Cortisol Increases Interstitial Collagenase Expression in Osteoblasts by Post-transcriptional Mechanisms*

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Glucocorticoids regulate both bone formation and bone resorption. In osteoblasts, they inhibit type I collagen synthesis; however, there is limited information about their effects on interstitial collagenase, the enzyme that degrades type I collagen. We used primary cultures of osteoblast-enriched cells from fetal rat calvariae (Ob cells) to study the effects of cortisol on collagenase expression. Northern blot analysis showed that cortisol increased collagenase transcript levels in a dose- and time-dependent manner, which was paralleled by an increase in immunoreactive metalloproteinase in the culture medium. Cortisol increased the half-life of collagenase mRNA from 6 to 12 h in transcription-arrested Ob cells. In contrast, cortisol modestly decreased collagenase gene transcription after 24 h of treatment. The up-regulation of collagenase by cortisol is osteoblast-specific, since the glucocorticoid decreased phorbol 12-myristate 13-acetate-induced collagenase mRNA expression in rat fibroblasts, a result that agrees with other studies of collagenase gene regulation in fibroblastic cells.

In conclusion, cortisol increases interstitial collagenase transcript levels by post-transcriptional mechanisms in osteoblastic cells. Our data demonstrate that glucocorticoids regulate collagenase gene expression in a novel tissue-specific manner, further highlighting the differences in gene regulation between osteoblastic and fibroblastic cells.

Glucocorticoids have marked effects on bone metabolism, regulating bone formation and bone resorption (1). In vitro studies have shown that glucocorticoids have complex effects on osteoblast gene expression, and these effects are dependent on the stage of osteoblast growth and differentiation and on the cell model and culture conditions used (2). Glucocorticoids induce cells of the osteoblastic lineage to differentiate into mature cells expressing the osteoblastic phenotype (3-5). However, their inhibitory actions on multiple aspects of osteoblastic function have a major impact on bone mass. Glucocorticoids inhibit cell replication, depleting a cell population capable of synthesizing bone collagen, and they inhibit α1(I) collagen expression by transcriptional and post-transcriptional mechanisms (6, 7).

Additionally, glucocorticoids regulate bone collagen degradation, although the effects have varied with the models and culture conditions used (1). Recently, glucocorticoids were shown to increase interstitial collagenase (matrix metalloproteinase 1) transcript levels in osteoblast cultures (8). This effect is observed only in osteoblasts; indeed, glucocorticoids inhibit transcription of interstitial collagenase in nonskeletal fibroblasts (9-12). This suggests novel tissue-specific regulation of collagenase by glucocorticoids. Matrix metalloproteinases and their inhibitors are considered active participants in the degradation of osteoid, and interstitial collagens are the only proteases known to initiate the degradation of type I collagen at neutral pH (13). Thus, up-regulation of osteoblast collagenase may play a role in the bone loss associated with pathological glucocorticoid excess. However, the mechanisms by which glucocorticoids stimulate the expression of collagenase in osteoblasts are unknown.

Investigations of the regulation of interstitial collagenase are critical for understanding the role glucocorticoids play in bone remodeling. Since glucocorticoids differentially regulate collagenase transcripts in osteoblasts and fibroblasts, determining the mechanisms by which this occurs will contribute to our knowledge of cell type-specific gene regulation. In this study, we examined the mechanisms of action of cortisol on interstitial collagenase synthesis in cultures of osteoblast-enriched cells from fetal rat calvariae (Ob cells).

MATERIALS AND METHODS

Culture Technique—The culture method used was described in detail previously (14). Parietal bones were obtained from 22-day-old fetal rats immediately after the mothers were sacrificed by blunt trauma to the nuchal area. This project was approved by the Institutional Animal Care and Use Committee of Saint Francis Hospital and Medical Center. Cells were obtained by five sequential digestions of the parietal bone, using bacterial collagenase (CLS II, Worthington Biochemical, Freehold, NJ). Cell populations harvested from the third to the fifth digestions were cultured as a pool at a density of ~10,000 cells/cm² and have been previously shown to have osteoblastic characteristics (14). In one set of experiments, fibroblastic cells derived from collagenase digestion of skin from 22-day-old fetal rats were cultured and tested (9). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with nonessential amino acids (Life Technologies, Inc.) and 10% fetal bovine serum (HyClone, Logan, UT) (14). Except for the nuclear run-off assay, primary cultures of Ob cells were used in all experiments, whereas skin fibroblasts were used after three or four passages. At confluence the cells were rinsed and transferred to serum-free medium for 24 h and then exposed to test or control medium in the absence of serum for 2-48 h. In experiments lasting longer than 24 h, the medium...
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was replaced with freshly prepared test and control solutions. Cortisol, cycloheximide, phorbol 12-myristate 13-acetate (PMA) and 5,6-dichloro-
robemidazole riboside (DRB) (all from Sigma) were dissolved in
absolute ethanol, and at dilutions of <1:10,000 an equal amount of
ehad was added to control cultures.

Northern Blot Analysis—Total cellular RNA was isolated with gua-
nidinium thiocyanate, at acid pH, followed by a phenol-chloroform (Sigma)
extraction as described (15). Equal amounts of RNA were denatured
and subjected to electrophoresis through formaldehyde-agarose gels
(16), and the RNA was blotted onto Gene Screen Plus as directed by the
manufacturer (DuPont). Restriction fragments containing a 2.6-kilo-
base (kb) rat interstitial collagenase cDNA (17) kindly provided by C.
Quinn (St. Louis, MO) and an 800-bp base pair rat glyceraldehyde-3-
phosphate dehydrogenase cDNA (18), (kindly provided by R. Wu,
Ithaca, NY) were labeled with [α-32P]dCTP (specific activity 3,000 Ci/
mmol; DuPont), by random-primed second strand synthesis (Prime-A-
Gene, Promega, Madison, WI) (19). Hybridizations were carried out at
42°C in 50% formamide, 5 × SSPE (750 mM sodium chloride, 50 mM
sodium phosphate, 5 mM EDTA), 5 × Denhardt’s solution, and 0.4%
SDS (Sigma). Post-hybridization washes were performed at 65°C in 1 ×
SSC (150 mM sodium chloride, 15 mM sodium citrate) and 0.1% SDS.

Autoradiograms were analyzed by densitometry, and collagenase RNA
levels were normalized to those of glyceraldehyde-3-phosphate dehy-
drogenase. Northern analyses shown are representative of two or more
cultures.

Nuclear Run-off Assay—Subconfluent cultures of Ob cells were
treated with trypsin, harvested, subcultured at a 1:6 dilution, and
allowed to grow to confluence as described previously (20). These first
passage Ob cells retain the osteoblastic phenotype and respond to
cortisol in a manner similar to that of primary cultures (7). At conflu-
ence, cells were serum-deprived and treated for 2–24 h, and nuclei were
isolated by Dounce homogenization in a Tris-Cl buffer containing 0.5%
Nonidet P-40. Nascent transcripts were labeled by incubation of nuclei
in a reaction buffer containing 500 μM each ATP, GTP, and CTP, 150
units of RNASin (Promega), and 250 μCi of [32P]UTP (3000 Ci/mM,
DuPont) (modified from Ref. 21). RNA was isolated by treatment with
DNase I and proteinase K, followed by ethanol precipitation. Linearized
plasmid vector DNA containing 1 μg of cDNA was immobilized onto Gene-
Screen Plus by slot blotting according to the manufacturer’s directions
(DuPont). The plasmid vector pGL2-Basic (Promega) was used as a
control for nonspecific hybridization, and cDNA for rat α1(I) collagen
was used as a positive control (22). Equal counts per minute of [32P]
RNA from each sample were hybridized to cDNA using the same
conditions as for Northern blot analysis and were visualized by au-
toradiography. The nuclear run-off assay shown is representative of three
experiments.

Transient Transfections and Reporter Gene Assays—A NotI/BglII rat
genic DNA fragment containing 2.1 kb of a recently cloned rat
interstitial collagenase promoter2 was used to drive expression of the
luciferase gene in the vector pGL2-Basic. A construct containing the
cytomegalovirus promoter driven β-galactosidase gene (pCMV-β-Gal,
Clontech, Palo Alto, CA) was used to control for transfection efficiency.
Ob cells were cultured to approximately 80% confluence and were
transiently co-transfected with the collagenase promoter-luciferase con-
pact and pCMV-β-Gal by calcium phosphate/DNA coprecipitation,
followed by glycerol shock (23). Cells were harvested, se-
run-deprived, and treated for 6 h with control medium or 1 μM cortisol.
Cell lysates were made using 1 × Reporter Lysis Buffer (Promega), and
luciferase activity was measured by injecting luciferase assay reagent
(Promega) into a portion of the cell lysate and counting photons using an
Optochem luminometer (MGM Instruments, Hamden, CT) according
to the manufacturer’s instructions. β-galactosidase activity was meas-
ured by incubating cell lysates with the chromogenic substrate for β-
galactosidase 3-(4-methoxyphenyl)-3-2′-(4-methoxyphenyl)-3′-(3-4-
methoxyphenyl)-2′,2-dicyclo-
(3.3.1,1.3)decyl-1)-phenyl-β-D-galactopyranoside (Galacton; Tropix,
Bedford, MA) with the modifications described (24). Luciferase activity
was normalized to β-galactosidase activity to control for slight varia-
tions in transfection efficiency.

Collagenase Immunoblot assay—Immunoreactive collagenase was deter-
mined by an enzyme-linked immunosorbant assay, as described previ-
ously (25, 27). Briefly, samples were incubated with antisera to rat
interstitial collagenase and added to the wells of microtiter plates
coated with 0.25 μg pure rat uterine interstitial collagenase. The plates
were incubated overnight at 4°C and washed; and the surface-bound
antibody was quantitated by the addition of goat antirabbit γ-globulin
conjugated with alkaline phosphatase. The bound alkaline phospha-
tase-dependent formation of p-nitrophenol was then determined spec-
trophotometrically. This assay detects collagenase at concentrations as
low as 1 ng/ml of culture medium.

Western Immunoblot Analysis—Ob cells were cultured as described
previously and media were stored at −80°C after the addition of
polyoxyethylene sorbitan monoauroate (Tween 20, Pierce) to a final
concentration of 0.1%. Proteins were separated by polyacrylamide gel
electrophoresis under denaturing, nonreducing conditions and trans-
ferred to Immobilon P membranes (Millipore, Bedford, MA) (26).
After blocking with 2% bovine serum albumin, the membranes were exposed
to a 1:1000 dilution of rabbit antiserum raised against rat collagenase (27,
28) and then to goat anti-rabbit IgG antiserum conjugated to horseradish
peroxidase. The blots were washed and developed with a horseradish
peroxidase chemiluminescence detection reagent (DuPont), visualized
by autoradiography on DuPont Reflection film employing Reflection
intensifying screens, and analyzed by densitometry. The Western blot
shown is representative of five cultures.

Statistical Analysis—Statistical differences were calculated by anal-
ysis of variance, and post hoc examination was performed by the Ryan-
Einst-Gabriel-Welsh F test (29, 30). Slopes were analyzed by the
method of Sokal and Rohlf (31).

RESULTS

Continuous treatment of Ob cells with 1 μM cortisol caused a
time-dependent increase in collagenase steady state tran-
scripts. Northern blot analysis showed that treatment with
cortisol increased collagenase transcripts 2-fold after 8 h, and
7-fold after 16 and 24 h (Fig. 1). This stimulatory effect on
collagenase transcripts was sustained for at least 48 h (not shown),
and cortisol treatment did not modify the abundance of the
1.9-kb glyceraldehyde-3-phosphate dehydrogenase tran-
script. The basal unstimulated level of collagenase transcripts
decreased with time in culture, possibly due to the inhibitory
effect of endogenous insulin-like growth factor 1. The cortisol-
mediated increase in collagenase mRNA levels was dose-de-
pendent and was observed at 0.1 and 1 μM cortisol (Fig. 2). In
parallel with its effects on collagenase transcripts, cortisol in-
creased levels of the protein in the culture medium of Ob cells
treated for 24 h. The concentration of immunoreactive metal-
loproteinase was below the limit of detection in control cultures
but was increased to 44 ± 6 ng/ml (mean ± S.E.; n = 4) in
cultures treated with 1 μM cortisol. Western blot analysis
showed a 5-fold increase in a 57-kDa immunoreactive protein
in the medium of cells treated with cortisol (Fig. 3). This band
Had the same mobility as the rat uterine procollagenase stand-
ard, while the lower molecular weight cross-reactive band,
which was not regulated by cortisol treatment, may represent

2 J. Jeffrey, unpublished data.

3 E. Canalis, unpublished data.
the presence of cortisol at 1 \( \mu M \) treated with 72 \( \mu M \) or glucocorticoid (GC) treated cultures was subjected to Northern blot analysis and hybridized with a \( ^{32}P \)-labeled rat collagenase cDNA; the blot was stripped and hybridized with a labeled rat glyceraldehyde-3-phosphate dehydrogenase cDNA. Transcripts were visualized by autoradiography, and collagenase mRNA is shown in the upper panel while glyceraldehyde-3-phosphate dehydrogenase mRNA is shown below. These results are representative of three cultures.

Another species of metalloproteinase.

To determine if the effect of cortisol on collagenase transcripts was dependent on protein synthesis, confluent cultures of Ob cells were treated with cortisol in the presence or absence of 3.6 \( \mu M \) cycloheximide, a dose known to inhibit protein synthesis in Ob cells by at least 85% (32). After 24 h of treatment, cycloheximide alone superinduced collagenase mRNA levels, suggesting that it may stabilize the transcript (33, 34). Co-treatment with cortisol reduced the superinduction of collagenase by cycloheximide (Fig. 4).

To determine if cortisol modified the stability of collagenase mRNA in Ob cells, the RNA polymerase II-specific inhibitor DRB was used to arrest transcription, and the decay of collagenase mRNA was monitored by Northern blot analysis (35, 36). Serum-deprived confluent cultures of Ob cells were exposed to control medium or to 1 \( \mu M \) cortisol for 4 h and then treated with 72 \( \mu M \) DRB for up to 12 h in the absence or presence of cortisol at 1 \( \mu M \). In transcription-arrested Ob cells, the half-life of collagenase mRNA was approximately 6 h, and cortisol increased the half-life of the transcript to approximately 12 h (Fig. 5, left panel). A similar increase in collagenase transcript stability was observed in Ob cells treated with cortisol for 12 h prior to the addition of DRB (Fig. 5, right panel). In both experiments, the slope for the collagenase mRNA decay in the cortisol-treated cells was significantly different from control (31). The decay of glyceraldehyde-3-phosphate dehydrogenase transcripts was the same in control and cortisol treated cultures (not shown). To determine if cortisol modified transcription of the collagenase gene, nuclear run-off assays were performed on nuclei from Ob cells treated with 1 \( \mu M \) cortisol for 2, 6, or 24 h. The levels of \( \alpha 1(I) \) collagen gene transcription were used as a control, since cortisol has been shown to decrease transcription of this gene (7). Cortisol did not alter transcription of the collagenase gene after 2 h (not shown) or 6 h (Fig. 6), although it did decrease \( \alpha 1(I) \) collagen gene transcription. After 24 h of treatment, cortisol caused a small decrease in transcription from the collagenase gene.

In human and rabbit fibroblastic cells, glucocorticoids antagonize the induction of collagenase by the protein kinase C agonist PMA (9–12). To determine if up-regulation of collagenase by cortisol in rat osteoblasts was a species-specific or a cell type-specific phenomenon, rat skin fibroblasts were treated for 6 and 24 h with 0.1 \( \mu M \) PMA in the presence or absence of cortisol at 1 \( \mu M \) (Fig. 7). Collagenase transcripts in untreated cells were almost undetectable, but treatment with PMA dramatically increased collagenase mRNA after 6 h. Co-treatment with cortisol antagonized this effect, and cortisol alone did not increase collagenase mRNA, documenting the specific nature of the cortisol effect in Ob cells. The ability of cortisol to augment
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or antagonize the PMA induction of collagenase transcripts in Ob cells also was tested (Fig. 8). After 2 h of treatment, PMA at 0.1 μM increased collagenase transcripts, while cortisol at 1 μM was modestly inhibitory. After 6 h, PMA increased collagenase mRNA levels 20-fold, and co-treatment with cortisol antagonized this effect by approximately 40%. Treatment with PMA for 24 h down-regulated osteoblast collagenase transcript levels to approximately 20% of the untreated control. In contrast, cortisol increased collagenase mRNA by 5-fold at 24 h, and co-treatment with PMA decreased this effect by 60–80%.

To further characterize the effects of PMA and cortisol on transcription of the collagenase gene in osteoblasts, Ob cells were transiently transfected with a construct containing a 2.1-kb fragment of the rat collagenase promoter driving expression of the reporter gene luciferase. Treatment of transfected cells with cortisol alone for 6 h caused a 25% decrease in luciferase activity (p < 0.05) (Fig. 9). Treatment of transfected cells with 0.1 μM PMA increased luciferase activity 2-fold, and co-treatment with 1 μM cortisol antagonized this effect.

**FIG. 6. Effect of cortisol at 1 μM on collagenase gene transcription.** Nuclei were isolated from control (C) or glucocorticoid (GC) treated Ob cells. In one experiment, cells were treated for 6 h, and in the other experiment they were treated for 24 h. Nascent transcripts were labeled in vitro with [32P]UTP, and the labeled RNA was hybridized to immobilized cDNA for α1(I) collagen, glyceraldehyde-3-phosphate dehydrogenase (GAPD), and rat collagenase. pGL2-Basic vector DNA (Promega) was used as a control for nonspecific hybridization. Transcripts were visualized by autoradiography. These results are representative of three experiments.

**FIG. 7. Effect of cortisol at 1 μM on PMA-induced collagenase transcripts in fibroblasts.** Rat skin fibroblasts were treated with control medium (C) or with PMA (P) at 0.1 μM, and the glucocorticoid cortisol (GC) at 1 μM for 6 or 24 h. Total RNA was isolated and subjected to Northern blot analysis with a 32P-labeled rat collagenase cDNA; the blot was stripped and rehybridized with a labeled rat glyceraldehyde-3-phosphate dehydrogenase cDNA. Transcripts were visualized by autoradiography, and collagenase mRNA is shown in the upper panel while glyceraldehyde-3-phosphate dehydrogenase mRNA is shown below. These results are representative of two cultures.

**FIG. 8. Effect of cortisol at 1 μM on PMA-induced collagenase transcripts in osteoblasts.** Ob cells were treated with control medium (C) or with PMA (P) at 0.1 μM and the glucocorticoid cortisol (GC) for 2, 6, or 24 h. Total RNA was isolated and subjected to Northern blot analysis with a 32P-labeled rat collagenase cDNA; the blot was stripped and rehybridized with a labeled rat glyceraldehyde-3-phosphate dehydrogenase cDNA. Transcripts were visualized by autoradiography, and collagenase mRNA is shown in the upper panel while glyceraldehyde-3-phosphate dehydrogenase mRNA is shown below. These results are representative of five cultures.

**FIG. 9. Effect of cortisol at 1 μM and PMA at 0.1 μM on collagenase promoter activity in transiently transfected Ob cells.** A 2.1-kb fragment of the rat collagenase promoter was used to drive expression of the luciferase gene in the promoter-less reporter plasmid pGL2-Basic. Ob cells were transiently co-transfected with collagenase promoter-luciferase plasmid and plasmid containing the cytomegalovirus promoter driving expression of the β-galactosidase gene. Transfected cells were treated for 6 h with control medium (C) or with medium containing glucocorticoid (GC), PMA, or PMA plus glucocorticoid (PMA + GC). Luciferase activity was normalized to β-galactosidase activity to control for slight differences in transfection efficiency. These results are representative of three experiments. *, significantly different from C, p < 0.01; **, significantly different from PMA, p < 0.01.

**DISCUSSION**

Glucocorticoids have significant effects on bone remodeling. Previous work demonstrated that they inhibit α1(I) collagen synthesis by transcriptional and post-transcriptional mechanisms, but there is limited information about their effects on collagen degradation and collagenase expression (1, 7). In the present study we demonstrated that cortisol causes a time- and dose-dependent stimulation of interstitial collagenase transcripts in cultures of Ob cells, which was paralleled by increased levels of immunoreactive collagenase in the culture medium. These effects were observed at doses of cortisol that modify parameters of osteoblastic differentiated function and at concentrations that were only slightly higher than physiological serum levels of cortisol (3–6). The same doses of cortisol modestly decreased transcripts for tissue inhibitor of metallo-
proteases (TIMP) 1 and did not affect the expression of TIMPs 2 and 3, suggesting that up-regulation of collagenase by glucocorticoids is important in increased extracellular matrix degradation.

Experiments using the RNA polymerase II inhibitor DRB demonstrated that cortisol stabilized collagenase mRNA in transcriptionally arrested Ob cells. In contrast, nuclear run-off assays and transient transfection of a rat interstitial collagenase promoter-reporter gene construct showed that collagenase gene transcription was slightly decreased by cortisol. These results indicate that cortisol increases collagenase expression by increasing transcript stability. The rat collagenase mRNA has a 1.2-kilobase 3'-untranslated region that is AU-rich and contains three repeats of the sequence AUUUA (17). In short lived mRNAs, such AU-rich sequences play a role in regulating transcript stability (reviewed in Ref. 37). The human and rabbit collagenase mRNAs contain repeats of AUUUA, and mutation of these motifs in the human transcript increases the stability of the RNA (38). It is probable that the glucocorticoid-mediated increase in Ob cell collagenase transcripts involves proteins interacting with such AU-rich regions of the mRNA (39).

Cortisol antagonized the induction of collagenase promoter activity by PMA in Ob cells. These effects are similar to those observed in fibroblasts (10–12), suggesting that osteoblasts and fibroblasts share a common mechanism for the regulation of collagenase transcription. Like the human interstitial collagenase gene, which is transcriptionally regulated by PMA and glucocorticoids, the rat collagenase promoter contains a TPA-responsive element (10–12, 40, 41). Components of the AP-1 transcription factor are activated by glucocorticoids, the rat collagenase promoter contains a TPA-responsive element (10–12, 40, 41). Components of the AP-1 and glucocorticoid repressors of collagenase gene transcription in fibroblasts can be mediated through antagonism of AP-1 (10–12).

Collagenase gene transcription and mRNA levels were down-regulated in Ob cells treated for 24 h with PMA. A nuclear run-off assay showed that after 24 h treatment with PMA, collagenase gene transcription was barely detectable. This is most likely due to depletion of protein kinase C activity following prolonged exposure to phorbol esters. In cells treated with PMA and cortisol for 24 h, the level of collagenase mRNA was intermediate between that of cortisol alone and PMA alone. This suggests that the effects of cortisol and PMA may be additive at this time point and that cortisol and PMA increase collagenase transcripts in Ob cells by distinct mechanisms.

The expression of collagenase in osteoblasts is up-regulated by a number of osteoeruptive agents, including parathyroid hormone, glucocorticoids and prostaglandins (8, 40, 42). The role of osteoblast-derived interstitial collagenase in the bone compartment is currently being explored, and there is increasing evidence for the coupling of osteoblastic function with osteoclastic bone resorption (reviewed in Ref. 43). Localized extracellular matrix degradation by osteoblast collagenase may provide a means for activated osteoclasts to adsorb to target bone surfaces. Collagenase may also play a role in regulating the availability of bone growth factors. For example, localized matrix degradation may release growth factors sequestered in the matrix, which may stimulate or inhibit osteoblastic function or may activate or be chemotactic for osteoclasts (44, 45). Insulin-like growth factors are among the most prevalent growth factors secreted by bone cells, and they stimulate the differentiation pathway of the osteoblast (45). Their activity can be modulated by insulin-like growth factor binding proteins, the abundance of which can be regulated by proteases, including Ca²⁺-dependent serine proteases and metzincin genes (46–49). A study characterizing a parathyroid hormone-regulated receptor for collagenase on a rat osteosarcoma cell line showed that this receptor is responsible for clearance of collagenase from the cellular environment (50). This suggests that the osteoblast maintains a tight control over collagenase activity and that promiscuous expression of collagenase would be undesirable. The importance of appropriately regulated osteoblast collagenase also is suggested by the development of collagenase-expressing bone tumors in transgenic mice overexpressing c-fos (51).

In conclusion, cortisol causes a cell type-specific increase in interstitial collagenase expression in osteoblasts, which is mediated by post-transcriptional mechanisms. These results further highlight the differences in gene regulation between osteoblasts and fibroblasts, two cell types that arise from a common precursor.

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