RhoA/ROCK Signaling Suppresses Hypertrophic Chondrocyte Differentiation*

Coordinated proliferation and differentiation of growth plate chondrocytes is required for normal growth and development of the endochondral skeleton, but little is known about the intracellular signal transduction pathways regulating these processes. We have investigated the roles of the GTPase RhoA and its effector kinases ROCK1/2 in hypertrophic chondrocyte differentiation. RhoA, ROCK1, and ROCK2 are expressed throughout chondrogenic differentiation. RhoA overexpression in chondrogenic ATDC5 cells results in increased proliferation and a marked delay of hypertrophic differentiation, as shown by decreased induction of alkaline phosphatase activity, mineralization, and expression of the hypertrophic markers collagen X, bone sialoprotein, and matrix metalloproteinase 13. These effects are accompanied by activation of cyclin D1 transcription and repression of the collagen X promoter by RhoA. In contrast, inhibition of Rho/ROCK signaling by the pharmacological inhibitor Y27632 inhibits chondrocyte proliferation and accelerates hypertrophic differentiation. Dominant-negative RhoA also inhibits induction of the cyclin D1 promoter by parathyroid hormone-related peptide. Finally, Y27632 treatment partially rescues the effects of RhoA overexpression. In summary, we identify the RhoA/ROCK signaling pathway as a novel and important regulator of chondrocyte proliferation and differentiation.

The development and growth of endochondral bones (such as ribs, vertebrae, and the long bones of vertebrate limbs) are regulated through the highly controlled rates of proliferation and hypertrophic differentiation of growth plate chondrocytes (1–3). In the growth plate, chondrocytes first undergo a series of cell divisions along the longitudinal axis of the growing bone, thereby forming characteristic columns of clonal cells. Chondrocytes then withdraw from the cell cycle and begin to increase their cell volume until reaching the fully differentiated state of hypertrophic chondrocytes. Transition from a proliferating to a hypertrophic phenotype involves numerous changes in gene expression, for example the induction of the collagen X (4, 5), matrix metalloproteinase 13 (6, 7), and bone sialoprotein, and matrix metalloproteinase 13. These effects are accompanied by activation of cyclin D1 transcription and repression of the collagen X promoter by RhoA. In contrast, inhibition of Rho/ROCK signaling by the pharmacological inhibitor Y27632 inhibits chondrocyte proliferation and accelerates hypertrophic differentiation. Dominant-negative RhoA also inhibits induction of the cyclin D1 promoter by parathyroid hormone-related peptide. Finally, Y27632 treatment partially rescues the effects of RhoA overexpression. In summary, we identify the RhoA/ROCK signaling pathway as a novel and important regulator of chondrocyte proliferation and differentiation.

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1 The abbreviations used are: BSP, bone sialoprotein; CDEP, chondrocyte-derived ezrin-like domain-containing protein; Luc, luciferase; MTM, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PTH, parathyroid hormone; PTHrP, PTH-related peptide; RCS, rat chondrosarcoma; RT, reverse transcription, wt, wild type.

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RhoA in ATDC5 cells results in enhanced cell proliferation and a marked delay in hypertrophic differentiation. In contrast, inhibition of ROCK signaling by a pharmacological inhibitor accelerates differentiation of ATDC5 cells and primary cells and partially rescues the effects of RhoA overexpression. These data suggest a novel and important function of Rho signaling in skeletal growth and development.

**EXPERIMENTAL PROCEDURES**

**Materials**—Timed pregnant CD1 mice (at 11.5 days postcoitus) were purchased from Charles River Laboratories. All cell culture media components were from Invitrogen or Sigma unless stated otherwise. Y27632, PTHrP were purchased from Calbiochem. All other reagents were of analytical grade from commercial suppliers. The RhoA expression plasmid used for generation of stable ATDC5 transfectants was obtained from the Guthrie cDNA Resource Center (www.cdna.org). The control expression vector pCDNA3.1+ was purchased from Invitrogen. Mutant and wild type RhoA expression vectors used in transient transfections were obtained from A. Hall. The reporter plasmids –1745CD1Luc (45) and pGHI2500ColX (46) have been described previously. The plasmid used for standardization, pRLSV40, was purchased from Promega. The RhoA antibody was from Cytoskeleton. ROCK1 and 2 antibodies were from Santa Cruz Biotechnology, and the β-actin antibody was from Sigma.

**Cell Culture**—ATDC5 cells were essentially cultured as described previously (47, 48). Cells were grown in Dulbecco’s modified Eagle’s medium and F-12 medium containing 5% fetal bovine serum at 37 °C under 5% CO2. Differentiation was initiated by the addition of 10 μg/ml insulin, 10 μg/ml transferrin, and 10 μg/ml selenium (ITS; Sigma). Rat chondrosarcoma (RCS) cells (49) were cultured as described previously (49, 50). Primary chondrocytes were isolated from the ribs of newborn mice as described previously (50, 51) and used within the first passage.

**Generation of Stable Transfectants**—ATDC5 cells were plated at 2 × 104 cells/ml in 6-well plates and transfected with pCDNA3.1+ (vector control) or the RhoA expression vector using FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. 24 h after transfection, selection was initiated using G418 (Invitrogen) at a concentration of 400 μg/ml. To avoid clonal artifacts, pools of transfected cells were collected and cultured as the parental ATDC5 cells but in the continued presence of G418. All experiments were performed three times, and representative experiments are shown.

**Micromass Cultures**—Micromass cultures from embryonic mouse limb buds were established and cultured as described previously (52). We have demonstrated recently that this culture system allows the reproducible hypertrophic differentiation of primary mouse limb bud cells in vitro (52).

**RNA Isolation and RT-PCR**—Total RNA was extracted from the cultures at the indicated time, using the Trizol reagent RNA extraction protocol according to manufacturer’s instructions (Invitrogen). cDNA was prepared from 1 μg of total RNA using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen) with (d)T12,14, and random hexamer primers (Amersham Biosciences). Amplification was performed in a 50-μl reaction mixture containing 1 μl of cDNA reaction and using AmpliTaq Gold polymerase (PerkinElmer Life Sciences). The primer sequences used for each PCR are outlined below. Initial denaturation was set at 95 °C for 1.5 min, annealing temperatures ranged from 60 to 65°C for 45–120 s, followed by extension at 72 °C. A final extension at 72 °C for 6 min concluded the reaction. PCR cycles varied from 20 to 37 cycles. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

**Real Time PCR**—Real time PCRs for collagen X and BSP were performed as described previously (52) using the TaqMan one-step RT-PCR kit (Applied Biosystems) in an ABI Prism 7500 HT Sequence Detector (PerkinElmer Life Sciences) with 40 cycles and an annealing temperature of 60 °C.

**Protein Isolation and Western Blotting**—For protein extraction, cells were washed twice in ice-cold phosphate-buffered saline and lysed in Cell Lysis Buffer (Cytoskeleton, Inc.). Protein concentrations were determined by BCA assays (Sigma) according to the manufacturer’s protocol. 40 μg of protein/lane was resolved on a 15% SDS-polyacrylamide gel, and proteins were transferred onto BioTrace™ polyvinylidene difluoride membranes ( Pall), which were incubated in blocking buffer (5% low fat milk power in Tris-buffered saline). Blocked filters were incubated with a 1:500 dilution of primary antibody in blocking buffer for 1 h and subsequently with a 1,8000 dilution of secondary antibody in blocking buffer for 1 h. Signal was visualized using enhanced chemiluminescence (ECL).

**Alkaline Phosphatase Activity and Mineralization Assays**—Alkaline phosphatase activity and mineralization were analyzed using staining methods and quantitative assays as described previously (52).

**Proliferation Assays**—For proliferation assays, ATDC5 cells were plated at 2 × 104 cells/ml in 96-well tissue plates for MTT assays and in 24-well plates for counting by hemocytometer. For MTT assay cells were incubated for the times indicated. 10 μl of MTT solution (Roche Applied Science) was added for the last 4 h. After solubilization, the formazan dye was quantitated using a scanning multwell spectrophotometer at 600 nm. The absorbance (A490 nm–A700 nm) revealed correlates directly with the cell number.

**Transient Transfections**—Primary chondrocytes were isolated from newborn mice as described previously (50, 51) and transfected with cyclin D1 and collagen X reporter vectors (50, 55), using 1 μg of reporter vector, 0.2 μg of expression plasmid (empty expression vector or RhoA expression vectors), and 0.1 μg of pRLSV40 for standardization. 48 h after transfections, cells were harvested, and firefly activity was deter-
FIG. 2. RhoA supports chondrocyte proliferation. A, RhoA protein levels were analyzed in untransfected (wt), vector-transfected, and RhoA-transfected ATDC5 cells by Western blotting. Cells transfected with the RhoA expression vector express higher levels of RhoA. Equal loading is demonstrated by probing for β-actin. B, increases in cell number during differentiation of untransfected, vector-transfected, and RhoA-transfected ATDC5 cells were examined by MTT assay. Equal cell numbers were plated, and differentiation was initiated on the same day (day 0) by addition of ITS. Cells were labeled with MTT at the indicated time points. After cell solubilization, $A_{600}$ nm was measured. Absorbance values correspond to cell numbers. The number of RhoA-overexpressing cells increases faster than the number of control cells and reaches higher maximal levels. One of two representative experiments is shown. C, the number of vector-transfected and RhoA-transfected ATDC5 cells was determined by direct cell counting. Equal cell numbers were plated initially, and differentiation was induced on the same day by the addition of ITS. At day 10 of differentiation, cells were trypsinized and counted in a hemocytometer. Cell numbers are shown as a percentage of the number of vector-transfected cells. The averages ± S.D. from three independent experiments (done in duplicate each) are presented. Overexpression of RhoA results in a greater than 30% increase in cell numbers (statistically significant, $p < 0.05$).

FIG. 3. RhoA overexpression suppresses induction of alkaline phosphatase activity and mineralization. A, induction of alkaline phosphatase activity during differentiation of untransfected (wt), vector-transfected, and RhoA-transfected ATDC5 cells was examined. Differentiation was induced on day 0 in all cell lines. Alkaline phosphatase activity was determined on the days indicated and normalized to protein content. Alkaline phosphatase activity increases more slowly and does not reach the same maximal levels in RhoA-overexpressing cells. One representative experiment (out of three independent experiments) is shown. B, accumulation of mineralization in untransfected, vector-transfected and RhoA-transfected differentiating ATDC5 cells was investigated. Differentiation was induced on day 0 in all cell lines. Mineralization was demonstrated at the indicated days by Alizarin red staining. After dye extraction, $A_{570}$ nm was measured to quantify mineralization. RhoA overexpression results in delayed and reduced mineralization. One representative experiment (out of three independent experiments) is shown.
mined and standardized to Renilla luciferase activity. For experiments with PTHrP, cells were transfected, serum-starved for 24 h, and stimulated with PTHrP (10^{-8} M) for 12 h before harvesting. Data shown represent the averages ± S.D. from three independent transfections, performed in duplicate each.

RESULTS

RhoA and ROCK1/2 Are Expressed throughout Chondrogenic Differentiation—We first analyzed the expression of RhoA and ROCK1/2 mRNAs during chondrogenic differentiation by RT-PCR analyses. All three genes are expressed throughout differentiation of ATDC5 cells (Fig. 1A) and primary mesenchymal limb bud cells in micromass cultures (Fig. 1B). Early chondrogenic differentiation and hypertrophy of ATDC5 cells are demonstrated by the expression of collagens II and X, respectively (Fig. 1A). A similar pattern of collagen gene expression is observed in primary micromass cultures (Fig. 1B). Next we examined the expression of the corresponding proteins in differentiating ATDC5 cells by Western blotting. RhoA, ROCK1, and ROCK2 proteins are detected throughout chondrogenic differentiation (Fig. 1C). These data demonstrate that the number of RhoA-overexpressing cells grows faster than the numbers of untransfected or vector-transfected cells (Fig. 2B). In addition, RhoA-overexpressing cells reach a higher cell density; although cell numbers plateau around day 12 in all lines, the values are approximately 25% higher in the RhoA-overexpressing cell lines. These results are confirmed by cell counting experiments that demonstrate a greater than 30% increase in cell number in RhoA-transfected cells (Fig. 2C). These data suggest that RhoA overexpression enhances the proliferation of chondrocytes and delays cell cycle exit.

RhoA Overexpression Delays Induction of Alkaline Phosphatase Activity and Mineralization—We next examined the effects of RhoA overexpression on hypertrophic chondrocyte differentiation. Alkaline phosphatase activity, a marker of hypertrophic differentiation, increases during differentiation of untransfected and vector-transfected ATDC5 cells (Fig. 3A). RhoA overexpression results in a marked delay in this decrease and markedly lower activity at the end of the culture period (30 days). Similarly, mineralization of the extracellular matrix, another marker of late chondrocyte differentiation, is clearly delayed and decreased by overexpression of RhoA (Fig. 3B). These data demonstrate that RhoA overexpression inhibits the induction of hypertrophic markers in differentiating ATDC5 cells.

RhoA Inhibits Hypertrophic Gene Expression—We studied the effects of RhoA overexpression on the expression of several marker genes of hypertrophic chondrocyte differentiation. RT-PCR analyses demonstrate that overexpression of RhoA leads to delayed onset of collagen X (Fig. 4A) and matrix metallopro-
teinase 13 (Fig. 4B) expression compared with control cells. Reduced expression of collagen X was confirmed by real time PCR analyses of gene expression (Fig. 4C). In addition, expression of the BSP gene, another marker of hypertrophic chondrocytes, is also decreased in RhoA-overexpressing cells, although not as much as for the collagen X gene (Fig. 4D). These findings confirm the inhibition of hypertrophic differentiation by RhoA signaling which is observed in alkaline phosphatase and mineralization assays.

RhoA Regulates Cyclin D1 and Collagen X Promoter Activity in Chondrocytes—We performed transient transfection studies in primary mouse chondrocytes to verify the roles of RhoA signaling in primary cells. Expression vectors for wild type and mutant RhoA proteins were cotransfected together with reporter plasmids for the cyclin D1 and collagen X promoters, which are active in proliferating (50, 55) and hypertrophic (46) chondrocytes, respectively. Cyclin D1 promoter activity is upregulated by overexpression of wild type RhoA and the activated Q63L mutant, whereas overexpression of dominant-negative T19N RhoA reduces cyclin D1 promoter activity by 39% (Fig. 5A). In contrast, collagen X promoter activity is reduced more than 2-fold by wild type and activated RhoA and strongly enhanced by dominant-negative RhoA (Fig. 5B). These data support our conclusion that RhoA signaling supports proliferation and inhibits hypertrophic differentiation in chondrocytes.

We and others have shown that PTH/PTHrP signaling stimulates cyclin D1 transcription in chondrocytes (55–57). Recent work has identified a Rho-specific activator, the guanine nucleotide exchange factor CDEP, as a target of PTH in chondrocytes (58, 59). We therefore asked whether RhoA signaling is
required for PTH/PTHrP-induced activation of the cyclin D1 promoter. 10^{-8} M PTHrP induces a strong (6-fold) activation of the cyclin D1 promoter in primary mouse chondrocytes, as described previously (55). However, overexpression of dominant-negative RhoA blocks this activation by 40%, indicating that RhoA signaling is required for maximal induction of cyclin D1 transcription by PTH/PTHrP. In accordance with these data, we demonstrate expression of CDEP mRNA throughout chondrogenic differentiation of micromass cultures and ATDC5 cells by RT-PCR (Fig. 5D). In both cell types, the highest levels of CDEP expression are seen at early stages where cell proliferation is strongest.

**ROCK Inhibition Accelerates Hypertrophic Differentiation**—The kinases ROCK1 and ROCK2 are among the most important effectors of RhoA (33). We made use of a pharmacological inhibitor of ROCK1/2, Y27632 (60), to block ROCK activity in wild type ATDC5 cells. Inhibition of ROCK signaling results in stronger staining for alkaline phosphatase activity (Fig. 6A).

These results were confirmed by quantitative analysis. ROCK inhibition causes a small, but reproducible increase in alkaline phosphatase activity (Fig. 6B). Similarly, mineralization is slightly enhanced by ROCK inhibition (Fig. 6C). In contrast, cell counts demonstrate that Y27632 treatment reduces the proliferation of RCS cells (49), which are commonly used as a model for proliferative chondrocytes, by 26% (Fig. 6D). Similar data were obtained using MTT assays in RCS and ATDC5 cells (data not shown, but compare Fig. 8). These data suggest that endogenous ROCK signaling is required for maximal proliferation of RCS and ATDC5 cells.

We next examined whether ROCK inhibition also affects hypertrophic gene expression. Treatment of ATDC5 cells with Y27632 results in accelerated induction of collagen X (Fig. 7A) and BSP (Fig. 7B) mRNA expression, as shown by real time PCR. Importantly, a similar enhancement of BSP expression is observed in primary micromass cultures (Fig. 7C), confirming that the effects of ROCK inhibition are not confined to the
ATDC5 cell line. In summary, these data show that endogenous ROCK signaling is necessary for maximal proliferation of chondrocytes and inhibits the induction of hypertrophic differentiation.

ROCK Inhibition Rescues Effects of RhoA Overexpression—
RhoA signals through ROCK-independent pathways, and ROCK proteins can be activated by signals other than RhoA (33). We therefore asked whether RhoA effects in ATDC5 cells are indeed mediated by ROCK signaling. We first examined whether Y27632 can rescue the effects of RhoA overexpression on proliferation and hypertrophic differentiation of ATDC5 cells. MTT assays (Fig. 8A) and cell counts (Fig. 8B) demonstrate that RhoA overexpression stimulates ATDC5 cell proliferation compared with untransfected and vector-transfected cells (as shown in Fig. 2). This effect is blocked to a large extent by inhibition of ROCK with Y27632. However, ROCK inhibition in control cells reduces cell proliferation further than in RhoA-expressing cells (Fig. 8, A and B), suggesting that Y27632 does not rescue the effects of RhoA overexpression completely and that ROCK-independent mechanisms are involved in the effects of RhoA on ATDC5 cell proliferation. Y27632 treatment also rescues the inhibitory effects of RhoA overexpression on alkaline phosphatase activity (Fig. 8C) and mineralization (Fig. 8D). Finally, inhibition of ROCK signaling causes a partial relief of RhoA repression of collagen X mRNA expression as demonstrated by RT-PCR (Fig. 9A) and real time PCR (Fig. 9B). These data indicate that signaling by endogenous ROCK proteins supports proliferation and inhibits hypertrophic differentiation of primary chondrocytes and ATDC5 cells downstream of RhoA; however, additional signaling pathways appear to be involved in the chondrocyte response to RhoA.

The intracellular signaling pathways controlling chondrocyte proliferation and differentiation, and thus endochondral bone growth, are largely unknown. We show here that both RhoA and ROCK1/2 are expressed throughout chondrogenic differentiation of primary cells and ATDC5 cells. Interestingly, protein levels of RhoA and ROCK1 increase at late (hypertrophic) stages of ATDC5 differentiation, an effect not seen at the RNA level. This discrepancy is most likely caused by posttranscriptional regulation of protein levels, e.g. through modulation of translation efficiency and/or protein stability. Using a combination of genetic and pharmacological approaches, we demonstrate that the RhoA/ROCK1/2 signaling pathway supports the proliferation of chondrocytes and delays hypertrophic differentiation as indicated by multiple parameters such as cell number, alkaline phosphatase activity, mineralization, and expression of hypertrophic marker genes. ROCK inhibition accelerates the differentiation of wild type ATDC5 cells and primary cells, indicating that endogenous RhoA/ROCK signaling plays an important role in suppressing hypertrophic differentiation. These conclusions are also supported by our cotransfection data, where we demonstrate that inhibition of endogenous RhoA by genetic means results in decreased cyclin D1 promoter activity and increased collagen X transcription in primary chondrocytes. Similarly, ROCK inhibition reduces proliferation of ATDC5 and RCS cells, suggesting a requirement for endogenous RhoA/ROCK signaling in normal cell cycle progression and/or in delaying the cell cycle exit during differentiation. Quantitative analyses demonstrate that inhibition of ROCK does not completely rescue all effects of RhoA overexpression,
suggesting that additional, ROCK-independent effector pathways are involved in RhoA signaling in cartilage. Numerous other effector pathways of RhoA have been described (61) and are potential candidates for mediating RhoA effects in chondrocytes.

The up-regulation of RhoA and ROCK1 protein levels at the hypertrophic stage is surprising in light of the biological effects observed (e.g., the suppression of hypertrophic differentiation by RhoA/ROCK signaling). However, it is possible that up-regulation of RhoA signaling components represents part of feedback mechanisms that delay cell cycle exit and onset of differentiation. Indeed, complex feedback loops (e.g., the Indian hedgehog/PTHrP loop) (1) have been shown to play crucial roles in controlling the timing of hypertrophic differentiation.

The mechanisms mediating RhoA effects on chondrocyte proliferation and differentiation are currently unknown. Rho GTPases are known to regulate the organization of the actin cytoskeleton (62). The close relationship between cell shape and behavior has been suggested (63, 64), and recent studies employing cartilage-specific disruption of the integrin-linked kinase and H9252 integrin genes in mice confirm this relationship in vivo (65–67). A regulatory role for the actin cytoskeleton in chondrocyte differentiation and in mediating these interactions between cell shape and behavior has been suggested (68, 69). The molecular mechanisms involved are not known, but Rho GTPases as main regulators of the actin cytoskeleton are prime candidates for such a role. However, Rho GTPases also perform numerous other cellular functions, for example in the regulation of gene expression and cell cycle progression in many cell types. Cell cycle genes play important roles in the regulation of chondrocyte proliferation and differentiation (summarized in Refs. 70 and 71); for example, the cyclin D1 gene is a target for multiple mitogenic stimuli in cartilage (50, 55–57, 72, 73), whereas the p21 (74–76), p57 (77, 78), p107 and p130 (79, 80) genes have been implicated in hypertrophic differentiation.

Our results indeed suggest regulation of chondrocyte cyclin D1 gene expression by RhoA signaling, but the involvement of additional effector pathways of RhoA is also likely.
target genes is likely. It will also be of great interest to examine whether Rho signaling controls the expression of known regulators of chondrocyte differentiation (such as components of the Indian hedgehog, fibroblast growth factor, bone morphogenetic protein, and PTH/PTHrP pathways) and whether such effects require changes in actin organization or are independent of cytoskeletal dynamics. These experiments are in progress in our laboratory.

In many biological systems RhoA signaling is antagonized by activity of the related GTPases Rac1 and Cdc42. Examples include neuron outgrowth (81, 82) and myoblast differentiation (83). The p38 mitogen-activated protein kinases are important effectors of these GTPases. Antagonistic regulation of chondrocyte proliferation and hypertrophy by RhoA/ROCK and Rac1/Cdc42/p38 signals is therefore plausible.

In summary, we have identified the GTPase RhoA and its effector kinases ROCK1/2 as novel regulators of chondrocyte proliferation and differentiation. This pathway therefore appears to play an important role in physiological cartilage development and potentially in growth-related diseases and skeletal disorders. Examination of the roles of RhoA and its effectors in vivo and the identification of upstream as well as downstream components of the RhoA-ROCK pathway will contribute further to a better understanding of the mechanism regulating the formation and function of the mammalian skeleton.

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