Full Paper

Predominant Promotion by Tacrolimus of Chondrogenic Differentiation to Proliferating Chondrocytes

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Abstract. Tacrolimus (FK506) has been used as a therapeutic drug beneficial for the treatment of rheumatoid arthritis in humans. In this study, we investigated the effects of FK506 on cellular differentiation in cultured chondrogenic cells. Culture with FK506 led to a significant and concentration-dependent increase in Alcian blue staining for matrix proteoglycan at 0.1 to 1,000 ng/ml, but not in alkaline phosphatase (ALP) activity, in ATDC5 cells, a mouse pre-chondrogenic cell line, cultured for 7 to 28 days, while the non-steroidal anti-inflammatory drug indomethacin significantly decreased Alcian blue staining in a concentration-dependent manner, without altering ALP activity. FK506 significantly increased the expression of mRNA for both type II and type X collagen, but not for osteopontin, in ATDC5 cells. Similar promotion was seen in chondrogenic differentiation in both mouse metatarsals and chondrocytes cultured with FK506. However, FK506 failed to significantly affect transcriptional activity of the reporter construct for either sry-type HMG box 9 (Sox9) or runt-related transcription factor-2 (Runx2), which are both transcription factors responsible for chondrocytic maturation as a master regulator. These results suggest that FK506 may predominantly promote cellular differentiation into proliferating chondrocytes through a mechanism not relevant to the transactivation by either Sox9 or Runx2 in chondrogenic cells.

Keywords: tacrolimus, ATDC5 cell, metatarsal, chondrocyte, type II collagen

Introduction

Tacrolimus (FK506) is an immunosuppressant with a wide spectrum of clinical usefulness for the treatment of a variety of diseases relevant to immunological abnormalities. For example, FK506 preferentially inhibits the production of inflammatory cytokines, such as tumor necrosis factor (TNF)-\(\alpha\) and interleukin (IL)-1\(\beta\), through suppression of T-cell activation in human peripheral blood mononuclear cells, without markedly affecting proliferation and differentiation of bone marrow cells (1). The immunosuppressant drugs FK506 and cyclosporine A inhibit the activity of the ubiquitously expressed Ca\(^{2+}\)/calmodulin–dependent protein phosphatase, calcineurin, through an interaction between the calcineurin subunit A and different immunophilins such as FK506-binding protein for FK506 and cyclophilins for cyclosporine A, which consequently leads to the inhibition of calcineurin-mediated dephosphorylation of the nuclear factor of activated T cells in T-lymphocyte cells (2, 3). The latter inflammatory cytokines including TNF-\(\alpha\) and IL-1\(\beta\) are believed to play a role crucial for the pathogenesis of rheumatoid arthritis (4, 5). In sera and synovial fluids of patients with rheumatoid arthritis, a marked increase is seen in IL-6 levels with concomitant production of the rheumatoid factor (6 – 8).

In patients with rheumatoid arthritis (9) and rats with adjuvant-induced arthritis (10), FK506 is shown to be more beneficial than methotrexate for the therapy of arthritis. In chondrogenic ATDC5 cells (3), chondrogenic differentiation is promoted by FK506, but not by cyclosporine A, in terms of matrix proteoglycan production and type II collagen (Col II) expression. The transcription factor sry-type HMG box 9 (Sox9) is inevitable for the mechanism underlying chondrogenesis...
as a master regulator (11), while in mice defective of
the osteoblastic master regulator runt-related transcrip-
tion factor-2 (Runx2), chondrogenesis is highly disturbed
in addition to osteoblastogenesis (12, 13). However,
little attention has been paid to the possible involvement
of these regulatory transcription factors for chondrocytic
differentiation, such as Sox9 and Runx2, in the
pharmacological usefulness of FK506 for the treatment
of rheumatoid arthritis to date.

In the present study, therefore, we have investigated
pharmacological profiles of FK506 in mechanisms
underlying the regulation of cellular differentiation
processes during chondrogenesis using mouse pre-
chondrogenic cell line ATDC5 cells, mouse chondral
metatarsals isolated before vascularization, and mouse
costal chondrocytes in culture.

Materials and Methods

Materials

The murine pre-chondrogenic ATDC5 cells were
purchased from RIKEN Cell Bank (Saitama). Pregnant
ddY mice were supplied by SANKYO LABO SERVICE
(Tokyo). Reporter plasmids for Runx2 and Sox9
were kindly donated by Dr. G. Karsenty (Baylor College
of Medicine, Houston, TX, USA) and Dr. B. de
Crombrugghe (Anderson Cancer Center, TX, USA),
respectively. A 4x48-p89-Luc construct consisted of 4
tandem copies of the Sox9 binding site element linked to
the minimal Col II gene promoter in the luciferase
reporter plasmid (14) was kindly provided by Dr. B. de
Crombrugghe, while p6OSE2-Luc containing 6 tandem
copies of the Runx2 binding site element linked to the
minimal osteocalcin gene promoter in the luciferase
reporter plasmid (15) is a generous gift from Dr. G.
Karsenty. FK506 (m.w. 822.03) was kindly given by
Astellas Pharma, Inc. (Tsukuba). Anti-digoxigenin
(DIG)-AP Fab fragments and CDP star were obtained
from Roche Diagnostics (Mannheim, Germany). Dual
Luciferase Assay System was provided by Promega
(Madison, WI, USA). Lipofectamine and Plus reagent
were purchased from Invitrogen (San Diego, CA, USA).
Other chemicals used were all of the highest purity
commercially available.

Procedures for ATDC5 cell culture

ATDC5 cells were cultured at a density of 1.0 × 10^4
cells/cm^2 in a 1:1 mixture of Dulbecco’s modified
eagle’s medium and Ham’s F-12 medium (DMEM/F12)
containing 5% fetal bovine serum (FBS), followed by
replacement with DMEM/F12 containing 5% FBS,
10 μg/ml transferrin, 3 × 10^-8 M sodium selenite, and
10 μg/ml bovine insulin, for the promotion of cell
differentiation, and subsequent culture at 37°C for
different periods up to 28 days under 5% CO2 as
described previously (16). Culture medium was changed
every 2 – 3 days.

Determination of mRNA expression by Northern blotting

Total RNA was extracted from cultured cells as
described previously (17). The extracted RNA was
resolved on a 1% formaldehyde/agarose gel and trans-
ferred onto positively charged nylon transfer membranes.
After fixing RNA to the blot by UV crosslinking, the
blotted membranes were prehybridized at 68°C for 1 h
and subsequently hybridized with denatured DIG-
labeled cRNA probes of Col II, type X collagen (Col X),
and osteopontin (OPN) at 68°C for 16 h. The membranes
were then washed and incubated with anti–DIG-AP-Fab
fragments, followed by incubation with CDP-star. The
membranes were then exposed to X-ray films for
appropriate periods to detect chemiluminescence for
subsequent quantification.

Determination of matrix proteoglycan accumulation
by Alcian blue staining

Cells were placed in 24-well plates and cultured for
different periods up to 28 days. Cells were rinsed with
PBS twice and then stained for 30 min with 1% Alcian
blue 8GS dissolved in 3% acetic acid. Cells were washed
with 3% acetic acid for 30 s 3 times, and then
photographed with an IMT-2-21 dissecting microscope
(Olympus, Tokyo). Staining intensity was finally
quantified using the computer program Scion image as
described previously (18).

Determination of chondrocytic maturation by alkaline
phosphatase (ALP) activity

On the day of the experiments, cells were harvested,
washed twice with cold phosphate-buffered saline, and
then sonicated in 0.1 M Tris-HCl buffer (pH 7.5)
containing 0.1% (v/v) Triton X-100. The assay buffer
composed of 0.05 M 2-amino-2-methylpropanol, 2 mM
MgCl2, and 10 mM p-nitrophenylphosphoric acid was
added at a volume of 200 μl into 10 μl of cell suspensions,
followed by reaction for 30 min at 37°C and
subsequent immediate determination of absorbance of
p-nitrophenol at 405 nm. Protein concentration was
determined with a Protein Assay Kit (Bio-Rad, Hercules,
CA, USA), and ALP activity was standardized on the
basis of both cellular protein content and incubation
time for the representation as pmol·min^-1·mg^-1 protein.
The activity was linearly increased with incubation time
up to 60 min under our experimental conditions, but
variable from experiment to experiment partly due to the
different batches of FBS used for culture.
**Procedures for embryonic metatarsal rudiment organ culture**

The three central metatarsal rudiments were isolated from ddY mouse embryos at 15.5 days post-gestation. Each of the middle three metatarsals was placed in a well of a 24-well plate containing 1 ml of organ culture medium: MEM supplemented with 0.05 mg/ml ascorbic acid, 1 mM β-glycerophosphate, and 0.25% FBS. These explants were grown at 37°C in a humidified 5% CO₂ incubator for a period up to 5 days. On the day of the experiments, the total area of each bone rudiment and the area of each middle mineralized part determined by Alizarin Red staining were individually measured for subsequent calculation of the mineralization ratio based on the area under the Olympus IMT-2-21 dissecting microscope. Similar results were invariably obtained with the calculation of mineralization ratios based on the lengths in place of the areas.

**Procedures for primary culture of mouse costal chondrocytes**

Cartilages were isolated from 1-day-old Std-ddY mice, followed by incubation at 37°C for 2 h in DMEM containing 0.25% collagenase and subsequent digestion of pellets obtained after centrifugation at 250 × g for 5 min with DMEM containing 0.25% collagenase for 3 h. Samples were then centrifuged at 250 × g for 5 min for collection of resultant pellets, followed by suspension in DMEM containing 10% FBS. Culture medium was changed to DMEM containing 10% FBS and 50 μg/ml ascorbic acid for subsequent culturing for different periods. Culture medium was changed every 2 days.

**Determination of gene transactivation by luciferase assay**

Reporter vectors were co-transfected with a SV40-Renilla luciferase construct by the lipofection methods using Lipofectamine/Plus Reagent in either the presence or absence of expression vectors of Sox9 and Runx2, which are both essential transcription factors required for chondrocyte maturation, into ATDC5 cells cultured in DMEM/F12 containing 5% FBS. Culture medium was changed to DMEM/F12 containing 5% FBS 1 h later, followed by further culture for an additional 20 h and subsequent medium change with DMEM/F12 containing 0.5% FBS. Four hours after medium change, cells were exposed to FK506 at different concentrations for 24 h, followed by cell harvest for determination of luciferase activity using specific substrates in a lumino-meter according to the Dual Luciferase Assay System. Transfection efficiency was normalized by determining the activity of Renilla luciferase. Approximately 20% – 30% of cells expressed green fluorescent protein (GFP) in ATDC5 cells transfected with the EGFP-C2 plasmid under the transfection condition used.

**Determination of mRNA expression by in situ hybridization**

Metatarsals were fixed with 10% formalin neutral buffer solution, followed by decalcification with 20% EDTA for 2 days and subsequent immersion in 30% sucrose overnight at 4°C. Metatarsals were then dissected for frozen sections with a thickness of 10 μm in a cryostat. In situ hybridization was carried out as described previously (18). In brief, sections were fixed with 4% paraformaldehyde freshly prepared in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature, followed by washing and treatment with Proteinase K. Sections were subjected to acetylation in 0.1 M triethanolamine/0.25% acetic anhydride, followed by washing and stepwise dehydration with ethanol for subsequent hybridization with a DIG-labeled cRNA probe at 65°C for 16 h. The area of positive signals was finally quantified using the computer program Scion image.

**Data analysis**

Results are all expressed as the mean ± S.E.M., and the statistical significance was determined by the two-tailed and unpaired Student’s t-test or one-way analysis of variance ANOVA with the Bonferroni/Dunnett post hoc test.

**Results**

**Effect of indomethacin on chondrocytic maturation in ATDC5 cells**

In order to evaluate chondrogenesis in vitro, ATDC5 cells were cultured in either the presence or absence of insulin required for cellular differentiation, followed by staining with Alcian blue to determine matrix proteoglycan accumulation. In the presence of insulin, the intensity of Alcian blue staining was drastically increased in proportion to the culture duration from 7 to 28 days (data not shown). An attempt was made to determine whether an anti-inflammatory drug used for the treatment of rheumatoid arthritis for years has particular pharmacological actions on chondrogenesis in vitro. For this purpose, cells were cultured in the presence of insulin together with the non-steroidal anti-inflammatory drug indomethacin at different concentrations. Sustained exposure to indomethacin led to a
significant decrease in Alcian blue staining in a concentration-dependent manner at concentrations of 0.1 – 1,000 ng/ml in cells cultured for 7 – 28 days (Fig. 2A). Although Alcian blue staining was drastically weakened in cells cultured in the absence of insulin throughout the culture period, FK506 was again effective in significantly increasing the staining intensity at concentrations over 10 ng/ml even in the absence of insulin (Fig. 2B). In cells cultured for 21 days under these conditions, in particular, FK506 almost doubled

Effect of FK506 on chondrocytic maturation in ATDC5 cells

Sustained exposure to FK506 led to a marked further increase in Alcian blue staining at a concentration range of 0.1 – 1,000 ng/ml in cells cultured in the presence of insulin for 7 – 28 days. Repetition and quantification clearly revealed that FK506 significantly promoted the temporal increases in Alcian blue staining at concentrations of 10 – 1,000 ng/ml throughout the culture period (Fig. 2A). Although Alcian blue staining was drastically weakened in cells cultured in the absence of insulin throughout the culture period, FK506 was again effective in significantly increasing the staining intensity at concentrations over 10 ng/ml even in the absence of insulin (Fig. 2B). In cells cultured for 21 days under these conditions, in particular, FK506 almost doubled...
Irrespective of the presence of insulin in culture medium, ALP activity was more than doubled during the culture from 7 to 14 days with a plateau thereafter up to 28 days (Fig. 3). However, sustained exposure to FK506 induced no significant changes in ALP activity in cells cultured for 7–28 days in either the presence (A) or absence (B) of insulin at concentrations effective in significantly increasing Alcian blue staining. Therefore, FK506 would modulate in vitro chondrogenesis through a mechanism different from that for indomethacin. Subsequent experiments were done in ATDC5 cells cultured in the presence of insulin.

**Effects of FK506 on chondrocytic marker gene expression in ATDC5 cells**

In order to evaluate the mechanism underlying the increased Alcian blue staining, cells were cultured with 1 μg/ml FK506 for different periods, followed by determination of mRNA expression of several chondrogenic markers on Northern blotting. As shown in Fig. 4A, a marked increase was invariably seen in
mRNA expression of the proliferating chondrocyte marker Col II, the hypertrophic chondrocyte marker Col X, and the calcified chondrocyte marker OPN in proportion to the culture period of 7 – 21 days. Sustained exposure to FK506 led to a marked increase in mRNA expression of both Col II and Col X, but not of OPN, in cells cultured for 14 days. Repetition and quantification of these experiments clearly revealed that FK506 significantly promoted the temporal increase in mRNA expression of both Col II and Col X in cells cultured for 7 – 14 days without significantly affecting that of OPN (Fig. 4B). Accordingly, FK506 could promote the rate of in vitro cellular differentiation from pre-chondrocytes to proliferating and hypertrophic chondrocytes, without altering differentiation from hypertrophic to calcified chondrocytes.

Effects of FK506 on chondrocytic maturation in metatarsals ex vivo

Metatarsals before vascularization were isolated from embryonic mice at 15.5 days after gestation, followed by organotypic culture for different periods up to 5 days in either the presence or absence of FK506 at different concentrations. In proportion to increasing culture days, marked increases were seen in both the total area of the cartilage rudiment and the area of the middle mineralized part in cultured metatarsals (data not shown). In metatarsals cultured with FK506 for 5 days, a marked increase was seen in the non-mineralization area with the mineralization area being unchanged (Fig. 5A). Repetition and quantification of these experiments revealed that FK506 significantly increased the total (Fig. 5B) and non-mineralization (Fig. 5C) areas without markedly affecting the mineralization area (Fig. 5D).

Metatarsals were then fixed with formalin for subsequent in situ hybridization analysis on Col II and Col X mRNA expression. Col II mRNA expression was highly localized in proliferating to prehypertrophic chondrocytic layers (Fig. 6A), while Col X mRNA was preferentially expressed by hypertrophic chondrocytes (Fig. 6B). Culture with FK506 led to a marked increase in the area of chondrocytes expressing Col II or Col X. However, repetition and quantification showed that FK506 significantly increased the area of Col II–positive cells without significantly affecting that of Col X–positive cells in cultured metatarsals. In line with the aforementioned expression profiles of marker genes in ATDC5 cells in vitro, accordingly, FK506 would predominantly promote cellular differentiation from pre-chondrocytes to proliferating chondrocytes, without
markedly altering that from proliferating to hypertrophic chondrocytes or from hypertrophic to calcified chondrocytes, in cultured metatarsals ex vivo.

**Effect of FK506 on chondrocytic maturation in primary cultured chondrocytes**

In order to confirm the promotion by FK506, costal chondrocytes were isolated from mouse ribs, followed by culture with FK506 for 3 – 28 days and subsequent determination of several marker genes. In contrast to ATDC5 cells, mRNA expression was gradually decreased for both Col II and Col X in proportion to increasing culture periods, with an increase in OPN mRNA expression, in primary cultured mouse chondrocytes (Fig. 7A). Sustained exposure to 1 μg/ml FK506 led to a significant increase in mRNA expression of
both Col II and Col X in chondrocytes cultured for 3 and 7 days, respectively (Fig. 7B). However, no significant changes were seen in OPN mRNA expression throughout the culture period up to 28 days.

Effects of FK506 on transcriptional activity in chondrocytic cells

An attempt was made to elucidate the possible involvement of transactivation mediated by particular transcription factors essential for chondrocytic differentiation in the mechanism underlying the promotion by FK506. These included Sox9 family members required for differentiation from pre-chondrocytes to proliferating chondrocytes and Runx2 needed for differentiation from proliferating to hypertrophic chondrocytes, respectively. For this purpose, mouse chondrocytes were cultured with 1 μg/ml FK506 for a period of 3 – 7 days, followed by quantitative determination of mRNA expression by RT-PCR analysis. FK506 failed to significantly affect mRNA expression of Sox5, Sox6, Sox9, or Runx2 in chondrocytes cultured for 3 – 7 days (Fig. 8A).

Transactivation was then evaluated in the reporter assay using the plasmid 4x48-p89-Luc, which consisted of 4 tandem copies of the Sox9 binding site element linked to the minimal Col II gene promoter in the luciferase reporter plasmid. The 4x48-p89-Luc plasmid was transiently transfected in either the presence or absence of Sox9 expression vector into ATDC5 cells for 24 h, followed by exposure to FK506 at different concentrations for 24 h and subsequent determination of luciferase activity. Although luciferase activity was drastically increased after the transfection of a Sox9 expression vector, FK506 failed to significantly affect the transcriptional activity determined by luciferase activity at the concentrations effective in significantly increasing Col II mRNA expression irrespective of the co-introduction of Sox9 (Fig. 8B). Similarly, transactivation by Runx2 was evaluated using the p6OSE2-Luc plasmid containing 6 tandem copies of the Runx2 binding site element linked to the minimal osteocalcin gene promoter in the luciferase reporter construct. Co-transfection with the Runx2 expression vector more than doubled luciferase activity, while exposure to FK506 did not significantly affect luciferase activity at the concentrations used independent of the introduction of Runx2 (Fig. 8C).

Discussion

The data presented in this study give rise to the idea that FK506 may preferentially promote cellular differentiation from pre-chondrogenic cells to proliferating chondrocytes expressing Col II toward consequent facilitation of differentiation from proliferating to hypertrophic chondrocytes expressing Col X as summarized in Fig. 9. The possibility that FK506 may directly promote differentiation from proliferating to hypertrophic chondrocytes in addition to the aforementioned promotion from pre-chondrocytes to proliferating chondrocytes is thus rather inconceivable. The transcription
factor Sox9 is a nuclear protein essentially required for converting mesenchymal stem cells condensed in bone marrows into chondrocytes, in association with other family members such as Sox5 and Sox6 (11). Both Sox5 and Sox6 are essential for promotion of transactivation of particular genes encoding cartilage-specific extracellular matrix, including Col II and Col X, as a heteromeric protein complex with Sox9, but defective of the DNA binding domain in contrast to Sox9 (11). In humans, mutations in a single allele of Sox9 result in a severe skeletal malformation syndrome such as campomelic dysplasia due to the haplo-insufficiency of Sox9 (19, 20). Furthermore, Sox9 heterozygous mutant mice exhibit similar skeletal anomalies as seen in campomelic dysplasia patients (21). In contrast, mutant mice overexpressing Sox9 show the delay of endochondral bone formation (14). These previous findings clearly indicate that Sox9 expression is tightly regulated during chondrogenesis. The expression of Sox9 is at least in part regulated by the ubiquitin-proteasome system in chondrocytes (14, 22).

Although the master regulator of osteoblastogenesis, Runx2, is weakly expressed by chondrocytes (23), maturational disturbance is seen in chondrogenesis in Runx2-deficient mice (12, 13). By contrast, Runx2 is highly expressed by cells in the perichondrium throughout skeletogenesis (15). Removal of the perichondrium leads to increased proliferation and promoted differentiation to hypertrophic chondrocytes in chicken tibial organotypic cultures (24, 25). Runx2 is recently shown to inhibit proliferation and hypertrophy of chondrocytes in a manner dependent on the expression in the perichondrium in growth plate cartilage (26). In the present study using ATDC5 cells defective of the perichondrium, however, FK506 promoted Alcian blue staining through a mechanism not related to transcriptional activity of either Sox9 or Runx2. As the chondrocytic master regulator Sox9 is directly responsible for Col II gene expression, the present findings are suggestive of an alternative mechanism other than transactivation mediated by Sox9 for the promoted expression of Col II by FK506 during chondrogenesis. The possible involvement of transactivation by Runx2 in mechanisms underlying the promotion by FK506 is also inconceivable by taking into consideration the findings obtained in reporter plasmid assays.

The possibility that FK506 may promote the expression of particular clock genes endowed to modulate chondrogenesis is not ruled out so far. For instance, parathyroid hormone induces retarded chondrogenesis through transient upregulation of the clock gene Period1 in ATDC5 cells (16). An alternative but hitherto unidentified speculation is that FK506 could alter expression profiles of particular membrane molecules responsible for negative and/or positive modulation of endocrine and paracrine signaling mechanisms for chondrocytic differentiation. These include signals mediated by parathyroid hormone (11), parathyroid hormone–related peptide (11, 27), Indian hedgehog (28, 29), leptin (30, 31), and glutamate (32–34). The possible correlation between promoted chondrogenesis and pharmacological usefulness for rheumatoid arthritis, however, is not clarified with FK506. The metatarsal culture system used in this study is well known to be an ex vivo model of chondrocyte developmental maturation, which undergoes normal patterns of both prolifer-
tion and differentiation for at least 5 days in culture (35). This allowed us to investigate the effects of FK506 in an experimental system where chondrocytes retain their normal architecture of round and columnar prehypertrophic and hypertrophic zones. The absence of mesenchymal cells, other than chondrocytes, from any of the cell layers, except those in bone collar or perichondrium of cultured metatarsals, is already confirmed by in situ hybridization in our previous study (36).

Recently, FK506 was shown to reduce adjuvant-induced arthritis in rats in vivo (10) and to promote cellular differentiation of ATDC5 cells cultured in the absence of insulin-like growth factor in vitro (3). In the present study, FK506 is for the first time demonstrated to promote the temporal expression profile of Col II and Col X mRNA with concomitant facilitation of matrix proteoglycan accumulation in ATDC5 cells cultured with insulin, in addition to promoted mRNA expression of both Col II and Col X in primary cultured mouse chondrocytes. The accelerated matrix proteoglycan accumulation could account for the usefulness of FK506 rather than indomethacin for the treatment of rheumatoid arthritis in humans. Both drugs would share common immunosuppressive effects but elicit different pharmacological actions on chondrocytic differentiation. Although retarded chondrogenesis could counteract the beneficial immunosuppression by indomethacin during the treatment of rheumatoid arthritis, indomethacin was also shown to inhibit nitric oxide–induced apoptotic cell death and differentiation arrest in a manner independent of cyclooxygenase activity in articular chondrocytes (37). The exact mechanism as well as the involvement of calcineurin for the promotion by FK506 of in vitro chondrogenesis, therefore, remains to be elucidated in future studies.

It thus appears that FK506 may promote chondrogenesis irrespective of the presence of insulin through a mechanism not related to transactivation of either Sox9 or Runx2 in chondrogenic ATDC5 cells. Predominant promotion by FK506 of Col II expression could be at least in part responsible for regeneration of extracellular matrix proteoglycan beneficial for the pain relief in particular articular cartilage in patients suffering from arthritis.

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