs) are classified into four families, HSP90, HSP70, HSP60, and the low molecular weight HSP family, based on their subunit molecular masses and structural homology.[5] The major heat shock protein, HSP70, has about 50% sequence homology in *Escherichia coli* and humans, and some domains show 96% homology.[6] Several of the major heat shock proteins are members of gene (protein) families that include proteins normally present and, in most cases, essential, for cell functions.[5,6]

Recently, it has been demonstrated that the synthesis of HSP70 is induced in cultured gastric mucosal cells by heat stress, and this protein has a cytoprotective function in vitro.[7] On the other hand, little is known about the expression of a 72-kDa heat shock protein (HSP72), a member of the HSP70 family, in the gastric mucosa under stress conditions, especially in vivo.[8] It has been shown that water-immersion stress produced several linear and dotted erosions in the fundic mucosal area, but not in the pyloric mucosal area.[9,10] In this study, we examined the differences in the expression levels and intracellular localization of HSP72, which molecule is known to contribute to cytoprotective functions, in the pyloric and the fundic mucosal area before and after water-immersion stress in vivo. We discuss the possible contribution of this protein to the difference in ulcer formation between the pyloric and fundic areas.

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Materials and methods

Animals

Male Sprague-Dawley rats (weighing 250–300 g) were kept in cages in a temperature-(23 ± 2°C) and humidity-(55 ± 5%) controlled room with a 12-h dark-light cycle before and during the experiment. The animals received a standard laboratory diet and water, provided ad libitum.

This experiment was approved by the Akita University Animal Care Committee.

Water-Immersion stress

The animals were placed in a restraint cage and immersed vertically, to the level of the xyphoid process, in a water bath (23°C).[11] They were sacrificed before and 0.5, 1, 6, 12, and 24 h (n = 6 at each time) after the initiation of the water-immersion stress; the stomach was then removed and fixed with 10 ml of 10% formalin. After 30-min fixation, the stomach was incised along the greater curvature. An ulcer index was determined, based on the total length of all mucosal lesions.[12,13] The ulcer index was evaluated separately in the pyloric and the fundic mucosal areas in each rat.

Sodium dodecyl sulfate (SDS)-Polyacrylamide gel electrophoresis

In each rat, part of the gastric mucosa in the pyloric and the fundic mucosal area was removed before formalin fixation, according to previously described methods.[13–15] Briefly, the mucosa was scraped with a slide glass, chopped finely with scissors, and homogenized with five volumes of ice-cold 25 mM Tris-Cl buffer (pH 7.5). The homogenate was then centrifuged at 18000 × g for 20 min. The supernatant (soluble fraction mainly containing the cytoplasmic fraction) was collected and the protein concentration was adjusted to 0.2 mg/ml. The protein concentration was measured by the method of Lowry et al.[16] The pellets were rehomogenized with the same buffer, containing 1.0% Triton X-100. The homogenate was then centrifuged at 18000 × g for 20 min, and the supernatant (insoluble fraction, mainly containing nuclear, mitochondrial, and membrane fractions) was collected, and the protein concentration of each sample was adjusted to 0.1 mg/ml. Samples were analyzed by 10% polyacrylamide gel electrophoresis, according to the method of Laemmli.[17] Gels were stained with 0.1% Coomassie brilliant blue (R-250) in a mixture of 25% isopropyl alcohol-10% acetic acid, and destained with 10% isopropyl alcohol-10% acetic acid.

Western blotting

Samples were electrophoresed on SDS-polyacrylamide gels, transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Nihon Millipore Kogyo, Tokyo, Japan) and processed as described by Towbin et al.[18] The membrane was incubated with anti-HSP72 antibody[5] (diluted 1:1000) and treated with horseradish peroxidase-conjugated anti-rabbit IgG (diluted 1:1500) (Bio-Rad, Richmond, Calif.). The peroxidase substrate was 3,3-diaminobenzidine tetrahydrochloride. The density of the immunologically stained bands was analyzed with a scanning densitometer. The relative density was calculated by the equation: Relative density (%) = density (each time)/density (0 h in the fundic mucosal area) × 100.[19]

Immunohistochemistry

Part of the gastric mucosa of each rat was used for immunohistochemical analysis. Tissue fixation and immunoperoxidase staining were performed as described previously.[20] Briefly, tissue pieces were fixed with periodate lysine-4% paraformaldehyde. The tissue sections were sliced (5 μm) with a cryostat. After the blocking of endogenous peroxidase with 0.3% H_2O_2 in methanol, the tissue sections were incubated with anti-HSP72 antibody, diluted 1:500 in 5% bovine serum albumin (BSA) for 12 h at 4°C. The sections then were incubated with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, Calif.) at room temperature for 1 h, and avidin-biotin-peroxidase complex (Vector) was applied at room temperature for 1 h. The sites of peroxidase activity were visualized with 0.25% 3,3-diaminobenzidine-tetrahydrochloride, containing 0.00035% H_2O_2.

Data analysis

Data were analyzed by one-way analysis of variance and, where appropriate, Student's t-test with Bonferoni's adjustment for multiple comparisons or Wilcoxon's rank sum test for unpaired (one-tailed test) data.

Results

Ulcer index of lesions in the gastric mucosa induced by water-immersion stress

Six-h exposure to water-immersion stress produced several linear and dotted erosions in the fundic mucosal area of the rat stomach, and the ulcer index was significantly higher 6, 12, and 24 h after the initiation of water-immersion stress compared to the level

at 0 h. However, no mucosal lesion was observed in the pyloric mucosal area even after 24-h exposure to water-immersion stress (Fig. 1A,B).

Specificity of antibody and expression of HSP72 after water-immersion stress

The specificity of anti-HSP72 antibody is shown in Fig. 2. Only the 72-kDa band was stained (Fig. 2, panel B). The expression of HSP72 before and after water-immersion stress is shown in Fig. 3. The constitutive expression (before water-immersion stress) of HSP72 was significantly higher in the pyloric mucosal area than in the fundic mucosal area. In the soluble fraction of the pyloric mucosal area, HSP72 was significantly increased 1 h after the initiation of water-immersion stress, peaking 6 h after the initiation of the stress. However, in the soluble fraction of the fundic mucosal area, HSP72 did not increase until 6 h after the initiation of water-immersion stress. In the insoluble fraction of the pyloric mucosal area, HSP72 was significantly increased even 0.5 h after the initiation of water-immersion stress, peaking at 6 h, whereas in the insoluble fraction of the fundic mucosal area, the increase

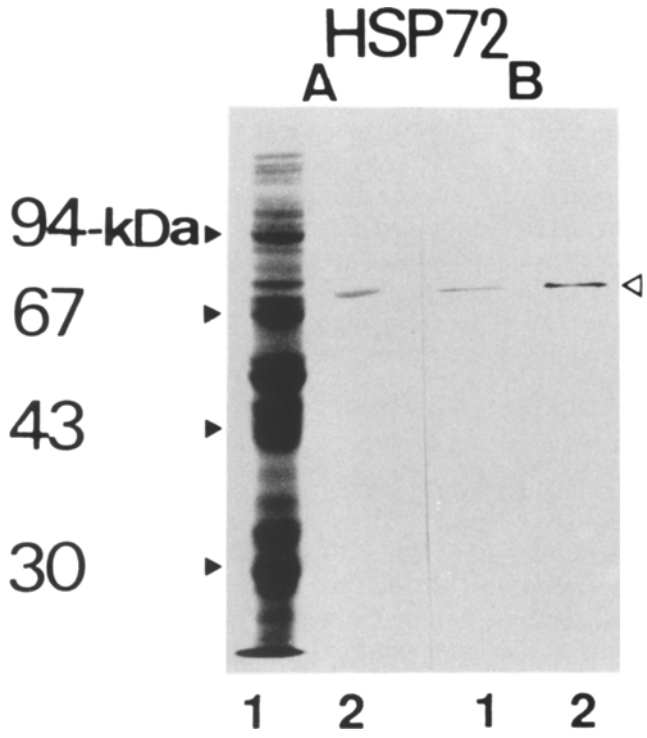


Fig. 2. Specificity of anti-72-kDa heat shock protein (*HSP72*) antibody. Samples were run on 10% sodium dodecylsulfate (*SDS*)-polyacrylamide gels and stained with Coomassie brilliant blue (*A*) or transferred to a polyvinylidene difluoride (*PVDF*) membrane and stained with anti-HSP72 antibody (*B*). Both panels; lane 1, soluble fraction of rat gastric mucosa; lane 2, purified HSP72

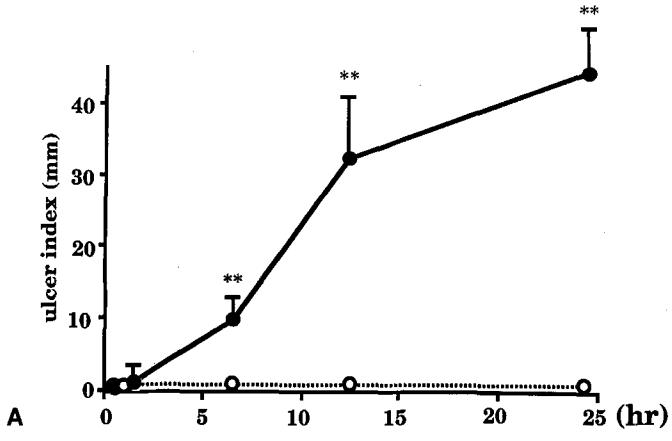
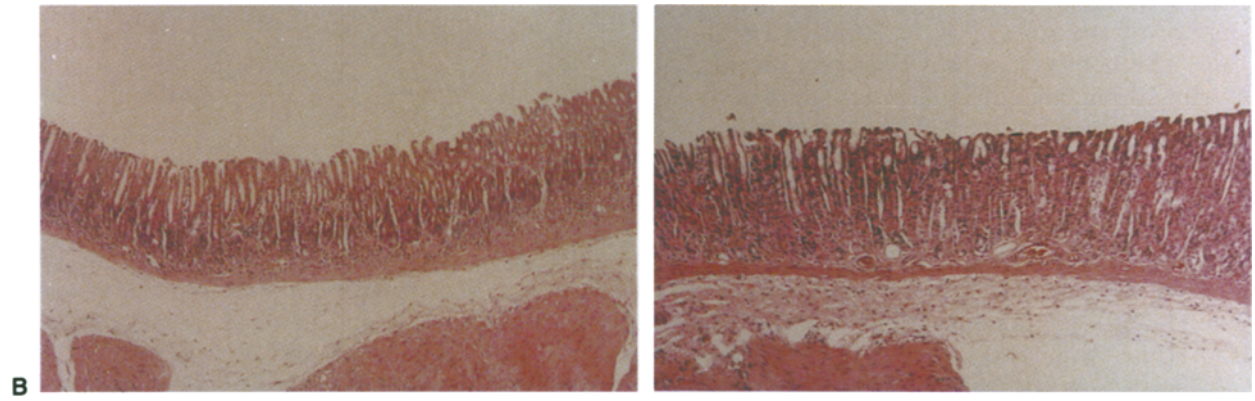


Fig. 1. **A** Ulcer index (length of ulcer) in the pyloric (*circles*) and the fundic (*dots*) mucosal area of the stomach induced by water-immersion stress. The ulcer index was significantly higher in the fundic mucosal area 6, 12, and 24 h after the initiation of water-immersion stress compared to the level at 0 h. However, no ulcer formation was induced in the pyloric mucosal area. $^{**}P < 0.01$ compared with 0 h. **B** Histological findings of the pyloric mucosal area before (*left*) and 12 h after (*right*) the initiation of water-immersion stress. H&E, ×100



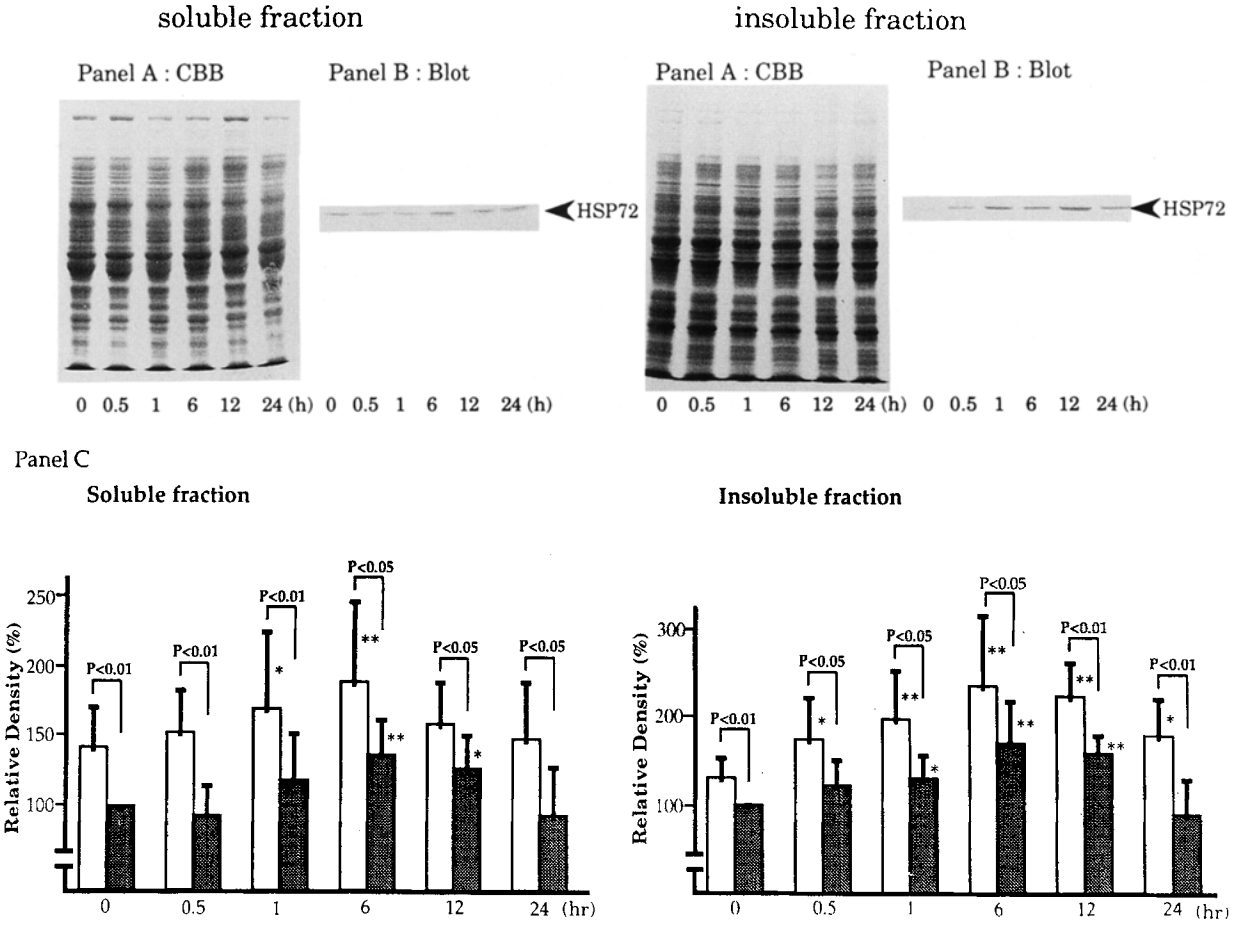


Fig. 3. Expression of HSP72 after water-immersion stress. Coomassie brilliant blue (*CBB*) staining of SDS-polyacrylamide gel electrophoresis (Panel A) and Western blot (Panel B). **C** Relative density of stained bands at different times. Values shown are means ± SE for six animals at each time period. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with 0 h. *Open columns*, pyloric mucosal area; *closed columns*, fundic mucosal area. *Bars* represent mean ± SE ($n = 6$)

of HSP72 was not significant until 1 h after the initiation of water-immersion stress, peaking 6 h after the initiation of the stress.

Immunohistochemical staining of anti-HSP72 antibody

An immunohistochemical study showed that HSP72 was constitutively expressed in the cytoplasm of the gastric mucosal cells, and that HSP72 increased in both the cytoplasm and nuclei of the pyloric mucosal cells after water-immersion stress. The staining in the nuclei, especially in the proliferative zone, was more intense in the pyloric mucosal cells than in the fundic mucosal cells (Fig. 4).

Discussion

Numerous methods for producing stress ulcers in animals, by means of physical or emotional stresses, have been reported and reviewed.[21,22] Most stress ulcers develop only in the fundic mucosal area (parietal cell area) of the stomach, while, in contrast, the pyloric mucosal area is not affected.[9,23] In our study, severe mucosal damage occurred in the fundic mucosal area of the stomach 6 h after the initiation of water-immersion stress. However, no mucosal lesions developed in the pyloric mucosal area even after exposure to water-immersion stress for 24 h. The water-immersion stress technique has been shown to induce several linear and dotted erosions in the fundic mucosal area of the rat stomach. Guth[24] showed that restraint stress caused a reduction in blood flow to the corpus (fundic mucosal area) but not to the antrum (pyloric mucosal area) in rats. Menguy and Masters[25] showed that the antral mucosa of rats was better able to sustain its energy metabolism than the fundic mucosa under conditions of ischemic stress. Kuwayama and Eastwood[23] showed that water-immersion stress inhibited epithelial proliferation in the fundic mucosa but not in the antral mucosa in rats. They conjectured that the failure of antral epithelial proliferation to decrease after stress

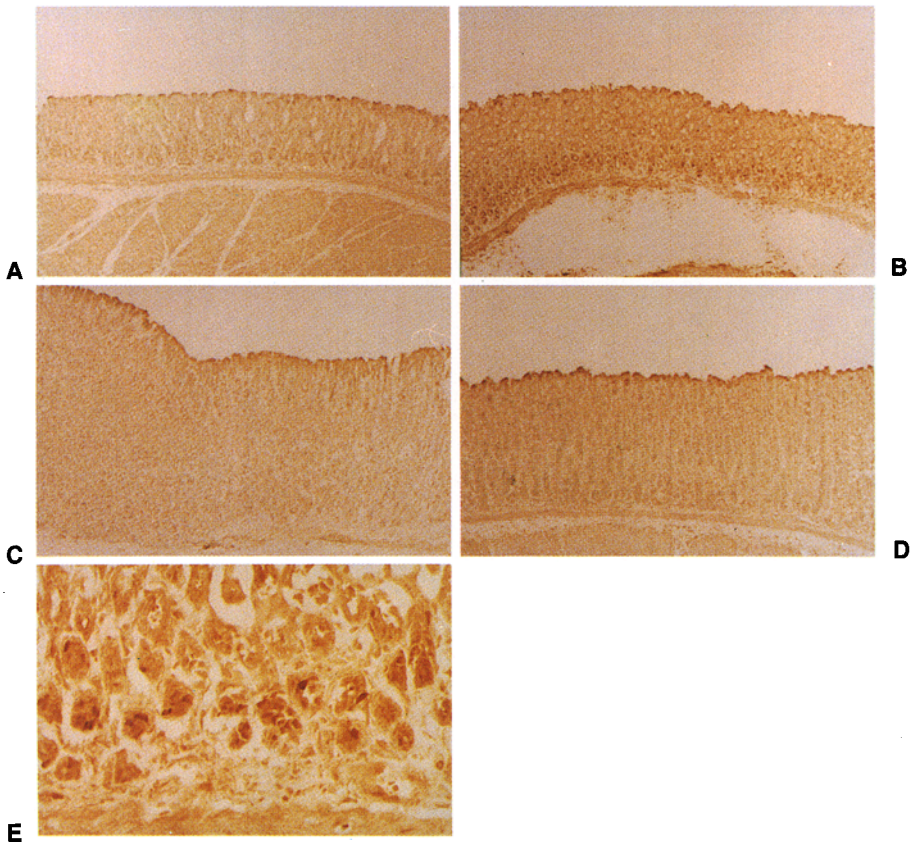


Fig. 4A–E. Immunohistochemical findings in the pyloric mucosal area **A** before and **B** 6 h after, and the fundic mucosal area **C** before and **D** 6 h after the initiation of water-immersion stress. (anti-HSP72 antibody staining, × 100). **E** High-power view of **B** shows staining in the nuclei (× 400). Intranuclear induction of HSP72 was more intense in the pyloric mucosal area than in the fundic mucosal area

may, in part, protect the antral mucosa from the development of mucosal erosions. These reports indicate that physiologic and biologic differences between the pyloric and the fundic mucosa may cause ulcers of different severity.

Recently, many reports have suggested that HSPs play important roles in cytoprotection against and tolerance to several environmental stress factors.[19,26,27] Pre-induction of the HSP70 family is reported to increase resistance to ethanol-induced damage in cultured gastric mucosal cells.[7] On the other hand, little is known about the expression and the function of a 72-kDa heat shock protein (HSP72) in the gastric mucosa under stressed conditions in vivo. In this study, we investigated the expression of HSP72 in the gastric mucosa before and after water-immersion stress. Our results showed that the constitutive expression (before water-immersion stress) of HSP72 in the pyloric mucosa was significantly higher than that in the fundic mucosa. In addition, the induction of HSP72 synthesis occurred prior to ulcer formation in the pyloric mucosal area (0.5 h in the soluble fraction, 1 h in the insoluble fraction). In the fundic mucosa, on the other hand, a significant increase in HSP72 was observed only after the formation of the mucosal lesions (6 h in the soluble fraction, 1 h in the insoluble fraction). Further, intracellular localization of HSP72 in the pyloric and the fundic mucosal areas differed under stress conditions. An immunohistochemical study showed that, in the pyloric mucosal area, especially in the proliferative zone, HSP72 increased both in the cytoplasm and the nuclei after the water-immersion stress. In the fundic mucosal area, on the other hand, the increase of HSP72 was observed only in the cytoplasm. Recent reports have suggested that HSP72 functions in the folding and oligometric assembly of intracellular proteins.[27,28] These functions are considered to protect cells from intracellular protein aggregation by binding the HSP with denatured proteins. Welch et al.[29] have stated that the intranuclear induction of HSP72 is essential for its cytoprotective functions in vitro. In our experiment, the increase of HSP72 was observed both in the soluble fraction (containing mainly the cytoplasmic fraction) and in the insoluble fraction (containing mainly the nuclear, mitochondrial, and membrane fractions). The evidence from the literature and our results, taken together, indicate that the quantity of the constitutive expression and the induction of HSP72 prior to ulcer formation is important for its cytoprotective function in vivo. In addition, the quantity of intranuclear expression of HSP72 prior to ulcer formation is also critical for

cytoprotection in the gastric mucosa. The differences noted in this study between the pyloric and fundic mucosal areas may indicate "biophysical" differences between these areas and may help to explain why pyloric mucosal cells are more tolerant to environmental stress than fundic mucosal cells.

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