Signaling mechanisms leading to regulation of proliferation and differentiation of the mesenchymal chondrogenic cell line RCJ3.1C5.18 in response to IGF-I

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Abstract

Since IGF-I is an important chondrocyte growth factor, we sought to examine the intracellular mechanisms by which it exerts two of its pivotal effects, stimulation of proliferation and differentiation. We used the mesenchymal chondrogenic cell line RCJ3.1C5.18, which progresses spontaneously to differentiated growth plate chondrocytes. This differentiation process could be enhanced by exogenous IGF-I. Pharmacological inhibition of the phosphatidylinositol-3 (PI-3) kinase by LY294002, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK)1/2 by U0126, the protein kinase (PK) A pathway by H-89 or KT5720, and the PKC pathway by bisindolylmaleimide suppressed IGF-I-stimulated cell proliferation. In contrast, IGF-I-enhanced early cell differentiation, as assessed by collagen type II and aggrecan gene expression, was not affected by MAPK/ERK1/2 pathway inhibition, but significantly diminished by inhibition of the PI-3 kinase, the PKC and the PKA pathway. Moreover, terminal differentiation of chondrocytes in response to IGF-I, as assessed by gene expression of alkaline phosphatase, Indian hedgehog, and collagen type X, were only interrupted by PI-3 kinase pathway inhibition. In conclusion, IGF-I exerts its differential effect on chondrocyte proliferation vs differentiation through the use of at least four partially interacting intracellular signaling pathways, whose activity is temporarily regulated. When chondrocytes progress from proliferating cells to early and terminal differentiating cells, they progressively inactivate IGF-I-related intracellular signaling pathways. This mechanism might be essential for the complex and cell stage-specific anabolic action of IGF-I in the growth plate.

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Introduction

The growth plate is a dynamic tissue in which cells undergo a developmental program from resting cells to proliferation, maturation, and hypertrophy, until they reach terminal differentiation and produce the mineralized matrix that supports endochondral bone formation (Kronenberg 2003, van der Eerden et al. 2003). Insulin-like growth factor-I (IGF-I) is a potent growth factor in the growth plate, exerting its actions by both endocrine and paracrine/autocrine mechanisms. When the growth hormone (GH)/IGF-I system is inactivated, very little growth occurs in the postnatal period. In fact, only 17% of postnatal growth in the mouse occurs independent of the GH/IGF-I axis (Lupu et al. 2001). Of the 83% of growth governed by the GH/IGF-I axis, IGF-I alone contributes 35%, much more than the 14% contributed by GH alone. Another large (34%) component of growth was found to be dependent on the presence of both GH and IGF-I.

It is known from cell culture (Ohlsson et al. 1994, Monsonego et al. 1995, Reinecke et al. 2000, Olney et al. 2004) and gene knockout experiments (Wang et al. 1999, 2004) that IGF-I stimulates both proliferation and differentiation of growth plate chondrocytes in vitro and in vivo. IGF-I exerts its biological effect by binding to the transmembrane type 1 IGF receptor, whose activation leads to the extensive tyrosyl-phosphorylation of insulin-receptor substrate-1, which acts as a docking protein for the downstream signal transduction pathways (Parrizas et al. 1997, Tsakiridis et al. 2001). Two canonical pathways, the phosphatidylinositol-3 (PI-3)-kinase and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK)1/2 pathway, and the IGF-I-activated protein kinase (PK) A and PKC intracellular cascades have been reported previously to mediate the mitogenic, differentiating and antiapoptotic response to IGF-I (Grey et al. 2003), but the relative contributions to the diverse cellular actions of IGF-I vary according to the cell type (Petley et al. 1998). For example, in brown adipocytes, skeletal muscle cells and Schwann cells, IGF-I-mediated cell differentiation is signaled through the PI-3-kinase pathway (Valverde et al. 1997, Kaliman et al. 1998, Ogata et al. 2004), whereas in human breast cancer cells the same pathway is mainly involved in IGF-I-stimulated mitogenesis (Dufourny et al. 1997). It is therefore necessary to
study IGF-I signaling in individual tissues to determine the role of each pathway in that specific cell type. The signaling cascades may be temporally separated or may have a cross-talk and partially overlap (Daaka et al. 1997, Stork & Schmitt 2002, Yoon et al. 2002). For example, in skeletal myoblasts, IGF-I initially induces proliferation and inhibits myogenesis (Coolican et al. 1997). Subsequently, it promotes the cell cycle exit and stimulates...

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**A**

**PI-3 kinase**

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PCNA
differentiation. Signaling through the MAPK/ERK1/2 pathway in these cells mediates the initial proliferative effect of IGF-I. On the other hand, preventing the late IGF-I-induced decrease in ERK1/2 phosphorylation blocks IGF-I-stimulation of myogenin mRNA, indicating that the time-dependent, opposing effects of IGF-I on skeletal muscle cell differentiation are mediated, at least in part, by biphasic but opposite effects on activation of the MAPK/ERK1/2 signaling pathway (Adi et al. 2002).

Little is known about the intracellular signaling pathways by which IGF-I exerts its effect on cell proliferation and differentiation in growth plate chondrocytes. In articular chondrocytes, IGF-I regulates differentiation, maintenance of the differentiated phenotype, and apoptosis via a PI-3 kinase pathway that modulates ERK, p38 kinase, and PKC signaling (Oh & Chun 2003). The role of the PKC pathway in both spontaneous and IGF-I-enhanced differentiation in growth plate chondrocytes has not been studied so far.

In the present study, we therefore examined the role of various IGF-I-activated signaling pathways in chondrocyte proliferation and differentiation. We used the mesenchymal RCJ3.1C5.18 (RCJ) cell line as a cell culture model, which is widely used for growth plate chondrocyte research (McEwen et al. 1999, Weksler et al. 1999, Cohen et al. 2006). RCJ cells derive from fetal rat calvaria (Grigoriadis et al. 1996, McDougall et al. 1996, Lunstrum et al. 1999). They undergo over 2 weeks of culture in the presence of dexamethasone a reproducible, time-dependent progression from chondroprogenitors to hypertrophic chondrocytes, accompanied by an upregulation of collagen type II and deposition of cartilage-specific proteoglycans in a sequence that mimics the phenotype of chondrocytes of the growth plate. Furthermore, they do not express IGF-I; therefore the action of this hormone can be studied without interference from endogenous IGFs (Spagnoli et al. 2001).

Materials and methods

Reagents

Minimum essential medium (MEM) was purchased from cc Pro (Neustadt, Germany), fetal calf serum from Paa Laboratories (Pasching, Austria), ascorbic acid, β-glycerophosphate and dexamethasone were from Sigma. Recombinant human IGF-I was supplied by Bachem (Weil am Rhein, Germany); LY294002 (LY), H-89, bisindolylmaleimide I (BIS), phorbol myristate acetate (PMA) and KT-5720 were obtained from Calbiochem (San Diego, CA, USA) and U0126 from Promega. [3H]-thymidine and enhanced chemiluminescent (ECL) reagents were provided from Amersham Pharmacia. The antibodies to phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2, phosphorylated Akt (p-Akt), total Akt, phosphorylated PKCθ (pPKCθ), β-actin, the horseradish peroxidase-conjugated (anti-rabbit and anti-mouse) antibodies were from Cell Signaling Technology (Frankfurt am Main, Germany), antibodies to proliferating cell nuclear antigen (PCNA), PKCζ, and PKAζ were purchased from Santa Cruz Biotechnologies (Santa Cruz, Heidelberg, Germany).

Cell culture

RCJ3.1C5.18 cells (kindly provided by Dr Anna Spagnoli, Department of Pediatrics, Vanderbilt University Medical Center, Nashville, Tennessee, USA) were grown at 37°C in humidified 5% CO₂ atmosphere in MEM (with Earle’s salts) supplemented with 1 mM NaCyt5-glutamine, 10 mM Hepes, 100 U/ml penicillin–streptomycin, 2 mM sodium pyruvate, 15% heat-inactivated fetal bovine serum, 10⁻⁷ M dexamethasone and studied within 25 passages. Cell viability was tested with the tetrazolium salt (MTT) assay. These cells sequentially acquire at 7 days of culture markers of chondrocytic differentiation (type II collagen) and progressively acquire at 10 and 14 days of culture markers of terminal differentiation (type X collagen and alkaline phosphatase; Lunstrum et al. 1999, Spagnoli et al. 2001, data not shown).

[3H]-Thymidine assay

The extent of thymidine incorporation into DNA was determined as uptake of radioactivity in precipitated material, as described previously (Kiepe et al. 2001).

Briefly, chondrocytes were grown in 96-well plates to confluence for 3 days; thereafter, the cultures were

Figure 1 The mitogenic effect of IGF-I in RCJ cells is mediated by signaling pathways involving the PI-3 kinase, the MAPK/ERK1/2, the PKA and, to a lesser extent, the PKC pathways. (A) RCJ cells were cultured until confluence, serum-starved for 12 h and stimulated by IGF-I (100 ng/ml) in the presence or absence of inhibitors of the respective signaling pathways (LY294002 (LY) for the PI-3 kinase pathway; U0126 for the MAPK/ERK1/2 pathway; an bisindolylmaleimide (BIS) for the PKC pathway; H-89 and KT-5720 for the PKA pathway) and the activator PMA for the PKC pathway for an additional 48 h. Control cells were cultured without IGF-I in the absence or presence of the same concentrations of inhibitors. [3H]-Thymidine incorporation into the acid-extractable pool was determined by scintillation counting and used as a measure of DNA synthesis, as described in Materials and methods. Data are means ± s.e. Statistics by ANOVA were for 12 parallel wells per group from three independent experiments. *P<0.05 vs control; †P<0.05 vs IGF-I. (B) Inhibition of the PI-3 kinase, the MAPK/ERK1/2, PKA and to a lesser extent, the PKC pathways reduces IGF-I-driven cell proliferation as assessed by PCNA protein content. RCJ cells were cultured until confluence, serum-starved for 12 h and stimulated by IGF-I (100 ng/ml) in the presence or absence of inhibitors of the different signaling pathways for an additional 12 h. PCNA protein content was evaluated by Western immunoblotting; representative autoradiographs of a total of three experiments are shown.
changed to serum-free medium. After 12 h, various concentrations of inhibitors with or without IGF-I were added to the medium and the incubation was continued for a further 48 h. The rate of chondrocyte proliferation was assessed by incubating the cells with 3 μCi/ml [3H]-thymidine for the final 4 h. Subsequently, cells were rinsed twice with PBS. The radiolabeled DNA was precipitated by acetic acid and dissolved in 1 M NaOH. [3H]-Thymidine incorporation into the acid-extractable pool as a measure of DNA synthesis was determined by scintillation counting.

Quantitative RT-PCR

For analysis of collagen type II, collagen type X, aggrecan, Indian hedgehog (Ihh), and alkaline phosphatase (ALP) mRNA abundance, cells were cultured in differentiating medium from day 4 (80–90% of confluence). After 3 days, at day 7 of culture, for investigating the expression of collagen type II and aggrecan, as markers for early differentiation (Sive et al. 2002), and at day 14, for investigating the expression of collagen type X, Ihh, and ALP, as markers for terminal differentiation (Lai & Mitchell 2005), cells were serum-starved for 12 h and stimulated with 100 ng/ml IGF-I for 12 additional hours, in the presence or absence of the indicated inhibitors, and then harvested. RNA was isolated by using the RNeasy mini columns (Qiagen) and reverse transcribed by using Moloney Murine Leukemia Virus reverse transcriptase and oligo(dt)/random hexamer primers (10:1) from Applied Biosystems (Darmstadt, Germany). For quantitative analysis, real time RT-PCR was performed using the ABI 7000 (Applied Biosystems) according to the manufacturer’s protocol. The following set of primers was chosen by the Primer Express program (Applied Biosystems) for collagen type II (forward, 5′-GGCAAGACCATCATGAGTACC-3′; reverse, 5′-CCCTATGTCACACCAAATTC-3′), for collagen type X (forward, 5′-GGTAAAGAGATTTCAGTAAGAGGAGAACA-3′; reverse, 5′-ACTTCTCATAGCCTGGCTTTCC-3′), for ALP (forward, 5′-AACAACCTCAGCTGACCTTCC-3′; reverse, 5′-TCATCTCTGCTTCCTTCC-3′), for Ihh (forward, 5′-CGAGCGAAATAAGTACGGACTACTG-3′; reverse, 5′-TCAAGACCGAGAAGCTCTGG-3′), and for 18S (forward, 5′-ATGTTGTGGACGAGGTATTCT-3′; reverse, 5′-GCTGAGCAGTTCCAGTGGC-3′). The software provided from the company allowed the quantitative detection of fluorescence by the incorporation of the substance SyberGreen into the amplification products. Amplification was performed in the presence of Universal Mastermix (PE Applied Biosystems) with SyberGreen to detect PCR products at the end of each amplification step, and the results were analyzed as already reported (Kiepe et al. 2006).

Western immunoblotting

For total cell homogenate cells were incubated with the indicated substances, scraped in 50 μl ice-cold lysis buffer containing a mixture of proteinase and phosphatase inhibitors, and cell extracts were treated as reported previously (Hömme et al. 2003, Kiepe et al. 2006). Cytosol membrane and nuclear factions of cells were prepared by previously described procedures (Hoeﬂich et al. 2004). Briefly, homogenates were centrifuged at 600 g for 20 min at 4 °C to pellet a crude nuclear fraction. The supernatant was recentrifuged at 100 000 g for 30 min at 4 °C to pellet membranes and the supernatant containing the cytosol. Nuclei and membranes were washed in Tris-buffered saline and lysed in lysis buffer containing a mixture of proteinase and phosphatase inhibitors. Protein amount was measured by the BCA method, and 20 μg protein were boiled (5 min) and electrophoresed on a 12% separating SDS–polyacrylamide gel. Separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Eschborn, Germany). The membranes were blocked in 5% milk for 1 h at room temperature, incubated overnight with the first antibody (dilution 1:2000 for PKCβ, dilution 1:4000 for p-Akt, β-actin and PCNA in 3% milk; dilution 1:2000 for p-Akt, pPKCβ, PKCβ, p-ERK1/2 and ERK1/2 in 3% BSA), washed extensively over a period of 30 min with Tris Buffer Saline-Tween 20 0.05%, and then incubated for 1 h with the secondary antibody (dilution 1:2000 in 3% milk), followed by further washing over a period of 30 min. The protein bands were visualized using an chemiluminescent detection system and Hyperfilm ECL film (Amersham Pharmacia Biotech) according to the manufacturer’s directions.

The successful separation of cytosol, cell membrane, and nuclear fractions was verified by incubating each cell fraction with specific marker proteins. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a marker for the cytosol (Sirover 1997), was abundant in the cytosolic fraction and nearly absent in the nuclear and membrane fractions. The membrane fraction was enriched with Na+-K+-ATPase, a known marker for cell membranes (Mrşny et al. 1988), and the nuclear fraction was identified by positive cAMP-response element binding protein staining, a known marker for the nucleus (Roesler et al. 1988; data not shown).

Statistical analysis

All experiments were performed at least thrice. For measurements with the ABI7000 system, samples were run in duplicate to account for technical and biological variability within and between experiments. Data are...
given as means ± s.e. All the data were examined for normal and non-Gaussian distribution by the Kolmogorov–Smirnov test. For comparison among normally distributed groups, one-way ANOVA followed by pairwise multiple comparisons (Student–Newman–Keuls method) or the Student’s *t*-test were used as appropriate. For non-normally distributed data, the non-parametric Kruskal–Wallis test followed by an all pairwise multiple comparison (Dunnett’s method) was used. *P*<0.05 was considered statistically significant.

**Figure 2** IGF-I enhances differentiation of RCJ cells. RCJ cells were grown until day 4, when they reached 80–90% of confluence. Then, cells were allowed to differentiate in medium supplemented with ascorbic acid (50 µg/ml) and β-glycerophosphate (10 mM). (A) Phase-contrast photomicrographs of proliferating (day 3) and differentiating (day 14) cells are shown (magnification ×40). (B) Cells were grown as described above. At day 7 of culture, cells were serum-starved for 12 h, and subsequently grown in the absence (left panel, magnification ×40) or presence of IGF-I (100 ng/ml) for an additional 12 h (middle panel, magnification ×40). The arrows indicate cartilage-like nodules. Right panel, a higher magnification (magnification ×400) of a typical cartilage nodule is shown. (C) Spontaneous and IGF-I-induced differentiation of RCJ cells was assessed by collagen type II mRNA expression, determined by real-time PCR, as a marker for early chondrocyte differentiation (left panel), and by sulfate proteoglycan accumulation, quantified by alcian blue staining, as a marker of terminal chondrocyte differentiation (right panel). The data in the left panel represent mean values (± s.e.) of a total of seven experiments, the data in the right panel of three typical experiments. Statistics by ANOVA. *P*<0.05 vs day 4; †*P*<0.05 IGF-I vs control.
Results

The mitogenic effect of IGF-I in RCJ cells is mediated by signaling pathways involving PI-3 kinase, MAPK/ERK1/2, PKC and PKA

To identify the intracellular pathways subserving the mitogenic action of IGF-I in chondrocytes, RCJ cells were treated with IGF-I alone or in the presence of specific pharmacological inhibitors of PI-3 kinase (LY294002), MAPK/ERK1/2 (U0126), PKC (BIS) and PKA (H-89 and KT-5720) signaling pathways. In addition, the effect of PMA, an activator of the PKC pathway, on cell proliferation was tested. Cell viability after 48 h of incubation with 25 μM LY294002 was approximately 80%, indicating that these substances were not cytotoxic at the indicated conditions. IGF-I at a concentration of 100 ng/ml stimulated proliferation of RCJ cells, as assessed by incorporation of [3H]-thymidine, three- to four-fold (Fig. 1A). The mitogenic effect of IGF-I was partially sensitive to the inhibition and activation of the PKC pathway and completely abolished by the respective inhibitors of the PI-3 kinase, MAPK/ERK1/2 and PKA kinase pathway (Fig. 1A). These data indicate that IGF-I-stimulated cell proliferation of RCJ cells is mediated through the PI-3 kinase, MAPK/ERK1/2 and PKA signaling pathways, whereas the PKC pathway plays only a minor role. Since the results obtained with the PKA inhibitor H-89 were similar to those obtained with KT5720, we decided to use only H-89 in the subsequent series of experiments.

Comparable results were obtained, when chondrocyte proliferation was assessed by PCNA protein content in cell lysate (Fig. 1B). PCNA is a nuclear protein required for cell-cycle progression and cellular proliferation. The PCNA content in RCJ cells exposed to IGF-I clearly increased. Coincubation with the respective inhibitors of the PI-3 kinase, MAPK/ERK1/2, or PKA pathways markedly reduced IGF-I-induced PCNA protein content, whereas coincubation with the PKC pathway inhibitor BIS had only a weak inhibitory effect.

IGF-I enhances differentiation of RCJ cells

The mesenchymal RCJ cell line spontaneously differentiates from displayed polygonal-shaped isolated chondrocytes to cartilage nodules over 4–14 days of culture in differentiating medium, in the presence of dexamethasone (Grigoriadis et al. 1996).

![Figure 3](image-url) IG F-I activates key signaling molecules of the PI-3 kinase and MAPK/ERK1/2 pathways. (A) Cells were cultured in differentiating medium until day 7 (left panel) and day 14 (right panel), serum-starved for 12 h and incubated for 1 h with IGF-I (100 ng/ml) in the presence or absence of 25 μM LY294002. Cell lysates were subjected to Western immunoblot analysis and the respective membranes were probed with specific antibodies against p-Akt and total Akt. Representative autoradiographs of a total of three independent experiments are shown. (B) After 12 h of starvation, cells were treated with IGF-I and the MAPK/ERK1/2 inhibitor U0126 for 1 h and the respective membranes were probed with specific antibody against p-Akt and total Akt. Representative autoradiographs of a total of three independent experiments are shown. (B) After 12 h of starvation, cells were treated with IGF-I and the MAPK/ERK1/2 inhibitor U0126 for 1 h and the respective membranes were probed with specific antibodies against p-ERK1/2 and total ERK 1/2 (right panel). Blots are representative for two independent experiments.
Differentiation of RCJ cells was promoted by incubating the cells with β-glycerophosphate and ascorbic acid from day 4 of culture, as described in Materials and methods. Differentiating cultures can be distinguished from proliferating cells in their tendency to become extensively nodulated, as shown in the light microscopy pictures (Lunstrum et al. 1999; Fig. 2A).

To evaluate changes in RCJ morphology due to the presence of IGF-I, differentiating cells were cultured in differentiating medium from day 4 of culture until day 7, followed by serum-deprivation for 12 h and treatment with IGF-I (100 ng/ml) for an additional 12 h. As shown in Fig. 2B, IGF-I enhanced nodule formation of differentiating chondrocytes. Furthermore, IGF-I treatment stimulated the gene expression of collagen type II, a marker of early chondrocyte differentiation two-fold compared with untreated cells (Fig. 2C, left panel). Exogenous IGF-I also enhanced the synthesis of sulfated proteoglycans, a marker of terminally differentiated chondrocytes, on day 11 and 14 of culture (Fig. 2C, right panel). These results indicate that IGF-I stimulates the chondrogenic differentiation of RCJ cells.

**IGF-I-enhanced early differentiation is mediated through the PI-3 kinase, PKA and PKC signaling pathways, IGF-I-enhanced terminal differentiation through the PI-3 kinase pathway**

Next, we sought to investigate whether IGF-I activates key signaling molecules of the respective pathways in differentiating chondrocytes. RCJ cells were cultured in differentiating medium from day 4 onwards. At the indicated time points, cells were serum-deprived for 12 h and subsequently treated with IGF-I in the

![Figure 4](https://www.endocrinology-journals.org/)

**Figure 4** IGF-I activates key signaling molecules of the PKC and PKA pathways. (A and B). At day 7 and day 14 of culture, cells were treated with IGF-I (100 ng/ml) and the respective inhibitors or PMA, an activator of PKC, for 30 min. The nuclear (N), membrane (M), and cytosolic (C) fractions were prepared by ultracentrifugation and immunoblotted with antibodies specific for PKCα (A) and PKAα (B). β-actin was used as a loading control. Blots are representative for three independent experiments.
Figure 5  IGF-I-enhanced early differentiation of RCJ cells, as assessed by collagen type II gene expression, is mediated through the PI-3 kinase, PKA and PKC signaling pathways. (A) RCJ cells were cultured in differentiating medium from day 4 onwards. At day 7 of culture, cells were serum-starved for 12 h and stimulated by IGF-I (100 ng/ml) in the presence or absence of specific inhibitors of the different signaling pathways (LY294002 (LY) for the PI-3 kinase pathway; U0126 for the MAPK/ERK1/2 pathway; bisindolylmaleimide (BIS) for the PKC pathway; H-89 for the PKA pathway). After 12 h, total RNA was extracted and collagen type II mRNA expression was measured by quantitative RT-PCR. Each experiment was repeated at least thrice. The columns represent the mean ± s.e. Statistics by ANOVA. *P < 0.05 vs control; †P < 0.05 vs IGF-I. (B) The MAPK/ERK1/2 inhibitor U0126 (2.5 μM) blocks ERK1/2 phosphorylation. After 12 h of starvation, cells were treated with IGF-I and the MAPK/ERK1/2 inhibitor U0126 for 1 h. The blot represents one typical experiment of a total of two. (C) Phosphorylation of ERK1/2 both under baseline conditions and in response to IGF-I decreases during the process of differentiation of RCJ cells. RCJ cells were cultured in differentiating medium from day 4 onwards. At the indicated time points, cells were serum-starved for 12 h and treated with or without IGF-I for 1 h. Cell lysates were subjected to Western immunoblotting and the membrane was probed with specific antibodies against the phosphorylated and unphosphorylated form of ERK1/2. The blot represents one typical experiment of a total of two.
presence or absence of specific pharmacological inhibitors of defined pathways for 12 h. As shown in Fig. 3A, the PI-3 kinase pathway inhibitor LY 294002 blocked completely the IGF-I-induced phosphorylation of AKT in a concentration of 25 μM on day 7 and day 14 of culture. These results indicate that IGF-I is capable of stimulating the PI-3 kinase pathway during the entire period of differentiation. U0126 specifically inhibited the MAP kinase pathway, not the PI-3 kinase pathway, and LY294002 specifically inhibited the PI-3 kinase pathway, not the MAP kinase pathway (Fig. 3B).

It has been shown previously that translocation from the cytoplasm to the cell membrane is required for the activation of PKC isoenzymes (Newton 1995) and PKA isoenzymes (Meinkoth et al. 1993). We therefore determined whether IGF-I stimulates the respective pathways by translocation of PKCα and PKAα cells were incubated with IGF-I, PMA as an activator of the PKC pathway and the respective inhibitors, followed by preparation of cytosolic, membrane, and nuclear fractions by ultracentrifugation and immunoblotting with specific antibodies. PKCα migrated at an approximate molecular mass of 80 kDa and was predominantly found in untreated cells at day 7 of culture in the cytosolic fraction (Fig. 4A). Treatment with IGF-I (100 ng/ml) or PMA (1 μM) for 30 min stimulated the translocation of PKCα from the cytosol predominantly to the cell membrane and to a lesser extent to the nuclear fractions (Fig. 4A, upper panel). The PKC inhibitor BIS blocked this translocation completely. In contrast, this translocation of PKCα from the cytosol to the cell membrane in response to IGF-I was clearly diminished on day 14 of culture (Fig. 4A, lower panel). PKAα migrated at an approximate molecular mass of 40 kDa and was also predominantly found in the cytosolic fraction in control cells. Stimulation with IGF-I on day 7 of culture led to a translocation of PKAα from the cytosol predominantly to the cell membrane; the PKA inhibitor H-89 reduced this translocation (Fig. 4B, upper panel). In contrast, this translocation of PKAα from the cytosol to the cell membrane in response to IGF-I was clearly diminished on day 14 of culture (Fig. 4B, lower panel). These results indicate that IGF-I activates the PKC and PKA pathways predominantly during early chondrocyte differentiation.

As shown in Fig. 5A, IGF-I-enhanced early differentiation as assessed by collagen type II mRNA expression...
was sensitive to the respective inhibitors of the PI-3 kinase, PKC and PKA pathways and not affected by MAPK/ERK1/2 inhibition. Comparable results were obtained for aggrecan gene expression as a marker for early chondrocyte differentiation (Fig. 6). The MAPK/ERK1/2 inhibitor U0126 was used at a concentration of 2.5 μM, which is capable of blocking ERK1/2 phosphorylation after 12 h of incubation (Fig. 5B). Consistent with these results is that the activity of the MAP kinase pathway as assessed by phosphorylation of ERK1/2 declined during the process of cell differentiation, both under baseline conditions and in response to IGF-I (Fig. 5C). Taken together, these results indicate that the PI-3 kinase and the G protein signaling pathways mediate the IGF-I-enhanced early differentiation of RCJ cells, while the MAPK/ERK1/2 pathway is not involved in this process.

Terminal differentiation of RCJ cells was assessed by ALP, Ihh, and collagen type X gene expression. At day 14 of culture, coincubation with the PI-3 kinase pathway inhibitor LY294002 significantly blocked the IGF-I-driven gene expression of ALP, Ihh, and collagen type X, while coincubation with the respective inhibitors of the other pathways did not blunt this IGF-I-effect (Figs 7–9). This observation that PKC and PKA pathways are no more active for IGF-I-signaling during terminal differentiation is supported by our previous observations that IGF-I on day 14 of culture is no more capable of activating the PKC and PKA pathways compared with day 7 of culture (Fig. 4A and B, lower panels). Taken together, these data indicate that of the four IGF-I-related signaling cascades investigated, only the PI-3 kinase pathway is required for early and terminal differentiation of RCJ cells in response to IGF-I.

Cross-talk between the PI-3 kinase, PKC and PKA pathways in response to IGF-I in differentiating RCJ cells

The data described above suggest that the differentiating action of IGF-I in RCJ cells is dependent upon several intracellular signaling molecules. To determine

Figure 7 IGF-I-enhanced terminal differentiation of RCJ cells, as assessed by collagen type X gene expression, is mediated through the PI-3 kinase signaling pathway, not through the MAPK/ERK1/2, PKC or PKA pathways. RCJ cells were cultured in differentiating medium from day 4 onwards. At day 14 of culture, cells were serum-starved for 12 h and stimulated by IGF-I (100 ng/ml) in the presence or absence of specific inhibitors of the different signaling pathways (LY294002 (LY) for the PI-3 kinase pathway; U0126 for the MAPK/ERK1/2 pathway; bisindolylmaleimide (BIS) for the PKC pathway; H-89 for the PKA pathway). After 12 h, total RNA was extracted and collagen type X mRNA expression was measured by quantitative RT-PCR. Each experiment was repeated at least thrice. The columns represent the mean ± S.E. Statistics by ANOVA. *P<0.05 vs control; †P<0.05 vs IGF-I.
potential interrelationships among these signaling molecules, the respective effect of a combination of these inhibitors on IGF-I-enhanced RCJ cell differentiation was examined. Consistent with the data shown in Fig. 5, coincubation with the PKC inhibitor BIS reduced the IGF-I-induced collagen type II mRNA expression by 40%, while coincubation with the PI-3 kinase inhibitor LY294002 suppressed IGF-I-induced collagen type II mRNA to a greater extent (80%) (Fig. 10A). Coincubation with these two inhibitors did not have an additive inhibitory effect on IGF-I-induced collagen type II mRNA expression. In contrast, coincubation of the PKA inhibitor H-89 with LY294002 diminished IGF-I-induced collagen type II mRNA expression to a greater extent (80%) (Fig. 10A). Coincubation with these two inhibitors did not have an additive inhibitory effect on IGF-I-induced collagen type II mRNA expression. In contrast, coincubation of the PKA inhibitor H-89 with LY294002 diminished IGF-I-induced collagen type II mRNA expression to a greater extent (80%) (Fig. 10A). Coincubation with these two inhibitors diminished IGF-I-induced collagen type II mRNA expression to a greater extent (80%) (Fig. 10A). Coincubation with these two inhibitors did not have an additive inhibitory effect on IGF-I-induced collagen type II mRNA expression (Fig. 10C), consistent with the hypothesis that these two pathways are independently activated by IGF-I. Furthermore, IGF-I enhanced the phosphorylated PKC subunit θ (Fig. 10D). This activation was sensitive not only to the inhibition of the PKC pathway with BIS as expected, but also sensitive to the inhibition of the PI-3 kinase with LY294002 (Fig. 10D).

**Discussion**

Growth plate chondrocytes are important target cells both for circulating and locally synthesized IGF-I. We therefore sought to define the intracellular signal transduction pathways, which mediate the differential regulation of chondrocyte proliferation vs differentiation in response to IGF-I. We found that IGF-I-driven chondrocyte proliferation is mediated by the PI-3 kinase, the MAPK/ERK1/2, and the PKA pathways, while the PKC pathway played only a minor role. We concede that results obtained from experiments in a cell line such as RCJ cells might not be applicable to the
growth of the whole animal. However, we have observed previously that IGF-I-enhanced cell proliferation in rat growth plate chondrocytes in primary culture is also mediated through the PI-3 kinase and the MAPK/ERK1/2 pathways (Kiepe et al. 2005). These congruent data indicate that our observations in RCJ cells apply to growth plate chondrocytes in general.

The involvement of the PKC and PKA pathways in IGF-I-driven mitogenesis of articular chondrocytes has been described previously (Taylor et al. 1988). It was recently reported that IGF-I signals mitogenesis and survival in osteoblastic cells through parallel, partly overlapping intracellular pathways involving PI-3 kinase, MAPK/ERK1/2, and Gγ2 subunits (Grey et al. 2003). These data indicate that IGF-I exerts its mitogenic effect in chondrocytes and osteoblasts by the use of comparable intracellular signaling pathways. In this context, it should be noted that the activation of different signaling pathways involved in IGF-I-mediated cell proliferation is clearly cell-type specific. For example, the proliferation of fetal brown adipocytes in response to IGF-I is mediated through activation of the MAPK/ERK1/2 pathway (Porras et al. 1998), whereas parallel PI-3 kinase and MAPK/ERK1/2 pathways subserve the mitogenic action of IGF-I in cardiomyocytes (Sundgren et al. 2003). Interestingly, in the skeletal myoblast cell line L6A1 only the MAPK/ERK1/2 pathway, not the PI-3 kinase pathway, is responsible for the mitogenic response of IGF-I (Samuel et al. 1999).

IGF-I is not only an important endocrine and paracrine/autocrine regulator of chondrocyte proliferation, but also enhances chondrocyte differentiation in vivo, because IGF-I knockout-mice exhibit smaller hypertrophic chondrocytes than their wild-type counterparts (Wang et al. 1999, 2004). These knockout data are consistent with previous in vitro data in primary rat chondrocytes (Ohlsson et al. 1994). We demonstrate here for the first time that IGF-I also enhances differentiation of RCJ cells. In our model, differentiation was assessed by cell morphology and marker molecules of early (collagen type II and aggrecan) and terminal (collagen type X, Ihh, and ALP) cell differentiation. Interestingly, IGF-I-enhanced early cell differentiation was mediated only through the PI-3 kinase, PKC and PKA pathways, while an intact MAPK/ERK1/2 pathway was not required. Consistent with
these data is our observation that the phosphorylation state of ERK1/2 decreased during spontaneous differentiation of RCJ cells, and IGF-I lost its capability to phosphorylate ERK1/2 after day 7 of culture (Fig. 5C). These data are in agreement with the findings of Phornphutkul et al. (2004) who observed minimal ERK response to IGF-I in hypertrophic ATDC5 cells, but the functional role of other IGF-related signaling pathways were not studied by these investigators. Taken together, these data indicate that the MAPK/ERK1/2 pathway is being inactivated both during spontaneous and IGF-I-mediated early differentiation of RCJ cells. The former finding is supported by the observation that in articular chondrocytes the phosphorylation pattern of ERK was inversely proportional to spontaneous cell differentiation as assessed by type II collagen expression (Zimmermann & Moelling 1999), indicating that also in this chondrocyte cell culture model the MAPK/ERK1/2 pathway is being inactivated during cell differentiation. These data are consistent with observations in human articular chondrocytes in which IGF-I enhanced differentiation as assessed by proteoglycan synthesis via the PI-3 kinase pathway (Starkman et al. 2005).

The IGF-I-driven gene expression of ALP, Ihh, and collagen type X, other marker molecules of terminal chondrocyte differentiation, were only mediated through the PI-3 kinase pathway, not the MAPK/ERK1/2 pathway (Figs 7–9). Hence it appears that chondrocytes, when they progress from the proliferative state to early and terminal differentiating cells, progressively inactivate several IGF-I-related intracellular signaling pathways beside the PI-3 kinase pathway (Figs 4 and 5C).

We observed that during differentiation of RCJ cells, there is a cross-talk between the IGF-I-induced activation of the PI-3 kinase pathway with the PKC pathway, but not between the other IGF-I-related pathways. Furthermore, inhibition of the PI-3 kinase pathway in RCJ cells reduced the expression of the pPKCq isoform, suggesting that PKC is downstream of the PI-3 kinase pathway. This observation is consistent with data in limb bud mesenchymal cells during micromass culture in which PI-3 kinase activation is required for the formation of condensations (Zimmermann & Moelling 1999).

Figure 10 Cross-talk between the PI-3 kinase, PKC and PKA pathways in response to IGF-I in differentiating RCJ cells. (A–C) RCJ cells were cultured in differentiating medium from day 4 onwards. At day 7 of culture, cells were serum-starved for 12 h and stimulated with IGF-I (100 ng/ml) in the presence or absence of specific inhibitors of the respective signaling pathways (LY294002 (LY) for the PI-3 kinase pathway, bisindolylmaleimide (BIS) for the PKC pathway, H-89 for the PKA pathway). After 12 h, total RNA was extracted and collagen type II mRNA expression was measured by quantitative RT-PCR. Each experiment has been repeated at least thrice. The columns represent the mean ± s.e.

Statistics by ANOVA. *P<0.05 vs control; †P<0.05 vs IGF-I; ‡P<0.05 vs IGF-I plus BIS; §P<0.05 vs IGF-I plus H-89; ¶P<0.05 vs IGF-I plus LY. (D) At day 7 of culture, cells were serum-starved for 12 h and incubated with IGF-I (100 ng/ml) for 1 h in the presence or absence of specific inhibitors of the different signaling pathways (LY for the PI-3 kinase pathway; BIS for the PKC pathway), at the indicated concentrations. Cell lysates were subjected to Western immunoblot analysis and the respective membranes were probed with specific antibodies against pPKCq and PKCq. A representative autoradiograph of a total of three independent experiments is shown.

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Cross-talks between the PI-3 kinase and MAPK/ERK1/2 signaling pathways have been reported. For example, in differentiating articular chondrocytes, IGF-I activates PI-3 kinase signaling, which is associated with an inhibition of the phosphorylation of ERK1/2 (Oh & Chun 2003). We observed in RCJ cells that the expression and activation of PKC-alpha in response to IGF-I (Oh & Chun 2003).

Cross-talks between the PI-3 kinase and MAPK/ERK1/2 signaling pathways have been reported.

Figure 11 Schematic model of intracellular pathways subserving the proliferative and differentiating actions of IGF-I in RCJ cells. Activation of the type 1 IGF receptor stimulates signaling through the parallel PI-3 kinase, MAPK/ERK1/2, PKC and PKA pathways. All the cascades regulate the IGF-I-enhanced proliferation of RCJ cells, although to a variable degree. PI-3 kinase, PKC and PKA pathways control early chondrocyte differentiation, whereas just the PI-3 kinase cascade has a role in the regulation of terminal differentiation. Most widely described interactions between intermediates are represented by solid thin arrows. It should be considered that not all steps occur in all tissues and that there may be cross-talk reaction between individual members of these pathways. Dashed thick arrows indicate pathways involved in the proliferation of RCJ cells according to our data, solid thick arrows pathways involved in cell differentiation.
IGF-I-induced phosphorylation of ERK1/2 was not affected by inhibiting either the PI-3 kinase or the PKC pathway (data not shown). These data suggest that the MAPK/ERK1/2 pathway in RCJ cells is independent of the PI-3 kinase or PKC pathway during early differentiation of RCJ cells. Taken together, these data indicate that during differentiation of RCJ cells, there is only a cross-talk between the IGF-I-induced activation of the PI-3 kinase pathway with the PKC pathway, but not between the other IGF-I-related pathways.

In conclusion, our data indicate that IGF-I exerts its differential effect on chondrocyte proliferation vs differentiation through the use of at least four partially interacting intracellular signaling pathways, whose activity is temporarily regulated. When chondrocytes progress from proliferating cells to early and terminally differentiating cells, they progressively inactivate IGF-I-related intracellular signaling pathways. This mechanism might be essential for the complex and cell stage-specific anabolic action of IGF-I in the growth plate.

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