Proliferation, not apoptosis, alters epithelial cell migration in small intestine of CFTR null mice

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Gallagher, Ann Marie, and Roberta A. Gottlieb. Proliferation, not apoptosis, alters epithelial cell migration in small intestine of CFTR null mice. Am J Physiol Gastrointest Liver Physiol 281: G681–G687, 2001.—Expression of a mutated cystic fibrosis transmembrane conductance regulator (CFTR) has been shown to enhance proliferation within CF airways, and cells expressing a mutated CFTR have been shown to be less susceptible to apoptosis. Because the CFTR is expressed in the epithelial cells lining the gastrointestinal tract and all CF mouse models are characterized by gastrointestinal obstruction, we hypothesized that CFTR null mice would have increased epithelial cell proliferation and reduced apoptosis within the small intestine. The rate of intestinal epithelial cell migration from crypt to villus was increased in CFTR null mice relative to mice expressing the wild-type CFTR. This difference in migration could be explained by an increase in epithelial cell proliferation but not by a difference in apoptosis within the crypts of Lieberkühn. In addition, using two independent sets of CF cell lines, we found that epithelial cell susceptibility to apoptosis was unrelated to the presence of a functional CFTR. Thus increased proliferation but not alterations in apoptosis within epithelial cells might contribute to the pathophysiology of CF.

Previously, Gottlieb and Dosanjh (9) showed that cultured C127 mouse mammary epithelial cells stably transfected with the ΔF508-mutated CFTR remained viable and were less susceptible to apoptosis induced by cycloheximide or etoposide than cells expressing the wild-type CFTR. Programmed cell death, or apoptosis, is a physiologically relevant process that occurs in regenerating tissues such as the lung and intestine. Cell turnover is a balance between proliferation and elimination, and cell proliferation has been shown to be greater in airway epithelia and nasal polyps in CF patients than in tissue from non-CF patients (12, 15). Although this phenomenon has traditionally been attributed to inflammation, the possibility that the proliferative differences might be due to differential expression of the CFTR has not been addressed.

Because all mouse models of CF (CFTR−/−) develop intestinal obstructions (10) and the CFTR is expressed in epithelial cells lining the gastrointestinal tract, we hypothesized that the CFTR−/− mouse intestine would show evidence of altered apoptosis and/or enhanced proliferation relative to mice expressing the wild-type CFTR. The hypothesis was tested in vivo using a knockout mouse model of CF. Mice expressing a mutant CFTR were found to have a greater rate of migration of epithelial cells up the crypt-villus axis within the small intestine. This increased rate could be attributed to an increase in the proliferative capacity of cells within the crypts of Lieberkühn but not to differences in apoptosis. Because these current results contradict the previous findings (9) of differences in apoptosis between cells transfected with the ΔF508 CFTR and the wild-type CFTR, studies were extended to further examine apoptosis in two independent cell culture models of CF.

METHODS

Animal Model

A mouse model of CF generated by Dr. Beverly Koller (University of North Carolina) was used for all studies (3). In this model (cftrmut/−/−), the CFTR gene has been disrupted by gene targeting, and as with all mouse models of CF, the

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animals develop intestinal obstructions and typically die within weeks of birth. However, by replacing the drinking water with Colyte (Schwarz Pharma, Milwaukee, WI), a commercially available electrolyte solution containing 6% polyethylene glycol, the mice remain alive past 18 mo of age (10). Animals were maintained within the custom breeding colony at The Scripps Research Institute with all animals receiving Colyte in place of drinking water. At the time of weaning, the genotypes of all mice were determined by isolation of tail DNA and PCR amplification of the CFTR alleles as described previously by Koller et al. (14). All animal studies adhered to approved institutional animal protocols and American Association for Accreditation of Laboratory Animal Care guidelines.

**Epithelial Cell Migration**

Bromodeoxyuridine (BrdU; Sigma Chemical, St. Louis, MO) was suspended in PBS at 20 mg/ml and administered via intraperitoneal injection at 150 μg/g body wt. The mice were killed by halothane overdose 2–48 h after BrdU injection. The small intestine was isolated and excised, rinsed in PBS, spayed open along its length, and rinsed twice to remove contents. The splayed intestine was fixed in Z-Fix (10% buffered formalin containing zinc; Anatech, Battle Creek, MI), coiled from proximal to distal end to form a “Swiss roll,” dehydrated, and embedded in paraffin for tissue sectioning. Tissue sections of Swiss rolls allow for examination of the entire length of the intestine in a single section. BrdU incorporation on Swiss rolls was analyzed by immunohistochemistry. Briefly, 3-μm-thick tissue sections were cleared of paraffin and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in methanol for 15 min at room temperature. After pepsin digestion (0.4 mg/ml in 0.1 N HCl, 15 min at 37°C), tissue was treated with 2 N HCl for 30 min at 37°C and rinsed in 0.1 M sodium borate (pH 8.5). Tissue sections were incubated with anti-BrdU (Sigma Chemical) at 1:500 in PBS containing 1 mg/ml IgG-free BSA; Caltag, Burlingame, CA) for 90 min at 37°C. Incubation with biotinylated goat anti-mouse IgG (1:2,000 in PBS containing 1 mg/ml IgG-free BSA; Caltag, Burlingame, CA) for 60 min at room temperature was followed by enzymatic labeling with horseradish peroxidase-conjugated streptavidin (1:1,000 in PBS/BSA, 30 min at room temperature; Caltag). Diaminobenzidine (0.04%, Sigma Chemical) in 0.05 M Tris, pH 7.4, and 0.01% H2O2 were used for color development. Tissues were counterstained with Gill’s hematoxylin (Fisher Scientific, Pittsburgh, PA), dehydrated through graded alcohols, and placed on a coverslip with Permount (Fisher Scientific, St. Louis, MO).

**Intestinal Epithelial Cell Proliferation**

Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) was evaluated within the crypts of control (nonirradiated, no BrdU) CFTR mice by an investigator blinded to animal identity. Tissues were acquired and processed as described above, and paraffin-embedded Swiss rolls were dewaxed and rehydrated through graded alcohols. Tissues were incubated with Target retrieval solution (DAKO, Carpinteria, CA) at 95°C for 20 min and at room temperature for an additional 20 min. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in methanol before incubation with anti-PCNA (clone PC10, DAKO) for 90 min at 37°C in 20% normal goat serum. After rinsing with PBS, tissue was incubated with biotinylated goat anti-mouse IgG (Caltag) suspended 1:2,000 in PBS containing 1 mg/ml IgG-free BSA. After 30 min incubation with horseradish peroxidase-conjugated streptavidin (Caltag), color was developed with SigmaFast diaminobenzidine (0.7 mg/ml diaminobenzidine in 0.06 M Tris buffer containing 0.2 mg/ml 3,3’-diaminobenzidine) in methanol (Pierce, Rockford, IL) and the color was developed with SigmaFast diaminobenzidine (0.7 mg/ml diaminobenzidine in 0.06 M Tris buffer containing 0.2 mg/ml 3,3’-diaminobenzidine) in methanol (Pierce, Rockford, IL) for 10 min at room temperature. Following fixation and sectioning as described above, intestinal tissue was deparaffinized, stained with hematoxylin, and placed on a coverslip with Permount. The average number of apoptotic nuclei was determined in a minimum of 50 crypts/animal in 18 CFTR +/- mice (8 control, 10 irradiated) and 14 each of +/- and +/- mice (5 control, 9 irradiated) and expressed as a percentage of the first 10 crypts. ANOVA was used to test whether irradiation increased intestinal cell apoptosis, and two-tailed t-tests were used to determine the statistical significance of irradiation with each genotype.

**Spontaneous and Induced Epithelial Cell Apoptosis**

In situ terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) has been found to be unreliable as a method of detecting apoptosis in the gastrointestinal tract (21). Therefore, a method previously described by Potten (21) which quantifies apoptosis by calculating the percentage of cells with condensed and/or fragmented nuclei within the first 10 cells following the Paneth cells in the crypts of Lieberkühn was used to quantify apoptosis in the small intestine of CFTR mice. Crypts were scored by an investigator blinded to the identity of animals that met the following criteria: 1) Paneth cells could be viewed, 2) the crypt lumen could be seen, and 3) a minimum of 10 nonoverlapping epithelial cells in the crypt column could be distinguished. Levels of spontaneous apoptosis were evaluated in control animals. Whole body cesium gamma irradiation was used to induce apoptosis in the small intestine of CFTR mice in an attempt to uncover genotypic differences in susceptibility to apoptosis. An initial dose-response curve using CFTR +/- mice revealed that significant epithelial cell apoptosis could be induced by 100 rads, with a complete loss of crypt-forming cells 5 h after whole body gamma irradiation with 100 rads. Higher doses of gamma irradiation did not result in detection of more apoptotic cells. Therefore, subsequent experiments were performed with a dose of 100 rads, and the animals were killed 3 h after gamma irradiation. Following fixation and sectioning as described above, intestinal tissue was deparaffinized, stained with hematoxylin, and placed on a coverslip with Permount. The average number of apoptotic nuclei was determined in a minimum of 50 crypts/animal in 18 CFTR +/- mice (8 control, 10 irradiated) and 14 each of +/- and +/- mice (5 control, 9 irradiated) and expressed as a percentage of the first 10 crypts. ANOVA was used to test whether irradiation increased intestinal cell apoptosis, and two-tailed t-tests were used to determine the statistical significance of irradiation within each genotype.
Cell Culture Models

C127 cell lines. C127 mouse mammary epithelial cells stably transfected with the wild-type CFTR or the CFTR with the deletion of phenylalanine at position 508 (C127 ΔF508) were obtained from Dr. G. M. Denning (Howard Hughes Medical Institute, University of Iowa College of Medicine) (4). These cells were grown in DMEM (GIBCO BRL, Rockville, MD) supplemented with 10% FBS (HyClone, Logan, UT), penicillin, streptomycin, and Fungizone (Sigma Chemical) and passaged 1:5 two times per week. The cell lines were grown in the presence of Genetin (500 μg/ml; GIBCO BRL).

CFT1 cell lines. CFT1 cell lines, which were derived from tracheal epithelial cells isolated from a 24-year-old CF patient homozygous for the ΔF508 mutation, were obtained from J. Yankaskas (University of North Carolina). The parental cell line (CFT1-C2) was used to establish the other three cell lines and has been immortalized with human papilloma virus. CFT1-LCFSN cells overexpress the wild-type CFTR introduced by retroviral vector (20). CFT1-ΔF508 cells express an additional copy of the CFTR with the ΔF508 mutation, and the CFT1-LC3 is a retroviral vector control cell line overexpressing the β-galactosidase gene. These cells were grown in Ham's F-12 (Fisher Scientific) supplemented with insulin (10 μg/ml), hydrocortisone (1 μM), endothelial cell growth supplement (ECGS; 3.75 μg/ml), epidermal growth factor (25 ng/ml), T3 (30 nM), transferrin (5 μg/ml), and cholera toxin (10 ng/ml) (all hormones were obtained from Sigma Chemical). All cells, except the CFT1-C2 cells, were grown in the presence of Genetin (500 μg/ml) and were passaged 1:3 two times per week.

Analyses of death

Membrane destabilization detected by YOPRO-1 uptake. Cells were plated at 10,000 cells/well into 96-well tissue culture plates. After 24 h, the medium was aspirated and replaced with 100 μl sterile PBS. Cells were ultraviolet (UV) irradiated at 0–8,000 J/m² using a Fisher Scientific UV crosslinker; the PBS was replaced with fresh medium, and the cells were incubated at 37°C with 5% CO₂. After 16 h, 25 μM YOPRO-1 (10 μl) was added to each well and incubated at 37°C for 10 min. YOPRO-1 is taken up by cells with an unstable membrane and binds to double-strand DNA; therefore, apoptotic or necrotic cells will be labeled with YOPRO-1 (13). With the use of a fluorescence plate reader (Bio-Tek Instruments, Winooski, VT), YOPRO-1 was found to be excited at 530 nm and emissions were detected at 480 nm. Cells were lysed with 10 μl of lysis buffer (125 mM EDTA and 2.5% Nonidet P-40) and incubated at 37°C with 5% CO₂ for 30 min after which fluorescence was evaluated to determine the total number of cells per well. The ratio of the first fluorescence (apoptotic and necrotic cells) to the second fluorescence (total cells) was taken to evaluate percent death and plotted against UV dose.

Nuclear condensation and fragmentation. Cells were plated at 30,000 cells/chamber into four-well chamber slides. One cell type was plated per chamber slide with cells plated in the first and fourth chambers only. After allowing the cells to adhere and reach ~80% confluence, the medium was aspirated and replaced with 500 μl sterile PBS. One chamber (containing control cells) was covered with an opaque material to prevent irradiation while the chamber slide was irradiated at 2,000 J/m². The position of the control and irradiated cells was randomized to ensure that the analysis was done under blinded conditions. The PBS was aspirated and replaced with fresh medium. Cells were incubated at 37°C with 5% CO₂ for 6 h after which 37% formaldehyde was added to each chamber (final concentration: 3.75% formaldehyde). During fixation, the slides were centrifuged at 1,200 rpm for 15 min to ensure minimal loss of cells during the fixation procedure. The cells were fixed for an additional 15 min, rinsed with PBS, and allowed to air dry. At the time of analysis, 30 μM Hoechst no. 33342 in PBS were added to each chamber, slides were coverslipped, and a minimum of 300 cells/chamber were analyzed for nuclear condensation and fragmentation by fluorescence microscopy. The percentage of fragmented and condensed nuclei from three separate experiments was obtained with the experimenter blinded to cell type and irradiation dose.

CFTR Adenovirus Infection

To confirm that the mutated CFTR was responsible for the resistance to apoptosis, C127 ΔF508 cells were infected with a CFTR adenovirus in an attempt to restore susceptibility to death in these cells. C127 ΔF508 cells were plated at 5,000 cells/well in a 96-well tissue culture plate and allowed to adhere overnight. Preliminary studies with the β-galactosidase adenovirus (Ad2/CMVβgal-4, gift from Genzyme, Framingham, MA) showed that these cells were fairly resistant to infection by this adenovirus and required 20,000 infective units/cell for 95% infectivity. Cells were infected with the 20,000 infective units of CFTR adenovirus (Ad2/CFTR-16, gift from Genzyme) suspended in DMEM containing 2% heat-inactivated FBS for 60 min at room temperature, followed by 48 h at 37°C with 5% CO₂. After this infection, cells were UV irradiated and evaluated with YOPRO-1 as described above to determine susceptibility to death. β-Galactosidase adenovirus-infected and uninfected cells were used as negative controls.

RESULTS

Mouse Model of CF

Mouse models of CF are consistent in their susceptibility to gastrointestinal obstruction; therefore, studies were designed to determine whether a nonfunctional CFTR alters the kinetics of epithelial cell migration within the gastrointestinal tract and ultimately might underlie the pathophysiology within the intestine.

The rate of migration of BrdU-labeled cells up the crypt-villus axis is faster in CFTR /− mice than in their CFTR +/+ littermates (P = 0.048). By 48 h, the distance that the epithelial cells from the CFTR /− mice have migrated is significantly greater than the distance of migration in the CFTR +/+ mouse intestine (P = 0.018) (Fig. 1). Because epithelial cell migration from the crypts of Lieberkühn up the intestinal villi is the net result of proliferation and apoptosis within the crypts, both components were evaluated separately to see if either or both are altered as a result of the expression of the mutant CFTR. An increase in epithelial cell migration might occur if apoptosis is reduced and/or proliferation within the crypts of Lieberkühn is increased.

Levels of spontaneous apoptosis are not different between CFTR genotypes, with CFTR /− mice exhibiting 5.4 ± 4.6% apoptosis within the crypts and CFTR +/− and CFTR +/+ mice presenting 6 ± 2.5% and 2.1 ± 2.1% apoptosis, respectively (Fig. 2). To deter-
To determine whether the difference in epithelial cell migration is the result of differences in crypt cell proliferation, epithelial cells within the crypts of Lieberkühn were evaluated for expression of PCNA. A significantly greater percentage of epithelial cells from CFTR \textsuperscript{−/−} mice (49.4 ± 6.6\% ) express PCNA than those from animals expressing at least one copy of the functional gene (CFTR \textsuperscript{+/−}, 36.8 ± 2.7\%; \( P = 0.003\)) (Fig. 3).

The results of the animal studies contradict the previous cell culture findings presented by Gottlieb and Dosanjh (9), in which cells expressing the mutated CFTR were more resistant to apoptosis than cells expressing the wild-type CFTR. Therefore experiments were designed to determine whether the cell culture results were misleading.

Mouse mammary epithelial cells expressing a mutated CFTR (C127 \textdelta F508) are more resistant to death induced by 1,000 or 2,000 J/m\textsuperscript{2} UV irradiation than cells expressing the wild-type CFTR (Fig. 4). With UV irradiation doses at or above 4,000 J/m\textsuperscript{2}, a similar percentage of cell death was seen in both cell types. This is consistent with the previous findings (9) that C127 \textdelta F508 cells are more resistant than C127 wild-type cells to induction of apoptosis by etoposide and cycloheximide.

To determine whether the difference in susceptibility to UV irradiation seen in the C127 cell lines is a result of differences in expression of a functional CFTR, C127 \textdelta F508 cells were infected by an adenovirus expressing the wild-type CFTR before induction of apoptosis. Although cells overexpressing the mutant CFTR may have a low level of CFTR activity (2), expression of the wild-type CFTR in the C127 \textdelta F508 cells did not increase the susceptibility of these cells to death relative to cells infected with the β-galactosidase adenovirus or uninfected cells (Fig. 5). These data suggest that expression of the functional CFTR does
not make C127 cells more susceptible to apoptosis than cells expressing a mutated CFTR. Instead, the data suggest that the observed difference in apoptosis is unrelated to the CFTR but instead may have been a property acquired during the clonal selection process.

To further assess whether the original data were misleading, a second set of cell lines was tested to determine susceptibility to apoptosis based on expression of a functional or mutated CFTR. In the CFT1 cell lines, at 16 h after UV irradiation, cell death increased in a dose-dependent manner to a similar extent across cell lines (Fig. 6). Similar data were obtained 6 h after UV irradiation using nuclear condensation and fragmentation as markers of apoptosis (Fig. 7). In addition, UV irradiation of CFT1 cell lines resulted in a similar time course of DNA degradation (data not shown) with DNA laddering present at 3 h after UV irradiation in all four cell lines. Furthermore, cleavage of caspase 3 (PharMingen, La Jolla, CA) and caspase 9 (New England Biolabs, Beverly, MA) after UV irradiation was not different across cell lines (data not shown). Thus the current data from the C127 and CFT1 cell lines suggest that the CFTR does not play a role in apoptosis.

DISCUSSION

Many CF patients and all mouse models of CF have a common pathology in their susceptibility to gastrointestinal obstruction. In the normal intestine, epithelial cells line the villi of the absorptive surfaces. These cells are continuously regenerated by proliferation in the crypts of Lieberkühn, differentiation, and migration up the villi. At the tips of the villi, the cells undergo apoptosis and are shed into the lumen (11, 24). In addition, Martin et al. (18) have shown that both proliferation and apoptosis occur in the stem cells within the crypts and that apoptosis within the crypts is likely to be an important part of the homeostatic process for tissue maintenance. Under normal conditions, stem cell proliferation and epithelial sloughing are in equilibrium. As we have shown in the current study, epithelial cells in CFTR<sup>−/−</sup> mice migrate up the crypt-villus axis at a greater rate than in animals expressing a functional CFTR. The epithelial cells from CFTR<sup>−/−</sup>...
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mice migrated 27% farther in 48 h than those from the CFTR +/− mice. This difference in rate is due to differences in proliferation and not apoptosis within the crypts of Lieberkühn with ~12% more cells with proliferative capacity in the CFTR −/− mice than the CFTR +/− mice. Because villus length is not different between the genotypes (data not shown), the increased number of cells proliferating within the crypt and migrating up the villi would lead to an increase in the mass of cells that are sloughed into the gastrointestinal tract.

The increased proliferative capacity within the CFTR-null mice may be attributed to a cellular defect within the cells as a result of a nonfunctional CFTR, although the mechanism by which this might happen is unknown. Potentially, differences in intracellular pH might account for the enhanced proliferation in CFTR −/− cells. Cytosolic pH is thought to be one of the factors that control the rate of proliferation (8) with cytoplasmic alkalinization constituting a signal for mitogenesis (1, 22, 27). In addition, cells with higher intracellular pH are more likely to be in the S, G2, or M phases of the cell cycle, whereas cells with lower pH are more likely to be in G1 (19). Cells expressing a mutated CFTR have been reported (5, 6) to have an elevated cytosolic pH, and overexpression of the wild-type CFTR but not a mutant CFTR results in growth arrest (23). Thus a mutation in the CFTR may result in an elevated intracellular pH, which stimulates the cell to undergo proliferation more readily and thus increase the rate at which epithelial cells of the crypts of Lieberkühn proliferate and migrate up the intestinal villi.

All the mouse models of CF show evidence of inflammation and intestinal obstructions (10); thus the possibility that the stimulus to enhanced proliferation within the intestine of the CFTR −/− mice might be due to differences in inflammation cannot be ruled out. Proliferation in nasal polyps (12, 16) and epithelium of bronchial airways (15) has been shown to be increased in CF patients and has been attributed to airway inflammation. Chronic inflammation has been shown to be present in the CF intestine with increased luminal content of immune cell constituents, secretory immunoglobulins, and cytokines (25). Therefore, inflammation within the gastrointestinal tract potentially can be a stimulus to enhanced proliferation.

Initial studies by Gottlieb and Dosanjh (9) showed decreased susceptibility to apoptosis in cells expressing the mutated CFTR relative to cells expressing the wild-type CFTR. In this initial study, cycloheximide and etoposide were used to induce apoptosis. The CFTR is an anion channel that when mutated does not allow for chloride to be transported across the plasma membrane. Because this is a transport problem, one concern with the use of any drug or chemical to induce apoptosis is a difference in the ability of the cells to transport the drug or chemical across the cell membrane. To avoid this potential problem in the current studies, UV irradiation was utilized as the apoptotic stimulus. Cells expressing the mutated CFTR were more resistant to apoptosis induced by UV irradiation than cells expressing the wild-type CFTR, which is in accordance with the findings of Gottlieb and Dosanjh (9). However, one control that was lacking from the original set of experiments was the introduction of the wild-type CFTR into these resistant cell lines to restore susceptibility to apoptosis. In the current studies, UV irradiation was utilized as the apoptotic stimulus. Cells expressing the mutated CFTR were more resistant to apoptosis induced by UV irradiation than cells expressing the wild-type CFTR, which is in accordance with the findings of Gottlieb and Dosanjh (9). However, one control that was lacking from the original set of experiments was the introduction of the wild-type CFTR into these resistant cell lines to restore susceptibility to apoptosis. In the current study, infection of C127 ΔF08 cells with an adenovirus expressing the wild-type CFTR failed to restore susceptibility to apoptosis. β-galactosidase adenovirus-infected cells were found to be more susceptible to death than both the uninfected and infected CFTR cells. The reason behind this increased propensity toward death is not known. The β-galactosidase construct used in these experiments was the nuclear-targeted protein, which, because of its location of expression, may make the cells more susceptible to the DNA-damaging effect of...
UV irradiation. To confirm the findings from the C127 cells, an independent set of cell lines was used to determine whether expression of a mutated or wild-type CFTR results in differences in susceptibility to apoptosis. Cells with a mutated CFTR were found to be no different in their sensitivity to UV irradiation than cells expressing the wild-type CFTR. This result was consistent with the findings in the C127 cells, suggesting that the CFTR does not play a role in a cell’s susceptibility to apoptosis.

In the CFTR-null mice, epithelial cell kinetics within the gastrointestinal tract differ from mice expressing at least one copy of the functional CFTR. The altered proliferation within the crypts of Lieberkühn is not accompanied by changes in apoptosis but results in an increased rate of cell migration up the villus. The increased number of cells reaching the intestinal lumen might contribute to the viscosity of the luminal contents and increase the likelihood of intestinal blockage, one of the pathophysiological hallmarks of CF. If epithelial cell proliferation and turnover is increased in parallel in the CF lung and pancreas, this might contribute to the viscosity of the bronchial and pancreatic secretions and exacerbate the symptoms of CF.

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