Parathyroid Hormone-(1–34) Enhances Aggrecan Synthesis via an Insulin-like Growth Factor-I Pathway*

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During endochondral bone formation, the growth plate chondrocytes proliferate, become hypertrophic, lose the cartilage phenotype, undergo mineralization, and provide a scaffold upon which subsequent longitudinal bone growth occurs. Parathyroid hormone (PTH), a calcium-regulating hormone, and parathyroid hormone-related peptide (PTHrP), which shares several properties with PTH, have profound effects on skeletal growth and new bone formation. In order to define further the mechanism by which PTH/PTHrP promotes the cartilage phenotype, chondrocytes isolated from the rib cages of developing rat embryos were evaluated for the biosynthesis of aggrecan. Cells treated with PTH-(1–34) for a 4-h period followed by a 20-h recovery period showed a significant increase in cartilage proteoglycan (aggrecan) synthesis in a dose-dependent manner. Only N-terminally intact PTH and PTHrP were effective in stimulating aggrecan synthesis. Addition of a neutralizing antibody to insulin-like growth factor-I (IGF-I) during PTH treatment resulted in the inhibition of PTH-stimulated aggrecan synthesis, whereas the addition of a neutralizing antibody to insulin-like growth factor-binding protein-2 (IGFBP-2) resulted in an increase in synthesis in both the control and PTH-treated cells. In addition, PTH treatment resulted in an increase in the mRNA for aggrecan, a reduction in IGFBP-3 mRNA, and no discernible changes in IGF-I mRNA levels, which was complemented by quantitative changes in IGFBP-3 and free IGF-I levels. The reciprocal relationship in the expression of aggrecan and IGFBP was further confirmed in chondrocytes from various gestational stages during normal development. Collectively, our results indicate that the effect of PTH may be mediated at least in part through the regulation of IGF/IGFBP axis, by a decrease in the level of IGFBP-3, and an increase in free IGF-I levels. It is likely that the local increase in IGF-I may lead to an increase in cartilage type proteoglycan synthesis and maintenance of the cartilage phenotype. The consequence of the prolonged maintenance may be to halt mineralization while a new scaffolding is created.

Endochondral bone formation is associated with a cascade of events that include condensation of undifferentiated mesenchyme, the subsequent differentiation of the core of the limb buds into cartilage, and maturation of chondrocytes into hypertrophic cells leading to new bone formation (1, 2). Thus, chondrocytes play a crucial role in bone formation, both by promoting the growth of the skeletal elements as well as by providing a scaffold upon which new bone is laid down. The differentiation of cells into discrete cell types is associated with the synthesis of a unique set of matrix molecules that characterize the differentiation stage of the cells and which may further influence subsequent differentiation processes (1). Chondrocytes from various regions of the growth plate exhibit morphological and functional differences that are characterized by distinct matrix molecules (3, 4). Improper assembly and organization of the growth plate leads to chondrodysplasias that are associated with skeletal abnormalities (5).

Multiple autocrine, endocrine, and paracrine factors have been shown to influence endochondral bone formation (6). These factors influence the proliferation of chondrocytes or differentiation or both. PTHrP has recently been demonstrated to be critical in embryonic skeletal development (7–9). When administered intermittently, both PTH, an agent that plays a crucial role in calcium homeostasis, and PTHrP, which shares several properties with PTH, stimulate new bone formation (10–12). Both proteins bind to a unique G-protein-coupled receptor (type I PTH/PTHrP receptor) that is present on a variety of cell types, including chondrocytes (13, 14). Mice with null mutations for the type I PTH receptor or for PTHrP develop skeletal abnormalities (7, 8). Thus, PTH and PTHrP play a crucial role in skeletal growth and maturation. In order to define further the mechanisms by which PTH/PTHrP may promote and prolong the expression of the cartilage phenotype, we evaluated the effects of PTH-(1–34) on the biosynthesis of cartilage proteoglycans. Upon treatment with PTH-(1–34), chondrocytes isolated from the rib cages of rat embryos showed a dramatic increase in aggrecan synthesis, which was associated with an increase in mRNA levels for aggrecan. The effects of PTH were reduced or blocked by a neutralizing antibody to IGF-I and mimicked by a neutralizing antibody to IGFBP-2. Further detailed analysis indicated that PTH-(1–34)-stimulated aggrecan synthesis was associated with a reduction in IGFBP-3 levels and an increase in free IGF-I levels. Thus, a local increase in IGF-I caused by PTH treatment may lead to an increase in cartilage type proteoglycan synthesis and maintenance of the cartilage phenotype. The consequence of the prolonged maintenance may be to halt mineralization while a new scaffolding is created.

EXPERIMENTAL PROCEDURES

Reagents—Rat PTH-(1–34), human PTH-(53–84), rat/human PTHrP-(1–34), bovine PTH-(3–34), bovine PTH-(7–34), rat PTH-(1–84), and rat/human PTHrP-(1–86) were purchased from Bachem, Torrance, CA. Human recombinant IGF-I and neutralizing antibodies to IGF-I, IGFBP, and insulin-like growth factor binding protein; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate.

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† The abbreviations used are: PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; IGF-I, insulin-like growth factor-I; IGFBP, insulin-like growth factor binding protein; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate.
IGF-II, and IGFBPs were from Upstate Biotechnology, Inc., Lake Placid, NY.

Cell Culture—Costal chondrocytes were isolated from rib cages of timed pregnant rat embryos. For most of the studies, gestational day 17/18 rat embryos were utilized. Rib cages were treated in sequence with digestion of 500 µg/ml trypsin, 200 µg/ml EDTA for 1 h at 37 °C followed by 2 mg/ml collagenase (CLS-2; Worthington) for 4 h at 37 °C. Cells were plated at 5 × 10^6/cm² in Ham's F-12 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and penicillin/streptomycin and were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

Only confluent primary cultures were used for this study.

Biochemical Characterization—Cultures grown in 24-well plates were washed free of serum with Dulbecco's phosphate-buffered saline. Treatments were added for the indicated intervals in low glucose Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 50 µg/ml ascorbic acid. The cultures were then washed extensively and allowed to recover for the indicated times in the same medium (without PTH). The cell layer (matrix) was extracted with 4 M guanidinium chloride, 80 mM sodium acetate, pH 6.0, 10 mM Na₂EDTA, 25 mM benzamidine hydrochloride, 100 mM 6-aminohexanoic acid, and 10 mM CHAPS. This extract and the labeled conditioned media were dialyzed against deionized water in 12,000-14,000 molecular weight cut-off tubing (Spectrapor, Spectrum Industries, Los Angeles, CA). The radioactive incorporation into these nondialyzable fractions was determined by scintillation counting.

**SDS-PAGE Analysis of Proteoglycan**—The radiolaabeled components were analyzed by electrophoresis on 3–15% SDS-polyacrylamide gels under denaturing and reducing conditions. Samples were loaded based on the products from an equal number of cells. After electrophoresis, the gels were saturated with fluor (Entensify; NEN Life Science Products), vacuum-dried, and exposed to x-ray film (Reflection; NEN Life Science Products). Molecular weights were determined by gel filtration standards (Bio-Rad). Relative band intensities were determined using a Fluor-S Multi-imager and Quantity One image acquisition and analysis software (Bio-Rad).

**Hyaluronate-Sepharose Affinity Chromatography**—In order to verify the aggregan nature of the molecules, sulfate-labeled samples were evaluated by chromatography on a hyaluronate-Sepharose column (15). In brief, human umbilical cord hyaluronic acid (Sigma) was purified from sulfated glycosaminoglycans by cetyl pyridinium chloride precipitation and was coupled to EAH-Sepharose (Amersham Pharmacia Biotech) using a published procedure (16). The proteoglycan samples were dissolved in 0.005 M phosphate buffer, pH 6.8, and were chromatographed on a column (5 × 0.24 cm) of hyaluronate-Sepharose equilibrated in the same buffer. The unbound material was removed by washing with 4 column volumes of the buffer, and the bound materials were eluted in sequence with 2 column volumes each of 0.5 and 4.0 M guanidinium chloride in 0.005 M phosphate, pH 6.8. The samples were extensively dialyzed against deionized water, lyophilized, and evaluated by SDS-PAGE/fluorography, as above.

**Chondroitinase Digestion**—The radiolaabeled samples (10^6 dpm each) were solubilized in 50 mM Tris-HCl, pH 8.0, containing 60 mM sodium acetate, plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM NaF-ethylenemide, and 10 mM Na₂EDTA) and were treated with chondroitinase ABC or AC lyase (0.025 units/ml; ICN, Costa Mesa, CA) for 40 min at 37 °C. The digested samples were dialyzed and evaluated by SDS-PAGE/fluorography.

**RNA Extraction and Northern Blot Analysis**—Total RNA from various treatments was extracted using Ultraspec RNA (Biotecx Laboratories, Houston, TX), and 30 µg each was subjected to electrophoresis on a 1% (w/v) agarose/formaldehyde gel and transferred to GeneScreen membranes (NEN Life Science Products). The probes for rat aggregan, IGF-I, and IGFBP-3 were generated by reverse transcription polymerase chain reaction and were subsequently cloned into a pCR II vector (Stratagene Placid, NY.  

**RESULTS**

**Effect of Rat PTH-(1–34) Treatment Time on Proteoglycan Synthesis**—Initially, we evaluated the effect of PTH-(1–34) on the chondrogenic phenotype in vitro by assessing proteoglycan synthesis. Costal chondrocyte cultures from day 18 rat embryos were treated for 4 or 20 h with 10 nM PTH-(1–34), were washed extensively to remove the PTH, and were allowed to recover for 20 or 4 h, respectively, in serum-free medium. During the last 4 h of the recovery period, the cultures were labeled with Na₃⁵SO₄, and the extracts of the cell layer + matrix were analyzed by SDS-PAGE/fluorography. The major polysaccharide molecules that migrated near the top of the gel are likely to be aggrecan. An additional band of approximate molecular size of 220 kDa was also identified and is likely to be biglycan, based on the relative migration positions in the gel and by comparison with globular molecular weight standards (17). PTH-(1–34) treatments (20 and 4 h + 20 h recovery) resulted in an increased incorporation of aggregan and the 220-kDa band into the cell layer/matrix (Fig. 1, lanes 4 and 6, (17)). PTH-(1–34) treatments (20 and 4 h + 20 h recovery) resulted in an increased incorporation of aggregan and the 220-kDa band into the cell layer/matrix (Fig. 1, lanes 4 and 6, (17)). PTH-(1–34) treatments (20 and 4 h + 20 h recovery) resulted in an increased incorporation of aggregan and the 220-kDa band into the cell layer/matrix (Fig. 1, lanes 4 and 6, (17)).

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Cultures were treated for 4 h with 10 nM PTH-(1–34), allowed to recover for either 4 or 18 h, and labeled with Na$_2^{35}$SO$_4$ during the last 4 h of each recovery period. The media and matrices were evaluated as described under “Experimental Procedures.” Lane 1, control; lane 2, PTH-(1–34).

**Effect of Recovery Time after PTH Treatment on Proteoglycan Synthesis**—In order to establish the optimal time of recovery needed to observe an effect on proteoglycan synthesis, chondrocytes were treated for 4 h with 10 nM PTH-(1–34), allowed to recover for various times in serum-free medium and finally labeled during the last 4 h of each recovery period. The results of 4- and 18-h recovery times are shown in Fig. 2. A time-dependent difference was observed in the distribution of the aggrecan and the 220-kDa band. Within 4 h of recovery, aggrecan was only barely detectable in the media and the cell layer, whereas a significant amount of the 220-kDa band was present in both the media and the cell layer. By 18 h of recovery, aggrecan synthesis was clearly evident in the cell layer. Within 4 h of recovery, PTH treatment resulted in only a slight increase in aggrecan levels (1.2-fold). After an 18-h recovery, there was a substantial increase in aggrecan (1.8-fold) and the 220-kDa band was evident. Within 4 h of recovery, there was a slight increase in the amount of aggrecan secreted into the medium (1.5-fold), but there was a dramatic increase (17-fold) in aggrecan incorporated into the cell layer. Similar results were obtained when cells were treated with two 24-h treatments of PTH (data not shown). The results suggest that chondrocytes not only retain the ability to respond to a second transient or prolonged treatment but that the response is greater than after a single transient exposure to PTH.

**Effect of Repetitive PTH Treatment on Proteoglycan Synthesis**—In order to evaluate whether chondrocytes treated transiently with PTH display a more pronounced effect after a second transient treatment with PTH, the cells were treated with two cycles of PTH (4 h treatment and 24 h recovery) followed by Na$_2^{35}$SO$_4$ label during the last 4 h. The results (Fig. 4) show that there was a significant increase in the aggrecan released into the medium (1.5-fold), but there was a dramatic increase (17-fold) in aggrecan incorporated into the cell layer. Similar results were obtained when cells were treated with two 24-h treatments of PTH (data not shown). The results suggest that chondrocytes not only retain the ability to respond to a second transient or prolonged treatment but that the response is greater than after a single transient exposure to PTH.

**Effect of PTH Analogs on Proteoglycan Synthesis**—To determine the specificity of the response, we compared the effects of PTH-(1–34) and PTH-(53–84), a fragment that does not stimulate cAMP accumulation but does stimulate alkaline phosphatase and osteocalcin production in osteoblasts (18, 19). In addition, PThrP-(1–34), which is similar to PTH-(1–34) in a variety of biological activities, was also evaluated. Cells were treated for 4 h with 10 nM each of PTH-(1–34), PTH-(53–84), or PThrP-(1–34). The treatments were removed; the cells were allowed to recover for 24 h, and the proteoglycan incorporation into the cell layer was evaluated after labeling during the last 4 h of recovery. Only PTH-(1–34) and PThrP-(1–34) increased proteoglycan synthesis (Fig. 5). PTH-(53–84) (see Fig. 5) and other fragments (PTH-(3–34) and PTH-(7–34)) that do not stimulate cAMP were ineffective.

**Characterization of Aggrecan**—In order to establish the identity of the newly synthesized proteoglycan, Na$_2^{35}$SO$_4$-labeled samples from control and PTH-treated cultures were tested for hyaluronate binding activity and susceptibility to chondroitinase digestion. Cells were treated with PTH-(1–34) using a repetitive treatment regimen (Fig. 4), and an aliquot of the cell layer/matrix extract was subjected to hyaluronate affinity chro-

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Guanidinium chloride extracts from PTH-treated cultures were dialyzed and subjected to affinity chromatography on a hyaluronate-Sepharose column, and the bound materials were eluted with 4 M guanidinium chloride. A similar sample was treated with chondroitinase ABC and AC lyase. The results were shown only for PTH-treated samples (Fig. 6, lanes 1–7). Similar results were obtained for untreated controls (data not shown). The high molecular weight band was eluted only by 1–7 M guanidinium chloride (Fig. 6, lane 3). In order to confirm further the proteoglycan nature of the Na$_2$SO$_4$ molecules, samples from PTH-treated cells were subjected to digestion with chondroitinase ABC or AC lyase. The results establish that both bands (Fig. 6, lanes 6 and 7) were digested by the enzymes and are therefore likely to be chondroitin sulfate-containing proteoglycans.

**PTH Effect on Proteoglycan Synthesis Involves IGF-I**—Previous studies have suggested that PTH effects on bone cells may involve IGF-I as a mediator and that IGFs may play a crucial role in cartilage proteoglycan metabolism (6, 20, 22). In order to determine if IGFs played a role in the PTH-mediated increase in proteoglycan synthesis and incorporation, proteoglycan synthesis was evaluated in the presence of neutralizing antibodies to IGF-I, IGF-II, or IGFBP-2. The results are shown in Fig. 7 as follows: (a) in comparison to control, PTH treatment resulted in a 2.1-fold increase in aggrecan synthesis; (b) antibody to IGF-I significantly reduced aggrecan synthesis in both the control and PTH-treated cells, whereas IGF-II antibody had no effect on either the control or PTH-treated cells. In the presence of IGFBP-2 antibody, there was a dramatic increase in aggrecan synthesis in the control and PTH-treated cells. These results suggest that free IGF-I that is likely to have been released from the binding protein may play a role in increasing aggrecan synthesis in both the control and PTH-treated cells.

**Expression of Aggrecan and IGFBP-3 during Various Stages of Chondrocyte Differentiation**—Since local IGF levels can be influenced by the amount of IGF-binding protein present, we wanted to evaluate whether PTH can influence binding protein levels and whether the effects of PTH are dependent on the developmental age of the cells. An initial 125I-IGF ligand blot analysis of chondrocyte-conditioned media from day 18 embryos revealed that the predominant binding protein present was IGFBP-3 (data not shown). We next asked whether a relationship exists between aggrecan and IGFBP-3 levels during normal chondrocyte differentiation. Rat costal chondrocytes from various stages of gestation were evaluated for proteoglycan synthesis (sulfate incorporation) and IGFBP-3 secretion (immunoradiometric assay). The results (Fig. 8) demonstrate a developmental stage-dependent reciprocal relationship between aggrecan and IGFBP-3 levels. The peak of aggrecan synthesis in this experiment was on day 18, whereas IGFBP-3 levels were at the lowest in the corresponding samples. These results are consistent with the suggestion that a reduction in IGFBP-3 may contribute to the normal aggrecan synthesis during development.

**PTH-(1–34) Regulation of Aggrecan mRNA in Chondrocytes from Various Gestational Age**—Because chondrogenic differentiation in rats occurs between day 17 and 18 and since PTH receptor expression is specific to developmental age, we next evaluated whether PTH-(1–34) exhibited stage-specific effects on aggrecan synthesis. Chondrocytes from various gestational days of development (days 17, 18, 20, and 21) were exposed to a repetitive treatment regimen with PTH-(1–34), and the total cellular RNA was isolated 2 h after the second treatment. Samples were subjected to electrophoresis and transferred to GeneScreen, and the membranes were hybridized with a ran-
Except Na₂

munoradiometric assay. A parallel set of cultures was treated the same medium. The media samples were assayed for IGFBP-3 levels by im-

tational days of development were treated for 24 h with serum-free FB

FBP-3 during development.

costal chondrocytes from various ges-

tracts was quantitated.

incorporation into the nondialyzable products of the guanidinium ex-

lyzable incorporation into the media was evaluated by SDS-PAGE/

bryos were treated with 10 nM PTH-(1–34), 100 ng/ml IGF-I, or

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tion in IGFBP-3/2 and a corresponding increase in free IGF-I.

We next asked whether PTH-(1–34) resulted in

Chondrocytes—

demonstrated that PTH and PTHrP decrease terminal

seal chondrogenesis and accompanying endochondral bone for-

and suggest that PTH may enhance the cartilage phenotype by

promoting cartilage-specific macromolecules.

Effect of PTH-(1–34) on IGF-I and IGFBP-3 Levels on Day 18 Chondrocytes—We next asked whether PTH-(1–34) resulted in

altered levels of free IGF-I and IGFBP-3 and whether exog-

enously added human IGF-I was effective in influencing PTH
effects on aggrecan synthesis. Chondrocytes from day 18 em-

bryos were treated with 10 nm PTH-(1–34), 100 ng/ml IGF-I, or

both, and aggrecan synthesis was evaluated by sulfate incor-

poration and SDS-PAGE analysis. The results confirmed that

PTH treatment resulted in a 2.5-fold increase in aggrecan

synthesis (Fig. 10A, lane 2). In the same cultures, PTH treat-

ment also resulted in a 35% increase in free IGF-I and a 22% 

reduction in IGFBP-3 levels (Fig. 10B) without affecting total 

IGF-I levels (data not shown). Surprisingly, exogenously added 

human IGF-I was not effective in eliciting aggrecan synthesis

(Fig. 10A), either alone (lane 2) or in combination with PTH

(lane 4). Similarly, there was no difference in free IGF-I and 

IGFBP-3 levels (Fig. 10B) between PTH and PTH + IGF-I-
treated cultures. These results further establish that PTH ef-

effects on aggrecan synthesis are associated with an increase in 

free IGF-I that is likely to be available due to a reduction in 

IGFBP-3 levels.

We next evaluated if the PTH effects on IGFBP-3 and IGF-I

were also observed at the mRNA level. Cultures obtained from 

day 18 embryos were treated with PTH-(1–34) for 4 h, and the 
total cellular RNA was isolated after a 2-h recovery and evalu-

ated by Northern blot. The results (Fig. 11) further establish 

that in comparison to control, PTH treatment resulted in the 

following: (a) an increase in aggrecan mRNA (80% increase); (b) 
a decrease in IGFBP-3 mRNA (60% reduction); and (c) a minor 

(24%) or no reduction in IGF-I mRNA levels. These data con-

firm that PTH treatment in vitro regulates aggrecan mRNA 
directly and aggrecan synthesis indirectly by reducing IG-

FBP-3 levels, ultimately regulating the phenotype of cells that 

are likely to be chondrogenic.

DISCUSSION

Longitudinal skeletal growth is the direct result of epiphy-

seal chondrogenesis and accompanying endochondral bone for-

mation. A variety of endocrine and growth factors regulate and 

fluence chondrocyte differentiation. Previous in vitro studies 
have demonstrated that PTH and PTHrP decrease terminal 
differentiation of chondrocytes (9, 29, 24). In this report, we 
demonstrate the following: (a) PTH-(1–34) treatment of rat 
costal chondrocytes resulted in an increase in aggrecan mRNA 
and aggrecan synthesis; (b) PTH effects on enhanced aggrecan 
synthesis were dependent on the stage of chondrocyte develop-

ment; and (c) increased synthesis was associated with a reduc-

tion in IGFBP-3 and a corresponding increase in free IGF-I. 
The results are consistent with the suggestion that PTH-(1–34) 
may prolong the maintenance of the chondrocyte phenotype by 

promoting the expression of cartilage-specific proteoglycan.
Parathyroid Hormone Stimulates Aggrecan Synthesis

Fig. 10. Effect of PTH-(1–34) and IGF-I on aggrecan synthesis and IGF-I/IGFBP-3 levels. Chondrocytes from day 18 embryos were treated with 10 nM PTH-(1–34), 100 ng/ml hIGF-I, or a combination of the two for 20 h. The media were removed and analyzed for the presence of free rat IGF-I and IGFBP-3 by immunoradiometric assays, whereas the cells were labeled with Na$_2$SO$_4$ for 4 h. The nondialyzable incorporation into the media was evaluated by SDS-PAGE/fluorography. A, aggrecan synthesis in response to control (lane 1), 10 nM PTH-(1–34) (lane 2), 100 ng/ml hIGF-I (lane 3), and 10 nM PTH-(1–34) + 100 ng/ml hIGF-I (lane 4). B, relative levels of free IGF-I (solid bars) and IGFBP-3 (gray bars) in response to the indicated treatments.

Fig. 11. Effect of PTH-(1–34) on IGF-I and IGFBP-3 mRNA levels. Day 18 costal chondrocytes were treated for 4 h with 10 nM PTH-(1–34), allowed to recover for 20 h, treated again with 10 nM PTH-(1–34), and then allowed to recover for 2 h, at which time total RNA was isolated. Thirty-microgram samples of each were separated on agarose gels, transferred to a membrane, hybridized with random labeled probes for aggrecan, IGFBP-3, IGF-I, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then exposed to x-ray film. Lane 1, control; lane 2, PTH-(1–34).

Proteoglycan synthesis was evaluated by sulfate incorporation studies and by steady-state mRNA analysis. The major sulfated proteoglycan was confirmed as aggrecan based on its ability to bind to hyaluronan and its digestion with chondroitinase ABC and AC. In addition, using Western blot, we have observed that the sulfated molecules were recognized by a polyclonal antibody against rat chondrosarcoma aggrecan core protein and also by a monoclonal antibody that recognizes the hyaluronate binding region (1-C-6 from Dr. B. Caterson, data not shown). These results confirm that the high molecular weight proteoglycan was indeed aggrecan. The lower molecular mass band (~220-kDa, based on globular molecular mass standards) was not characterized but is likely to be biglycan (17). Since this molecule was digested by both chondroitinase ABC and AC, it is likely to be chondroitin sulfate-substituted rather than a dermatan sulfate molecule. Taken together, these results establish that PTH treatment resulted in increased aggrecan synthesis.

The increase in aggrecan synthesis by chondrocytes was dependent on the concentration of PTH, with 10 nM eliciting a maximal response. The increase in aggrecan synthesis was observed when the cells were treated for 20 or 4 h followed by a 16-h recovery in the absence of PTH. Previous studies using adult rabbit chondrocytes have demonstrated an effect of PTH only after a 24-h treatment and not within a 4-h treatment (25). These differences may be a reflection of species or developmental age or both. For our studies, we have utilized chondrocytes from rat embryos at different gestational ages, whereas the cells used by Kato et al. (25) were obtained from 400-g rabbits. Furthermore, there are significant differences in receptor density for PTH (23) and in PTH responsiveness during development (Fig. 9).2

Treatment of chondrocytes with PTH for 4–6 h, followed by a recovery time of 18–24 h, resulted in optimal aggrecan synthesis, suggesting that PTH may initially stimulate factors that regulate aggrecan synthesis. Although several candidates can play such a role, our studies have focused on IGF-I as a potential mediator of PTH action on aggrecan synthesis, since previous studies have suggested such a role for IGF-I in mediating PTH action on bone cells (21, 22). IGF action can be regulated both by synthesis of IGF or by local release of IGF from the IGF-IGFBP complex. Several observations presented in this study demonstrate the latter possibility. First, a neutralizing antibody to IGF-I, when included in the treatment medium, caused a significant decrease in the levels of aggrecan and the 220-kDa band in control and PTH-treated cells. A neutralizing antibody to IGF-II had no such activity. Conversely, inclusion of a neutralizing antibody to IGFBP-2 resulted in a dramatic increase in aggrecan and biglycan levels in both control and PTH-treated cells. In addition, quantitative determination of mRNA levels for aggrecan, IGF-I, and IGFBP-3 (Fig. 11) and measurement of free IGF-I and IGFBP-3 levels (Fig. 10) further confirm that PTH stimulation of aggrecan may be associated with a reduction in IGFBP-2 and/or -3. Although there are several IGFBPs, for this study, we have focused on IGFBP-3 (Figs. 8, 10, and 11) and IGFBP-2 (Fig. 7), because our initial ligand blot analysis indicated that IGFBP-3 was the major band.3 Thus, a reduction in IGFBPs may release free IGF-I at the local level, leading to increased aggrecan synthesis. It is interesting to note that a reduction in IGFBPs may play a role in aggrecan synthesis during normal cartilage development, as a reciprocal relationship exists between aggrecan synthesis and IGFBP levels (Fig. 10) further confirm that PTH stimulation of aggrecan may be associated with a reduction in IGFBP-2 and/or -3. Although there are several IGFBPs, for this study, we have focused on IGFBP-3 (Figs. 8, 10, and 11) and IGFBP-2 (Fig. 7), because our initial ligand blot analysis indicated that IGFBP-3 was the major band.3 Thus, a reduction in IGFBPs may release free IGF-I at the local level, leading to increased aggrecan synthesis. It is interesting to note that a reduction in IGFBP-2 may play a role in aggrecan synthesis during normal cartilage development, as a reciprocal relationship exists between aggrecan synthesis and IGFBP levels.

3 C. A. Frolik, A. K. Harvey, and S. Chandrasekhar, unpublished data.
can and IGFBP levels (Fig. 8). Collectively, these results suggest that aggrecan synthesis in normal chondrocytes is dependent on local IGF-I levels and availability and that PTH may enhance aggrecan synthesis by increasing free IGF-I and decreasing IGFBP production (Figs. 10 and 11).

It is not clear why the exogenously added human IGF-I was not effective in eliciting aggrecan synthesis (Fig. 10). Several conditions were attempted, including “serum starving” the cells for 24–48 h before IGF-I was added or by adding IGF-I during the recovery time after a 4- or 20-h PTH treatment (data not shown). One possible explanation is that rat cells are not responsive to human IGF-I.

It is important to recognize, however, that IGF-I/IGFBP-3-mediated aggrecan synthesis is not the only mechanism by which PTH may influence aggrecan synthesis. PTH(1–34) appears to have a direct effect on aggrecan synthesis, since a 4–6-h PTH exposure resulted in an increase in aggrecan mRNA accumulation (Fig. 9). PTH activities on target cells are stimulatory and PTHrP inhibits the normal transition to hypertrophy resulting in prehypertrophic chondrocytes (7, 13, 14). Overexpression of PTHrP receptors may play a crucial role in regulating chondrocyte maturation.

The role of IGF-I in chondrocyte proteoglycan synthesis has been well documented (27–30). Furthermore, PTH has been shown to alter local IGF-I levels in calvaria (21, 22). Our results demonstrate that PTH effects on embryonic (gestational days of 18 days or less) chondrocytes may be mediated by similar local control mechanisms. Since proteoglycans have generally been suggested to cause the inhibition of mineralization, a potential role for PTH is to delay the onset of hypertrophy of the cartilage by stimulating aggrecan synthesis (23). The delay in hypertrophy maturation of collagen may allow cells to add more cartilage, which can be subsequently mineralized for serving as a scaffold for new bone. This would require a very precise regulation of PTH/PTHrP action, but it is not known whether such a regulation occurs in vivo. PTH/PTHrP receptor is expressed in prehypertrophic chondrocytes (7, 13, 14). Overexpression of PTHrP inhibits the normal transition to hypertrophy resulting in various skeletal dysplasias (31, 32), whereas ablation of PTHrP results in a decrease in chondrocyte proliferation and premature differentiation (33–36). Similarly, deletion of the PTH/PTHrP receptor results in accelerated chondrocyte maturation (8). Thus, a temporal and spatial regulation of PTH/PTHrP receptors may play a crucial role in regulating chondrocyte maturation.

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