Altered expression of RXFP1 receptor contributes to the inefficacy of relaxin-based anti-fibrotic treatments in systemic sclerosis

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ABSTRACT

Objective. Relaxin is a potent anti-fibrotic hormone that has been tested to ameliorate fibrosis in systemic sclerosis (SSc), but with controversial results. The aim of the study is to sequence relaxin receptor gene RXFP1 and to assess its mRNA expression and protein levels in the skin of SSc patients and healthy subjects.

Methods. Fibroblasts were isolated from unaffected/affected skin samples of (n=16) limited-cutaneous-SSc-(LcSSc) and from affected ones of (n=4) diffuse-cutaneous-SSc-(DcSSc) patients. Fibroblasts from healthy subjects were used as controls. Sequencing of exonic target regions of interest for RXFP1 gene was performed, coupled with mRNA transcript variant analysis. RXFP1 mRNA and protein levels were assessed by quantitative-real-time-PCR-(qRT-PCR) and by immunocytochemistry-(ICC). Alpha-smooth-muscle-actin-(α-SMA) synthesis induced by transforming-growth-factor-beta-1-(TGF-β1) stimulation was investigated in all fibroblasts with and without pre-treatment with serelaxin (a recombinant form of human relaxin-2 targeting the receptor RXFP1).

Results. Sequencing of RXFP1 gene showed no relevant mutations in all fibroblast populations. The analysis of mRNA transcripts revealed the presence of 13 different mRNA isoforms of RXFP1 (7 coding and 6 non-coding) upregulated in LcSSc/DcSSc-affected samples and not in LcSSc-unaffected and in healthy ones. On the contrary, ICC demonstrated the absence of RXFP1 in LcSSc/DcSSc-affected fibroblasts and the presence in LcSSc-unaffected and in healthy ones. To prove these findings, serelaxin pre-incubation was unable to counteract TGF-β1-driven upregulation of α-SMA in LcSSc/DcSSc-affected fibroblasts only, but not in LcSSc-unaffected and healthy ones.

Conclusion. The absence/altered expression of relaxin receptor RXFP1 in the affected fibroblasts of SSc patients could explain the inefficacy of relaxin-based anti-fibrotic treatments in the disease.

Introduction

Systemic sclerosis (scleroderma, SSc) is a rare, heterogeneous disease with a high associated mortality, characterised by progressive fibrosis of the skin and internal organs such as lungs, heart, kidneys and gastrointestinal tract, coupled to widespread macro- and microvascular alterations (1). SSc occurs in susceptible individuals as defined by genetic studies and it is stimulated by initialising events, although these initiating factors are poorly understood at present (2). The main abnormalities of the disease are related to the connective tissue, in which the excessive production of collagen and other extracellular matrix proteins are responsible of a progressive and irreversible fibrosis (3). There is no resolutive cure for SSc, although the existing therapeutic strategies are able to keep the symptoms under control (4). In fact, vascular alterations are well managed through the use of prostacyclin analogues in secondary Raynaud Phenomenon (RP) (5), and through the use of endothelin receptor antagonists (ERA) or phosphodiesterase type 5 inhibitors (PDE-5i) for pulmonary arterial hypertension (6, 7). Autoimmune response is also kept under control through the immunosuppressive drugs (8). Only fibrosis remains untreated, since there are no treatments able to strongly interfere with the development of this process (9).

In order to treat SSc-related fibrosis, relaxin (RLX), a dimeric peptide hor-
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mone belonging to the family of insulin-like peptides (10), has been used in the past to ameliorate the fibrotic process in SSc (11). In fact, besides functional association with reproduction, RLX plays other physiological roles, including the inhibition of collagen biosynthesis and/or the stimulation of collagen breakdown (12). However, one of the major mechanisms of the RLX anti-fibrotic effect is the antagonism of transforming-growth-factor-beta (TGF-β) signalling (13). In particular, in vitro studies showed that RLX binding to its receptor (RXFP1) results in the activation of the protein kinase ERK1, with downstream activation of endothelial nitric oxide synthase (eNOS) and increased nitric oxide production (14). This in turn activates soluble guanylyl cyclase and cGMP production (14). This pathway has been shown to inhibit the phosphorylation of Smad2/3, resulting in decreased TGF-β signalling (14). There are three different isoforms of RLX (H1, H2 and H3) in humans (15). Scientists agreed on the fact that H2-RLX is the isoform responsible for reducing organ fibrosis (16). These evidences justified the use of human H2-RLX in SSc. In fact, preclinical animal models showed encouraging results: H2-RLX treatment, or H2-RLX delivered by adenovirus, effectively reduced cardiac fibrosis induced by β-adrenergic stimulation in rodents (17), while RLX knockout mice developed pulmonary fibrosis that was reversed after RLX treatment (18). On the contrary, despite animal models were promising, clinical trial outcomes were not so convincing: although a phase II trial by RLX subcutaneous infusion was encouraging, a larger phase III clinical trial demonstrated that RLX neither improved the total skin thickness score or pulmonary function nor reduced the functional disability in SSc patients (19). Recently, scientists discovered that fibroblasts, keratinocytes, endothelial and smooth muscle cells from affected skin of SSc patients do not express the wild type form of the RLX receptor RXFP1 (20), and therefore the clinical inefficacy of RLX-based treatments could be partially explained.

Based on clinical and in vitro findings, the aim of the study is to investigate the reasons of the alteration of RLX receptor in the affected skin of SSc patients by sequencing the exonic target regions of interest for gene RXFP1 to evaluate the presence of possible mutations in fibroblasts derived from SSc patients and healthy subjects. As further confirmation, the potential of serelaxin (a recombinant form of human H2-RLX) to reduce fibrotic conditions was tested in vitro in SSc affected fibroblasts and in healthy fibroblasts stimulated with TGF-β.

Materials and methods

Patients enrollment, skin biopsy, fibroblast isolation and culture

Patients (n=16) affected by limited-cutaneous-SSc (LeSSc) and (n=4) by diffuse-cutaneous-SSc (DeSSc) in accordance with the description of LeRoy et al. (21) and who fulfilled the 2013 American College of Rheumatology/European League Against Rheumatism diagnostic criteria for SSc (22) were enrolled into the study. We performed skin biopsies by using a 3-mm punch on (mid-forearm) affected skin graded as 2 according to the modified Rodnan skin score (mRSS) (23). Unaffected areas (upper-arm) of skin from the same patients with LeSSc and from healthy subjects (n=10) were also taken. The unaffected skin was defined by clinical palpation (graded as 0 according to the mRSS) and by histological examination that excluded SSc-related lesions. All patients and healthy subjects gave their fully informed, voluntary, written consent according to the principles of the Declaration of Helsinki and in compliance with the ethics committee of the University of Siena, whose institutional review board approved the entire study protocol with code CEL_10465. The major demographic and clinical characteristics of the patients enrolled are shown in Table I.

Fibroblasts were isolated from skin specimens by enzymatic digestion. Briefly, explants were de-epidermised using a dispase solution (dispase activity 14 U/ml; Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37°C and then were dissolved into a collagenase III solution (2.4 U/ml; Sigma-Aldrich) for 30 minutes. Fibroblasts obtained were passaged twice and cultured at a density of 1x10⁵ cells per flask in Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with penicillin (100 U/ml; Sigma-Aldrich), streptomycin (100 µg/ml; Sigma-Aldrich), amphotericin B (0.25 µg/ml; Sigma-Aldrich), glutamine (2 mM; Sigma-Aldrich), and 10% fetal bovine serum (FBS, Sigma-Aldrich), followed by incubation at 37°C in an atmosphere of 5% CO₂ and 95% air until confluence (1 week) in 75-cm² flasks (BD Costar, Cambridge, MA, USA). Viability was estimated by trypan blue staining (Sigma-Aldrich). Fibroblasts were used at third passage (P3) for all the in vitro experiments.

mRNA isolation and quantitative real-time polymerase chain reaction

Fibroblasts were collected in TRIzol reagent (Sigma-Aldrich). Total RNA was extracted following the manufacturer’s instructions. The total RNA content of the samples was quantified by measuring the absorbance at 260 nm using an UltrSpec 2000 spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The RNA was then reverse-transcribed using a random hexamer MultiScribe enzyme (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reactions (qRT-PCR) were run in the StepOne Real-Time PCR System (Applied Biosystems) using TaqMan chemistry (Invitrogen, Carlsbad, CA, USA). Two microliters of complementary DNA in a final volume of 20 µl were amplified using the 20× Assays-on-Demand gene expression assay mix (Applied Biosystems). Specific primers were designed based on the reported sequences (National Center for Biotechnology Information Primer Bank): RXFP1: 5’-GGTGGAGCACACGCTTG-3’ (forward) and 5’-AAGAACCAGAGGCTAAGC-3’ (reverse). TaqMan probes, specific primers and ribosomal 18S, selected as a housekeeping gene, were purchased from Applied Biosystems. Messenger RNA (mRNA) levels were normalised to those of 18S.
Sequencing of RXFP1 gene

Genomic DNA was isolated from cultured fibroblasts at P3 obtained from both unaffected and affected skin of 20 SSc patients and from 10 healthy subjects using NucleoSpin Tissue Kit (Macherey-Nagel, Bethlehem, CA, USA). The libraries were prepared in accordance with the TruSeq Custom Amplicon Low Input Library Prep Reference Guide (Document no. 1000000002191 v.03, March 2016). Index tags were added by amplification to the extension-ligation product. The purified libraries were validated using the Agilent 4200 TapeStation to check size distribution. Coverage assessment was performed using the “coverageAnalysis” plug-in (v.4.2.1.4) that gives information about the amplicon read coverage. Sequencing of exonic target regions of interest (CDS+UTR) with variant analysis was completed with Illumina MiSeq Reporter v.2.5.1 for all samples. Reads were aligned against the entire reference genome (GRCh37/ hg19) using the Burrows-Wheeler Aligner (BWA), which aligns relatively short nucleotide sequences against a long reference sequence. Variants were called using the “variantCaller” plug-in (4.2.1.0). The plug-in identifies all nucleotide variants and provides read counts and frequencies for each variant, type of variant, homozygous or heterozygous state of the variant, gene name, numbers of forward (Allele Cov+) and reverse sequences (Allele Cov-) containing the variant, the homopolymer length including the variant identify and results of some bioinformatics prediction software. The integrative genomics viewer (IGV) tool was used for visualisation of amplicons sequences generated. Finally, the wANNOVAR software (http://wannovar.usc.edu/) was used to obtain detailed information about nomenclature, presence in dbSNP database and bioinformatic analysis of all variants called by the variantCaller plug-in.

Direct sequencing of the purified PCR products was performed in both directions (PE Big Dye Terminator Cycle Sequencing Kit) on an ABI Prism 310 genetic analyser (PE Applied Biosystems, Forest City, CA, USA) for 24 h. Final concentration and timing for TGFβ-1 and Serelaxin stimulations were chosen after dose- and time-response experiments performed before (Fig. 1).

Immunocytochemistry (ICC)

Fibroblasts were fixed in a 4% paraformaldehyde solution (Sigma-Aldrich) for 10 min at room temperature. Then, cells were washed 3 times with phosphate buffer solution (PBS; Sigma-Aldrich) and permeabilised with 0.1% Triton X-100 (Sigma-Aldrich) for 5 minutes and then blocked with 1% bovine serum albumin (BSA)/10% normal goat serum/0.3M glycine in 0.1% PBS-Tween (all from Sigma-Aldrich) for 1h. The cells were then incubated overnight at +4°C with rabbit monoclonal to alpha smooth muscle Actin (α-SMA)-Alexa Fluor® 488 conjugated (Abcam, Cambridge, UK) at 1:100 dilution in PBS containing 1% BSA. Cells were then washed twice in PBS and incubated for 10 min with 4',6-diamidino-2-phenylindole (DAPI) solution (diluted 1:1000 in PBS) (Abcam, Cambridge, USA). The libraries were prepared using the Agilent 4200 TapeStation to check size distribution. Coverage assessment was performed using the “coverageAnalysis” plug-in (v.4.2.1.4) that gives information about the amplicon read coverage. Sequencing of exonic target regions of interest (CDS+UTR) with variant analysis was completed with Illumina MiSeq Reporter v.2.5.1 for all samples. Reads were aligned against the entire reference genome (GRCh37/ hg19) using the Burrows-Wheeler Aligner (BWA), which aligns relatively short nucleotide sequences against a long reference sequence. Variants were called using the “variantCaller” plug-in (4.2.1.0). The plug-in identifies all nucleotide variants and provides read counts and frequencies for each variant, type of variant, homozygous or heterozygous state of the variant, gene name, numbers of forward (Allele Cov+) and reverse sequences (Allele Cov-) containing the variant, the homopolymer length including the variant identify and results of some bioinformatics prediction software. The integrative genomics viewer (IGV) tool was used for visualisation of amplicons sequences generated. Finally, the wANNOVAR software (http://wannovar.usc.edu/) was used to obtain detailed information about nomenclature, presence in dbSNP database and bioinformatic analysis of all variants called by the variantCaller plug-in.

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Table I. Demographic and clinical characteristics of patients at the time of biopsy collection.

<table>
<thead>
<tr>
<th>Patients Subset</th>
<th>Age (years)</th>
<th>Gender (M/F)</th>
<th>Disease duration (years)</th>
<th>ANA/ENA</th>
<th>mRSS</th>
<th>Organ involvement</th>
<th>Therapy</th>
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<tr>
<td>1</td>
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<td>12</td>
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<td>17 Lung</td>
<td>ERAs, Vitamin D</td>
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<td>2</td>
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<td>5</td>
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<tr>
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<tr>
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<td>15</td>
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<td>14 Lung</td>
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<tr>
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<td>11</td>
<td>Positive/Scl-70</td>
<td>17 Lung</td>
<td>ERAs, Prostanoids</td>
</tr>
</tbody>
</table>

ANA: antinuclear antibodies; ENA: extractable nuclear antigens; CenpB: Centromere protein B; mRSS: modified Rodnan skin score; ERAs: endothelin receptor antagonists; PPIs: proton pump inhibitors; Scl-70: topoisomerase I.
UK) before imaging. Negative control was performed using PBS/1% BSA buffer instead of primary antibody. The analysis of α-SMA expression was performed in each experimental condition evaluating intensity fluorescent levels in the same number of cells by light microscopy (magnification × 10) using Opera Phenix™ High-Content Screening System (PerkinElmer Inc, Waltham, MA, USA).

**Statistical analysis**
Data were evaluated by GraphPad Prism 7th software for Windows. Analysis of variance (ANOVA) was performed by Kruskal-Wallis test for multiple groups. Significance was set at \( p<0.05 \). Data are expressed as arbitrary units (AU) means ± standard deviations (SD) of three technical replicates for ICC and as gene of interest (GOI)/18S relative normalised ratio for qRT-PCR of three technical replicates.

**Results**
**RXFP1 mRNA expression**
Figure 2 shows the mRNA levels of RXFP1 in healthy and diseased fibroblasts. Overall, expression levels seem higher in the LcSSc/DcSSc-affected fibroblasts compared to LcSSc-unaffected ones \( (p<0.01) \) and to healthy ones \( (p<0.01) \). The single donor analysis demonstrated that the upregulation of mRNA levels in the affected fibroblasts is consistent among all the recruited donors (Fig. 1A). However, it is important to underline that primers used are not specific for the wild type transcript, but they do recognise all the other 12 transcript variants (6 coding and 6 non-coding). Therefore, the mRNA levels shown are the sum of the wild type and the other transcript variants.

**Sequencing of RXFP1 gene**
Variant analysis allowed the identification of three potentially pathogenic variants in three different SSc samples among which a stop gain variant: (RXFP1:NM_001253728:exon3:c.G205A:p.V69M), (RXFP1: NM_001253727:exon3:c.C211T:p.Q71X),(RXFP1:NM_001253728:exon10:c.C779G:p.P260R). The first two variants were only found in the affected LeSSc/DcSSc fibroblasts while the third variant was in both LeSSc-unaffected and LeSSc/ DcSSc-affected fibroblasts, leading to hypothesise a second hit process. None of the identified variants was confirmed by Sanger sequencing. Variants of unknown significance were also identified in the 3’UTR region in affected as well as in unaffected fibroblasts, but not confirmed by Sanger. All together these data allow to conclude that the RXFP1 mRNA level difference among affected and unaffected fibroblasts identified in SSc patients cannot be explained by variants in the genomic sequence which could play a role in modulating the expression level.

**Fibroblast treatment with serelaxin**
Figure 3 shows the immunocytochemistry of α-SMA with the relative densitometric values. In untreated conditions, α-SMA is overexpressed in LcSSc-affected fibroblasts compared to healthy dermal fibroblasts stimulated with three different concentrations of TGFβ-1 (5-10-20 ng/mL) at three different time points (4-16-24 hours). αSMA relative densitometric (mean ± SD) levels in healthy dermal fibroblasts untreated, stimulated with TGFβ-1 (5 ng/mL) only, with different concentrations of Serelaxin (1-10-100-500 ng/mL) and with Serelaxin (1-10-100-500 ng/mL) pre-incubations (1 h) before the addition of TGFβ-1 (5 ng/mL).
LcSSc-unaffected (p<0.01) and healthy ones (p<0.01). TGFβ-1 stimulation is responsible of α-SMA increase in healthy and LcSSc-unaffected fibroblasts, while no increase is detected in LcSSc-affected fibroblasts which seem not-responsive to TGFβ-1 stimulation because they already express high levels of α-SMA. Pre-treatment with serelaxin is able to counteract the TGFβ-1 driven upregulation of α-SMA in LcSSc-unaffected (p<0.05) and healthy (p<0.01) fibroblasts, but not in LcSSc-affected ones.

**Discussion**

The first *in vitro* studies related to the relationship between RLX and fibrosis were conducted on scleroderma: RLX treatment of healthy and diseased dermal fibroblasts resulted in a significant decrease in collagen secretion and increased collagen degradation (24, 25). Further evidences for a possible implication for RLX in SSc came from *in vivo* mouse models that showed reduced lung collagen and epithelial thickening (26), decreased cardiac fibrosis and myocardial apoptosis (27), reduced renal collagen (28) and reduced hepatic fibrosis (29). Unfortunately, the use of RLX for the treatment of SSc in humans generated ambiguous results. In fact, early studies generally demonstrated a benefit of partially purified porcine RLX for many SSc-related symptoms: in fact, in various tissues, RLX increases angiogenesis and cell motility through upregulation of vascular endothelial growth factor, matrix metalloproteases, and nitric oxide, thus counteracting the dysregulated angiogenesis in SSc (30). However, further
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studies were interrupted due to concerns about the safety of impure RLX preparations in humans (31, 32). In the last decade, despite it was demonstrated that serum RLX levels are higher in SSC patients compared to healthy subjects (33), placebo-controlled clinical trials were performed in SSC patients using highly pure recombinant RLX, but no significant improvement in skin thickness and in lung parameters were seen (34, 35). A possible reason for the failure may be the advanced degree of SSC in the patients enrolled in the trials (moderate to severe scleroderma), particularly in light of the finding that RLX treatment was not able to reverse advanced stages of fibrosis in the animal models (36). Additional explanations may include downregulation of RLX signalling at the doses used, or the development of RLX autoantibodies, which were detected at both low and high doses (34). Therefore, there are ongoing attempts to find an alternative to the use of pure recombinant RLX, such as synthetic peptides that act as RLX receptor RXFP1 agonists (37). However, due to a short half-life and low plasmatic stability, synthetic peptides require continuous delivery through intravenous injections or by an osmotic subcutaneous pump (38).

Therefore, the novel therapeutic strategies moved the attention on the RXFP1 receptor, rather than focusing on the RLX recombinant protein. Regarding the RXFP1 receptor, our previous study conducted in 2012 demonstrated that RXFP1 protein levels were absent in the fibroblasts derived from the lesional skin of LcSSc/DcSSc patients, but they were normally expressed in the fibroblasts derived from the unaffected skin portions of the same LcSSc patients in the same extent as those ones expressed in fibroblasts derived from healthy subjects (20).

On the contrary, in the present study we demonstrated that RXFP1 mRNA levels are higher in LcSSc/DcSSc-affected fibroblasts compared to LcSSc-unaffected ones and to healthy ones. Therefore, to try to explain this discrepancy between mRNA and protein levels in the same samples, we decided to sequence the exonic target regions of interest of the gene RXFP1 to exclude or identify the presence of possible mutations. To the best of our knowledge, this is the first study in which the sequencing of the RXFP1 gene has been performed in SSC patients: this allowed the identification of three potentially pathogenic variants that unfortunately were not confirmed by Sanger and therefore could not explain the genetic and phenotypic differences between affected-LcSSc and unaffected-LcSSc fibroblasts within the same patient and, more in general, between healthy and diseased LcSSc/DcSSc fibroblasts. The RXFP1 receptor belongs to the leucine-rich repeat (LRR)-containing G-protein coupled receptor (GPCR) LGR subfamily (39). Recent studies demonstrated that GPCR expression and intracellular trafficking are modulated by protein-protein interactions, and that alternative splice variants and naturally occurring mutant forms of the GPCRs can influence the expression and function of their respective wild type (WT) receptors (40, 41). In humans, three novel splice variants of the RXFP1 gene were identified in the decidua of fetal membranes (42). It has been demonstrated that all the splice variants decreased the 50% effective concentration of WT RXFP1, showing that they reduced the optimal efficacy of the WT receptor and exerting a dominant negative effect on its function by compromising the constitutive homodimerisation, maturation and cell surface delivery (43). This data could explain our findings, in which the total RXFP1 mRNA levels in affected-LcSSc fibroblasts are higher than those found in the unaffected-LcSSc ones of the same patients, suggesting the possible interference of the other splice variants in the maturation and cell membrane delivery of the wild type form of the receptor, resulting in the ineffectiveness of recombinant RLX to bind the receptor. In fact, the Seralaxin pre-treatment in affected-LcSSc/DcSSc fibroblasts is not able to counteract the pro-fibrotic effect of TGF-β in inducing αSMA, probably due to the lack of the RXFP1 receptor in the surface of those cells. Another possible explanation is that SSc fibroblasts, once differentiated into myofibroblasts, tend to keep the phenotype by making the fibrotic process irreversible (44). This is partially true because it has been recently demonstrated, at least in vitro, that TGF-β induced pro-fibrotic response of SSc fibroblasts can be abrogated by the simultaneous inhibition of c-Abl and Src kinases (45).

Conclusions
In conclusion, taken together, these data demonstrated that the altered RXFP1 receptor expression in SSC fibroblasts in not a consequence of RXFP1 gene mutations, but rather the resultant of post transcriptional modifications leading to a non-functional WT receptor. Therefore, researchers should pay attention to the present study before designing new clinical trials involving RLX and its receptor in SSC.

This study presents some limitations. The first limitation is technical and related to the absence of specific primers that can distinguish the WT RXFP1 mRNA transcript from the other isoforms. The second limitation is due to the lack of studies related to the influence of sex on the anti-fibrotic actions of relaxin (13), and the data collected in this study are mainly generated in women. The third limitation is related to the cohort studied enriched for limited disease. A more balanced cohort (with more diffuse patients) would have been more suitable since these patients have a prominent fibrotic component.

References
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