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Isolation of a collagenase cDNA clone and measurement of changing collagenase mRNA levels during induction in rabbit synovial fibroblasts


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ABSTRACT To facilitate our studies on the mechanisms controlling collagenase production at a molecular level in rabbit synovial fibroblasts, we have constructed a cDNA library using mRNAs isolated from cells induced with crystals of monosodium urate monohydrate. We have screened this library with cDNA probes made from induced and control mRNAs populations. From among 30 clones that hybridized preferentially to the induced-cell probe, 4 contained collagenase sequences. The largest, a clone of 650 base pairs, was identified by its ability to hybrid select a mRNA that could be translated in a cell-free system into a product that was precipitable with monospecific antibody to collagenase. Using the clone to probe blots of RNA from induced cells, we detected the appearance of a collagenase mRNA of 2.7 kilobases within 5 hr of addition of urate. The level of collagenase mRNA continued to increase for 35–40 hr, when it was 60 to 90 times more abundant in induced cells than in control cells. The increase in mRNA levels correlated with an increase in immunoreactive collagenase protein that was detectable in culture medium by 10 hr.

Mammalian collagenases (EC 3.4.24.7) have been isolated from a variety of cells and tissues, including polymorphonuclear leukocytes (1–3), monocytes/macrophages (4, 5), skin fibroblasts (6, 7), resorbing uterus (8), bone (9, 10), and cornea (11, 12). (For review, see ref. 13.) An additional and abundant source of collagenase is proliferating rheumatoid synovium and it is now accepted that synovial cell collagenase plays a large role in mediating the joint destruction that accompanies rheumatoid disease (14). To study the mechanisms governing the control of collagenase synthesis by synovial cells, we have developed a model system that uses monolayer cultures of rabbit synovial cells. These cells secrete negligible quantities of collagenase unless they are stimulated with agents such as the tumor promoter phorbol myristate acetate (15) or crystals of monosodium urate monohydrate (16). Collagenase activity, measured in a fibril assay (17), is detectable in culture medium 24 hr after addition of these stimuli and by 30–36 hr is present in levels comparable with those achieved in primary cultures of human rheumatoid synovial cells (18).

It has been shown that increased production of collagenase in rabbit synovial fibroblasts is correlated with an increase in the level of translatable mRNA for this protein (19). To further study the regulation of collagenase gene expression, we have constructed a cDNA clone for rabbit synovial fibroblast collagenase and here we report its isolation and characterization and its use to quantify collagenase mRNA levels in cells at intervals after addition of an inducer.

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MATERIALS AND METHODS

Cell Cultures. Monolayer cultures of normal rabbit synovial fibroblasts were established as described (15, 19). For experiments, cells were grown to confluence in 100-mm- or 150-mm-diameter culture dishes and then placed in serum-free Dulbecco's modified Eagle's medium/0.2% lactalbumin hydrolysate in the presence or absence of crystals of monosodium urate monohydrate at 350 μg/ml (16, 19). At appropriate times, medium was harvested and assayed for collagenase enzyme activity or for detection of collagenase protein by immunoblotting techniques (see below).

Preparation and Translation of mRNA. RNA was extracted from cells by the procedure described earlier (19). Poly(A)+ mRNA was selected by two cycles of oligo(dT)-cellulose chromatography (20). RNA was passed through an oligo(dT)-cellulose column three times in 50 mM Tris-HCl, pH 8/0.5 M NaCl/0.1% NaDodSO4 and washed in the same buffer, and the poly(A)+ RNA was eluted with 50 mM Tris-HCl, pH 8/0.1% NaDodSO4.

mRNA was translated in the wheat germ cell-free system with [35S]methionine (935 Ci/mmol; 1 Ci = 37 GBq; Amer sham). One microgram of poly(A)+ mRNA was translated in a 25-μl reaction mixture as detailed (19). Translation products were visualized by autoradiography of 7.5 or 10% polyacrylamide gels (19, 21). The method used previously for immunoprecipitation of the translated proteins was modified to minimize nonspecific sticking and was carried out as follows: a 100-μl translation mixture was diluted to 800 μl with 0.1 M NaCl/5 mM EDTA/50 mM Tris-HCl, pH 7.5/0.5% Triton X-100. Ten microliters of nonimmune IgG (0.5 mg/ml) was added for 30 min at room temperature, and then 40 μl of protein A-Sepharose (Sigma) was added. After centrifugation (12,000 × g, 0.5 min, room temperature), IgG monospecific for rabbit synovial fibroblast collagenase (a gift of Carol Vater and Edward Harris, Jr.) (22) or nonimmune IgG was added to the supernatant and the mixture was incubated at 4°C overnight. The antigen–antibody complexes were isolated with protein A-Sepharose. After 1 hr, the Sepharose beads were washed twice with 1 M NaCl/5 mM EDTA/50 mM Tris-HCl, pH 7.5/0.5% Triton X-100, twice with 0.1 M NaCl/1 mM EDTA/10 mM Tris-HCl, pH 7.5/1% Triton X-100/0.5% deoxycholate (Sigma)/0.1% NaDodSO4, once with 0.1 M NaCl/5 mM EDTA/50 mM Tris-HCl, pH 7.5/0.5% Triton X-100, and finally with water. Antigen–antibody complexes were dissociated from the Sepharose by boiling in NaDodSO4 gel electrophoresis sample buffer (19, 21) and immunoprecipitated material was visualized after gel electrophoresis and autoradiography (19, 21).

Preparation of a cDNA Library. A cDNA library was constructed as described by Crabtree and Kant (23). Poly(A)+

Abbreviations: bp, base pair(s); kb, kilobase(s).
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1981
RNA was isolated from rabbit synovial fibroblasts that had been induced with urate crystals. Avian myeloblastosis virus reverse transcriptase (J. Beard, Life Sciences) was used to polymerize a complementary strand of DNA and, after strand separation by boiling, a second strand of DNA was synthesized with the Klenow fragment of DNA polymerase I (Bethesda Research Laboratories). S1 nuclease (Bethesda Research Laboratories) digestion removed single-stranded cDNA with a mean size of 750 base pairs (bp). The cDNA was fractionated by size on an A-150M column (Bio-Rad) in 10 mM Tris-HCl, pH 7.5/1 mM EDTA and then dried with [3H]dCTP (Amersham) using terminal deoxynucleotidyltransferase (Boehringer Mannheim). It was hybridized to pBR322 that had been cleaved with Pst I (Bethesda Research Laboratories) and labeled with [3H]dGTP. Both insert and plasmid had tails of 10-20 nucleotides at their 3' ends. Calcium-shocked Escherichia coli. HB101 was transfected (24) with the hybridized plasmid and 167 transformants were selected on the basis of tetracycline resistance and ampicillin sensitivity.

Identification of a cDNA Clone for Collagenase. The transformants were screened by colony hybridization (25). Duplicate filters were prepared and hybridized with a single-stranded [32P]cDNA probe that had been synthesized, using reverse transcriptase, from control cell mRNA or urate-stimulated cell mRNA. Thirty colonies were selected that hybridized more strongly to the induced probe than to the control probe. These colonies were screened by hybrid selection based on the method of Riccardi, et al. (26). Nitrocellulose filters that contained recombinant plasmid DNA were prepared as follows: 4 µg of recombinant DNA was suspended in 100 µl of H2O, and 50 µl of 1 M NaOH was added. After boiling for 2 min, 30 µl of 2 M NaOAc (pH 5.5) and 40 µl of 1 M HCl were added. The DNA was precipitated by addition of 2.5 vol of ethanol. The pelleted DNA was suspended in 10 µl of H2O. After the suspension had been boiled for 1 min and quick frozen, 5 µl of 3 M NH4OAc was added. Four micrograms of DNA was then doped onto a small square of nitrocellulose and allowed to dry overnight. The filters were baked for 2 hr at 80°C under vacuum. After being washed three times with distilled water, each filter was then prehybridized in 50 µl of 50% formamide/0.1 M Pipes, pH 6.4/0.5 M NaCl/poly(A) (Sigma) (100 µg/ml) at 38°C for 1 hr. For hybridization selection, conditions were the same with the addition of 4 µg of poly(A)" RNA, for 2-4 hr. The filters were then washed five times at 60°C with 0.15 M NaOAc/0.015 M Na citrate, pH 7.0/containing 0.5% NaDodSO4, twice at room temperature with 10 mM Tris-HCl, pH 7.9/2 mM EDTA, and finally boiled for 1 min in 100 µl of H2O to release the hybridized RNA. The RNA was precipitated by adding 1 µg of tRNA, 7 µl of 3 M NH4OAc, and 270 µl of ethanol. The pellet was washed once with 80% ethanol, dried, and resuspended in an appropriate volume for translation.

Restriction Mapping. Restriction enzymes were purchased from Bethesda Research Laboratories. Restriction digestion of plasmid DNA was carried out at 5-10 units/µg of DNA for 5 hr at 37°C in the buffers specified by the supplier. Digestions were carried out with whole plasmid DNA and gel-purified 1350-bp EcoRI and 575-bp Pst I digestion fragments. The DNA was precipitated, and the relative positions of the restriction sites were defined by various simultaneous multiple digestions. The restriction fragments were visualized on 8% polyacrylamide gels by staining with ethidium bromide.

DNA Gel- and Dot-Blot Analysis. RNA was isolated from rabbit synovial fibroblasts at various times after induction of urate crystals. Twenty-five- and 50-µg samples of RNA from each time point were denatured by treatment with glyoxal (10 mM NaPO4, pH 6.6/63% dimethyl sulfoxide/1 M glyoxal, 50°C, 1 hr) and electrophoresed through 1.5% agarose in 10 mM NaPO4 (pH 6.6). RNA was transferred to nitrocellulose as described by Thomas (27). For dot-blot analysis, 1, 2, 4, or 10 µg of whole cell RNA was spotted onto nitrocellulose (27).

After prehybridization, the filters were hybridized for 16 hr (28) with 103 cpm of gel-purified cloned insert per ml (specific activity, 10-18 cpm/µg). The hybridization probe was made by nick-translation (P-kit; New England Nuclear). The results after hybridization and autoradiography were quantified by cutting out appropriate regions of the filters and determining their radioactivity in a scintillation spectrophotometer. Background values were obtained from equivalent areas from each filter that did not contain any immobilized RNA.

Immunoblotting Technique. Proteins in 1 ml of culture medium were precipitated by addition of cold trichloroacetic acid to a final concentration of 5%, suspended in sample buffer, and electrophoresed into a 7.5% polyacrylamide gel as described (29). Proteins were then electrophoretically transferred to nitrocellulose filters (Trans-Blot Cell, Bio-Rad) in Tris/glycine/methanol buffer (25 mM Tris-HCl, pH 8.3/192 mM glycine/20% methanol) overnight at 4°C, 30 V (V/cm).

To detect immunoreactive collagenase, we first blocked the filters with 3% bovine serum albumin 50 mM Tris-HCl, pH 7.4/150 mM NaCl/5 mM EDTA for 1 hr at 37°C. This solution was replaced with buffer without bovine serum albumin but with 0.05% Tween 20 and monospecific antibody to collagenase. After overnight incubation at room temperature, the filters were washed three times with buffer containing Tween and then allowed to react with a second antibody, rabbit anti-sheep IgG (Accurate Chemicals, Westbury, NY) linked to horseradish peroxidase for 4 hr at room temperature. After three additional washes, the filters were treated with substrate [diaminobenzene dihydrochloride (Sigma), 5 mg/ml, in 10 mM Tris-HCl buffer/0.02% cobalt chloride], and the color was allowed to develop for 5 min.

Fibril Assay for Collagenase. Culture medium was assayed for collagenolytic activity (17) with fibrils of radiolabeled reconstituted guinea pig collagen (30, 31). Latent collagenase was activated by incubation with aminophenylmercuric acetate (32, 33). One unit of collagenase degraded 1 µg of collagen per min per ml of culture medium at 37°C.

RESULTS

We used colony hybridization to screen our cDNA library of 167 transformants for the presence of a cDNA insert corresponding to a mRNA found in greater abundance in induced than in control cells. Duplicate filters of the transformed colonies were prepared and hybridized to radiolabeled cDNA probes that had been synthesized with reverse transcriptase from induced or control cell mRNAs. Colonies that hybridized more strongly to the induced probe were chosen for further screening. Duplicate filters that had been hybridized with the control probe or with the induced probe are shown in Fig. 1 A and B, respectively. The arrows indicate examples of colonies that hybridized weakly or not at all with the control probe but gave a strong signal when hybridized to the induced probe. With this technique, 30 candidate colonies were chosen to be screened further for the presence of collagenase sequences.

To facilitate the screening procedure, groups of five clone DNAs from the induced library were assayed by hybrid selection. The hybrid-selected mRNAs were eluted and translated in a wheat germ cell-free translation system and the products were visualized by gel electrophoresis and autoradiography. Using this procedure, we were able to select
mRNAs that directed the synthesis of protein corresponding in molecular weight to preprocollagenase (19, 21). The results from a typical hybrid-selection screening are shown in Fig. 2A. One group of five cDNAs (lane 1) selected a mRNA coding for a protein corresponding in Mr to preprocollagenase while the second group of five cDNAs (lane 2) and DNA from pBR322 (lane 3) did not.

Subsequent analysis of the individual clones in lane 1 revealed that one clone, designated H9, hybrid selected a mRNA whose translation product was immunoprecipitable with monospecific antibody to collagenase but not with nonimmune IgG (Fig. 2B). Specifically, the figure compares the mobility on gel electrophoresis of the protein (Mr, 59,000) synthesized by nonselected mRNAs from induced cells (lane 2) with that selected by H9 (lane 4) and identifies this protein as preprocollagenase by immunoprecipitation (lane 5).

The cDNA insert from this clone was then characterized. Restriction digests of the clone for collagenase revealed that it was \( \approx 650 \text{ bp} \) long, having a map as shown in Fig. 3. Using the cDNA insert from the H9 clone, we screened the remainder of the library and identified three additional clones containing sequences coding for collagenase, all of which were shorter than the H9 insert.

To analyze levels of mRNA induction and to determine a size for the collagenase mRNA, we carried out RNA filter hybridizations. Cultures were treated with urate crystals and harvested at various times. From these cultures, medium was saved at each time point for assay of collagenase enzyme activity and whole cell RNA was prepared for hybridization analyses. The result of an RNA gel-blot analysis is shown in Fig. 4. No collagenase mRNA was detected at 0 hr, while increasing amounts of a 2.7-kb RNA could be seen...
starting at 5 hr. Quantitation of the mRNA by both gel- and dot-blot analyses indicated that induction resulted in a 60- to 90-fold increase in mRNA levels during the first 30 hr (Fig. 4B).

The induction of collagenase message in these cells was paralleled by an increase in collagenase protein in culture medium as detected by protein blot analysis. Proteins in medium were separated by polyacrylamide gel electrophoresis and then transferred to nitrocellulose. Collagenase was measured with an enzyme-linked immunoadsorbent assay using monospecific antibody to collagenase and a second antibody linked to horseradish peroxidase. Collagenase was detectable in culture medium by 10 hr (Fig. 5). Three proteins were apparent, having Mr values of 61,000, 57,000, and 53,000. The Mr 61,000 band represents a glycosylated form of collagenase that is found in culture medium but not in translation products (21, 34). The Mr 57,000 band is procollagenase (19, 21) and the Mr 53,000 band is as yet not fully characterized. Initial experiments suggest that it may represent a degradation product.

In contrast, no collagenolytic activity in culture medium was detectable until 20 hr, when 0.04 ± 0.036 units of enzyme were present. By 30 and 35 hr, this had increased to 0.19 ± 0.054 and 0.667 ± 0.065 units, respectively.

**FIG. 5.** Detection of immunoreactive collagenase by protein blot analysis. Aliquots of culture medium from the experiment described in Fig. 4 were analyzed by NaDodSO4 gel electrophoresis. The proteins were transferred to nitrocellulose filters and exposed to monospecific antibody for rabbit synovial fibroblast collagenase. A second antibody linked to horseradish peroxidase was then added and color was developed by the addition of the substrate diaminobenzene dihydrochloride. Lane C: cells were incubated for 40 hr in the absence of urate.

**DISCUSSION**

In this paper, we report the isolation of a cDNA clone containing an insert complementary to rabbit synovial fibroblast collagenase mRNA. This clone hybridized to a mRNA that directed the synthesis of protein identified as proprocollagenase by its molecular weight and by its ability to be precipitated by monospecific anticolagenase antibodies (Fig. 2). Hybridization analyses of RNA blots (Fig. 4) indicated that the size of the collagenase mRNA is 2.7 kb. Since only about 1800 nucleotides are needed to actually code for the protein, roughly one-third of the mRNA (including the poly(A) tail) represents noncoding regions.

Quantitation of the RNA gel-blot and dot-blot filter results suggested a minimum 7-fold induction of collagenase mRNA sequences within 5 hr after addition of urate crystals. The increase continued for at least 35–40 hr, at which time there was a 60- to 90-fold induction in the level of collagenase mRNA in the cells and a several hundred fold increase in collagenase enzyme activity in the culture medium. This induction period is considerably longer than what has been reported for plasminogen activator (35, 36). This neutral proteinase is similar to collagenase in that it is active in the extracellular space and its production can be transcriptionally regulated by a variety of experimental and physiological stimuli. With plasminogen activator, maximal stimulation of enzyme activity by vasopressin or calcitonin (35) or by phorbol esters (36) occurred within 6 hr and represented a 15- to 20-fold enhancement compared with controls.

Earlier studies measuring the time of appearance of collagenase activity in culture medium showed a lag period of ~24 hr after addition of a stimulus (15). In the hours immediately following this 24-hr period, there was a sudden and dramatic increase in the amount of collagenase activity detectable in culture medium. Our previous experiments correlated the appearance of collagenase activity in the culture medium at ~24 hr with the appearance of translatable collagenase mRNA within the cells (19).

In the studies presented here, using the more sensitive techniques of RNA and protein blot analyses, we have shown that collagenase mRNA can be detected as early as 5 hr and continued to increase for at least 35–40 hr. Even though immunoreactive collagenase appeared shortly there-
Thus, which synthesized on Mr to process. Through cultures ligand-linked glycosylation occurs to give a species of M, 61,000 (21, 34). Similar to human skin procollagen (7), synovial cell procollagenase is rapidly synthesized and is secreted within 60 min (34). Furthermore, the inhibition of secretion of the synthesized collagenase by monensin-treated cultures suggests that collagenase is secreted by means of a pathway through the Golgi (34, 37).

Less well understood are events occurring during the induction process. A feature common to most, if not all, stimulii of collagenase synthesis is membrane perturbation, such as occurs during cell fusion (38) or phagocytosis (16) or more subtly, via specific membrane receptors (39). After this initial event, but preceding the initiation of transcription, there may be a series of changes marking an "activated" cell, as has been described for neutrophils (40). In general these include a change in membrane potential, mobilization of cellular phospholipids, an influx of Ca++ rearrangement of the cytoskeleton, and the generation of cAMP. Phospholipid metabolism (15, 16, 41), redistribution of microfilaments (42), and an increase in cAMP (15, 43) have been associated with increased production of collagenase by synovial cells.

The rabbit synovial fibroblast system that we have described is an attractive model for studies on mechanisms controlling collagenase synthesis. The cells can be induced to produce collagenase through a number of different agents (15, 16, 42, 44) and collagenase synthesis by induced cells can be inhibited with steroids (18, 45-47) and retinoids (46, 47).

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