The Raf-1/MEK/ERK Pathway Regulates the Expression of the p21<sup>Cip1/Waf1</sup> Gene in Chondrocytes<br><br>(Received for publication, January 8, 1999, and in revised form, July 15, 1999)  

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The gene encoding the cyclin-dependent kinase inhibitor p21<sup>Cip1/Waf1</sup> is up-regulated in many differentiating cells, including maturing chondrocytes. Since strict control of chondrocyte proliferation is essential for proper bone formation and since p21 is likely involved in this control, we initiated analyses of the mechanisms regulating expression of p21 in chondrocytes. p21 expression and promoter activity was strongly increased during the differentiation of chondrogenic MCT cells. We have identified a 68-base pair fragment conferring transcriptional up-regulation of the p21 gene in chondrocytes. The activity of this fragment required active Raf-1 in MCT cells as well as in primary mouse chondrocytes. Inhibition of downstream factors of Raf-1 (MEK1/2, ERK1/2, and Ets2) also repressed the activity of the 68-base pair fragment in MCT cells. The chemical MEK1/2 inhibitor PD98059 reduced protein levels of p21 in MCTs and primary mouse chondrocytes. These data suggest that signaling through the Raf-1 pathway is necessary for the optimal expression of p21 in chondrocytes and may play an important role in the control of bone formation.

Axial and appendicular skeletal elements are formed by endochondral ossification, a process that involves formation of cartilage precursors and subsequent replacement by bones (reviewed in Ref. 1). This transition from cartilage to bone occurs within the growth plate, where longitudinal bone growth is controlled. Growth plate chondrocytes undergo a series of rapid cell divisions before they withdraw from the cell cycle and differentiate to large, hypertrophic cells. These hypertrophic chondrocytes undergo apoptosis, their extracellular matrix is degraded, and they are replaced by osteoblasts that produce a bone matrix. Both proliferation and differentiation-associated enlargement of growth plate chondrocytes contribute to bone growth. Analyses of transgenic and knockout mice have demonstrated that disturbance of the fine balance between chondrocyte proliferation and differentiation can cause skeletal defects such as skeletal dysplasias (2–4). Deregulation of chondrocyte proliferation may also be involved in the pathogenesis of chondrosarcomas (5). However, the molecular processes regulating chondrocyte proliferation and differentiation remain largely unknown.

Disruptions of several mouse genes involved in growth control have been shown to cause severe deformities and growth abnormalities of the skeleton with frequently lethal consequences. Examples are the inactivation of the genes encoding the transcription factors ATF-2 and c-Fos (6, 7), the antiapoptosis protein Bel-2 (8), the thyroid hormone α and vitamin D receptors (9–11), the fibroblast growth factor receptor 3 (12), the parathyroid hormone-related peptide (13, 14), the p57<sup>Kip2</sup> gene (15), and the double knockout of the p107 and p130 genes (16).

The p57, p107, and p130 genes belong to the large group of genes controlling progression through the cell cycle. Our current knowledge of the role and regulation of cell cycle genes in chondrocytes has been reviewed recently (17). p107 and p130 are closely related to the retinoblastoma protein (pRb) (reviewed in Ref. 18). These proteins, called pocket proteins, are capable of inhibiting cell cycle progression when they are present in their hypophosphorylated forms. The cyclin-dependent kinases (CDKs)<sup>1</sup> phosphorylate (and thereby inactivate) the pocket proteins. CDK activity is regulated by at least three mechanisms. The CDKs must associate with their partners, the cyclins, to be active. In addition, activation of CDKs requires phosphorylation of some amino acid residues and dephosphorylation of others. As well, CDK activity can be inhibited by members of two groups of CDK inhibitors: the Cip/Kip group, consisting of p21<sup>Cip1/Waf1</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup>, and the Ink group, consisting of p16<sup>Ink4a</sup>, p16<sup>Ink4a</sup>, and p15<sup>Ink4b</sup> (reviewed in Ref. 19).

Simultaneous inactivation of the p107 and p130 genes results in delayed chondrocyte differentiation, leading to deformed bones, shortened limbs, and perinatal death (16). A similar phenotype was observed in p57<sup>Kip2</sup> null mice (15). These data suggest that loss of these negative regulators of cellular proliferation delays the exit from the cell cycle that normally occurs during chondrocyte differentiation. A second member of the Cip/Kip group of CDK inhibitors, p21<sup>Cip1/Waf1</sup>, was recently shown to be up-regulated in differentiated, hypertrophic chondrocytes (20), consistent with its putative role in exit from the cell cycle during differentiation. p21<sup>Cip1/Waf1</sup> was originally cloned as an inhibitor of cyclin-dependent kinases, as a gene up-regulated in cellular senescence, and as a p53-responsive gene that was able to confer cell cycle arrest in response to activation of p53 upon DNA damage and other stresses (21–25). p21 expression during development is often high in differentiated, postmitotic cells, and appears to be largely independent of p53 (26). On the other hand, p53, a tumor suppressor, is involved, for example, in the nerve growth factor-induced activation of the p21 promoter during differentiation of PC12 cells (27). Although p21−/− mice show normal

* This work was supported by grants from the Medical Research Council of Canada, the Arthritis Society, and the Alberta Cancer Foundation (to F. B.) and by postdoctoral fellowships from the Deutsche Forschungsgemeinschaft and Deutscher Akademischer Austauschdienst (to P. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: CDK, cyclin-dependent kinase; bp, base pair; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; WT, wild type.
development, indicating that the role of p21 in terminal differentiation is redundant, fibroblasts from p21-deficient mice are deficient in their ability to arrest in the G1 phase of the cell cycle (28). In addition, keratinocytes from p21−/− mice have been shown to display enhanced proliferative capacity, reduced expression of differentiation markers, and enhanced tumorigenesis in vivo (29). These results imply that p21 plays an important role in the terminal differentiation of certain cell types.

We have investigated the expression of p21 in the differentiating chondrogenic cell line MCT (30) as well as in primary mouse chondrocytes in order to identify the mechanisms regulating p21 expression in chondrocytes. Our data indicate that a 68-bp pair fragment of the p21 promoter, containing binding sites for Ets family transcription factors and p53, is responsible for the up-regulation of p21 in these cells. ERK1/2 phosphorylation increases in parallel with p21 induction, suggesting that the Raf-1 pathway (which activates Ets family transcription factors), rather than p53, is necessary for this up-regulation of p21. Inhibition of Raf-1, MEK1/2, ERK1/2, or Ets2 activity blocks the induction of p21 promoter activity during chondrocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Reagents and Materials**—Cell culture media, sera, antibiotics, glutamine, and Lipofectin were purchased from Life Technologies, Inc. The Dual Luciferase Assay Kit was from Promega. The p21 (C19) and ERK (D2) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), the actin antibody was from Roche Molecular Biochemicals, and the phosphospecific ERK antibody (number 9105) was from New England Biolabs. The MEK inhibitor PD98059 was purchased from Calbiochem.

**Plasmids**—Raf-1 expression plasmids have been described recently and were provided by Drs. U. Rapp and S. Ludwig (31). The dominant negative ERK expression plasmid pCMV-p41mapk (K/A) was kindly provided by Dr. R. Davis (32). Ets2 expression constructs were obtained from Dr. R. Pestell (33). Construction of plasmid pG1 p21 H2320 employed a HindIII fragment from WWMbuc (a plasmid containing the human p21 promoter; Ref. 21) cloned into the HindIII site of pG2 basic (Promega). Digestion of pG1 plasmid p21 H2320 with SacI or SmaI and religation yielded the plasmids pG1 p21 S2260 and pG1 p21 S2260, respectively. Digestion of pG1 plasmid p21 H2320 or pG1 p21 S2260 with NeI and PstI and religation yielded the plasmids pG1 p21 H/NP and pG1 p21 S−N/P, respectively. Numbering of nucleotides of the p21 promoter was done according to Ref. 21.

**Cell Culture and Transfections**—MCT cells were cultured as described (30) in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (50 units/ml), streptomycin (50 μg/ml) at 32 °C under 5% CO2. The evening before transfections, 1 × 10⁵ cells were seeded into each well of a 24-well plate. The next morning, transfections were performed with Lipofectin according to the manufacturer’s protocol. Briefly, each well was transfected with 1.5 μg of reporter gene construct and 0.5 μg of pRLSV40 (Promega) to standardize for transfection efficiency using 1.5 μl of Lipofectin for 4 h at 32 °C. For cotransfections, 1.5 μg of reporter plasmid was co-transfected with 1.0 μg of Raf-1 or ERK expression plasmid or with empty expression vector and 0.3 μg of pRLSV40. After transfections, cells were cultured for further 24 h at 32 °C and then lysed with Passive Lysis Buffer (Promega) according to the manufacturer’s protocol. For inhibition of MEK1/2 activity, cells were transfected as above and cultured for 6 h after transfection in the presence of 10% fetal bovine serum prior to the addition of 20 μM PD98059 or an equivalent volume of Me2SO (for controls) for the last 18 h of incubation.

**Isolation and Transfection of Primary Chondrocytes**—Primary chondrocytes were isolated from newborn mice as described (34, 35). Chondrocytes were plated at a density of 3 × 10⁵ cells/10-cm plate, incubated over night to a density of 40%, and treated with Me2SO or 20 μM PD98059 for 18 h in order to analyze p21 protein expression. Cells were then harvested for Western blot analyses as described below. Transfections were performed as described above.

**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). 10 μl of lysate was assayed first for firefly luciferase and then for Renilla luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity. Data represent the average and S.D. of three independent experiments, each done in triplicate. Statistical analyses of all luciferase values were performed using Student’s t test. Significant differences were established as p < 0.05.

**Western Blot Analyses**—MCT cells were cultured at 32 °C to 40% confluence and then cultured for a further 24 h at 32 or 37 °C and lysed in SDS loading buffer for analyses of p21 expression. MCT cells were transferred to 37 °C at 40% confluence and incubated for 18 h in the presence of 20 μM PD98059 or an equal volume of Me2SO. Primary chondrocytes were cultured to 40% confluence and then incubated for 18 h in the presence or absence of PD98059 as above. The amount of protein corresponding to 5 × 10⁶ cells was separated on SDS gels and transferred to Hybond-C membranes (Amersham Pharmacia Biotech). Proteins binding to the primary antibodies were detected with a horseradish peroxidase-conjugated secondary anti-rabbit antibody (Santa Cruz Biotechnology) and visualized with the enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech).

**Northern Blot Analyses**—MCT cells were cultured as above and harvested for isolation of RNA as described (36). 10 μg of total RNA were separated on 0.8% denaturing agarose gels, blotted onto Hybond-N+ membranes (Amersham Pharmacia Biotech), and hybridized to a digoxigenin-labeled p21 probe. Digoxigenin labeling of the mouse p21 cDNA (37), hybridization, washing, and detection (using CDP-Star) were performed with reagents from Roche Molecular Biochemicals according to the manufacturer’s instructions.

**RESULTS**

**Expression of p21 in Chondrogenic MCT Cells**—Expression of p21 in growth plate chondrocytes has recently been shown to be linked to maturing and hypertrophic chondrocytes (20). In order to identify possible cell culture models for the study of the regulation of p21 gene expression in chondrocytes, we analyzed the levels of p21 protein during differentiation of MCT cells. MCT cells are derived from murine chondrocytes immortalized with a temperature-sensitive SV40 large T antigen. At 32 °C they show moderate expression of several markers of maturing and differentiated, hypertrophic chondrocytes. Expression of these markers strongly increases upon transfer to 37 °C and is paralleled by an immediate stop in proliferation and subsequent cell death within 96 h (Ref. 30; data not shown). Levels of p21 increased strongly after 24 h at 37 °C (Fig. 1A). p21 mRNA expression also increased significantly upon transfer to 37 °C (Fig. 1B). Transient transfections of a plasmid encoding the firefly luciferase reporter gene driven by a 2320-bp (base pair) fragment of the human p21 promoter (plasmid pG1 p21 H2320) showed a similar pattern of activity. Whereas the p21 promoter conferred promoter activity similar to the herpes simplex thymidine kinase promoter in MCT cells cultured at 32 °C, it increased 6-fold after transfer to 37 °C (Fig. 1C).

**Activity of p21 Promoter Fragments in MCT Cells**—We transfected several deletion constructs of the 2320-base pair (bp) fragment of the p21 promoter (Fig. 2A) into MCT cells at 37 °C in order to analyze activities in differentiating chondrocytes. Deletion of a 68-bp fragment at the 5′-end of the construct (plasmid pG1 p21 S2260) resulted in a 75% decrease in promoter activity, compared with plasmid pG1 p21 H2320 (Fig. 2B). Deletion of an internal fragment of 1874 bp (plasmid pG1 p21 H N/P) had almost no effect on promoter activity. However, deletion of the 5′-68-bp fragment and the internal fragment together (plasmid pG1 p21 S−N/P) reduced promoter activity to less than 10% of that of the 2320-bp promoter fragment. The activity of pG1 p21 S−N/P was very similar to that of the 5′-68-bp fragment and the internal fragment together. Luciferase activity was normalized to Renilla luciferase activity. Data represent the average and S.D. of three independent experiments, each done in triplicate. Statistical analyses of all luciferase values were performed using Student’s t test. Significant differences were established as p < 0.05.

We investigated the role of the 68-bp fragment on p21 promoter up-regulation at 37 °C during MCT differentiation by determining the activities of plasmids pG1 p21 H2320 and pG1 p21 S2260 at both temperatures (Fig. 2C). Whereas the full-length promoter displayed 6-fold higher activity at 37 °C, the promoter fragment missing the 68 nucleotides showed only a...
expression of the p21 gene was examined at the protein level by Western blotting. MCT cells were incubated for 24 h at 32 or 37 °C. The activity of the Raf-1 pathway also causes reduced levels of p21 protein. MCT cells were incubated for 24 h at 37 °C in the presence of Me2SO or PD98059. Western blot analyses showed that inhibition of MEK1/2 with PD98059 induced a dramatic decrease in p21 protein levels (Fig. 6).

**ERK1/2 Phosphorylation Increases during MCT Differentiation**—Our results implied that the activity of the Raf-1/MEK/ERK pathway increases during MCT differentiation. To verify this, the phosphorylation status of ERK1/2 (which reflects the activity of MEK1/2) in MCT cells cultured at 32 and 37 °C was investigated using Immunoblot analyses with a phosphospecific ERK1/2 antibody. The amount of phosphorylated ERK1 increases 3.2-fold after 24 h at 37 °C (Fig. 7). Immunoblot analyses with a nonphosphospecific ERK1/2 antibody revealed that equal amounts of ERK1 protein were present at both temperatures. With both antibodies, ERK2 could only be detected after prolonged exposure of the blots with either of the antibodies (data not shown).

**The Effect of the c-Ets2 Transcription Factor on p21 Promoter Activity**—The transcription factor c-Ets2 has been shown to be expressed in chondrocytes (42) and to be a target of the Raf-1/MEK1/ERK1/2 pathway (43). We cotransfected the plasmid pGL p21 H2320 with expression constructs for different forms of c-Ets2 (33, 43) into MCT cells at 32 °C (data not shown) and at 37 °C (Fig. 8A). Overexpression of WT c-Ets2 at 37 °C increased the activity of the promoter 2.7-fold, whereas overexpression of c-Ets2 (EtslacZ) caused a reduction of 56% in promoter activity (Fig. 8B). Overexpression of a dominant negative version of c-Ets2 (EtslacZ) caused a reduction of 56% in promoter activity. p21 promoter activity was stimulated by c-Ets2 at 32 °C to a similar degree.

We next examined whether c-Ets2 is necessary for Raf-1 activation of the p21 promoter (Fig. 8B). The plasmid pGL p21 H2320 was cotransfected with expression vectors for activated Raf-1 (BXXB) and the different forms of c-Ets2. Stimulation of the p21 promoter by activated Raf-1 was enhanced by WT c-Ets2, but the nonphosphorylatable mutant (Ets-2 A72) and the dominant negative form of c-Ets2 inhibited this effect by 47% and 69%, respectively.

**The Raf-1 Pathway Is Necessary for p21 Expression in Primary Chondrocytes**—We next wanted to determine whether
the effects of the Raf-1 pathway on p21 expression that we had observed in the MCT cell line were also present in primary chondrocytes. We transfected pGl p21 H2320 and pGl p21 S2260 together with wild type Raf-1, constitutively active Raf-1 (BXB), and dominant negative Raf-1 (C4B) (Fig. 9). Overexpression of wild type or activated Raf-1 caused a 2.3- and 2.9-fold increase in p21 pGl H2320 activity, respectively, whereas overexpression of the dominant negative form of Raf-1 inhibited promoter activity of this fragment by 65%. In contrast, pGl p21 S2260 displayed only marginal responses to ectopic Raf-1.

To verify the role of the Raf-1 pathway in p21 protein expression in primary chondrocytes, we incubated primary mouse chondrocytes with Me2SO or 20 μM PD98059 for 18 h and harvested for Western blot analyses (Fig. 10). PD98059 caused a 3.8-fold reduction in p21 protein levels in primary chondrocytes.

DISCUSSION

We have identified a 68-base pair fragment of the p21Cip1/Waf1 promoter that is responsible for the regulation of p21 promoter activity in both the chondrogenic cell line MCT (30) and primary mouse chondrocytes. Deletion of this fragment resulted in an approximately 70% decrease in promoter activity in both cell types (Figs. 2 and 9). In contrast, pGl p21 S2260 displayed only marginal response to ectopic Raf-1.

To verify the role of the Raf-1 pathway in p21 protein expression in primary chondrocytes, we incubated primary mouse chondrocytes with Me2SO or 20 μM PD98059 for 18 h and harvested for Western blot analyses (Fig. 10). PD98059 caused a 3.8-fold reduction in p21 protein levels in primary chondrocytes.
effect on the activity of the p21 promoter in MCT cells. However, deletion of this fragment clearly enhances the effect of the deletion of the 68-bp fragment. These data suggest that response elements for the same pathways or transcription factors are present in both fragments. Binding sites for both Ets family transcription factors and p53 have been reported in both the 68-bp and the NsiI/PstI fragment (34, 35). Deletion of the NsiI/PstI fragment has no effect on promoter activity as long as the major response element in the 68-bp fragment is present, but deletion of both promoter fragments together causes strong reduction of promoter activity.

Transcription factors of the Ets family are common targets of MAP kinase pathways (44, 45). In addition, Raf-1 (an activator of MAP kinases) and p21 are both up-regulated in maturing and differentiated chondrocytes in vivo (20, 40). Here we show that Raf-1 and its downstream kinases MEK1/2 and ERK1/2 are necessary for the activity of the 68-bp p21 promoter fragment in differentiating MCT cells. This effect is probably mediated by the Ets site within this portion of the p21 promoter (38), since overexpression of c-Ets2, which is expressed in chondrocytes in vivo (42), had similar effects as overexpression of Raf-1 (Figs. 4 and 8). More importantly, dominant negative c-Ets2, or a form of c-Ets2 that cannot be phosphorylated by ERK1/2, repressed the stimulatory effect of activated Raf-1 on p21 promoter activity, suggesting that endogenous c-Ets2 (or a closely related factor) is necessary for the transduction of the Raf-1 signal. c-Ets2 can also activate p21 promoter activity at 32 °C where p53 is inactivated by the simian virus 40 large T antigen (data not shown), suggesting that these effects of c-Ets2 are independent of p53. Similarly, overexpression of wild type or activated Raf-1 can also activate the p21 promoter at 32 °C, providing further evidence for a p53-independent mechanism.

The importance of c-Ets2 in skeletal development has recently been demonstrated in transgenic mice, where overexpression of this transcription factor caused skeletal defects similar to those found in Down’s syndrome (46). p21 might be one of the targets genes of c-Ets2 in chondrocytes responsible for this phenotype, although additional target genes probably contribute to the defects, since p21-deficient mice do not display obvious skeletal phenotypes (28). The absence of skeletal defects in p21-deficient mice can probably be explained by the redundancy among the genes encoding CDK inhibitors. At least three other CDK inhibitors (p16, p27, p57) have been shown to be expressed in chondrocytes (15, 47, 48) and may substitute for p21 function in its absence.

The phosphorylation of ERK1 increases during MCT differentiation. Protein extracts from MCT cells cultured for 24 h at 32 or 37 °C were analyzed for ERK1/2 protein content and phosphorylation by Western blot. Whereas similar levels of total ERK1 protein were detected at both temperatures, the levels of phosphorylated ERK1 increased sharply at 37 °C. In both cases, ERK2 was detectable only after extended exposure of the blot (data not shown). Western blot for actin showed that equal levels of protein were loaded.
probably responsible for the activation of MEK1/2 in differentiating MCT cells. Recently, two groups reported that very strong activation of transfected Raf-1 in fibroblasts was able to induce expression of p21 (49, 50). However, we demonstrate that the activities of endogenous Raf-1, MEK1/2, and ERK1/2 are required for maximal expression of the p21 gene in chondrocytes. In addition, we have, for the first time, located the cis-active element in the p21 promoter responsible for the majority of these effects in chondrocytes. The coexpression of p21 and Raf-1 in vivo (20, 40) makes it very likely that the regulatory relationship between both that we have demonstrated in vitro exists in vivo as well.

Since deletion of the 68-bp fragment causes a larger decrease in p21 promoter activity than inhibition of the Raf-1/MEK/ERK pathway (Figs. 2 and 4), additional mechanisms must contribute to the activity of this fragment. One candidate for this function is the p53 response element (21, 39), which overlaps with the Ets binding site. However, since the shift from 32 to 37 °C results in inactivation of the SV40 large T antigen and thus activation of p53, it remains to be examined whether involvement of p53 in the control of the p21 promoter in chondrocytes is of physiological relevance. Alternatively, the Ets binding site might be responsible for the observed activity in a manner independent of the Raf pathway. A contribution of additional, so far unidentified cis-active elements cannot be excluded as well.

The construct pGI p21 S2260, which does not contain the 5' Ets binding site, still demonstrated mild transcriptional responses to overexpression of the different Raf-1 constructs (Fig. 4), as well as to PD98059 and overexpression of dominant negative Raf-1 (C4B). After 24 h at 37 °C, cells were harvested, and cytosolic firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield relative luciferase activity. Whereas wild type c-Ets2 synergizes with Raf-1 to activate the p21 promoter, both dominant negative and nonphosphorylatable c-Ets2 repress the activating function of Raf-1.

### FIG. 8. The effect of Raf-1 is mediated by Ets family transcription factors. A, pGI p21 H2320 was transfected (together with pRlSV40) into MCT cells, together with empty expression vector (vector), expression vectors for WT c-Ets2 or a mutant c-Ets2 that cannot be phosphorylated by ERK1/2 (Ets A72), or dominant negative c-Ets2 (Ets lacZ). After 24 h at 37 °C, cells were harvested, and cytosolic firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield relative luciferase activity. Only wild type c-Ets2 could activate the p21 promoter. B, pGI p21 H2320 was cotransfected with activated Raf-1 (BXB) into MCT cells, together with empty expression vector (vector) or expression vectors for the different forms of c-Ets2 as in A. After 24 h at 37 °C, cells were harvested, and cytosolic firefly luciferase activity was measured and standardized to Renilla luciferase activity to yield relative luciferase activity. Whereas wild type c-Ets2 synergizes with Raf-1 to activate the p21 promoter, both dominant negative and nonphosphorylatable c-Ets2 repress the activating function of Raf-1.

### FIG. 9. The p21 promoter is regulated by Raf-1 in primary chondrocytes. Primary mouse chondrocytes were incubated for 18 h at 37 °C in the presence of 20 μM PD98059 (+) or an equal volume of Me2SO (DMSO) (–). p21 protein expression was determined using Western blot analyses. Equal gel loading was demonstrated using an antibody against actin.

### FIG. 10. PD98059 inhibits expression of p21 protein in primary mouse chondrocytes. Primary mouse chondrocytes were incubated for 18 h at 37 °C in the presence of 20 μM PD98059 (+) or an equal volume of Me2SO (DMSO) (–). p21 protein expression was determined using Western blot analyses. Equal gel loading was demonstrated using an antibody against actin.
MEK/ERK pathway uses additional mechanisms to increase p21 levels, such as translational control or regulation of mRNA or protein stability. Alternatively, promoter elements not present in the 2320-bp promoter fragment used may be responsible for this effect.

Raf-1 can be activated by many different signals, including growth factors acting through receptor tyrosine kinases (reviewed in Ref. 42). Among these, insulin, insulin-like growth factors, and fibroblast growth factors are known to regulate chondrocyte proliferation and differentiation (reviewed in Ref. 1). In addition, integrin signaling has been shown to activate Raf-1 (52), and β1 integrin function has been shown to be essential for chondrocyte differentiation (53). Finally, transforming growth factor β-1 and bone morphogenetic proteins have been shown to activate Raf-1 in some cell types (54, 55) and are also known to regulate skeletogenesis (1). Experiments are under way to identify which of these signals contribute(s) to the regulation of Raf-1 activity in chondrocytes.

The effect of ectopic Raf-1 on p21 promoter activity (Figs. 4 and 9) as well as the effects of the MEK inhibitor PD98059 on p21 protein expression (Figs. 6 and 10) are very similar in MCT cells and primary mouse chondrocytes. The 68-bp fragment is responsible for the effects of the Raf-1 pathway on the p21 promoter in both cell types. Therefore, it is very likely that the complete pathway connecting Raf-1 to the p21 gene has been maintained in the MCT cell line. The data obtained with primary chondrocytes suggest that the Raf-1 pathway plays an important role in the regulation of p21 expression in cartilage in vivo.

In addition to its role in the regulation of p21 gene expression, Raf-1 is also necessary for the transcription of the type X collagen gene in hypertrophic chondrocytes (41). Type X collagen is the classical marker for these differentiated, postmitotic cells. Since it is essential for the optimal expression of both p21 and collagen X, we suggest that Raf-1 may play an important role in the coordination of cell cycle withdrawal and the onset of differentiation-specific gene expression during chondrocyte maturation.

In summary, we have identified the Raf-1/MEK/ERK pathway as the major regulator of p21 gene transcription in differentiating chondrocytes. These data suggest a new role for this pathway in the control of chondrocyte differentiation and bone growth. Future studies will have to address the cellular mechanisms regulating Raf-1 activity in chondrocytes as well as a potential role for p21 or the Raf-1 pathway in skeletal disorders. In addition, it will be of great interest to examine the function of the Raf-1 pathway during endochondral ossification in vivo.

Acknowledgments—We are grateful to Drs. B. de Crombrugghe and V. Lefebvre for MCT and RCS cells, Dr. B. Vogelstein for the plasmid WWPluc, Dr. R. Davis for the dominant negative ERK expression vector, and Drs. U. Rapp and S. Ludwig for Raf-1 expression plasmids.

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