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Raf Signaling Stimulates and Represses the Human Collagen X Promoter Through Distinguishable Elements

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Abstract Endochondral bone growth is regulated through the rates of proliferation and differentiation of growth plate chondrocytes. While little is known about the intracellular events controlling these processes, the protein kinase c-Raf, a central component of the cellular signal transduction machinery, has recently been shown to be expressed only by differentiated, hypertrophic chondrocytes. The involvement of c-Raf in the transcriptional regulation of the hypertrophic chondrocyte-specific collagen X gene was investigated using cotransfections of collagen X reporter plasmids and expression vectors for mutant c-Raf proteins. Both activated and dominant-negative forms of c-Raf reduced the activity of the collagen X promoter to approximately 30%. The element mediating the repressing effect of activated c-Raf was located between nucleotides -2864 and -2410 of the promoter, whereas the effect of the dominant-negative form of c-Raf was conferred by the 462 nucleotides immediately upstream of the transcription start site. Inhibition of MEK1/2 and ERK1/2, downstream components of Raf-signaling, also caused repression of basal collagen X promoter activity. These data suggest that c-Raf regulates collagen X promoter activity positively and negatively through different cis-acting elements and represent the first evidence of c-Raf activity described in hypertrophic chondrocytes. J. Cell. Biochem. 72:549–557, 1999.

Key words: collagen X; chondrocytes; c-Raf; endochondral ossification; promoter; MEK1/2; ERK1/2

Longitudinal growth of endochondral bone is controlled by the coordinated proliferation and differentiation of growth plate chondrocytes [for review see Erlebacher et al., 1995; Mundlos and Olsen, 1997]. In recent years, gene disruption experiments in mice as well as the identification of genes involved in human skeletal diseases have implicated a large number of growth factors and their receptors in the regulation of chondrocyte proliferation and/or differentiation, including parathyroid-hormone related peptide [Amizuka et al., 1994; Karaplis et al., 1994], insulin-like growth factors (IGF)1 and 2 [Baker et al., 1993], fibroblast growth factor (FGF) receptor 3 [Deng et al., 1996], thyroid hormone receptor α [Fraichard et al., 1997], and vitamin D receptor [Li et al., 1997; Yoshizawa et al., 1997]. Interruption of the fine balance between proliferation and differentiation in these experiments usually results in growth retardation, bone deformation, and/or perinatal death, indicating that very complex and stringent regulation of bone growth is crucial for normal development. However, little is known about the intracellular processes and signal transduction pathways connecting the extracellular signals to the control of chondrocyte gene expression.

The collagen X gene is exclusively expressed by differentiated, hypertrophic chondrocytes [Reichenberger et al., 1991]. Mutations in the human collagen X gene have been found to cause Schmid Metaphyseal Chondrodysplasia, a heritable skeletal disorder [Wallis, 1993; Warman et al., 1993]. Partially overlapping phenotypes are observed in transgenic mice harboring a dominant-negative collagen X gene [Jacenko et al., 1993] and in collagen X-null mice [Kwan et al., 1997]. Aside from its essential role in skeletal development, collagen X serves as the classical marker for hypertrophic chondrocytes. Analyses of the mechanisms regulating the expression of the collagen X gene should therefore allow insights into the processes controlling chondrocyte differentiation. Regulation of expression of the chicken collagen X gene occurs at the level of transcription

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[LuValle et al., 1989, 1993]. We have recently identified positive and negative elements regulating the transcription of the human collagen X gene [Beier et al., 1996, 1997]; however, the transcription factors and mechanisms controlling the activity of these elements have not been identified.

The c-Raf protein kinase is a central component of the cellular signal transduction machinery [reviewed in Daum et al., 1994]. c-Raf can be activated by a variety of receptors, including transmembrane tyrosine kinase receptors (i.e., insulin receptor, IGF1 receptor, and FGF receptor 3), receptors containing seven transmembrane domains [Daum et al., 1994], and integrin receptors [Chen et al., 1996]. c-Raf is essential for the regulation of cellular proliferation and differentiation in *C. elegans*, Drosophila, and mammals [Daum et al., 1994]. Activated c-Raf phosphorylates and thereby stimulates the dual-specificity kinases MEK1 and MEK2 (MAP/ERK Kinase 1 and 2), which in turn phosphorylate the MAP (mitogen-activated protein) kinases ERK1 and ERK2 (extracellular signal-regulated kinase 1 and 2). Substrates for ERK1/2 include other kinases as well as a variety of transcription factors, including several factors of the Ets family [reviewed in Treisman, 1996]. The raf-MEK-ERK pathway therefore forms a pathway connecting extracellular signals to the control of gene expression at the level of transcription.

In the growth plate of endochondral bones, c-Raf is expressed by differentiated, hypertrophic chondrocytes, whereas it is undetectable in proliferating chondrocytes [Kaneko et al., 1994]. The function of c-Raf in hypertrophic chondrocytes remains unclear. The similarity in the expression patterns of collagen X and c-Raf in the growth plate prompted us to investigate the possibility that c-Raf is involved in the transcriptional regulation of the collagen X gene. Our results show that c-Raf regulates the activity of the human collagen X promoter both negatively and positively through distinguishable elements. The repressing effect of c-Raf is conferred by a 5′-fragment recently identified as a silencer element, whereas the enhancing effect of c-Raf and its downstream components is mediated by the nucleotides immediately upstream of the transcription initiation site. These experiments are the first to show biological activity of c-Raf in hypertrophic chondrocytes.

MATERIALS AND METHODS

Reagents and Materials

Cell culture media, sera, antibiotics, Glutamine, and Lipofectin were purchased from GIBCO BRL (Gaithersburg, MD). The Dual Luciferase Assay Kit and the plasmid pRlSV40, encoding Renilla luciferase for standardization under control of the SV40 promoter, were obtained from Promega (Madison, WI). The human type X collagen promoter plasmids have been described recently [Beier et al., 1997]. Raf expression plasmids were generously provided by S. Ludwig and U. Rapp and have been described recently [Bruder et al., 1992; Ludwig et al., 1996]. The dominant-negative ERK expression plasmid pCMV-p41mapk (K/A) was kindly provided by R. Davis. The MEK inhibitor PD098059 [Alessi et al., 1995; Dudley et al., 1995] was purchased from New England Biolabs (Beverly, MA).

Cell Culture and Transfections

MCT cells were cultured as described [Beier et al., 1997; Lefebvre et al., 1995] in DMEM medium supplemented with 10 % FBS (fetal bovine serum), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 mg/ml) at 32°C under 5% CO_2. The evening before transfections, 6×10^4 cells were seeded into each well of a 24-well plate (in medium containing 10% FBS). The next morning transfections were performed under serumfree conditions with Lipofectin according to the manufacturer's protocol. Briefly, each well was transfected with 1.5 mg of reporter gene construct and 0.25 mg of pRlSV40 (Promega) using 1.5 ml of lipofectin for 4 h at 32°C. For cotransfections, 1.5 mg of reporter gene constructs were cotransfected with 1.5 mg of expression plasmid or empty expression vector, and 0.25 mg of pRlSV40. After transfections, cells were cultured for further 30 to 40 h at 32°C in medium containing 10% FBS and then lysed with Passive Lysis Buffer (Promega) according to the manufacturer's protocol. For inhibition of MEK activity, cells received 20 mM of PD098059 (in DMSO) 2 h after transfection until lysis; control cells received DMSO. Every transfection was repeated at least three times with each of two different plasmids preparations; results are shown for one representative series of three independent transfections. Statistical analyses

were performed using Student's *t*-Test. Significant differences were established as $P < 0.01$.

Luciferase Assays

Luciferase assays were performed with the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions in a Turner TD-20e luminometer (Promega). Ten ml of lysate was assayed first for firefly luciferase (representing the activity of the collagen X promoter fragments) and then for Renilla luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity.

RESULTS

Effect of Mutant c-Raf Forms on Collagen X Promoter Activity

In order to investigate whether c-Raf influences the activity of the human collagen X promoter, we performed cotransfection experiments of the reporter plasmid pGlXH3000 (Fig. 1) with expression plasmids for different forms of c-Raf. Transfections were done in MCT cells, a mouse chondrogenic cell line that has been shown to express collagen X as well as other markers of hypertrophic chondrocytes [Lefebvre et al., 1995]. pGlXH3000 contains 2864 nucleotides of the human collagen X promoter, including described enhancer and silencer elements [Beier et al., 1997; Thomas et al., 1995], fused to the luciferase reporter gene. Cotransfection of pGlXH3000 with an expression vector for wild type c-Raf caused a reduction in luciferase activity to 41.7 % of the values obtained when the empty expression vector (kRSPA) was cotransfected with pGlXH3000 (Fig. 2). Cotransfection with the plasmid BXB, which encodes a constituitively active form of c-Raf, decreased the activity of the type X collagen promoter to 29.2% of the control (Fig. 2). Inhibition of endogenous c-Raf by cotransfection with the vector C4B, encoding a dominant-negative form of c-Raf, also decreased the activity of pGlXH3000 to 32.4% (Fig. 2).

Identification of Elements in the Collagen X Promoter Conferring Effects of c-Raf

Since both overactivation and inhibition of c-Raf decreased the activity of the full-length collagen X promoter, we asked whether both effects could be mediated by the same cis-acting element(s) in the promoter. To answer this question, we performed cotransfection experiments of the c-Raf expression vectors with shorter collagen X promoter constructs (Fig. 1) described recently [Beier et al., 1997]. Cotransfection with c-Raf BXB inhibited only the largest collagen X promoter fragment, pGlXH3000. Re-

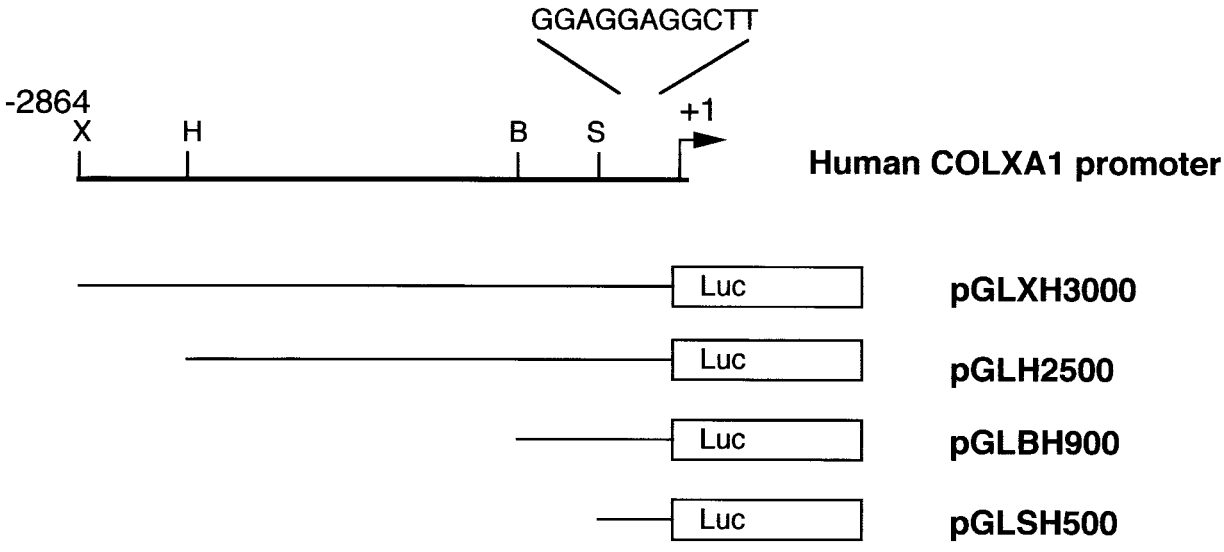

Fig. 1. Collagen X promoter constructs. Different fragments of the human collagen X promoter were cloned into the luciferase promoter vector pGl2 basic [Beier et al., 1997]. The common 3′- end of all constructs is a Hind3 restriction site 30 nucleotides downstream of the transcription initiation site [Reichenberger et al., 1992]. The 5′-ends are 2864 (pGlXH3000), 2410 (pGlH2500), 963 (pGlBH900), and 462 (pGlSH500) nucleotides upstream of the transcription initiation site. The potential binding site for Ets family transcription factors conserved among human, mouse, bovine, and chicken collagen X genes [Beier et al., 1996] is shown on top. Restriction sites are given as abbreviations: X, Xho1; H, Hind3, B, BamH1, S, Ssp1.

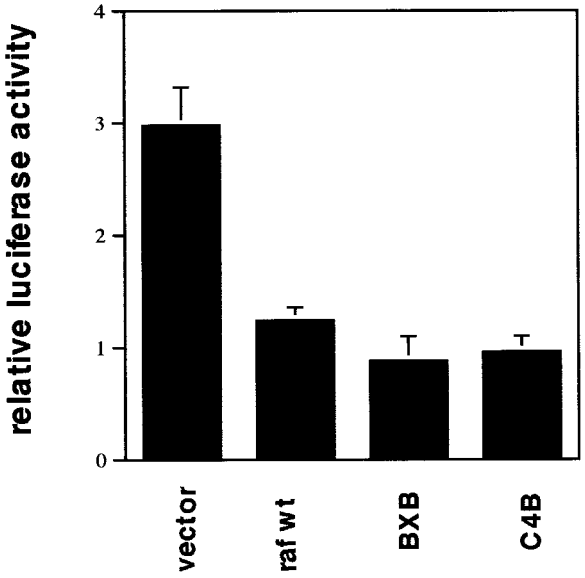

Fig. 2. Effects of overexpression of wild type and mutant c-Raf on collagen X promoter activity. The collagen X reporter plasmid pGlXH3000 was cotransfected with the empty expression vector kRSPA (vector), and expression vectors for wild type c-Raf (raf wt), constituitively active c-Raf (BXB), and dominant-negative c-Raf (C4B). Thirty hours after transfections, cells were harvested, firefly luciferase activity was measured, and standardized to Renilla luciferase activity to yield the relative luciferase activity. The average of three independent experiments and standard deviations are shown. Overexpression of all three forms of c-Raf inhibit the activity of pGlXH3000.

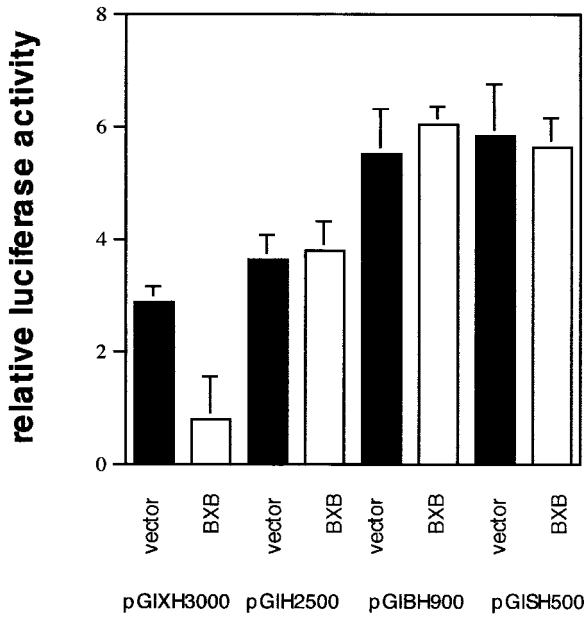

Fig. 3. Effects of expression of constituitively active c-Raf on the activity of different collagen X reporter plasmids. The collagen X reporter plasmids pGlXH3000, pGlH2500, pGlBH900, and pGlSH500 were cotransfected with the empty expression vector kRSPA (vector) or the expression vector for constituitively active c-Raf (BXB). Thirty hours after transfections, cells were harvested, firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. The average of three independent experiments and standard deviations are shown. Overexpression of constituitively active c-Raf inhibits only the largest collagen X promoter fragment in pGlXH3000, whereas the activity of the shorter fragments are not affected.

porter constructs containing shorter fragments of the collagen X promoter, pGlH2500 (containing 2410 nucleotides of collagen X promoter sequence), pGlBH900, and pGlSH500, showed no significant differences between cotransfections with BXB and controls (Fig. 3; Table I). Similar results were obtained in cotransfection experiments of the reporter constructs with the wild type c-Raf expression vector (Table I). These data suggest that repression of collagen X promoter activity by c-Raf is mediated by sequences located between position -2864 and -2410. Computer analyses revealed no sequence elements in this promoter fragment which could bind transcription factors activated by the c-Raf pathway (data not shown).

In contrast, cotransfection with the dominant-negative c-Raf expression vector C4B inhibited the activity of all four collagen X promoter fragments to a similar degree. This suggests that sequences in the 462 nucleotides directly upstream of the transcription initiation site (corresponding to the shortest construct tested) require c-Raf activity for full activity (Fig. 4; Table I).

Effects of MEK1/2 and ERK1/2 on the Basal Collagen X Promoter

We performed cotransfection experiments using the collagen reporter constructs together with a dominant-negative expression vector for ERK1/2, to test whether characterized downstream targets of c-Raf are involved in the regulation of the collagen X promoter. Inhibition of endogenous ERK activity yielded a decrease in the activity of all four promoter constructs to 30–35% of control values (Fig. 5; Table I). Inhibition of MEK1/2 with the specific chemical inhibitor PD098059 repressed the activity of all four promoter constructs to a similar degree (Fig. 6; Table I), confirming the importance of the raf-MEK-ERK pathway for the activity of the basal type X collagen promoter using a biochemical approach.

DISCUSSION

The similar expression patterns of c-Raf and collagen X in the endochondral growth plate

TABLE I. Effects of Modulators of the c-Raf/MEK/ERK Pathway on the Activity of Collagen X Reporter Plasmids[a]

Reporter	c-raf wt	BXB	C4B	dn ERK	PD098059
XH3000	41.7 ± 4.2	29.2 ± 7.3	32.4 ± 4.2	31.3 ± 2.5	42.7 ± 3.3
H2500	99.0 ± 9.3	110.0 ± 10.6	33.3 ± 10.1	34.0 ± 9.3	29.9 ± 3.9
BH900	98.9 ± 15.3	109.4 ± 5.5	36.2 ± 8.2	33.9 ± 6.7	27.6 ± 3.1
SH500	103.5 ± 1.5	96.6 ± 9.1	35.1 ± 3.4	31.8 ± 2.7	36.1 ± 5.2

[a]The collagen X reporter plasmids pGIXH3000 (XH3000), pGIH2500 (H2500), pGIBH900 (BH900), and pGISH500 (SH500) were either cotransfected with empty expression vectors, expression vectors for wild type c-Raf (c-Raf wt), constituitively-active c-Raf (BXB), dominant-negative c-Raf (C4B), and dominant-negative ERK (dn ERK), or incubated (after transfection) in the presence or absence of 20 μM PD098059. Thirty to 40 hours after transfections, cells were harvested, firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. The relative luciferase activity obtained in the presence of the modulators of the c-Raf/MEK/ERK pathway is shown as the percentage of the activity obtained in the respective controls (empty expression vectors for cotransfection experiments, DMSO for incubation with PD098059). The average of three independent experiments is shown. Whereas wild type and constituitively active c-Raf only inhibit the activity of the largest promoter fragment in pGIXH3000, dominant-negative c-Raf, dominant-negative ERK, and the MEK inhibitor PD098059 repress the activity of all tested collagen X promoter fragments. The average of three transfection experiments and standard deviations are shown.

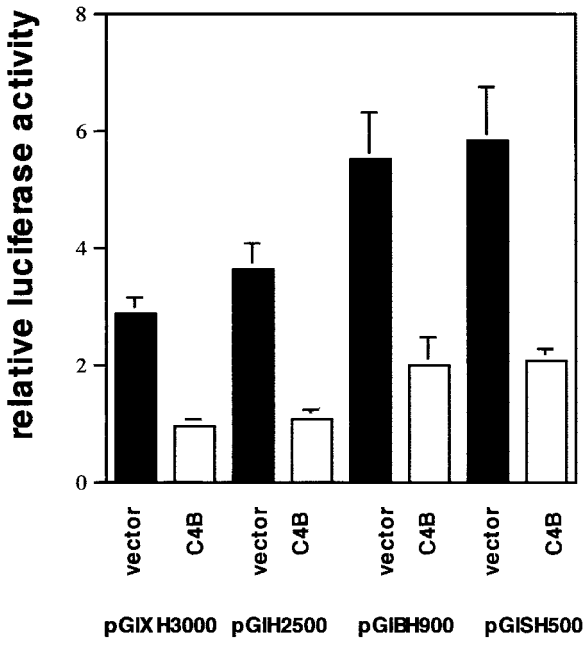

Fig. 4. Effects of expression of dominant-negative c-Raf on the activity of different collagen X reporter plasmids. The collagen X reporter plasmids pGIXH3000, pGIH2500, pGIBH900, and pGISH500 were cotransfected with the empty expression vector kRSPA (vector) or the expression vector for dominant-negative c-Raf (C4B). Thirty hours after transfections, cells were harvested, firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. The average of three independent experiments and standard deviations are shown. Inhibition of c-Raf by expression of a dominant-negative form represses the activity of all four collagen X reporter plasmids.

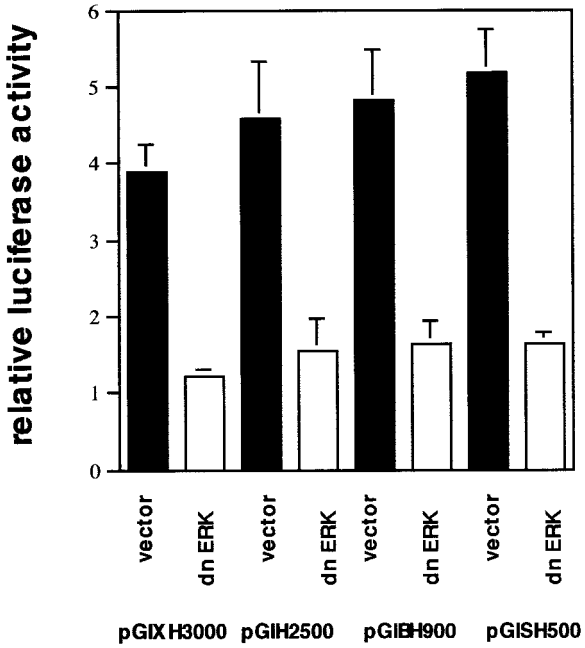

Fig. 5. Effects of expression of dominant-negative ERK on the activity of different collagen X reporter plasmids. The collagen X reporter plasmids pGIXH3000, pGIH2500, pGIBH900, and pGISH500 were cotransfected with empty expression vector (vector) or an expression vector for dominant-negative ERK1 (dn ERK). Forty hours after transfections, cells were harvested, firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. The average of three independent experiments and standard deviations are shown. Inhibition of ERK activity decreases the activity of all tested collagen X promoter fragments.

prompted us to ask whether c-Raf is involved in the transcriptional control of the collagen X gene. Surprisingly, both the inhibition of endogenous c-Raf and the addition of exogenous, activated c-Raf severely reduced the activity of the collagen X promoter (Fig. 2). However, c-Raf targets different regions of the promoter to result in positive and negative regulation.

Introduction of ectopic wild type or constituitively active c-Raf into MCT cells repressed the

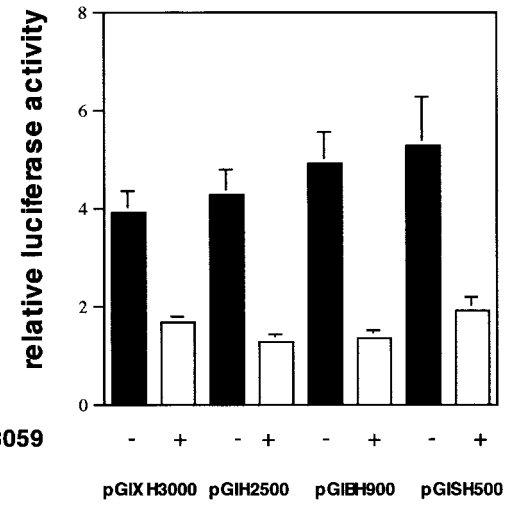

Fig. 6. Effects of the MEK inhibitor PD098059 on the activity of different collagen X reporter plasmids. The collagen X reporter plasmids pGlXH3000, pGlH2500, pGlBH900, and pGlSH500 were transfected into MCT cells. Two hours after transfection cells received 20 mm PD098059 (+) or DMSO (-). Thirty-eight hours after transfections, cells were harvested, firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield the relative luciferase activity. The average of three independent experiments and standard deviations are shown. Inhibition of MEK activity causes a significant reduction in the activity of all four collagen X reporter plasmids.

activity of the collagen X promoter through a fragment located between positions -2864 and -2410 (relative to the start site of transcription; Fig. 3). We have previously identified the same fragment as a silencer element inhibiting the expression of the collagen X gene in fibroblasts and immature chondrocytes [Beier et al., 1997]. The magnitude of the silencer effect and the repression by c-Raf is very similar, suggesting that the c-Raf pathway could be responsible for the silencer effect. Since c-Raf is apparently not expressed in proliferating, immature chondrocytes, it can not be involved in the repression of the collagen X promoter in these cells. However, several other kinases such as A-Raf, B-Raf, c-Mos, and MEKKs can activate the kinases downstream of c-Raf, MEK1/2, and ERK1/2 [reviewed in Lewis et al., 1998]. Ectopic expression of c-Raf could mimic these signals and contribute to the repression of collagen X promoter activity.

Inhibition of endogenous c-Raf by expression of a dominant-negative form of c-Raf represses the collagen X promoter through the basal 462 basepairs (Fig. 4). Inhibition of either MEK1/2 or ERK1/2 (downstream targets of c-Raf) cause very similar effects on promotor activity as inhibition of c-Raf (Figs. 5, 6). This is not surprising since these kinases are the predominant or possibly exclusive in vivo targets of c-Raf [Daum et al., 1994] and are therefore expected to be necessary for the transduction of the c-Raf signal to the target promoters. We have described a potential binding site for transcription factors of the Ets family, which are common targets of MAP kinase pathways [Treisman, 1996], within this 462 bp region [Beier et al., 1996]. Computer analyses did not show any additional sequence elements in this region of the promoter which could bind known targets of the c-Raf pathway (data not shown). As shown recently [Beier et al., 1996], the Ets site is the only potential transcription factor binding site conserved among the chicken, mouse, bovine, and human collagen X genes and presumably plays an important role in the activity of the proximal collagen X promoter. Further experiments will have to address whether the potential Ets binding site is the actual target sequence of the raf pathway, and which transcription factors are involved.

It appears puzzling that the c-Raf pathway should contribute to both positive and negative regulation of the collagen X promoter. However, it has been shown that differences in intensity or duration of c-Raf activation can cause opposite biological effects. For example, modest activation of c-Raf in NIH3T3 fibroblasts causes activation of cyclin D1 expression and proliferation, whereas high-intensity activation of c-Raf induces expression of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ and cell cycle arrest [Sewing et al., 1997; Woods et al., 1997]. A possible molecular basis for this phenomena is that the kinases downstream of c-Raf can activate different transcription factors, such as Ets factors, different Fos-related proteins, and c-myc [Lewis et al., 1998]. These transcription factors might have different thresholds for activation by the c-Raf pathway, which could explain why ectopic, activated c-Raf has different effects on gene expression than the endogenous c-Raf. Since inhibition of endogenous c-Raf, as well as of endogenous MEK1/2 and ERK1/2, clearly represses the activity of the collagen X promoter, the primary role of c-Raf in the transcriptional regulation of the collagen X gene appears to be stimulatory. The observed negative regulatory role of c-Raf might be used for the fine regulation of collagen X promoter activ-

ity in response to very strong activation of c-Raf. However, since all three forms of c-Raf inhibit the activity of the largest collagen X promoter fragment, it can not be excluded that the presence of increased amount of c-Raf protein interferes with other signaling pathways necessary for the activity of this fragment. While, to our knowledge, no such function of c-Raf has been reported in the literature, this mechanism remains a possibility. Since the repression of activity of the shorter collagen X promoter fragments are specific for the dominant-negative form of c-Raf, and can be reproduced by inhibition of MEK1/2 and ERK1/2, this repression can clearly be attributed to inhibition of the c-Raf pathway.

Our data do not exclude the possibility that activation of the c-Raf pathway induces MCT cell differentiation first, and upregulation of collagen X promoter activity is a secondary event. However, the presence of a binding site for Ets transcription factors in the collagen promoter suggests that the c-Raf pathway activates the collagen X promoter directly. In addition, the MEK inhibitor PD98059 causes a downregulation of collagen X promoter activity within 24 hours, whereas other parameters of MCT differentiation (such as cell proliferation) are not affected within this time frame (data not shown). These points make the above mentioned possibility unlikely.

In summary, these data suggest a dual role for c-Raf (or related factors) in the regulation of the human collagen X promoter. Raf proteins may be involved in the repression of the promoter in cells not expressing type X collagen. More importantly, raf signaling is required for full activity of the collagen X promoter in hypertrophic chondrocytes.

The extracellular signals that induce c-Raf activity in hypertrophic chondrocytes are currently unknown. c-Raf can be activated by growth factors acting through tyrosine kinase receptors [Daum et al., 1994]. Among these, insulin, IGFs, and FGFs are known to play a role in the regulation of chondrocyte differentiation [Cancedda et al., 1995]. Members of the TGFβ (transforming growth factor) family such as TGFβ-1 and BMP-4 (bone morphogenetic protein 4) have also been shown to activate c-Raf [Reimann et al., 1997; Xu et al., 1996]. In addition, integrin signaling can activate c-Raf and the MAP kinase pathway [Chen et al., 1996] and has been demonstrated to be essential for differentiation of chondrocytes [Hirsch et al., 1997]. Experiments are under way to identify the physiological activators of c-Raf in hypertrophic chondrocytes.

Collagen X is not the only hypertrophic chondrocyte-specific gene regulated by c-Raf. The cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ has been shown to be upregulated in hypertrophic chondrocytes [Stewart et al., 1997] and is likely involved in exit from the cell cycle during differentiation. We have found that inhibition of the c-Raf pathway strongly represses the expression of p21 in MCT cells, whereas introduction of activated c-Raf increases p21 promoter activity [unpublished communication]. c-Raf signaling may therefore represent a mechanism that connects the exit from the cell cycle with the onset of differentiation-specific gene expression in growth plate chondrocytes.

Although c-Raf has been most often associated with the control of cellular proliferation and survival, it is also required for the differentiation of certain cell types in invertebrates and vertebrates [Daum et al., 1994]. Within the growth plate, c-Raf clearly appears to be involved in the establishment of the differentiated, hypertrophic phenotype of chondrocytes. This model is supported by the expression pattern of c-Raf in the growth plate [Kaneko et al., 1994] as well as by its role in the expression of the p21 and collagen X genes.

The data presented here are, to our knowledge, the first to address the function of an important signal transduction pathway in hypertrophic chondrocytes. While much work remains to be done to characterize both the upstream factors activating c-Raf in chondrocytes and the transcription factors and specific cis-acting elements mediating the action of the raf pathway, we show here that c-Raf can both inhibit and activate the expression of the collagen X promoter and may function as an integrator of several extracellular signals regulating collagen X expression.

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