Parathyroid hormone (PTH) and its related peptide regulate endochondral ossification by inhibiting chondrocyte differentiation toward hypertrophy. However, the intracellular pathway for transducing PTH/PTH-related peptide signals in chondrocytes remains unclear. Here, we show that this pathway is mediated by mitogen-activated protein kinase (MAPK) p38. Incubation of hypertrophic chondrocytes with PTH (1–34) induces an inhibition of p38 kinase activity in a time- and dose-dependent manner. Inhibition of protein kinase C prevents PTH-induced p38 MAPK inhibition, whereas inhibition of protein kinase A has no effect. Thus, protein kinase C, but not protein kinase A, is required for the inhibition of p38 MAPK by PTH. Treatment of hypertrophic chondrocytes by PTH or by p38 MAPK inhibitor SB203580 up-regulates Bcl-2, suggesting that Bcl-2 lies downstream of p38 MAPK in the PTH signaling pathway. Inhibition of p38 MAPK in hypertrophic chondrocytes by either PTH, SB303580, or both together leads to a decrease of hypertrophic marker type X collagen mRNA and an increase of the expression of prehypertrophic marker cartilage matrix protein. Therefore, inhibition of p38 converts a hypertrophic cell phenotype to a prehypertrophic one, thereby preventing precocious chondrocyte hypertrophy. Taken together, these data suggest a major role for p38 MAPK in transmitting PTH signals to regulate chondrocyte differentiation.

During endochondral ossification, chondrocytes undergo a differentiation process including proliferation, maturation, hypertrophy, and apoptosis (1). Cells from these different stages of differentiation synthesize specific molecules, for example, type X collagen is synthesized specifically by hypertrophic chondrocytes (2, 3), whereas cartilage matrix protein (CMP)/matrilin-1, is a marker of prehypertrophic chondrocytes (1). Parathyroid hormone (PTH) and parathyroid hormone-related peptide (PTHrP) are major regulators of the chondrocyte differentiation process. PTH and PTHrP both bind to PTH receptors, which belong to G protein-coupled receptors (4). In a growth plate, PTH receptors are expressed at specific stages during chondrocyte differentiation, with the highest level at the prehypertrophic to hypertrophic transition (5). This suggests that PTH receptors may be important for the regulation of chondrocyte differentiation from the prehypertrophic state to the hypertrophic state. Indeed, overexpression of PTHrP or its constitutively active receptor in growth plates of transgenic mice delays endochondral bone formation (6), whereas mice with ablation of the PTH/PTHrP receptor gene develop skeletal dysplasia because of accelerated chondrocyte hypertrophy (7–9). Thus, the PTH/PTHrP receptor plays a fundamental role in the control of endochondral bone formation by transducing signals inhibiting chondrocyte hypertrophy. However, the intracellular signaling mechanism for PTH/PTHrP to regulate chondrocyte differentiation remains unknown.

The PTH/PTHrP receptor may exert its biological action via at least two signaling pathways, i.e. elevating intracellular cAMP, thereby activating cAMP-dependent protein kinase A (PKA) or activating phospholipase C that results in an increase in intracellular free calcium and activation of protein kinase C (PKC) (10). It has also been shown that Bcl-2, an anti-apoptosis molecule, is also involved in the PTH/PTHrP signaling pathway in chondrocytes (11). In this study, we will determine the involvement of PKA and PKC pathways in transducing PTH/PTHrP signals to regulate chondrocyte differentiation. Furthermore, the signaling molecules downstream of PKA or PKC that mediate PTH/PTHrP regulation of chondrocyte differentiation will be defined. We will present the evidence that a stress-induced mitogen-activated protein kinase (MAPK) p38 is a central link to connect PTH signaling to the regulation of chondrocyte hypertrophy.

Recent studies indicate that stress-induced MAPK superfamily, which includes p38 MAPK and c-Jun NH2-terminal kinase (JNK), is important for regulating cell differentiation and apoptosis by transmitting extracellular signals to the nucleus (12). However, the roles of JNK and p38 MAPK in the regulation of chondrocyte terminal differentiation—hypertrophy are not known. p38 MAPK and JNK are serine and threonine protein kinases that are activated by osmotic pressure, stress, and cytokines (13). In addition, several growth factors and G protein-coupled receptors may also activate p38 MAPK and JNK (14). The functional difference between p38 MAPK and JNK can be distinguished using a selective kinase inhibitor SB203580 that inhibits only p38 but not JNK activities (15). The aim of this study is to test whether stress-activated MAPKs are involved in the regulation of chondrocyte hypertrophy by PTH. If so, which specific MAPK pathway regulates chondrocyte differentiation? We show here that in primary chick hypertrophic chondrocytes PTH inhibits p38 MAPK activity in a dose- and time-dependent manner but has
little effect on JNK activity. The inhibition of p38 MAPK activity by PTH appears to be mediated by PKC. Furthermore, we demonstrate that inhibition of p38 MAPK activity is a signaling mechanism responsible for regulation of chondrocyte terminal differentiation by PTH/PTHR.

EXPERIMENTAL PROCEDURES

Materials—PTH (1–34), myelin basic protein (MBP), calphostin C, and thyrin were purchased from Sigma. Dibutyryl-cAMP and H89 were obtained from Calbiochem (La Jolla, CA). SB203580 was from BIOMOL (Plymouth Meeting, PA). Electrophoresis reagents were obtained from Bio-Rad. Agarose-conjugated anti-phosphotyrosine antibody (clone 4G10) and c-Jun (169) GST were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-p38 MAPK, anti-JNK1, anti-BCL-2, and protein A/G were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Monoclonal antibodies including anti-CMP and anti-link protein were prepared as described previously (16). Horseradish peroxidase-linked anti-rabbit/mouse secondary antibodies were obtained from Pierce. [γ-32P]ATP was purchased from PerkinElmer Life Sciences. Ham’s F-12 medium was from Life Technologies, Inc.

Cell Culture and Preparation of Cell Lysates—Primary chondrocyte cultures were established as described previously (1). Briefly, hypertrophic chondrocytes were obtained from 17-day-old embryonic chickens. Chondrocytes were obtained from the caudal part of the cartilage. We chose 17-day-old embryonic chickens because the cephalic part of sternum cartilage contains early hypertrophic chondrocytes that just started to differentiate by PTH/PTHrP. We chose 17-day-old embryonic chickens because the cephalic part of sternum cartilage contains early hypertrophic chondrocytes that just started to differentiate by PTH/PTHrP. Furthermore, we demonstrate that inhibition of p38 MAPK activity is a signaling mechanism responsible for regulation of chondrocyte terminal differentiation by PTH/PTHR.

To demonstrate whether PTH, which suppressed hypertrophy and hypertrophic cell populations of cells, as assessed by Western blot analysis of p38 MAPK activity in hypertrophic chondrocytes. Thus, PTH inhibits p38 MAPK Activity and Tyrosine Phosphorylation—To determine whether PTH activity correlates with chondrocyte hypertrophy, we examined the basal level of p38 MAPK activity in both prehypertrophic and hypertrophic chondrocytes. As shown in Fig. 1A, the basal p38 MAPK activity in hypertrophic chondrocytes was at least 10-fold higher than that of prehypertrophic cells (prehypertrophic, 1 μg of protein). The basal p38 MAPK activity was increased in hypertrophic chondrocytes, thus correlating with the differentiation state of chondrocytes. The difference of p38 MAPK activity between prehypertrophic and hypertrophic cells was not due to the difference of the expression levels of p38 MAPK protein, because there was no significant difference between the p38 protein levels from these two populations of cells, as assessed by Western blot analysis of p38 MAPK (Fig. 1A).

To determine whether p38 MAPK activity, which suppressed hypertrophy of chondrocytes (9), had an effect on the kinase activity of p38 MAPK, hypertrophic chondrocytes were treated with PTH (1–34) for various time periods or with various dosages (Fig. 1B). An inhibition of p38 MAPK by 100 nM PTH was observed at 10 min of treatment, and the inhibition lasted until 60 min when the kinase recovered to the basal level (Fig. 1, B and C). Dose response study of PTH indicated that both 1 and 100 nM PTH treatment of chondrocytes for 10 min inhibited p38 MAPK significantly, but 0.01 nM PTH did not (Fig. 1, B and C). To further confirm the inhibitory effect of PTH on p38 MAPK activity, tyrosine phosphorylation of p38 MAPK was determined during the time course of PTH treatment. Because p38 MAPK activation requires phosphorylation of both threonine and tyrosine residues in the catalytic domain, the level of p38 MAPK activity reflection of the extent of kinase activation (17). As shown in Fig. 1D, tyrosine phosphorylation of p38 MAPK was decreased after 10 min of PTH treatment and returned to the basal level after 60 min. This was in agreement with the time course of the PTH inhibition of p38 MAPK activity in hypertrophic chondrocytes. Thus, PTH inhibi-
Hypertrophic chondrocytes were incubated with either 100 nM PTH for the indicated time periods or with various dosages of PTH for 10 min. Equal amount of cell lysates was immunoprecipitated (IP) with an anti-tyrosine antibody 4G10. The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and blotted with an anti-p38 MAPK antibody. A representative immunoblot shows similar results were obtained from three experiments.

PKC but Not PKA Is Involved in Inhibition of p38 MAPK by PTH—To determine whether inhibition of p38 MAPK by PTH was mediated by the PKC or PKA pathway, hypertrophic chondrocytes were incubated with specific inhibitors of PKC or PKA pathways. As shown in Fig. 3, inhibition of PKC by calphostin C, a selective PKC inhibitor, did not alter basal p38 activity (Fig. 3A, compare Cal.C with Control). However, calphostin C completely abolished PTH-mediated inhibition of p38 MAPK activity (Fig. 3A, compare Cal.C+PTH with PTH). This suggested that PKC was required for PTH-induced inhibition of p38 MAPK activity. In contrast, treatment of cells with either

**Fig. 2. PTH does not affect JNK activity.** Hypertrophic chondrocytes were treated with 100 nM PTH for the indicated time periods. Cell lysate was incubated with an anti-JNK1 antibody, and the immune complex was collected. The JNK activity assay was performed in the presence of [γ-32P]ATP with GST-e-Jun as substrate. The kinase activity was quantified using a PhosphorImager. The graph is the summary of three independent experiments.

To test whether the inhibition of p38 MAPK activity by PTH involved Bcl-2, a recently identified component of the PTH pathway regulating chondrocyte differentiation (11), the protein levels of Bcl-2 in hypertrophic chondrocytes were determined by Western blot analysis. PTH treatment at 100 nM stimulated Bcl-2 protein expression, whereas treatment with 100 ng/ml thyroxine, a chondrocyte differentiation inducer (21), did not alter the Bcl-2 protein level (Fig. 4A). Thus, PTH specifically stimulated the Bcl-2 protein level in hypertrophic chondrocytes.

Inhibition of p38 MAPK Is Associated with Up-regulation of Bcl-2—To determine whether the inhibition of p38 MAPK activity by PTH led to the up-regulation of Bcl-2, 10 μM SB203580, a specific inhibitor of p38 kinase activity; or a combination of both PTH and SB203580. The up-regulation of Bcl-2 by PTH (Fig. 4B, PTH) can be mimicked by the inhibition of p38 MAPK by SB203580 (Fig. 4B, SB203580). In addition, a similar extent of the increase of Bcl-2 level was observed when cells were treated with both SB203580 and PTH (Fig. 4B, SB+PTH). These data suggested that inhibition of p38 MAPK was involved in PTH-regulated Bcl-2 expression.

Specific Inhibition of p38 MAPK Activity by SB 203580 in Hypertrophic Chondrocytes—Although it is known that SB203580 at 10 μM, the concentration used in this study, inhibits activity of p38 MAPK but not that of JNK or ERK (22), it has been reported recently that SB203580 at the 5–10 μM range could inhibit interleukin-2-induced PKB (Akt) activation in CT6 cells (18). To determine whether SB203580 also inhibited PKB activation in hypertrophic chondrocytes, hypertrophic chondrocytes were incubated with SB203580 at various concentrations, and kinase activities of both p38 MAPK and PKB were determined. As shown in Fig. 5, SB203580 inhibited p38 MAPK in a dose-dependent manner. In contrast, no significant inhibition of PKB activity was observed in these SB203580-treated cells. This result was verified by an in vitro assay, in which lysates of hypertrophic chondrocytes were incubated with SB203580 for 5 min, followed by determination of PKB activity. No significant inhibitory effects of SB203580 on PKB activity was observed (Control, 3207 ± 72; 1 μM SB203580, 2984 ± 124; 10 μM SB203580, 3023 ± 97; arbitrary units, n = 3). Thus, the optimal concentration of SB203580 for
specific inhibition of p38 MAPK may depend on cell types. In hypertrophic chondrocytes, 10 μM SB 203580 inhibited p38 MAPK activity but not PKB activity.

**PTH Differentially Regulates CMP and α1 type X mRNA Expression**—To determine the downstream effects of PTH regulation of chondrocyte hypertrophy, we quantified the levels of mRNA of type X collagen, a marker of hypertrophic chondrocytes (2, 3), and that of CMP/matrilin-1, a marker of prehypertrophic chondrocytes (1). Real-time quantitative RT-PCR indicated that α1 type X mRNA was significantly decreased after 1 day of treatment of hypertrophic chondrocytes with 100 nM PTH, with maximal inhibition achieved after 3 days (Fig. 6). Thus, PTH inhibited chondrocyte hypertrophy. In contrast, PTH induced a significant increase of CMP mRNA after 1 day of treatment of hypertrophic chondrocytes with 100 nM PTH, with maximal inhibition achieved after 3 days (Fig. 6). Thus, PTH inhibited chondrocyte hypertrophy. In contrast, PTH induced a significant increase of CMP mRNA after 1 day of treatment of hypertrophic chondrocytes with 100 nM PTH, with maximal inhibition achieved after 3 days (Fig. 6). This suggested that PTH converted hypertrophic chondrocytes to the prehypertrophic phenotype, of which CMP was the marker. This change of CMP mRNA level, however, was transient. After 3 days of treatment with PTH, the CMP mRNA level returned to the basal level (Fig. 6).

**Inhibition of p38 MAPK Is Associated with Up-regulation of CMP by PTH**—Inhibition of p38 MAPK activity by SB203580 stimulated the CMP mRNA level to a similar extent as treatment with both PTH and SB together (Fig. 8A). Treatment with PTH alone also stimulated the CMP mRNA level but to a lesser degree (Fig. 8A). This suggested that inhibition of p38 MAPK could be achieved by PTH and some unknown elements, which led to up-regulation of the CMP mRNA level. In agreement with the mRNA data, a parallel increase of CMP protein level was observed by the same treatment (Fig. 8B, CMP). In contrast, although the level of link protein, a protein expressed throughout chondrocyte differentiation process, was up-regulated by PTH, this stimulatory effect was not achieved by either SB treatment alone or SB and PTH treatment together (Fig. 8B, LP). This indicated that other signaling mechanism that did not involve p38 MAPK might contribute to the PTH stim...
ulation of LP expression. Furthermore, the up-regulation of CMP by PTH, SB, or PTH and SB treatment together (Fig. 8, A and B, CMP), as well as the down-regulation of type X collagen by these treatment (Fig. 7), corresponded to the inhibition of p38 MAPK activity during these treatment (Fig. 8 C). This further supported the conclusion that the inhibition of p38 MAPK activity was responsible for both up-regulation of CMP and down-regulation of type X collagen by PTH.

DISCUSSION

The endochondral ossification process, which consists of chondrocyte proliferation, maturation, hypertrophy, and apoptosis, requires precise regulation of these differentiation events. It has become clear in recent years that a major regulator of chondrocyte hypertrophy is the PTH/PTHrP signaling pathway (7–9). Surprisingly, very little is known about the intracellular molecules that transduce PTH/PTHrP signals to regulate chondrocyte hypertrophy. In this study, we present the first evidence that inhibition of chondrocyte hypertrophy by PTH is mediated by an inhibition of p38 MAPK activity. We propose a model in which p38 MAPK is a central link in the PTH/PTHrP signaling pathway to regulate chondrocyte hypertrophy by connecting PKC in the upstream and Bcl-2 in the

**FIG. 4. Inhibition of p38 MAPK is associated with up-regulation of Bcl-2 by PTH.** A. hypertrophic cells were treated with 100 nM PTH (PTH), 100 ng/ml thyroxine (TH), or untreated (Control) for 3 days. Cell lysates were then prepared for Western blot analysis with an anti-Bcl-2 antibody. B, to determine the possible role of p38 MAPK in PTH-mediated Bcl-2 expression, chondrocytes were preincubated with 10 μM SB203580 (SB), a selective p38 MAPK inhibitor, for 2 h before 100 nM PTH was added for additional 3 days. Equal amount of cell lysate was loaded to a SDS-polyacrylamide gel and blotted with an anti-Bcl-2 antibody. The immunoreactive proteins were detected by the ECL system for Western blot. A representative blot is shown for each set of experiments. Bar graphs show the averages of quantified data from three independent experiments. *, p < 0.01, significant difference compared with control.
downstream (Fig. 9). The PTH-p38 MAPK pathway prevents precocious chondrocyte hypertrophy by simultaneously inhibiting type X collagen, a marker of hypertrophic chondrocytes, and stimulating CMP, a marker of prehypertrophic chondrocytes.

It has been shown previously that PTH and PTHrP, which bind to a common PTH/PTHrP receptor (4), employ at least two pathways for transducing signals, i.e., activation of PKA via a Gs-coupled mechanism, or activation of PKC via a Gq-coupled mechanism (10). In addition, both PKA and PKC have been reported to regulate the p38 MAPK pathway, and the specificity of regulation seems to vary depending on cell types (17, 23, 24). In this study, we have shown that it is the PKC pathway that is responsible for transducing PTH signals to inhibit p38 MAPK activity in hypertrophic chondrocytes. PKC has also been implicated in regulating chondrogenesis of mesenchymal cells (25). Our data provide evidence that p38 MAPK maybe a downstream signaling molecule in PKC-regulated chondrocyte differentiation.

We have also shown that inhibition of p38 MAPK activity is responsible for transducing PTH signals to prevent chondrocyte hypertrophy. The activity of p38 is elevated more than 10-fold in hypertrophic chondrocytes. Treatment with PTH inhibits such elevation, thereby preventing hypertrophy. This
inhibition is specific, because PTH has no effect on the activities of JNK, another major stress-activated MAPK. Both p38 and JNK exist in chondrocytes, and they are activated by interleukin-1 and TNF-α, which may lead to chondrocyte apoptosis and cartilage degradation (26). We have shown here that inhibition of p38 MAPK alone is sufficient to prevent chondrocyte terminal differentiation to hypertrophy. Inhibition of p38 MAPK, even in the absence of PTH signaling, causes downstream effects including up-regulation of Bel-2 and CMP and down-regulation of type X collagen. This suggests that p38 may play a role not only in transmitting the PTH signals but also in the general control of chondrocyte hypertrophy.

Our study also connects the PTH-p38 MAPK pathway to the regulation of Bel-2 level in hypertrophic chondrocytes. Previous transgenic experiments have shown that Bel-2 is a component of the PTH signaling pathway (11). We show here that the Bel-2 level is regulated by the p38 MAPK activity. Bel-2 is an anti-apoptosis molecule that maintains cellular calcium homeostasis (27, 28). Furthermore, it has been shown that chondrocyte calcium metabolism regulates its hypertrophic phenotype (29, 30) and that chondrocyte hypertrophy is closely associated with apoptosis (31). However, it was not known how chondrocyte hypertrophy is related to apoptosis and calcium metabolism. Our finding that p38 MAPK regulates both Bel-2 and chondrocyte hypertrophy through PTH signaling may reconcile all of these findings.

It is interesting to note that the PTH-p38 MAPK pathway not only inhibits the hypertrophic marker type X collagen but also stimulates the prehypertrophic marker CMP. This indicates that PTH prevents chondrocyte terminal differentiation not only by inhibiting hypertrophy but also by actively converting a hypertrophic phenotype to a prehypertrophic one. This is consistent with the finding that hypertrophic chondrocytes can be converted into prehypertrophic chondrocytes (1, 32) in a “retro-differentiation” process (1). However, this reversal is transient, as evidenced by the temporal stimulation of CMP in hypertrophic cells by PTH. A possible explanation for this phenomenon may be the desensitization of PTH/PTHrP receptors, which is common for G protein-coupled receptors upon prolonged exposure to ligands (33, 34). Receptor desensitization could be due to phosphorylation of the receptors induced by agonist and mediated by PKA, PKC, or other G protein-coupled receptor kinases (34, 35). For example, it has been demonstrated that phosphorylation of PTH/PTHrP receptors by prolonged PTH incubation desensitizes the receptors, probably via a G protein receptor kinase (34). Indeed, in PTHr overexpression transgenic mice, endochondral ossification is delayed but eventually occurred (6). Our data suggest that one way to overcome such transient nature of retro-differentiation is to consistently inhibit the downstream p38 MAPK activity.

In summary, our study presents an important intracellular pathway for PTH signaling in hypertrophic chondrocytes. This pathway links extracellular PTH/PTHrP signals to intracellular regulatory molecules, including PKC, p38 MAPK, and Bel-2, and ultimately to the regulation of chondrocyte differentiation and synthesis of extracellular matrix molecules such as type X collagen and CMP. The elucidation of this pathway may have strong implications for our understandings of skeletal development, which is potently regulated by PTH/PTHrP.

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FIG. 9. Summary of signaling cascades in PTH-mediated chondrocyte differentiation. E, extracellular; M, cell membrane; C, cytoplasm; N, nucleus.