Posttranscriptional Effect of Insulin-like Growth Factor-I on Interleukin-1β–induced Type II–secreted Phospholipase A_2 Gene Expression in Rabbit Articular Chondrocytes

Claire Jacques,*‡ Gilbert Béréziat,* Lydie Humbert,* Jean-Luc Olivier,* Marie-Thérèse Corvol,‡ Joëlle Masliah,* and Francis Berenbaum*§

**Biochemistry and Molecular Biology Laboratory, Faculty of Medicine of St. Antoine, University Pierre and Marie Curie, 75012 Paris, France; ‡Growth Pathology and Development Laboratory, Faculty of Medicine of Necker, University René Descartes, Institut National de la Santé et de la Recherche Médicale U30, 75015 Paris, France; and §Department of Rheumatology, Hôpital St. Antoine, 75012 Paris, France*

Abstract

Large amounts of type II–secreted phospholipase A_2 (type II $sPLA_2$) are secreted into inflammatory synovial fluid and they are believed to induce the synthesis of lipid mediators by articular chondrocytes. Preliminary experiments showed that insulin-like growth factor-I, which counteracts cartilage degradation in arthritis, inhibits interleukin-1β–induced type II $sPLA_2$ gene expression in rabbit articular chondrocytes (Berenbaum, F., G. Thomas, S. Poiraudeau, G. Béréziat, M.T. Corvol, and J. Masliah. 1994. *FEBS Lett.* 340: 51–55). The present study showed that IL-1β induced the sustained synthesis of prostaglandin E_2 and a parallel increase in type II $sPLA_2$ gene expression (assessed by enzymatic activity and Northern blot analysis), but no increase in cytosolic PLA_2 gene expression (assessed by Northern and Western blot analysis) or cytosolic PLA_2 activity in rabbit articular chondrocytes. IGF-I inhibited both IL-1β–stimulated PGE_2 synthesis and type II $sPLA_2$ gene expression, but had no effect on cytosolic PLA_2 gene expression. Nuclear run-on experiments revealed that IL-1β stimulated the transcription rate of type II $sPLA_2$ gene, giving rise to long-lived mRNA in cells treated with actinomycin D. IGF-I did not affect transcription rate, suggesting that it acts as a post-transcriptional step. Sucrose density gradient analysis of the translation step showed no effect of IGF-I on the entry of type II $sPLA_2$ mRNA into the polysomal pool or on its distribution into the various polysomal complexes, suggesting that IGF-I does not act on the translation of the mRNA. Lastly, IGF-I strongly decreased the half-life of IL-1β–induced type II $sPLA_2$ mRNA (from 92 to 12 h), suggesting that IGF-I destabilizes mRNA. These data demonstrate that IL-1β stimulates the transcription rate of the type II $sPLA_2$ gene and gives rise to a very stable mRNA. In contrast, IGF-I decreases the half-life of the type II $sPLA_2$ message. (*J. Clin. Invest.* 1997. 99:1864–1872.) Key words: inflammation • phospholipase A_2 • messenger RNA • insulin-like growth factor-I • chondrocyte

Introduction

Phospholipases A_2 (PLA_2s)[1] catalyze the hydrolysis of the *sn*-2 fatty acyl ester bond of phospholipids to yield a free fatty acid, very often arachidonic acid, and a lysophospholipid (1, 2). The hydrolysis of phospholipids by a PLA_2 is the first step in prostaglandin synthesis, the second one being the oxidation of free arachidonic acid by a cyclooxygenase. Two of the main classes of PLA_2 involved in lipid mediator synthesis, cytosolic PLA_2 ($cPLA_2$) (3, 4) and the PLA_2 secreted during inflammatory reactions (type II $sPLA_2$) (5, 6) have been cloned. The rapid lipid mediator synthesis (in a few minutes) induced by membrane receptor stimulation has been linked to stimulation of $cPLA_2$ by protein kinase cascades (7) and calcium-induced $cPLA_2$ translocation to cell membranes (3). The actual way in which type II $sPLA_2$ acts is still uncertain. It may act directly on perturbed cell membranes (8), or via the recently cloned $sPLA_2$ receptors (9).

The importance of type II $sPLA_2$ in arthritis has been inferred from experiments in which purified or recombinant human type II $sPLA_2$ was injected intraarticularly or in an air pouch (10-12). Its direct action has been recently challenged by Morgan et al. (13), who found no inflammatory effect of intradermal injections of human type II $sPLA_2$ in the rat. However, others have found that intradermal injections exacerbated inflammation in an experimental rat model arthritis (14). The proinflammatory interleukin-1β induces the synthesis and secretion of type II $sPLA_2$ in rabbit articular chondrocytes (15) and the activation of cyclooxygenase-2 (16). We have demonstrated (17) that this effect is inhibited by insulin-like growth factor I, a polypeptide structurally and functionally similar to proinsulin that enhances matrix synthesis in normal cartilage in vivo (18) and in vitro (19). IGF-I receptor, IGF-I mRNA, and IGF-I protein have all been found in articular chondrocytes (20, 21), suggesting the presence of an autocrine/paracrine regulation system. The present study was therefore carried out to determine whether IL-1β and/or IGF-I influence type II $sPLA_2$ gene expression at a transcriptional and/or posttranscriptional level. The results indicate that IL-1β increases the transcription rate of the type II $sPLA_2$ gene, producing a long living mRNA, whereas IGF-I greatly decreases

Address correspondence to Francis Berenbaum, Laboratory of Biochemistry and Molecular Biology, URA CNRS 1283, CHU Saint-Antoine, 27, rue Chaligny, 75012 Paris, France. Phone: 33 1 40 01 14 90 or 33 1 49 28 25 20; FAX: 33 1 40 01 14 95 or 33 1 49 28 25 13. E-mail: francis.berenbaum@sat.ap-hop-paris.fr

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1. *Abbreviations used in this paper:* PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $sPLA_2$, secreted PLA_2.

the half-life of the type II $sPLA_2$ mRNA, without having any effect on translation.

Methods

Chondrocyte culture and stimulation. 4-wk-old rabbits were killed and the shoulders, knees, and femoral heads were dissected out under sterile conditions (22). The articular cartilage was removed, cut into small pieces, and digested at 37°C with 0.05% testicular hyaluronidase in HAM's F12 medium (Flobio, Courbevoie, France) for 15 min, and then with 0.25% trypsin for 30 min, and lastly with 0.2% clostridial collagenase for 90 min. Hyaluronidase, collagenase, and trypsin were purchased from Boehringer Mannheim, Meylan, France. The resulting suspension of chondrocytes was seeded into 75-cm^2 flasks (4×10^5 cells per flask) in HAM's F12 medium supplemented with 10% fetal calf serum (Boehringer Mannheim), 10 IU/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37°C in 5% CO_2 and the culture medium was changed every 2–3 d. The cells reached confluency within 6–7 d and were placed in quiescent medium (without fetal calf serum) 24 h before the experiments. Recombinant human IL-1β (Immugenex, Boston, MA) and human IGF-I (Genzyme by Tebu, le Perray en Yvelines, France) were stored at −80°C until used. The concentrations of IL-1β and IGF-I used in this study were those determined in a dose-response assay (17).

Prostanglandin E_2 assay. Supernatants from stimulated cells were collected and the prostaglandin E_2 concentrations were measured by a specific enzyme radioimmunoassay kit (DuPont-NEN, Wilmington, DE) according to the manufacturer's protocol. Prostaglandin E_2 production was evaluated in duplicate serial dilutions of the sample and amounts read off a standard curve of prostaglandin E_2.

Preparation of rabbit articular chondrocyte membranes and cytosolic fractions. The cells were scraped off in 40 mM Tris buffer, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, 1 mM PMSF, and 1 μg/ml leupeptin and sonicated. The homogenate was centrifuged at 100,000 *g* for 1 h, the supernatant (cytosol) was collected and the pellet (membrane) was resuspended and sonicated for 20 s in 100 mM Tris buffer, pH 8.5, and stored at −80°C.

Phospholipase A_2 assay. $sPLA_2$ activity was measured by the selective fluorometric assay of Radvanyi et al. (23) as modified by Pernas et al. (24). The phospholipase A_2 activity secreted into the medium was measured on 100-μl samples using 2 nmol fluorescent substrate 1-palmitoyl-2 (10-pyrenyldecanoyl)-*sn* glycero-3-monomethyl phosphatidic acid (Interchim, Montluçon, France). 100% hydrolysis of the substrate was measured using 0.1 U PLA_2 from Naja Naja (Sigma Chemical Co., Saint Quentin Fallavier, France). The hydrolysis of substrate in the presence of fresh culture medium was used as negative control and was deducted from the PLA_2 activity.

The $cPLA_2$ assay measured the hydrolysis of [1-^{14}C]arachidonic acid from the substrate L-3 phosphatidylcholine, 1-stearoyl-2[1-^{14}C]arachidonyl: 200 GBq/mmol, 54 mCi/nmol (Amersham, les Ulis, France). This substrate, in chloroform-methanol, was taken to dryness under a stream of nitrogen, resuspended in 10 μl diethyl ether and dispersed in 100 mM Tris HCl, pH 8.5, by sonication. The substrate was added to the assay mixture, which contained, in a final volume of 250 μl, 100 mM Tris-HCl, pH 8.5, 5 mM $CaCl_2$, 0.1% bovine serum albumin, and 50 μg cytosol proteins. The reaction mix was incubated at 37°C for 30 min and the reaction was terminated by adding chloroform-methanol. Lipids were extracted (25), and resolved by thin-layer chromatography on silica gel plates using chloroform: methanol:water (65:25:4) as solvent. The spots were visualized with iodine vapor; the spots corresponding to phosphatidylcholine and free fatty acid were scraped off and their radioactivity was determined.

Immunoblot analysis of $cPLA_2$. Cytosol proteins (100 μg) were subjected to SDS-PAGE (10% acrylamide) under reducing conditions. The proteins were transferred electrophoretically to nitrocellulose membrane (Bio Blot-NC: Schleicher & Schuell, Dasser, Germany), by electrophoresis for 3 h at 4°C at 200 mA (Hoefer Scientific Instruments, San Franscisco, CA). The blot membrane was washed at room temperature in blocking buffer containing phosphate-buffered saline, 3% nonfat dried milk, and 0.1% Tween 20, and then incubated for 2 h in the same buffer containing anti–human $cPLA_2$ rabbit polyclonal IgG (1:50; Santa Cruz Biotechnology, by Tebu). The membrane was washed and incubated with a goat anti–rabbit IgG coupled to peroxidase (1:1,000; Bio-Rad, Ivry Sur Seine, France) for 1 h at room temperature. The membrane was washed again and immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham). 1 μg cytosolic proteins from sf9 cells infected with $cPLA_2$. PVL1393 plasmid was used as positive control.

Cloning of a 290-bp fragment of rabbit $sPLA_2$ cDNA. A pair of oligonucleotides CS10 5′-GTGGCAGAGGATCCCCCAAGG-3′ and CS11 5′-GCAAC<u>TG</u>GGCGT*G*<u>T</u>TCCCT<u>C</u>TGCA-3′ were synthesized and used as sense and antisense primers in a reverse transcription–PCR procedure to clone a 290-bp fragment of rabbit cDNA. The CS10 oligonucleotide corresponds to the [210; 232] region of the rat type II $sPLA_2$ cDNA (26), which perfectly matches the [287; 307] region of the human sequence (6). The CS11 oligonucleotide was deduced from the rat [472; 498] and human [544; 570] sequences of $sPLA_2$ cDNA. The nucleotides corresponding to the rat sequence are underlined, those corresponding to the human sequence are in italics. The nucleotides that are common to the rat and human sequences are in normal characters. Total RNA from IL-1–stimulated chondrocytes (1 μg; see below) was reverse transcribed by 5 U Thermus thermophilus polymerase for 15 min at 55°C in a 20-μl reaction mixture of 10 mM Tris-HCl, pH 8.3, 90 mM KCl, 1 mM $MnCl_2$, 1.25 mM dNTP, and 0.5 μm CS11. Thereafter, 10 pmoles CS10, 10 μl PCR buffer (100 mM Tris-HCl, pH 8.3, 1 M KCl, 20 mM $MgCl_2$, 7.5 mM EGTA, and autoclaved water) were added to the reverse transcription reaction mixture to bring the volume to 100 μl. Samples underwent a preliminary denaturation step at 95°C for 5 min followed by 35 cycles of PCR (denaturation 95°C, annealing 55°C, and extension 72°C, each step for 1 min). The 290-bp PCR product was purified by electroelution and sequenced on an ABI 373A automatic sequencer using the CS10 and CS11 oligonucleotides and the dye terminator FS DNA sequencing kits from Perkin-Elmer Corp. (Beaconsfield, UK).

Northern blot analysis. Total RNA was isolated from cultured chondrocytes using guanidium isothiocyanate (27). The total RNA content was measured by spectrophotometry, and its integrity was assessed by agarose gel electrophoresis; 15 μg total RNA per lane were separated on 1% agarose/2.2 M formaldehyde gels and transferred to nylon filters (Hybond N; Amersham). The membranes were prehybridized for 15 min, and then hybridized at 65°C for 2 h with the rapid-hyb-buffer kit (Amersham) using type II $sPLA_2$ and $cPLA_2$ cDNA probes. The probe for $sPLA_2$ was obtained from human placenta as described by Pernas et al. (24), and that for $cPLA_2$ from U937 cell mRNAs (4). The probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol, giving specific activities at around 2×10^9 cpm/μg cDNA; Amersham), using a random-primed labeling system (Amersham). The hybridized membranes were washed with 0.1% SDS in 2× SSC (150 mM NaCl, 17 mM sodium citrate) at room temperature for 15 min, followed by two washes with 0.1% SDS in 0.1× SSC at 65°C for 15 min each, and autoradiographed with Hyperfilm (Amersham) at −80°C for 18–72 h. The blots were successively hybridized using type II $sPLA_2$ cDNA, $cPLA_2$ cDNA, and then to 28S rRNA probes labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, as described by Barbu and Dautry (28). The filters were washed in 0.1% SDS in 0.01× SSC at 85°C before rehybridization. The relative abundance of the mRNA was calculated by quantitative scanning of autoradiograms using a laser densitometer (2202 Ultrascan; LKB, Uppsala, Sweden).

Type II $sPLA_2$ mRNA stability. The mRNA half-lives were measured by adding actinomycin D (1 μg/ml) to cells at the time of peak steady state IL-1β–induced type II $sPLA_2$ mRNA accumulation (24 h). RNA was isolated from samples of cells taken at several times after the addition of actinomycin D and RNA blot analysis was performed

as described above. The data were graphed as the percent of RNA remaining versus the time after addition of actinomycin D, and the slopes of the linear portions of the decay curves were calculated by linear regression to determine the mRNA decay constants. mRNA half-lives were calculated with the formula $t_{1/2}$ = ln 2/mRNA decay constant (29).

Nuclear run-on analysis. Nuclei were prepared from chondrocytes by a modified procedure of Marzluff et al. (30). Cytochalasin B (5 μg/ml) (Sigma Chemical Co.) was added 15 min before cell lysis because of the massive cytoskeleton in this cell type. Chondrocytes were then resuspended in 0.32 M sucrose, 3 mM $CaCl_2$, 2 mM Mg acetate, 0.1 mM PMSF, 0.25 mM benzamidine, 10 mM Tris-HCl, pH 8, and homogenized with 50 strokes in a Dounce homogenizer. The pellets were collected by centrifugation at 300 *g* for 10 min and resuspended in 2 M sucrose, 5 mM Mg acetate, 0.1 M EDTA, 10 mM DTT, 0.1 mM PMSF, 0.25 mM benzamidine, 10 mM Tris-HCl, pH 8, and homogenized again with 50 strokes in a Dounce homogenizer. The homogenates were centrifuged at 20,000 *g* for 45 min at 4°C and the nuclei were suspended in a storage buffer containing 40% glycerol, 50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, and 1 mM EDTA. Suspensions were checked under the microscope for completeness of cell lysis. Nuclear run-on experiments were performed essentially as described by Antras et al. (31). Nuclei (1.5×10^7) were incubated in a 200-μl reaction mixture containing 20% glycerol, 100 mM Tris-HCl, pH 7.9, 50 mM NaCl, 0.4 mM EDTA, 1.2 mM DTT, 0.1 mM PMSF, 0.35 mM $(NH_4)_2SO_4$, 4 mM $MnCl_2$, 10 mM creatine phosphate, 10 μg/ml creatine phosphokinase, 500 U/ml RNasin, 1 mg/ml heparin sulfate, 1 mM each ATP, GTP, and CTP, and 250 μCi of [α-^{32}P]UTP (3,000 Ci/mmol) (Amersham) at 30°C for 30 min. The mixture was then treated with 2 U RNase-free DNase I (Boehringer Mannheim) for 10 min at 37°C, followed by incubation for 30 min at 37°C with 1% SDS and 100 μg/ml proteinase K. Nascent RNAs were extracted in 3 vol 8 M guanidine-HCl, pH 5, containing 20 mM sodium acetate, 1 mM DTT, 0.5% (wt/vol) lauroylsarcosine. Yeast tRNA (50 μg) was added to each sample and RNA was precipitated by adding 0.6 vol of ethanol for 12 h at −20°C. The precipitate was collected by centrifugation at 12,000 *g* at −10°C for 30 min, and dissolved in 0.5 ml 7 M guanidine HCl, pH 7, containing 20 mM sodium acetate, 20 mM EDTA, 1 mM DTT, 0.5% (wt/vol) lauroylsarcosine. 25 μl 2 M sodium acetate (pH 4.5) was added, followed by 0.6 vol ethanol, and transcripts were allowed to precipitate for 2 h at −20°C. This last step was repeated once. Equal numbers (∼ 40×10^6) of cpm were hybridized in 1 ml 50% formamide, 3× SSPE (1× SSPE: 0.18 M NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.7), 2.4% SDS, 1× Den-

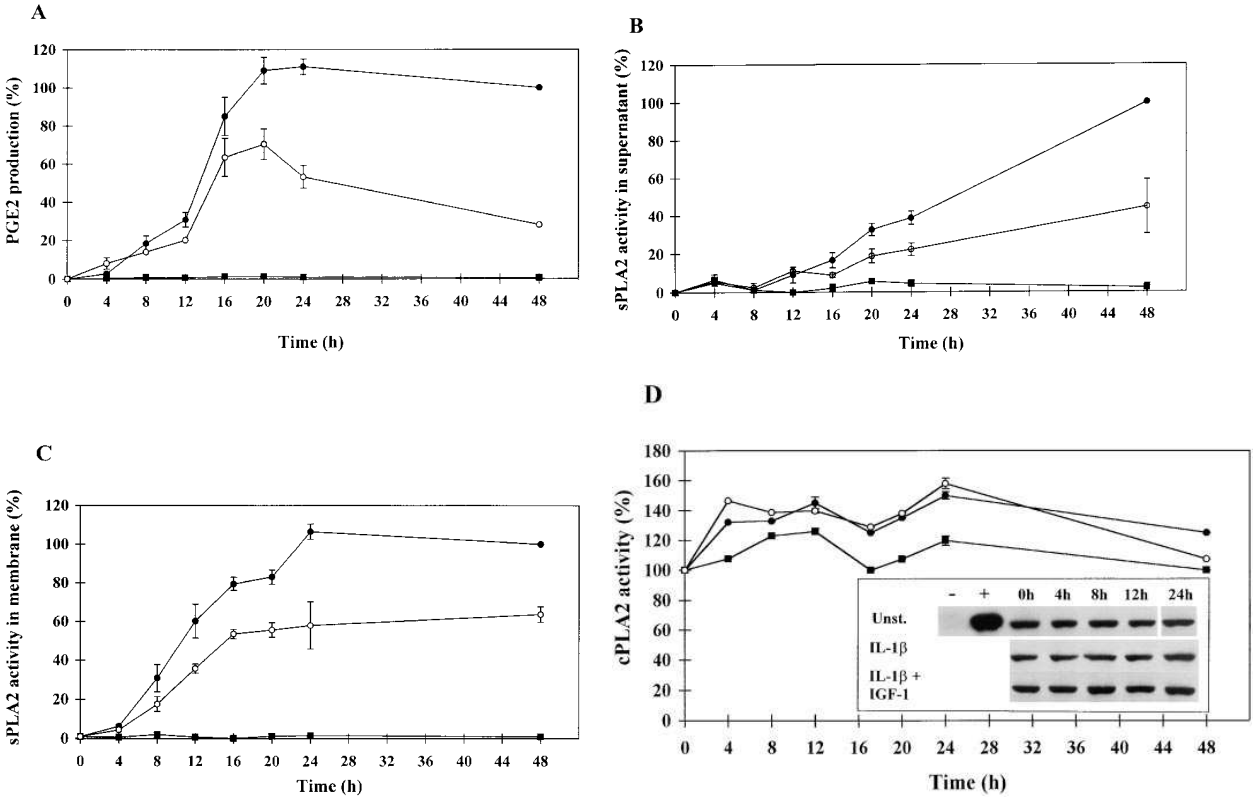

Figure 1. IL-1β stimulates PGE_2 synthesis and $sPLA_2$ activity in supernatant and membranes in rabbit articular chondrocytes. IGF-I inhibits the IL-1β effect. Rabbit articular chondrocytes in primary culture were not stimulated (■), or stimulated with 10 ng/ml IL-1β with (○) or without (●) 25 ng/ml IGF-I for up to 48 h. (*A*) PGE_2 production. PGE_2 was measured by radioimmunoassay in 100-μl aliquots of medium. 100% is the PGE_2 production by cells incubated with IL-1β for 48 h (272±18 ng/10^6 cells). (*B*) $sPLA_2$ activity secreted into the medium. The enzymatic activity was measured with a fluorescent substrate as indicated in Methods. 100% is the activity after incubation with IL-1β for 48 h (24±2.5 nmol/min per 10^6 cells). (*C*) High ionic strength–releasable membrane $sPLA_2$ activity; membranes from disrupted cells were incubated in 1 M NaCl for 30 min and centrifuged. PLA_2 activity was measured in the supernatant as in *B*. 100% is the activity after incubation with IL-1β for 48 h (10.5±2.1 nmol/min per 10^6 cells). (*D*) Cytosolic PLA_2 activity. The enzymatic activity was measured in the supernatant from disrupted cells using a specific *sn*-2[^{14}C]arachidonyl-PC substrate as indicated in Methods. 100% is the activity after incubation with IL-1β for 48 h (525±25 pmol/min per 10^6 cells). (*D*, *inset*) Autoradiogram of the representative Western blot for $cPLA_2$ content (100 μg total cytosol proteins). Line −: negative control (high molecular weight standard mixture SDS-6H), line +: positive control (sf9 cells infected with $cPLA_2$ DVL1393 plasmid). Results are means±SEM of two to four separate experiments for each time point.

hardt's, 200 μg/ml each DNA and tRNA for 72 h to 10 μg of each linearized plasmid immobilized on nitrocellulose filters (linearized puc 19 plasmid containing either $cPLA_2$ cDNA, type II $sPLA_2$ cDNA, GAPDH cDNA, or no insert). The filters were washed twice with 2× SSPE at 37°C for 15 min and incubated for 30 min at 37°C in 2× SSPE containing 5 μg/ml RNase A. Final washings were carried out in 2× SSPE, 0.5% SDS for 30 min at 42°C and in 0.5× SSPE, 0.5% SDS for 30 min at 42°C.

Sedimentation velocity analysis in sucrose gradients. Sucrose gradient and sedimentation velocity analyses were performed by the procedure of Chen et al. (32). Briefly, confluent cells were removed by trypsinization, harvested by centrifugation (1,200 *g* for 5 min at 4°C), and washed twice with ice-cold phosphate-buffered saline containing 50 μg/ml cycloheximide. Cells were lysed by incubation for 10 min in 1 ml ice-cold lysis buffer containing 0.25 M sucrose, 20 mM Hepes, pH 7.5, 250 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 100 μg/ml cycloheximide, 0.5% (vol/vol) Triton X-100, and 0.2 mg/ml heparin. The lysate was centrifuged for 10 min at 4°C in a microcentrifuge, and a 0.5-ml aliquot of the resulting supernatant, representing material from $\sim 4 \times 10^7$ cells, was layered onto a sucrose gradient (see below). Linear 15–50% (wt/vol) sucrose gradients (12.3 ml) were prepared with a two-chamber gradient former and pumped into centrifuge tubes. The nonsucrose component of the gradient solution was the same as the homogenizing buffer, except that the concentration of KCl was 500 mM and that of heparin was 0.5 mg/ml. Cytoplasm extract (0.5 ml) was layered onto each tube and the tubes were centrifuged at 40,000 *g* for 110 min at 4°C in an SW 40 rotor (Beckman Instruments, Inc., Fullerton, CA). Gradient fractions (0.85 ml) were collected, and the absorbance (254 nm) of each fraction was measured. RNA was then extracted from each fraction as described above. mRNA was determined by Northern blot analysis.

Results

Kinetics of type II $sPLA_2$, $cPLA_2$ activities, and PGE_2 synthesis in the presence of IL-1β and IGF-I. Rabbit articular chondrocytes secrete type II $sPLA_2$ when stimulated with IL-1β (13). The kinetics of this process were determined in parallel with the $cPLA_2$ activities and PGE_2 synthesis, and the effect of IGF-I was examined. To ensure the stability of the rabbit articular chondrocyte phenotype, experiments were performed in primary culture cells. The polygonal shape of the cells in culture was checked every day under a phase contrast microscope. The amount of type II collagen mRNA in confluent cells was measured in representative flasks (33).

Incubation of rabbit articular chondrocytes with IL-1β (10 ng/ml) for up to 48 h induced the gradual production of PGE_2, which became apparent at 8 h and was maximal at 24 h (Fig. 1 *A*). Type II $sPLA_2$ secretion started later, and was still increasing after 48 h (Fig. 1 *B*). Membranes of IL-1β–stimulated rabbit articular chondrocytes treated with 1 M NaCl released PLA_2 activity after 4 h, which peaked at 24 h (Fig. 1 *C*). No activity was released from the membrane of resting cells. These data suggest that type II $sPLA_2$ accumulated at first in membranes, and then is excreted in the media. By contrast, $cPLA_2$ activity increased only modestly over that of controls after incubation with IL-1β for 4 h, and then remained unchanged for 48 h (Fig. 1 *D*), while the $cPLA_2$ content measured by Western blot (Fig. 1 *D*, *inset*) remained unchanged.

IGF-I (25 ng/ml) alone had no effect on PGE_2 synthesis, $cPLA_2$ activity, or type II $sPLA_2$ secretion at any time (data not shown), but it inhibited the IL-1β–induced synthesis of PGE_2 and type II $sPLA_2$ (both membrane associated and secreted into the medium) (Fig. 1), whereas it had no effect on basal or IL-1β–stimulated $cPLA_2$ activity (Fig. 1 *D*). The induction of type II $sPLA_2$ activity in chondrocyte membranes by IL-1β and its inhibition by IGF-I paralleled the production of PGE_2 (compare Fig. 1, *C* and *A*).

Kinetics of the effect of IL-1β on type-II $sPLA_2$ mRNA and $cPLA_2$ mRNA level in the presence and in the absence of IGF-I. An mRNA of the expected length ($\sim$ 750 bases) was detected using human type II $sPLA_2$ cDNA as probe. Primers corresponding to conserved regions of rat and human $sPLA_2$

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      310                                                        360
        AlaThrAspArgCysCysValThrHisAspCysCysTyrLysArgLeuGlu
Human   TGCAACGGATCGCTGCTGTGTCACTCATGACTGTTGCTACAAACGTCTGGAG
        :::::: :: :::::::::: :  : ::::::::: ::::::   :::::::::
Rabbit  TGCAACAGACCGGTGCTGTCTTGCCCATGACTGCTGCTACCGCCGTCTGGAG
          -  -  -  -  -  - LeuAla -  -  -  -  - Arg -  -  -

        LysArgGlyCysGlyThrLysPheLeuSerTyrLysPheSerAsnSerGlyS
Human   AAACGTGGATGTGGCACCAAATTTCTGAGCTACAAGTTTAGCAACTCGGGGA
        ::::::  :::::::::::: :: :::::::::::: :: ::::    :
Rabbit  AAACGTAAATGTGGCACCAAGTTCCTGAGCTACAAATTCAGCATGAGCCGTG
         -  - Lys -  -  -  -  -  -  -  -  -  -  - Met - ArgG

        erArgIleThrCysAlaLysGlnAspSerCysArgSerGlnLeuCysGluCy
Human   GCAGAATCACCTGTGCAAAACAGGACTCCTGCAGAAGTCAACTGTGTGAGTG
        ::: :::::::::::  :::::::::::: :: :: :: :: :::::::: ::
Rabbit  GCAAAATCACCTGTGGGAAACAGGACTCTTGTAGGAGCCAGCTGTGTGAATG
        lyLys -  -  - Gly -  -  -  -  -  -  -  -  -  -  -  -

        sAspLysAlaAlaAlaThrCysPheAlaArgAsnLysThrThrTyrAsnLys
Human   TGATAAGGCTGCTGCCACCTGTTTTGCTAGAAACAAGACGACCTACAATAAA
        :::::: :: :: :::  :::::::::::: :::  :   : :::::  :
Rabbit  TGATAAAGCGGCAGCCTACTGTTTTGCTAGGAACCGGGAAAGCTACAGCAGG
          -  -  -  -  - Tyr -  -  -  -  - ArgGluSer - SerArg

                                    543
        LysTyrGlnTyrTyrSerAsnLysHis
Human   AAGTACCAGTACTATTCCAATAAACAC
        :::::::::: :::  :::: :: : :
Rabbit  AAGTACCAGTTCTACCCCAACAATCGC
         -  -  - Phe - Pro - AsnArg
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Figure 2. Sequence of the 235-bp cloned fragment of the $sPLA_2$ rabbit cDNA and comparison with the corresponding human sequence. 77% of the sequences were homologous. Comparison of the rabbit and human sequences was performed using the Fasta program of Lipman and Pearson provided by BISANCE service (59). The translated amino acid sequence is shown on the top of the human nucleotidic sequence. The divergent amino acids in rabbit $sPLA_2$ were shown under the rabbit cDNA sequence. The His^{48}, Asp^{49}, Asp^{99}, Ala^{102}, and Ala^{103} that are indicated by italic characters are components of the catalytic site (6).

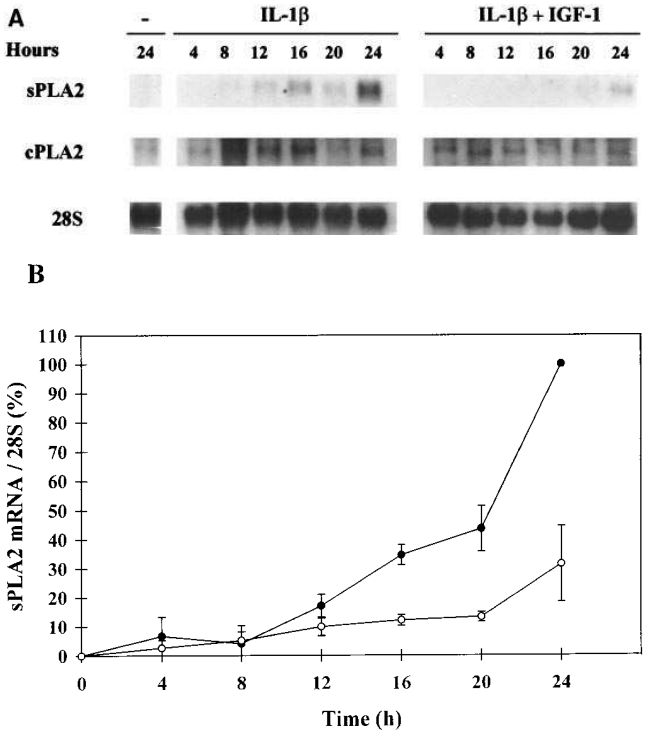

Figure 3. IL-1β induces type II $sPLA_2$ mRNA and IGF-I decreases the steady state level induced by IL-1β in rabbit articular chondrocytes. (*A*) Autoradiogram showing a representative experiment of the time course responses to 10 ng/ml IL-1β and 10 ng/ml IL-1β plus 25 ng/ml IGF-I. Total RNA was isolated from articular chondrocytes and 15 μg were subjected to Northern blot with cDNA probes for $sPLA_2$, $cPLA_2$, 28S rRNA. (*B*) Densitometric analysis of $sPLA_2$ mRNA autoradiograms (●, IL-1; ○, IL-1 + IGF-I). Data from densitometric scanning are normalized to the 28S signal. Results are means±SEM from three to five separate experiments for each time point.

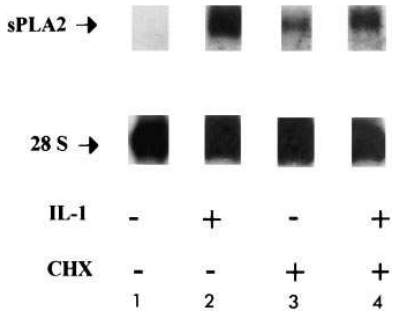

Figure 4. Cycloheximide induces type II $sPLA_2$ mRNA and decreases the steady state level induced by IL-1β in rabbit articular chondrocytes. Autoradiogram of Northern blot analysis with cDNA probes for $sPLA_2$ or 28S rRNA. Articular chondrocytes were incubated with serum-free medium (lane *1*), IL-1β 10 ng/ml (lane *2*), cycloheximide (CHX) 35 μM (lane *3*), or IL-1β 10 ng/ml plus CHX 35 μM (lane *4*). Total RNA was isolated and 15 μg were subjected to Northern blot. The autoradiogram is representative of three separate experiments.

cDNA were used to amplify a 290-bp fragment from IL-1–stimulated chondrocyte RNA. This reverse transcription–PCR product was sequenced and found to be 77% identical to human $sPLA_2$ type II cDNA (Fig. 2). This great similarity allowed us to use the human cDNA coding sequence as a probe. No type II $sPLA_2$ was detected in control cells by Northern blot, but it appeared within 4 h in response to IL-1β and its concentration gradually increased up to 24 h. IGF-I alone did not induce type II $sPLA_2$ mRNA (data not shown), but strongly decreased the response to IL-1β (Fig. 3, *A* and *B*). By contrast, the baseline concentration of $cPLA_2$ mRNA was not altered by IL-1β or by IGF-I for up to 24 h (Fig. 3 *A*).

The inhibitory control of the type II $sPLA_2$ gene in unstimulated cells depends on ongoing protein synthesis, since adding cycloheximide to the incubation medium elicited the synthesis of type II $sPLA_2$ mRNA (Fig. 4). De novo protein synthesis was also required for the full effect of IL-1β on type II $sPLA_2$ mRNA, since cycloheximide also greatly decreased the amount of IL-1β–stimulated type II $sPLA_2$ mRNA (Fig. 4).

Effect of IL-1β on the transcription rate of type-II $sPLA_2$ gene and the influence of IGF-I on this effect. The absence of a basal level of type II $sPLA_2$ mRNA and its considerable increase in response to IL-1β suggested that this cytokine increased the rate of type II $sPLA_2$ gene transcription. This is consistent with the total blocking of the IL-1β effect on type II $sPLA_2$ mRNA expression by actinomycin D (data not shown). Nuclear run-on assays were therefore performed to check if IL-1β modified the transcription of the type II $sPLA_2$ gene and to investigate the target of IGF-I.

Confluent rabbit articular chondrocytes were stimulated with IL-1β (10 ng/ml) alone, or with IL-1β plus IGF-I (25 ng/ml) for 24 h. The cells were lysed and chondrocyte nuclei were isolated from unstimulated (controls) and stimulated cells. Initiated transcripts were elongated in vitro using [^{32}P]UTP. Radiolabeled nuclear RNA was hybridized to pUC 19 (as negative control), type II $sPLA_2$ cDNA, $cPLA_2$ cDNA, and GAPDH cDNA immobilized on nitrocellulose membrane (Fig. 5 *A*). The autoradiogram data showed that the type II $sPLA_2$/GAPDH ratio was increased fourfold (Fig. 5 *B*), whereas the $cPLA_2$/GAPDH ratio was uneffected by incubation with IL-1β for 24 h, indicating that IL-1β stimulated type II $sPLA_2$, but did not stimulate $cPLA_2$ gene transcription. In contrast, IGF-I, which strongly inhibited the IL-1β–stimulated production of type II $sPLA_2$ mRNA level and type II $sPLA_2$ synthesis and secretion, had no effect on the transcription rate of type II $sPLA_2$ gene (Fig. 5, *A* and *B*). Therefore, IGF-I may act post-transcriptionally.

Effect of IGF-I on the half-life of IL-1β–induced type II $sPLA_2$ mRNA. The action of IGF-I on the effect of IL-1β on type II $sPLA_2$ mRNA was investigated by measuring type II mRNA in the presence of actinomycin D, which blocks DNA transcription and gives an accurate measurement of mRNA half-life over a short period. The amounts of type II $sPLA_2$ mRNA in rabbit articular chondrocytes incubated for 24 h with IL-1β alone or IL-1β plus IGF-I were measured after blocking RNA synthesis by actinomycin D. The type II $sPLA_2$ mRNA induced by IL-1β was very stable, with a half-life of 92 h (Fig. 6). IGF-I shortened the type II $sPLA_2$ mRNA half-life dramatically to 12 h (Fig. 6). This, plus the inability of IGF-I to reduce IL-1β–stimulated transcription rate (Fig. 5), indicate that this agonist stimulates signaling pathways that lead to the destabilization of type II $sPLA_2$ mRNA, or that inhibit a stabilizing effect of IL-1β.

Influence of IGF-I on the average number of ribosomes per IL-1β–induced type II $sPLA_2$ mRNA and the relative amount of type II $sPLA_2$ mRNA in the polysomal fractions. The inhibition of IL-1β–induced type II $sPLA_2$ gene expression by

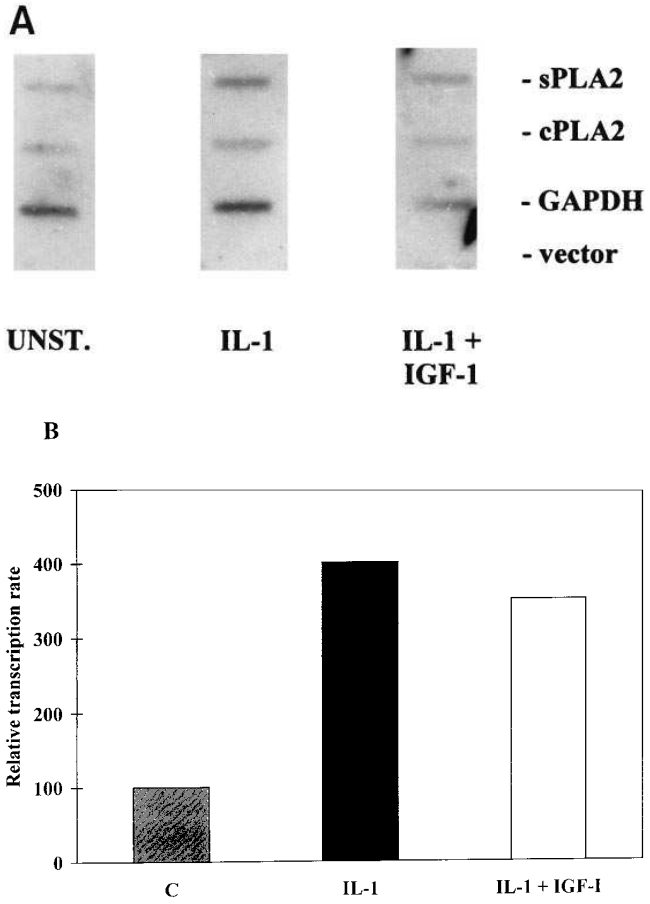

Figure 5. IL-1β increases and IGF-I does not modify the transcription rate of type II $sPLA_2$ gene in rabbit articular chondrocytes. (*A*) Autoradiogram showing effect of IL-1β and IGF-I (one representative experiment of three). Nuclei from control (*UNST.*), IL-1β- (10 ng/ml), or IL-1β plus IGF-I (25 ng/ml) -treated chondrocytes (24 h) were isolated and nuclear run-on assays were performed in the presence of [^{32}P]UTP for 30 min. The nascent ^{32}P-labeled transcripts were hybridized to slots of filter-bound type II $sPLA_2$, $cPLA_2$, GAPDH, and pUC 19 DNA (*vector*). (*B*) Densitometric analysis showing the transcription rate of type II $sPLA_2$ in control cells (*grey bar*), and in cells stimulated with IL-1β alone (*solid bar*), or IL-1β plus IGF-I (*open bar*). The transcription rate of type II $sPLA_2$ is normalized to the transcription rate of GAPDH.

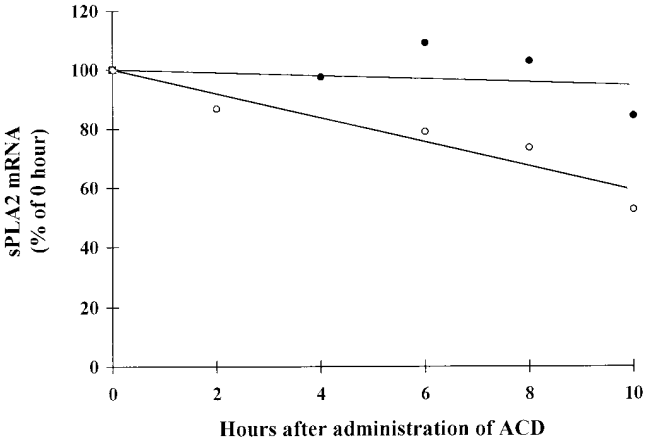

Figure 6. IGF-I decreases the stability of IL-1β–induced type II $sPLA_2$ mRNA in rabbit articular chondrocytes. Densitometric analysis of autoradiograms of Northern blots with a cDNA probe for type II $sPLA_2$ and a 28S rRNA probe, after adding the transcription inhibitor actinomycin D (*ACD*). Articular chondrocytes in primary culture were incubated with 10 ng/ml IL-1β (●), or with IL-1β plus 25 ng/ml IGF-I (○) for 24 h followed by the addition of vehicle or ACD, 5 μg/ml. Total RNA was then isolated after 2, 4, 6, 8, and 10 h of ACD treatment. 15 μg total RNA was placed in each lane. One of three separate experiments expressed as a percentage of levels observed at the time of ACD or vehicle addition.

IGF-I was further examined by comparing extracts of chondrocytes incubated with IL-1β and IL-1β plus IGF-I using sucrose density gradient ultracentrifugation. This technique explored (*a*) the fractional entry of type II $sPLA_2$ mRNA into the polysomal pool, and (*b*) the rate of translation or elongation of the translated product. The distribution of total RNA on the sucrose density gradient in extracts of IL-1β–stimulated and IL-1β plus IGF-I–stimulated cells were similar (Fig. 7 *A*). Based on previous studies (32), it was inferred that fractions 1–3 contained unassembled ribosomal subunits and non–ribosomal-associated mRNA (mRNPs), whereas fractions 4–15 contained the translating polysomal complexes with the lower numbered, less dense, fractions containing fewer ribosomes per mRNA than the higher numbered, more dense fractions. Northern blot analysis revealed that the distribution of type II $sPLA_2$ mRNA among the polysomal fractions 4–15 for IL-1β and IL-1β plus IGF-I–stimulated cells were similar (Fig. 7 *B*). Hence, IGF-I did not influence the average number of ribosomes per type II $sPLA_2$ message. There were also similar relative amounts of type II $sPLA_2$ mRNA in the polysomal fractions of the gradient from chondrocytes stimulated with IL-1β alone and with IL-1β plus IGF-I (Fig. 7 *C*). This suggests that IGF-I did not affect the distribution of type II $sPLA_2$ mRNA between the subpool of mRNA not being translated and the subpool of type II $sPLA_2$ mRNA associated with polysomes.

Discussion

IL-1β causes the time-dependent induction of the type II $sPLA_2$ gene in rabbit articular chondrocytes. This induction results in increased specific mRNA and the appearance of enzyme activity in chondrocyte membranes and in the incubation medium (Fig. 1). The cDNA of type II $sPLA_2$ indicates that it has the characteristics of secreted proteins (5, 6), and lacks the propeptide sequence of pancreatic type I $sPLA_2$s (34). It is therefore secreted as a fully active enzyme. Our results demonstrate that the newly synthesized enzyme, in rabbit articular chondrocytes, induced by IL-1β is associated with cell membranes, probably of the secretory pathway, before being secreted into the medium (Fig. 1). This contrasts with the situation in several immunoinflammatory cells such as platelets (5, 35), mastocytes (36, 37), and neutrophils (38), in which the enzyme is stored in secretory granules and quickly secreted upon cell stimulation. The actual location of membrane-bound type II $sPLA_2$ and the molecular mechanism of this association are not fully understood. The enzyme may lie on the outer surface of the cytoplasmic membrane by interacting with peptidoglycans (39), or by binding to specific receptors (9).

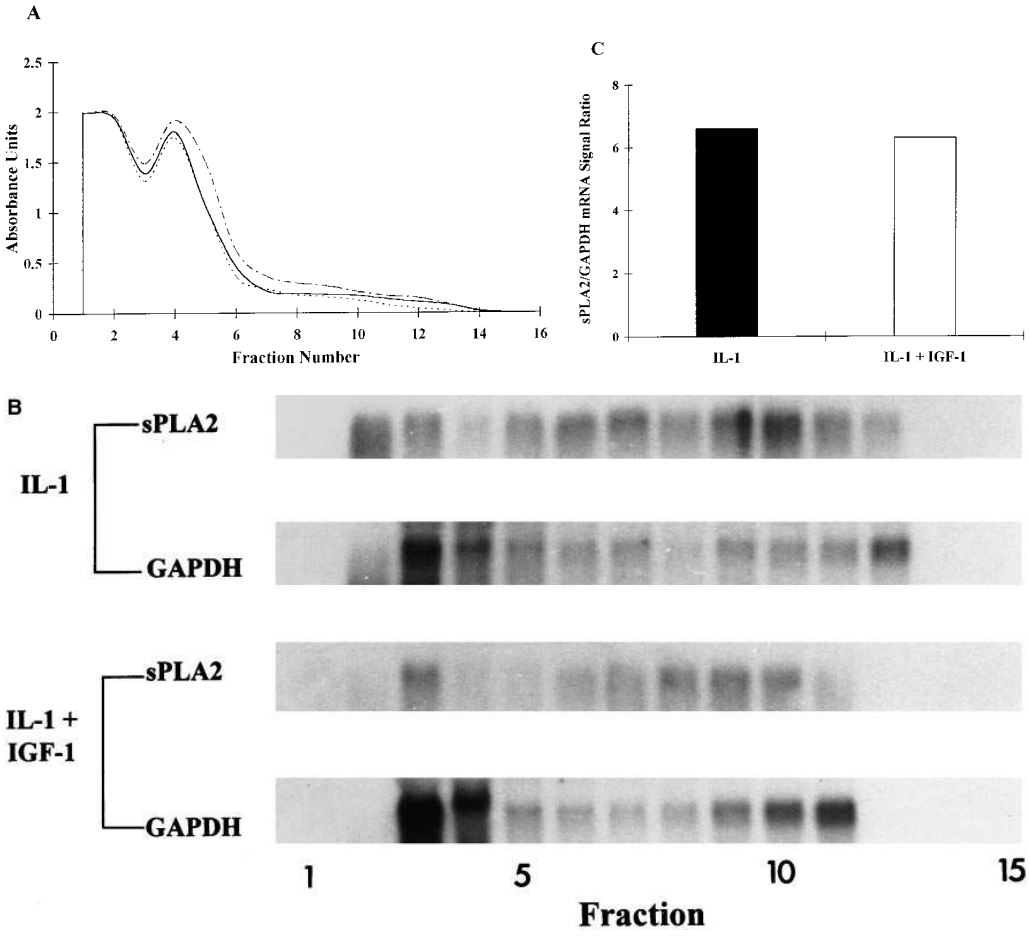

Figure 7. IGF-I does not modify the relative amount of IL-1β–induced type II $sPLA_2$ mRNA in the polysomal fraction or the number of ribosomes per IL-1β–induced type II $sPLA_2$ mRNA in rabbit articular chondrocytes. Cytoplasmic extracts of rabbit articular chondrocytes stimulated with 10 ng/ml IL-1β with or without 25 ng/ml IGF-I, or unstimulated controls were analyzed by centrifugation through a sucrose gradient. (*A*) Each gradient fraction was collected and the absorbance at 254 nm measured (unstimulated cells, *dashed line*; IL-1β, *solid line*: IL-1β and IGF-I, *dashed-dotted line*). The density of the gradient increases from left to right. (*B*) Typical autoradiogram showing Northern blot analysis of total RNA isolated from equal volumes of each fraction using cDNA probes for type II $sPLA_2$ and for GAPDH. (*C*) Densitometric analysis of the autoradiograms. The $sPLA_2$ signals (normalized to GAPDH) in the polysome-containing fractions (fractions 4–15) were summed and divided by the sum of the nonpolysomal fractions (fractions 1–3).

The ability of this enzyme to hydrolyze the membranes of mammalian cells is disputed. Type II $sPLA_2$ has been shown to play a role in arachidonic acid and eicosanoid synthesis in various proinflammatory cells (40–42) and in a murine fibroblast overexpressing human type II $sPLA_2$ (24). However, macrophages (43) and platelets were found to be refractory (35, 44, 45). Nevertheless, type II $sPLA_2$ was recently shown to generate lysophosphatidic acid in membrane microvesicles shed from activated cells, including human platelets (8). IL-1β could induce the synthesis of type II $sPLA_2$ and the "membrane rearrangement" required for $sPLA_2$ action in rabbit articular chondrocytes, as proposed by Kudo et al. (46) in mastocytes stimulated by TNFα.

Several groups have reported the secretion of PLA_2 by rabbit articular chondrocytes (15, 17, 47). We and others have shown that the PLA_2 activity and type II $sPLA_2$ protein in human biological fluid are closely correlated (48, 49). Thus, in the absence of specific antibodies against rabbit type II $sPLA_2$ and with no cross-reactivity between the PLA_2 secreted by rabbit articular chondrocytes and either human- or rat-specific anti–type II $sPLA_2$ antibodies (data not shown), we conclude that $sPLA_2$ activity in the medium reflects the amount of type II $sPLA_2$ protein. This report shows that IL-1β stimulates transcription (Fig. 5). No type II $sPLA_2$ mRNA was detected under resting conditions by Northern blotting (Fig. 2), but run-on experiments revealed basal transcriptional activity (Fig. 5 *A*). This indicates that primary type II $sPLA_2$ transcripts are poorly processed into mature mRNA in unstimulated chondrocytes, and/or that newly synthesized mRNA is rapidly degraded. This degradative process needs ongoing protein synthesis since the basal mRNA level appeared when the chondrocytes were grown on cycloheximide-supplemented medium (Fig. 4), indicating that type II $sPLA_2$ gene expression is actively repressed in unstimulated cells. IL-1β might also act posttranscriptionally in rabbit articular chondrocytes since it induced an extremely stable type II $sPLA_2$ mRNA (half-life $\sim$ 92 h) (Fig. 6). The transcriptional regulatory activity of IL-1β has been partly linked to activation of nuclear factor-κB/I-κB (50) and activator protein-1 (51). However, responsive elements for nuclear factor-κB or activator protein-1 action were not found in the −1614/+806 part of the human type II $sPLA_2$ promoter (52). At least part of the effect of IL-1β on type II $sPLA_2$ gene expression is probably indirect via the induction of other regulatory genes. This indirect action is supported by the way cycloheximide inhibits the increase on IL-1β–induced type II $sPLA_2$ mRNA in chondrocytes (Fig. 4).

Insulin-like growth factor I inhibits both the synthesis of type II $sPLA_2$ and the production of PGE_2 (Fig.1). This suggests that the physiological role of IGF-I is to act as an antiinflammatory cytokine, regulating the synthesis of inflammatory mediators in articular chondrocytes. The rate at which IGF-I inhibits PGE_2 production was similar to that of its inhibitory effect on membrane-associated PLA_2 activity, whereas IGF-I had no action on $cPLA_2$ activity. This provides more evidence that type II $sPLA_2$ is involved in IL-1β–stimulated PGE_2 synthesis in these cells. Other growth factors also inhibit IL-1β–induced type II $sPLA_2$ gene expression (e.g., PDGF, reference 53, and TGFβ, reference 54), in mesangial cells. But TGFβ did not inhibit $sPLA_2$ and PGE_2 synthesis in parallel (55). The effect of IGF-I on $sPLA_2$ gene expression does not result from inhibition of transcription (Fig. 5). Hence, it must affect mRNA stability, translational efficiency, or both. Growth factors could influence translational efficiency by the phosphorylation of eukaryotic initiation factors (56). Gradient analysis of the translational step provided no evidence for any effect of IGF-I on the fractional entry of the type II $sPLA_2$ mRNA into the polysomal pool, or on the rate of translation or elongation, since there were no differences in the distribution of type II $sPLA_2$ mRNA in the polyribosome profile. As the initiation of translation depends on the interaction between eukaryotic initiation factors and the extreme 5′ terminal sequences, our results suggest that the extreme 5′ UTR sequence of type II $sPLA_2$ mRNA does not contain elements that bind to eukaryotic initiation factors and/or associated proteins.

The data reported here show that IGF-I markedly shortens the half-life of type II $sPLA_2$ mRNA (Fig. 6). The mRNA half-life is the result of a complex set of events, including its organization into the nucleoparticle, the informosome, and its interaction with the ribosomal machinery (57). Our results suggest that the main way in which IGF-I inhibits IL-1β–induced $sPLA_2$ gene expression involves the molecular events that stabilize the mRNA before its entry into polysomes. 3′ UTR mRNA sequences are believed to be crucial for mRNA decay (58), but there is as yet no evidence of such sequences in the type II $sPLA_2$ mRNA, or of factors in chondrocytes that could account for the changes in its stability in response to IL-1β or IGF-I. Discovery of such regulatory factors could help define the signaling pathways of physiological molecules and lead to the design of a new strategy for developing specific antiinflammatory drugs.

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