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Advances in Brief

Suppression of Saccharin-induced Mutagenicity by Interferon-α in Human RSa Cells

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Abstract

Saccharin is an artificial sweetener commonly used in the formulation of foods and beverages. Sodium saccharin-induced mutagenicity is detectable in human RSa cells by estimation of cloning efficiency of ouabain-resistant mutant cells and determination of K-*ras* codon 12 mutation in genomic DNA, analyzed by PCR and differential dot-blot hybridization. However, in this study no phenotypic or genetic mutations were detected in RSa cells cultured with human IFN (HuIFN)-α before sodium saccharin treatment. The suppressive effect was lessened by transient treatment with antipain immediately after sodium saccharin treatment. Elevation of antipain-sensitive protease activity was found, furthermore, in RSa cells cultured with HuIFN-α and subsequently treated with sodium saccharin. Thus, antipain-sensitive protease induction in cells tested here may be involved in suppression of the mutagenicity of saccharin by HuIFN-α.

Introduction

Hypermutable prokaryotes and nonhuman eukaryotes have played an important role in the search for mutagens and/or carcinogens.

The human cell line RSa also exhibits hypermutability and is, therefore, useful in searching for mutagens (1, 2). Previously, we found the mutagenicity of saccharin by detection of phenotypic changes in RSa cells that showed increased resistance to ouabain lethality after culture with sodium saccharin (3). When RSa cells were treated with sodium saccharin at concentrations up to 22.5 mg/ml for 24 h, more than 33 Oua^{R2} mutants/10^5 surviving cells were detected (3). Moreover, in addition to quantification of Oua^R phenotypic mutation rate, we attempted to devise a convenient method for detection of saccharin-induced genetic mutation in RSa cells. Recently, we found induction of base substitution mutations of K-*ras* codon 12 in the genomic DNA of chemical-treated RSa cells, assessed by PCR and differential dot-blot hybridization (4, 5).

Saccharin is commonly used as an artificial sweetener in the formulation of foods and beverages. In addition to the findings of our previous studies outlined above, the mutagenic potential of the chemical has already been suggested in prokaryotes and nonhuman eukaryotes (3). However, saccharin has not yet been classified as a proven mutagen (3, 6, 7). Interestingly, we have found that frequencies of UV (far UV light, mainly 254 nm wavelength) irradiation-induced phenotypic mutation are reduced in cells pretreated with HuIFN-α before irradiation (8). Therefore, we considered the possibility of modulation of the saccharin-induced mutagenicity by unknown cellular conditions. Whether HuIFN-α is capable of modulating the mutagenic potential of saccharin in RSa cells is intriguing. Thus, we comparatively evaluated the mutagenic potential of saccharin between HuIFN-α-pretreated and nonpretreated RSa cells by analysis of Oua^R phenotypic mutation and K-*ras* codon 12 mutation in genomic DNA.

Materials and Methods

Agents. Sodium saccharin was obtained from Tokyo Kasei Co., Ltd. (Tokyo, Japan). Recombinant HuIFN-αA (2.0×10^8 IU/mg protein), provided by Nippon Roche Co., Ltd. (Kamakura, Japan), was described elsewhere (9). Natural HuIFN-α (5.0×10^8 IU/mg protein) and anti-HuIFN-α polyclonal antibody (1.0×10^6 neutralizing units/mg protein) were described elsewhere (10). Antipain, elastatinal, and DTNB were purchased from Nacalai Tesque (Kyoto, Japan).

Cells and Culture Conditions. The human cell line RSa was described elsewhere (11, 12). The medium used for cell culture was EMEM (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and antibiotics (100 μg streptomycin/ml and 100 units penicillin G/ml). Cells were cultured by incubation with the medium at 37°C in a humidified atmosphere containing 5% CO_2.

HuIFN-α, Sodium Saccharin, and Antipain Treatment. Treatment of RSa cells with HuIFN-α or with sodium saccharin was performed according to the method described previously (3, 9). Briefly, logarithmically growing cells were plated at a density of 1.0×10^6 cells/100-mm culture dish, and after 24 h they were replenished with fresh MEM or the same medium containing HuIFN-α (50 IU/ml). HuIFN-αA was generally used, unless specifically noted. HuIFN-α preparations were used after incubation with or without solutions containing anti-HuIFN-α antibodies capable of neutralizing 100-fold higher levels of HuIFN-α than that tested, as described previously (10). After culturing for 24 h, the medium was removed from the dish, and the attached cells were washed three times with PBS. After washing, the cells were exposed to various concentrations of sodium saccharin in EMEM. Twenty-four h later, the EMEM with or without sodium saccharin was removed, and the cells were washed twice with PBS. The plates were then replenished with culture medium either containing or not containing antipain, and incubated. For antipain exposure, the incubation was performed for 6 h, followed by washing cells with PBS and subsequent culture, as described elsewhere (13). After 2 days of sodium saccharin treatment, cells were plated for an Oua^R mutation test. To detect a K-*ras* codon 12 mutation, cells were incubated with the medium for 6 days after sodium saccharin treatment. For analysis of protease activity, cells pretreated with or without HuIFN-α were cultured with EMEM containing or not containing sodium saccharin for appropriate periods (up to 24 h).

Cell Proliferation Assay. The cell proliferation assay was carried out as described previously, except for inoculation of 1.0×10^6 cells/100-mm culture dish as described above (14). Viable cells were determined by the trypan blue dye exclusion test and counted with a hemocytometer.

Oua^R Mutation Test. The quantitative assay for induced frequency of Oua^R cells was carried out by the method described in our previous papers (1, 3).

Briefly, cells prepared as described above were plated at a density of 5.0×10^4 cells/100-mm culture dish (six dishes for each point) in medium containing 1.0×10^{-7} M ouabain. At the same time, cells were seeded at 1.0×10^3 cells/100-mm culture dish (three dishes for each point) for determination of cloning efficiency. Every second day the medium was changed, and then 14 days later, colonies containing 50 or more cells were counted. The mutant frequency was determined by dividing the total number of mutant colonies by the total number of cells plated, corrected by the cloning effi-

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[2] The abbreviations used are: Oua^R, ouabain-resistant; HuIFN, human IFN; EMEM, Eagle's MEM; DTNB, 5, 5′-dithiobis(2-nitrobenzoic acid); IC_{50}, the concentration of enzyme activity to one-half that of the control.

ciency, and expressed as mutants/10^4 surviving cells. Results are expressed as the means of values obtained from two independent experiments.

For the assay of Na^+/K^+-ATPase activity, preparation of cell membrane fractions and assay of the enzyme activity were performed as described elsewhere (1). The specific activity of Na^+/K^+-ATPase was estimated as micromoles of released Pi/mg protein of the fractions/h, and the activity of each membrane fraction in the presence of ouabain was expressed as a relative ratio against the activity of controls (without ouabain).

Detection of K-*ras* Codon 12 Mutation by PCR and Differential Dot-Blot Hybridization. Mutations at codon 12 of K-*ras* were detected according to the method described previously (4). Briefly, genomic DNA was extracted from cells by a standard proteinase K/SDS/phenol chloroform procedure. Target sequences of sample DNA were amplified *in vitro* by means of PCR by using primers 5′-GACTGAATATAAACTTGTGG-3′ and 3′-GCTTATAC-TAGGTTGTTATC-5′, and the amplified DNA was dot-blotted onto nylon membranes. After prehybridization and hybridization with the digoxigenin-11-dUTP-3′end-labeled K-*ras* codon 12 normal or mutant probes, the membranes were washed, blocked with blocking reagent, reacted with polyclonal sheep anti-Dig Fag conjugated to alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany), and colored with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate solutions (Boehringer). Photographs were taken for permanent records.

Assay of Protease Activity. Protease samples were prepared from cell lysates principally according to the method described elsewhere (9). Cell lysates were dissolved in a lysis buffer composed of 50 mM HEPES-NaOH (pH 7.5), 1 mM EDTA, 0.1% MEGA-8, and 5 μM chroloquine, as described elsewhere (13). The protease samples were incubated in ^{125}I-fibrin-coated tubes at 37°C for 30 min, with or without protease inhibitors. The tubes were not coated with plasminogen, in contrast to our reported previously method in which the tubes were precoated with this agent in addition to radiolabeled fibrin (9). Inhibitors alone at the concentrations used did not affect fibrinolysis in the fibrin-coated tubes. Reactions were performed in triplicate, and each experiment was repeated at least twice. Results are expressed as the total amount of radioactivity/10^5 cells, after subtracting the radioactivity of a sample containing lysates from mock-treated cells (500 cpm).

Other Conditions. All of the experiments were performed under dim light or a yellow lamp (National FL 20S-Y-F; Matsushita Electric Industrial Co., Ltd., Osaka, Japan).

Results and Discussion

Comparison of sodium saccharin-induced mutagenicity in RSa cells with and without HuIFN-α treatment was first tested by calculating numbers of Oua^R mutant cells relative to the total number of surviving cells. However, mutation frequency is modulated by cell survival conditions, and RSa cells are sensitive to HuIFN-α lethality when inoculated dispersed onto dishes (12). Thus, cell selection by the lethal effect was avoided by confluent inoculation of RSa cells, whereby proliferation rates were almost the same between cells pretreated with 50 IU/ml HuIFN-αA for 24 h and nonpretreated cells (Fig. 1*A*). Further combination of 25 mg/ml sodium saccharin did not influence the growth rate between HuIFN-α-pretreated and nonpretreated cells, although sodium saccharin treatment itself inhibited proliferation (Fig. 1*A*). In Oua^R mutation tests, cloning efficiency of RSa cells treated with 50 IU/ml HuIFN-αA before the sodium saccharin treatment was also similar to that of cells without the HuIFN-α pretreatment at all sodium saccharin doses tested (up to 25 mg/ml; Fig. 1*B*).

Sodium saccharin treatment (up to 25 mg/ml) resulted in an increase in frequency of Oua^R mutation up to about ten mutants/10^4 surviving cells (Fig. 1*C*). In contrast, HuIFN-αA (50 IU/ml) pretreatment showed no such elevation of Oua^R mutation rate, with an incidence of 0–0.73 Oua^R mutants/10^4 surviving cells (Fig. 1*C*). Pretreatment with natural HuIFN-α (50 IU/ml) also suppressed the induction of Oua^R mutation by sodium saccharin (Fig. 1*C*). In contrast, pretreatment with anti-HuIFN-α antibody inhibited the suppressive effect of natural HuIFN-α on the mutagenicity of sodium saccharin, although pretreatment with the antibody alone had no such affect (Fig. 1*C*). Thus, under the same cell survival conditions with and without HuIFN-α pretreatment, sodium saccharin-induced Oua^R mutation in RSa cells was suppressed by HuIFN-α, irrespective of whether recombinant or natural HuIFN-α was used.

Suppressive effect of HuIFN-α on sodium saccharin-induced mutagenicity in RSa cells was tested additionally by analysis of mutations at K-*ras* codon 12 in the genomic DNA by using PCR and differential dot-blot hybridization. Hybridization signals with mutant probes were clearly detected in cells after 6 days of sodium saccharin treatment at concentrations ranging from 20 to 25 mg/ml (Fig. 2), as reported previously (4, 5). However, signals with mutant probes were not detected in cells precultured with HuIFN-αA (50 IU/ml) before sodium saccharin treatment, although hybridization signals with the

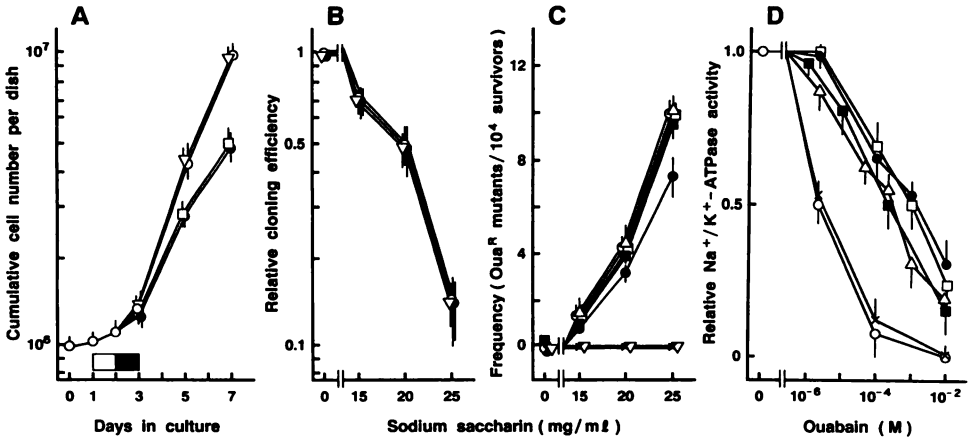

Fig. 1. Effects of HuIFN-α and antipain on cell proliferation (*A*), cloning efficiency (*B*), and Oua^R mutant frequency (*C*) of sodium saccharin-treated RSa cells and the effects of ouabain on Na^+/K^+-ATPase activity (*D*). The 50 IU/ml HuIFN-α pretreatment and subsequent treatment with sodium saccharin after treatment with and without 0.01 mM antipain were performed as described in "Materials and Methods." *A*: □, period of HuIFN-α pretreatment; ■, period of sodium saccharin treatment; ○, control without HuIFN-α or sodium saccharin treatment; ▽, HuIFN-αA pretreatment; □, 25 mg/ml sodium saccharin treatment alone; ●, HuIFN-αA pretreatment and 25 mg/ml sodium saccharin treatment. *B*, cloning efficiency of control cells without sodium saccharin, HuIFN-α, or antipain treatment was 10%, which was arbitrarily assigned a value of 1.0. ○, nontreatment with HuIFN-α; ●, HuIFN-αA pretreatment; ▽, HuIFN-αA pretreatment and antipain treatment. *C*, ○, nontreatment with HuIFN-α or antipain; ▽, HuIFN-αA pretreatment; ×, natural HuIFN-α pretreatment; ■, natural HuIFN-α plus anti-HuIFN-α antibody treatment; △, antipain treatment; ●, HuIFN-αA pretreatment and antipain treatment; □, anti-HuIFN-α antibody treatment. *D*, the activity of controls without ouabain was 3.3–4.3 μ moles Pi/mg protein/h △, □, and ■, cells selected as Oua^R cell clones after HuIFN-αA pretreatment and 25 mg/ml sodium saccharin treatment after antipain treatment; ●, cells selected as Oua^R cell clones after 25 mg/ml sodium saccharin treatment; ○, nonmutagenized cells without HuIFN-α, sodium saccharin, or antipain treatment or Oua^r selection; ×, cells treated with HuIFN-αA pretreatment after 25 mg/ml sodium saccharin; *bars*, SE.

normal probe were found in all of the tested samples (Fig. 2). Thus, HuIFN-α pretreatment before sodium saccharin treatment appeared to have a suppressive effect on not only Oua^R mutation but also on mutation of K-*ras* codon 12.

When HuIFN-α suppresses UV-induced Oua^R mutation, antipain, known as a protease inhibitor, prevents the suppressive effect when cells are cultured with medium containing the inhibitor immediately after UV irradiation (13). This preventive effect of antipain was reproduced in the present study in tests of sodium saccharin-induced mutagenicity. When HuIFN-αA-pretreated RSa cells were transiently cultured with medium containing 0.01 mM antipain immediately after treatment with sodium saccharin (up to 25 mg/ml), the frequency of Oua^R mutation increased exhibiting several mutants/10^4 surviving cells (Fig. 1*C*). The increased levels recovered nearly to those in cells without the HuIFN-α pretreatment (Fig. 1*C*), despite the lack of an effect on cloning efficiency by this combination treatment (Fig. 1*B*). However, Oua^R mutation was not induced by antipain treatment alone without exposure to sodium saccharin (Fig. 1*C*).

Twenty Oua^R cell clones were selected randomly after HuIFN-α pretreatment and sodium saccharin exposure after antipain treatment, and, thereafter, ouabain-sensitivity of Na^+/K^+-ATPase in membrane preparations from each clone was examined. As shown in Fig. 1*D*, the Na^+/K^+-ATPases from the three Oua^R cell clones were less inhibited by ouabain (1.0×10^{-6}-1.0×10^{-2} M) than those from untreated cells or those from cells pretreated with HuIFN-α after sodium saccharin treatment. The IC_{50} for ouabain against the ATPase from the nonmutagenized cells was approximately 2.0×10^{-6} M (Fig. 1*D*). However, the IC_{50} values of ouabain on this enzyme from the three Oua^R cell clones were all more than 2.0×10^{-4} M, similar to that from Oua^R cells cloned after treatment with sodium saccharin alone, approximately 1.0×10^{-3} M (Fig. 1). Another 17 Oua^R cell clones also had Oua^R ATPase activity, with IC_{50} values of more than 2.0×10^{-4} (data not shown). Thus, in the Oua^R cell clones tested, increased resistance to ouabain cell killing appeared because of the refractoriness of Na^+/K^+-ATPase to the chemical.

Sodium saccharin-induced hybridization signals with K-*ras* codon 12 mutant probes were also detectable by transient treatment with 0.01 mM antipain, even in RSa cells pretreated with HuIFN-αA (Fig. 2). The antipain treatment alone again showed no mutagenic potential, as demonstrated by the absence of clear hybridization signals with mutant probes after treatment with 0.01 mM antipain but without the HuIFN-α and sodium saccharin (Fig. 2). Furthermore, the combination treatment with antipain did not show any modulation of sodium saccharin-induced hybridization signals with mutant probes in cells not pretreated with HuIFN-α (Fig. 2).

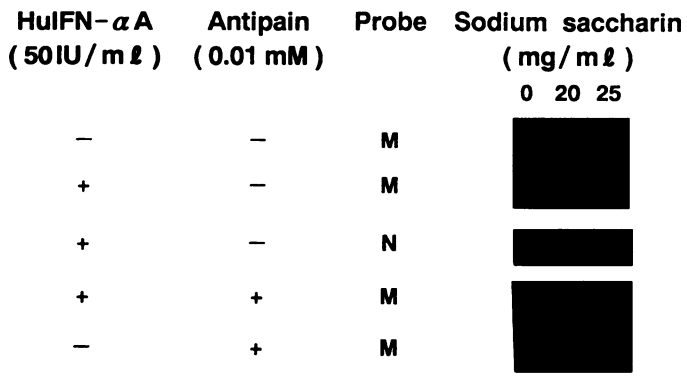

Fig. 2. Effects of HuIFN-α and antipain on detection of K-*ras* codon 12 mutations in RSa cells after 6 days of treatment with sodium saccharin. Preparation of genomic DNA, and PCR and differential dot-blot hybridization by using digoxigenin-labeled mutant (*M*) and normal (*N*) probes were carried out as described in "Materials and Methods." +, treatment with the indicated agent; –, nontreatment with the indicated agent.

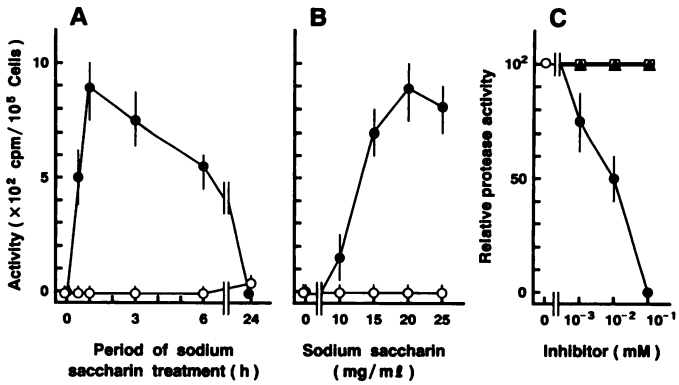

Fig. 3. Protease activity in extracts of RSa cells after HuIFN-α treatment following sodium saccharin treatment and effects of protease inhibitors on the activity. Preparation of cell lysates and measurement of the protease activity were performed as described in "Materials and Methods." *A*, protease activity in extracts prepared from cells at indicated times of 20 mg/ml sodium saccharin treatment. ○, sodium saccharin treatment alone; ●, 50 IU/ml HuIFN-αA pretreatment and sodium saccharin treatment. *B*, protease activity in extracts prepared from cells 1 h after treatment with various doses of sodium saccharin. ○, nonpretreatment with HuIFN-α; ●, 50 IU/ml HuIFN-αA pretreatment and sodium saccharin. *C*, cell lysates were prepared from RSa cells pretreated with 50 IU/ml HuIFN-αA for 24 h and then treated with 20 mg/ml sodium saccharin for 1 h. The activities were expressed as relative values, taking that without the addition of inhibitors (900 cpm/10^5 cells) as 100. ○, without inhibitors; ●, antipain; □, DTNB; ▲, elastatinal; *bars*, SE.

A transient elevation of antipain-sensitive protease activity was detectable in cells treated with HuIFN-α and then irradiated with UV (13). Therefore, inhibition of antipain for the suppressive effect of HuIFN-α on sodium saccharin mutagenicity raised the question of whether antipain-sensitive protease activity is also induced in RSa cells treated with HuIFN-α and then sodium saccharin. There was no elevation of the protease activity levels in RSa cells during 20 mg/ml sodium saccharin treatment up to 24 h (Fig. 3*A*) or at any doses up to 25 mg/ml after 1 h of treatment (Fig. 3*B*). However, the elevation of protease activity was detected in 50 IU/ml HuIFN-αA-pretreated RSa cells only 30 min after the sodium saccharin treatment and reached the maximal levels after 1 h (Fig. 3*A*). The elevation continued to be detectable until 6 h after sodium saccharin treatment but was not detectable at 24 h (Fig. 3*A*). The elevated levels were most evident at 20 mg/ml sodium saccharin (Fig. 3*B*), although HuIFN-α treatment itself did not induce the protease activity, as demonstrated at 0 mg/ml sodium saccharin (Fig. 3*B*). The induced protease activity was gradually inhibited *in vitro* with increasing concentrations of antipain (0.001–0.1 mM; Fig. 3*C*. The IC_{50} of antipain was about 0.01 mM (Fig. 3C). In contrast, the activity was less inhibited by other protease inhibitors, such as elastatinal and DTNB, at all concentrations tested (Fig. 3*C*). This antipain specificity of the protease activity appeared in accordance with that of the protease activity induced in RSa cells pretreated with HuIFN-α and then irradiated with UV (13).

Many problems remain unresolved concerning the molecular mechanisms of sodium saccharin-induced mutagenicity in hypermutable human RSa cells. Nevertheless, the present study suggested that there may be cellular conditions modulating mutagenic potential of sodium saccharin as well as UV mutagenicity. As for variation in the susceptibility to UV-induced mutagenicity among cell cycle phases in synchronized RSa cells, the frequency of Oua^R mutation has already been found to be greatest in the latter one-half of the G_1 (11). Sodium saccharin treatment at G_1 also induces the greatest mutagenicity among the cell cycle phases examined for the time of the chemical treatment, whereas HuIFN-α pretreatment before the sodium saccharin treatment from early G_1 results in nondetectable mutagenicity.[3]

[3] Unpublished data.

Flow cytometric analysis has revealed that the G_1 cell cycle distribution in proliferating RSa cells was more than one-half of the population (12). Thus, more detailed analyses at G_1 are now being performed for clarification of the mechanisms of sodium saccharin-induced mutagenicity and the suppressive effects of HuIFN-α.

Recovery of Oua^R and K-*ras* codon 12 mutation by antipain from HuIFN-α-induced suppression suggests that antipain-sensitive cellular functions, probably including the inhibitor-sensitive protease activity, may play some roles in determining the mutagenic potential of sodium saccharin. It will be intriguing to determine how the protease activity elevated by HuIFN-α pretreatment leads to suppression of sodium saccharin-induced mutagenicity. From RSa cells we have already established variant cells with the ability to induce the antipain-sensitive protease activity after UV irradiation without HuIFN-α treatment (15, 16). The variant cells show hypomutability for Oua^R and K-*ras* codon 12 mutations after UV irradiation (15, 16) and sodium saccharin treatment.[4] The antipain-sensitive protease induction may be causually related with the suppression of sodium saccharin-induced mutagenicity. Whether the protease induced in UV-irradiated variant cells is similar to that in RSa cells pretreated with HuIFN-α and then irradiated with UV is currently being examined. HuIFN-α is a cytokine present in normal human serum (17–20). Thus, when we investigate whether sodium saccharin-induced mutagenicity occurs in the whole body, it might be better to consider HuIFN-α-modulated effects on the mutagenic potential.

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