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Serum Induction of the Collagen X Promoter Requires the Raf/MEK/ERK and p38 Pathways

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The collagen X gene is expressed exclusively by differentiated, hypertrophic chondrocytes. The mechanisms controlling collagen X expression remain largely unknown. Here we show that collagen X promoter activity can be induced by serum stimulation of chondrogenic MCT cells. The serum response is conferred by a 462 nucleotide promoter fragment. Both the c-Raf/MEK/ERK and p38 MAP kinase pathways are required for this effect, whereas phosphatidylinositol-3-kinase and protein kinase A repress promoter activation. These data are the first to demonstrate serum inducibility of the collagen X promoter and to identify signal transduction pathways involved.

Key Words: **collagen X; chondrocytes; c-Raf; p38; ERK.**

Longitudinal growth of endochondral bones is regulated by the coordinated proliferation and differentiation of growth plate chondrocytes (recently reviewed in [1]). Maintenance of the fine balance between proliferation and differentiation of growth plate chondrocytes is crucial for normal development of the skeleton, since disruption of this balance can cause skeletal deformities, dwarfism, and frequently death (reviewed in [2, 3]). However, little is known about the intracellular mechanisms controlling chondrocyte proliferation and differentiation.

The collagen X gene is the classical marker gene for differentiated, hypertrophic chondrocytes [4]. Mutations in the human collagen X gene cause Schmid Metaphyseal Chondrodysplasia, a heritable skeletal disorder [5, 6]. Similar phenotypes have been described in transgenic mice harboring a dominant-negative collagen X gene [7] and in collagen X-null mice [8]. Since the collagen X gene is exclusively expressed by hypertrophic chondrocytes, studies of the regulation of collagen X expression could reveal mechanisms regulating chondrocyte differentiation into hypertrophic cells.

Although it has been shown that regulation of expression of the chicken collagen X gene occurs at the level of transcription [9, 10], the signaling pathways and transcription factors controlling collagen X promoter activity remain unknown. We have shown previously that the c-Raf/MEK/ERK pathway is necessary for maximal collagen X promoter activity in chondrogenic MCT cells [11], which display several characteristics of hypertrophic chondrocytes [12]. Here we show that collagen X promoter activity is induced by serum and that this induction involves the c-Raf/MEK/ERK pathway, as well as the related p38 pathway.

MATERIALS AND METHODS

Materials. The human collagen X promoter plasmids pGlXH3000, pGlH2500, pGlBH900, and pGlSH500 have been described [12, 13]. Expression vectors for dominant-negative c-Raf [14] and dominant-negative ERK, JNK, and p38 were generously provided by Drs. U. Rapp and R. Davis, respectively. The chemical inhibitors PD98059, SB202190, wortmannin and H-89 were purchased from Calbiochem and dissolved in DMSO.

Cell culture, transfections, and luciferase assays. Cell culture of MCT cells [11], transfections, luciferase assays, and statistical analyses of luciferase data were done as described [12]. Luciferase data are given as the average of three independent transfection experiments. After transfections, cells were cultured in serum-free medium for 48 hours. This medium was then replaced with medium containing the indicated amount of FBS, and cells were harvested for luciferase assays at the indicated time points. For cotransfections, 1 μg of collagen X reporter plasmid was cotransfected with 0.2 μg of pRlSV40 (Promega; for standardization), and 0.3 μg of empty expression vector or expression vectors for dominant-negative c-Raf, ERK, JNK, or p38.

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Abbreviations used: DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated kinase; MEF, myocyte enhancer factor; MEK, MAP/ERK kinase.

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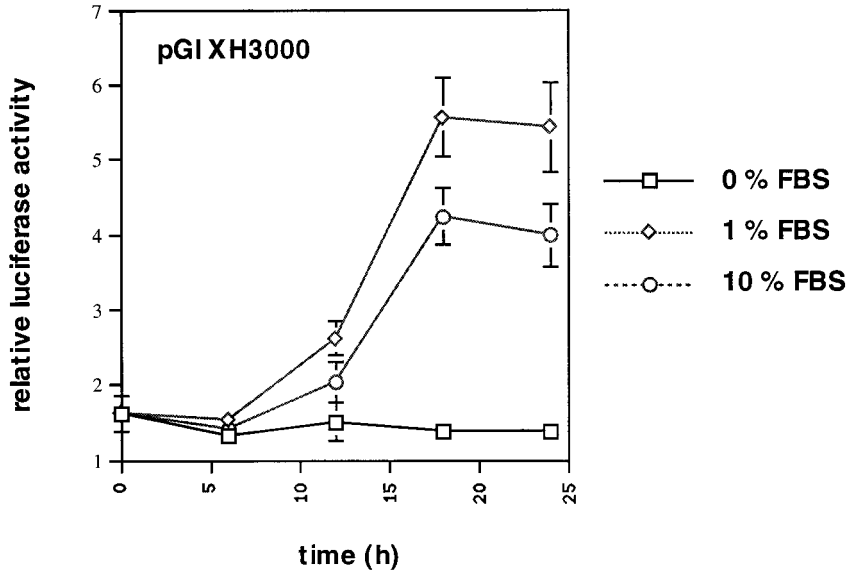

FIG. 1. Serum induction of the collagen X promoter. MCT cells were transfected with pGlXH3000 and pRlSV40, serum-starved for 48 hours, and restimulated with medium containing 0, 1, or 10% FBS. After 0, 6, 12, 18, and 24 hours, cells were harvested and firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. Average and standard deviations from three independent experiments are shown.

RESULTS

Collagen X promoter activity is induced by serum. Serum has been shown to regulate chondrocyte differentiation and collagen X mRNA expression in a dose-dependent manner [11, 15]. We examined the effects of serum on collagen X promoter activity by transfection of the plasmid pGlXH3000, containing 2864 nucleotides of the collagen X promoter [12, 13], into MCT cells that express collagen X [11]. Cells were incubated in serum-free medium for 48 hours. Medium was then exchanged for medium containing 0, 1, or 10% FBS. Cells were harvested at 0, 6, 12, 18, and 24 hours of serum stimulation for determination of luciferase activity. Stimulation of promoter activity was detected after 12 hours with 1 and 10% FBS and reached maximal activity at 18 hours (Fig. 1). 1% FBS was 40% more effective in stimulation of collagen X promoter activity then 10% FBS.

Medium containing 1–2% FBS yields optimal stimulation of collagen X promoter activity. Next we wanted to determine the optimal serum concentration for stimulation of collagen X promoter activity. MCT cells were transfected with pGlXH3000, serum-starved for 48 hours, and restimulated with 0, 0.1, 0.5, 1, 2, 5, and 10% FBS. After 18 hours, cells were harvested for measurement of luciferase activity (Fig. 2). Whereas 0.1 and 0.5% FBS had relatively little effect on collagen X promoter activity, 1% and 2% induced an approximate 4-fold increase in promoter activity. This effect was weaker with higher FBS concentrations which only induced promoter activity approximately 2-fold.

The basal collagen X promoter confers serum induction. We transfected several different collagen X promoter constructs [12, 13] into MCT cells to analyze

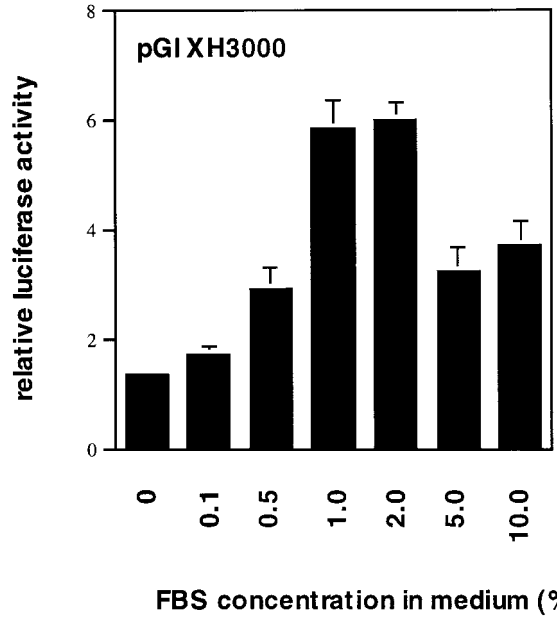

FIG. 2. Effect of serum concentration on collagen X promoter activity. MCT cells were transfected with pGlXH3000 and pRlSV40, serum-starved for 48 hours, and restimulated with medium containing 0, 0.1, 0.5, 1, 2, 5, or 10% FBS. After 18 hours, cells were harvested and firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. Average and standard deviations from three independent experiments are shown.

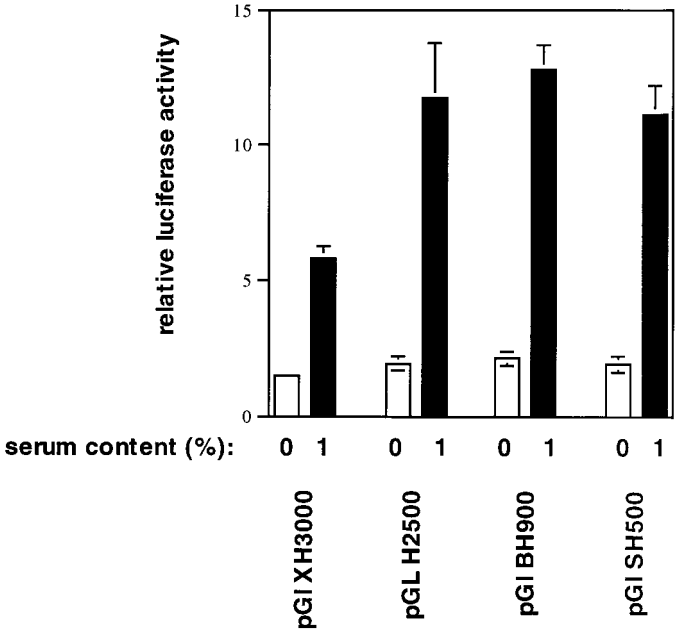

FIG. 3. Effect of serum stimulation on collagen promoter fragments. MCT cells were transfected with pRlSV40 and pGlXH3000, pGlH2500, pGlBH900, or pGlSH500, serum-starved for 48 hours, and restimulated with medium containing 1% FBS. After 18 hours, cells were harvested and firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. Average and standard deviations from three independent experiments are shown.

which part of the promoter is responsible for serum induction. All tested promoter fragments were induced by stimulation with 1% FBS for 18 hours (Fig. 3). The plasmids pGlH2500, pGlBH900, and pGlSH500 showed a 5- to 6-fold induction, compared to a 3.8-fold induction of pGlXH3000.

The c-Raf/MEK/ERK and p38 pathways are necessary for serum induction of the collagen X promoter. Since the plasmid pGlSH500, which is responsive to serum, requires the c-raf/MEK/ERK pathway for maximal activity [12], we next tested whether this pathway is involved in the serum stimulation of the collagen X promoter. pGlXH3000 was cotransfected with either empty expression vector or an expression vector for a dominant-negative form of c-Raf (C4B; [14]) into MCT cells. After serum starvation and restimulation with 1% FBS for 18 hours, luciferase activities were determined. The dominant-negative form of c-Raf caused a 2.5-fold reduction in serum-induced promoter activity (Fig. 4). A similar result was obtained when an expression vector for a dominant-negative form of ERK1, a downstream target of c-Raf signaling, was cotransfected with pGlXH3000. In contrast cotransfection with dominant-negative JNK, a related MAP kinase, did not affect collagen X promoter activity significantly. Inhibition of a third MAP kinase, p38, with a dominant-negative form caused a 30% reduction in collagen X promoter activity.

PhosphatidylinositoI-3-kinase and protein kinase A repress serum induction of the collagen X promoter. To further analyze the signaling pathways mediating serum induction of the collagen X promoter, pGlXH3000 was transfected into MCT cells, cells were serum starved, and the effects of stimulation with 1% FBS for 18 hours in the presence of chemical inhibitors for several kinases was examined. 20 μM PD98059, which inhibits MEK1/2, and 1 μM SB202190, which inhibits p38, reduced collagen X promoter activity by 61% and 46%, respectively (Fig. 5). In contrast, 1 μM H-89, an inhibitor of protein kinase A, and 100 nM wortmannin, an inhibitor of Phophatidylinositol-3-Kinase, caused an increase in 83% and 31% in pGlXH3000 promoter activity, respectively (Fig. 5).

DISCUSSION

Previous work had shown that serum regulates expression of collagen X mRNA in a dose-dependent manner in MCT cells and primary rat chondrocytes [11, 15]. Whereas high concentrations of serum repress collagen X expression, low concentrations have stimulatory effects [15]. We demonstrate here that these effects are

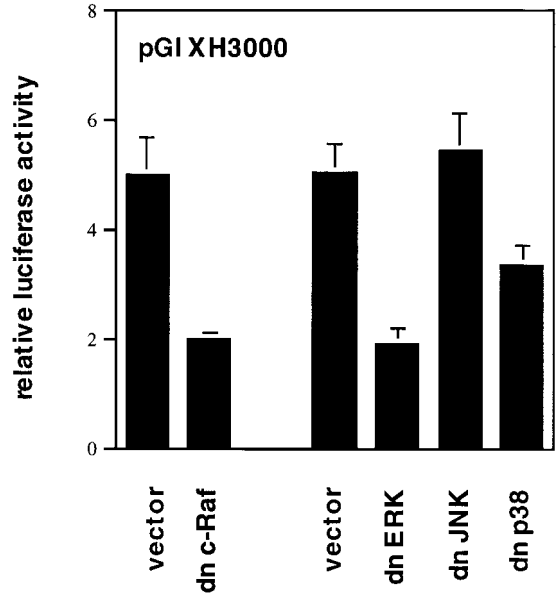

FIG. 4. Effect of dominant-negative c-Raf, ERK1, JNK, and p38 on serum stimulation of the collagen X promoter. MCT cells were transfected with pGlXH3000, pRlSV40, and empty expression vectors or vectors for the expression of dominant-negative c-Raf, ERK1, JNK, or p38. After transfection, cells were serum-starved for 48 hours, and restimulated with medium containing 1% FBS. After 18 hours, cells were harvested and firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. Average and standard deviations from three independent experiments are shown.

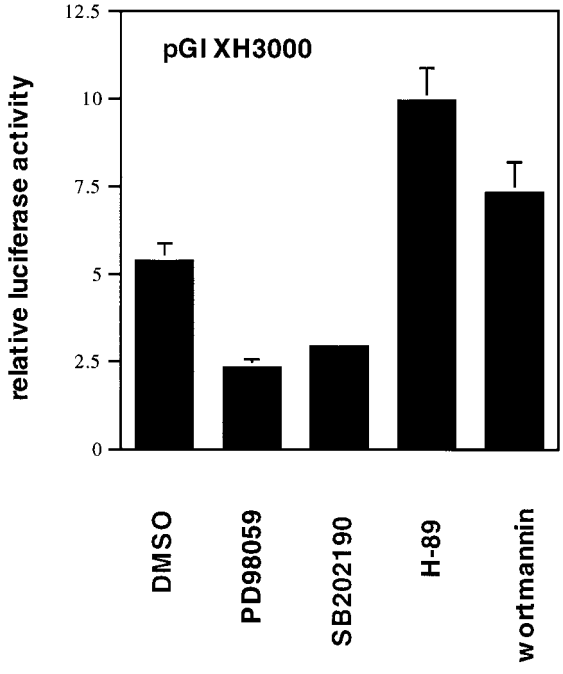

FIG. 5. Effect of chemical inhibitors on serum stimulation of the collagen X promoter. MCT cells were transfected with pGlXH3000 and pRlSV40, serum-starved for 48 hours, and restimulated with medium containing 1% FBS. At the time of serum stimulation, DMSO or 20 μM PD98059, 1 μM SB202190, 1 μM H-89, or 100 nM wortmannin were added. After 18 hours, cells were harvested and firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. Average and standard deviations from three independent experiments are shown.

due—at least in part—to activation of collagen X promoter activity. Our experiments show that 1–2% serum had optimal effects on collagen X promoter activity. This effect was seen in the smallest construct tested, which contains 462 nucleotides of the human collagen X promoter. The largest promoter fragment, in pGlXH3000, displayed reduced activation by serum compared to the other three constructs. This effect is most likely due to the presence of silencer elements in this promoter fragment [13].

The 462 nucleotide basal collagen X promoter requires the c-Raf/MEK/ ERK pathway for maximal activity [12]. We show that this pathway is also necessary for the serum-induction of the collagen X promoter. In addition, a second MAP kinase pathway, p38, is necessary for maximal serum induction of the collagen X promoter. Both ERK and p38 could act through a putative binding site for Ets family transcription factors which is conserved among the chicken, mouse, bovine, and human collagen X promoters [16]. However, a binding site for MEF-2-related transcription factors has been identified in the mouse collagen X promoter [17], and several MEF-2 proteins are among the transcription factors phosphorylated by p38 [18]. Experiments are underway to identify the cis-active elements in the collagen X promoter targeted by the ERK and p38 pathways.

In contrast to the described MAP kinases, Phosphatidylinositol-3-Kinase and Protein Kinase A appear to repress the activity of the collagen X promoter, since their inhibition causes increased promoter activity. These data suggest that a wide variety of signal transduction pathways contribute to the regulation of collagen X transcription. It remains to be determined whether or not these pathways act directly on the collagen X promoter, and what role they play in the regulation of chondrocyte proliferation and differentiation.

In summary, our data show for the first time that the collagen X promoter is induced by serum stimulation, and that the c-Raf/MEK/ERK and p38 MAP kinase pathways are necessary for maximal induction. These data suggest a surprisingly complex regulation of collagen X transcription and form a basis for the complete elucidation of the mechanisms controlling collagen X expression and chondrocyte differentiation during normal and pathological skeletal development.

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