Rat Collagenase

CLONING, AMINO ACID SEQUENCE COMPARISON, AND PARATHYROID HORMONE REGULATION IN OSTEOBLASTIC CELLS

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We have isolated clones for rat collagenase from a rat osteoblastic cell cDNA library. These clones have been sequenced and the amino acids deduced. The calculated molecular weight is 51,352 for the proenzyme and 42,229 for the active enzyme. The deduced amino acid sequence was compared to those previously reported for: 1) human collagenase, 2) rat transin 1 (stromelysin), 3) human stromelysin, and 4) rabbit collagenase. The number of amino acids conserved was 47, 47, 50, and 47%, respectively. We also compared the collagenase mRNA and protein in different rat cells (osteoblast, uterine smooth muscle, synovial fibroblast) and determined that in rat uterine cells the message is slightly larger, although collagenase protein in all three cell types is identical in size. Parathyroid hormone dramatically induces the 2.9-kilobase collagenase mRNA in the rat osteoblastic cells, UMR 106-01. Nuclear run-on studies in UMR 106-01 cells demonstrated a 4–8-fold induction in the rate of synthesis of collagenase mRNA at 2 and 4 after parathyroid hormone treatment, with steady state levels of mRNA increased 100-fold at 4 h. Thus, parathyroid hormone regulation of the collagenase gene in UMR 106-01 cells is in part transcriptional.

Collagenase plays a crucial role in the remodeling and development of collagenous structures in the extracellular matrix (1, 2). In the mammal, postpartum involution of the uterus results in the rapid degradation of collagen by collagenase. In rats, appearance of collagenase in the uterus is evident only during active involution (3–5), not in late pregnancy nor after degradation is complete (6). Collagenase activity has also been implicated in the continuous process of bone renewal (10, 11) which involves bone resorption by osteoclasts and bone production by osteoblasts and is necessary for calcium homeostasis and bone repair. Although traditionally osteoblasts have been implicated only in bone formation, a current model postulates that, in response to resorption stimulators, they cease synthesis of collagen and secrete neutral proteases such as collagenase (12) which degrade the osteoid layer of the bone surface allowing osteoclasts to resorb the mineralized bone.

Parathyroid hormone (PTH) is one of several agents which increase collagenase secretion by both normal and transformed rat osteoblastic cells (13–17). However, the mechanism by which PTH stimulates collagenase production in osteoblasts is not well understood. To study PTH action in osteoblast function, our laboratory has utilized the rat osteosarcoma cell line, UMR 106-01, which is osteoblastic in phenotype and produces high levels of Type I collagen, the major protein of bone matrix. In addition, these cells secrete a neutral collagenase (matrix metalloprotease I) and inhibitors of collagenase in response to PTH treatment (18). Collagenase release is also stimulated to a lesser extent in these cells by 1,25-(OH)2 vitamin D3, prostaglandin E2, retinoic acid, and epidermal growth factor.

Our current study compares the collagenase protein and mRNA produced in rat uterine myometrial smooth muscle cells, synovial fibroblasts, and UMR 106-01 osteosarcoma cells by Western and Northern blotting, respectively. Until now, no nucleic acid probe has been available to examine collagenase gene expression in the rat. The human and rabbit collagenase genes have previously been cloned (19, 41), however, there is not enough nucleotide sequence homology to allow the use of either as a probe for collagenase in rat cells. Therefore, we describe the isolation of a rat collagenase cDNA clone and compare the deduced amino acid sequence to those previously published for other closely related zinc-binding metalloproteases (18–20). By Northern blot analysis of steady state mRNA levels and nuclear run-on studies, we have determined that PTH regulates collagenase expression, at least in part, by altering the rate of transcription in UMR 106-01 cells.

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1 The abbreviation used is: PTH, parathyroid hormone.

2 W. T. Roswit, N. C. Partridge, and J. J. Jeffrey, unpublished data.
DISCUSSION

We have determined that the collagenase mRNA in the UMR 106-01 cells is 2.9 kilobases in size, slightly larger than the human fibroblast collagenase (2.5 kilobases; Ref 19) and human stromelysin transcripts (2.3 kilobases; Ref 20) and very much larger than the closely related rat transin (stromelysin) gene (1.9 kilobases; Ref 18). This difference in message size has been attributed mainly to size differences in the 3' untranslated region between the two genes (18-20).

The collagenase transcript from rat uterine endometrial cells appears slightly larger than the message in either UMR 106-01 or rat synovial fibroblasts by Northern blot analysis (Fig. 7). Western blot analysis (Fig. 8) suggests that identical protein products are expressed in all three cell types. Therefore, the difference in message size is probably due to a difference in the size of the 3'-untranslated region, possibly related to a differential regulation of gene expression, such as alternative splicing, or simply a difference in the size of the poly(A) tail. We are now in the process of cloning the rat uterine gene to determine which of these is the case.

Steady state mRNA levels along with nuclear run-on studies have shown that the inductive effect of PTH on collagenase gene expression is, in part, transcriptional in the UMR 106-01 cells. This stimulation is rapid, causing in different experiments, a 4-8-fold increase in the rate of synthesis at 2 and 4 h after PTH treatment which results in approximately a 100-fold increase in the amount of mRNA accumulated. Since the increase in the rate of synthesis does not completely account for the mRNA accumulated, PTH probably also increases collagenase mRNA stability. We are currently designing experiments to address this issue.

We have identified four highly conserved regions in the rat collagenase protein. Two of these regions, the amino acids before the tryptophan activation site of the proenzyme and the zinc-binding region of the active site, are hydrophobic and have been identified previously (38, 39). However, the two remaining regions (amino acids 139-145 and 175-183 of the rat proenzyme) have not been recognized until now. Amino acids 139-145 (ADIMISF) could form a hydrophobic pocket while amino acids 175-183 (GDAHFDDDE) are in a very hydrophilic region. Both regions could possibly be binding sites for protein-protein interactions. An obvious choice for these protein-protein interactions would be the collagenase inhibitor (tissue inhibitor of metalloproteinase) binding region; however, previously reported data suggest that this interaction occurs elsewhere in the molecule (43).

We speculate that the aspartic acid of region 139-145 (ADIMISF) occupies the third zinc coordinate site suggested by Springman et al. (44). They identified Cys in the first conserved region and His and His (numbers refer to human fibroblast collagenase) of the conserved zinc-binding region of the active site as possibly occupying zinc coordinate sites L4, L1, and L2, respectively, in the proenzyme. Their model states that Cys is replaced by H2O in the active form of the enzyme. They also speculate that a His or Glu will occupy coordination site L3. We have identified a conserved hydrophobic region that contains an Asp residue. Alkaline phosphatase has an Asp in one of the zinc coordination sites, the L1 position. Asp identified here is 59 amino acids from His, a number in the long spacer range (20-120 amino acids) observed in catalytic zinc sites (45).

The highly conserved hydrophilic region, amino acids 175-183 (GDAHFDDDE), that we have identified could be involved in receptor binding. We have evidence for a specific cell surface receptor in UMR 106-01 cells which mediates the re-uptake of collagenase. Currently, studies are in progress to characterize this receptor.

The UMR 106-01 rat osteosarcoma cell line has been well characterized and proven to be a good model system for studying hormonal regulation of the bone remodeling process. We have isolated a rat collagenase cDNA clone, an important tool for studying regulation of extracellular matrix remodeling which is necessary for repair, and in the case of bone, calcium homeostasis. We have demonstrated that collagenase induction by PTH occurs primarily at the transcriptional level in UMR 106-01 cells. Currently, we are utilizing our cDNA clone to further these studies.

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REFERENCES


isolation of CMH clone

Based on our steady-state results, preliminary data reported above, we had a CMH library constructed as described (10; see also legend of Fig. 3). After screening, we obtained a 5' end cDNA clone (11), which was used to isolate a CMH clone by degenerate oligonucleotide primed polymerase chain reaction (11) using the primer CMH. This clone was sequenced in both directions (11) and hybridized to the human fibroblast collagenase gene (11). The calculated molecular weight for the collagenase carboxyl terminal (11) was 11,350 and 10,185, respectively. Sequencing was accomplished on both strands using the strategy shown in Figure 3. The nucleotide sequence was determined using the dideoxynucleotide sequence analysis method (11).

Figure 3. Restriction map of the rat collagenase CMH clone. a) 12.1 kb clone (CMH5A) and 1.6 kb clone (CMH6D). Both clones were isolated from a λgut11 library constructed with mRNA from PRM2 fibroblasts (11). b) Collagenase-collagenase gene.

Sequence analysis (continued)

In conclusion, we have isolated a CMH clone that is coding for a protein with homology to the human fibroblast collagenase gene. The sequence analysis shows that the CMH clone is coding for a protein with homology to the human fibroblast collagenase gene (11). The calculated molecular weight for the collagenase carboxyl terminal (11) was 11,350 and 10,185, respectively. Sequencing was accomplished on both strands using the strategy shown in Figure 3. The nucleotide sequence was determined using the dideoxynucleotide sequence analysis method (11).

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Comparison of rat collagenase cDNA

To determine if the cDNA expressed by different rat cells is identical in size and composition, we hybridized our 1000 cDNA probe with a filter containing poly(A) RNA isolated from rat uterine endometrial and UMR 106-01 cells. The RNAs were hybridized to the probe in a solution of formamide, and the hybrid bands were visualized with a light microscope. The results are shown in Figure 1, and they indicate that the cDNA expressed by different rat cells is identical in size and composition.
Figure 7. Northern blot analysis of rat collagenase. Western analysis and enzyme activity assays were performed as described in Methods. Lane 1: rat aortic fibroblast collagenase (concentrated from 200 μl of crude medium); Lane 2: 500 μg of purified rat uterine collagenase. Lane 3: 500 ng of purified UMR A+ collagenase protein.

PTH induction of collagenase mRNA

A duplicate of the Northern blot shown in Figure 3, of poly(A)+ RNA from PTH treated UMR 106-01 cells, was probed with the 1.9 kb EcoRI fragment of UMR A+ collagenase. The 1.9 kb mRNA species that hybridized in this probe (Figure 5a) was identical in size to the transcript seen with the aliguloseptide probes. A densitometric analysis (data not shown) of the Northern blot in Figure 5a indicated that there was about a 2-fold increase in steady state mRNA present 4 h after treatment with PTH, the abundance falling to approximately half the 4 h level by 24 h. Also evident was a low level of expression at 0 h (no PTH), which was very obvious on overexposure of the autoradiogram. This time course of mRNA induction was consistent with the enzyme activity increase in the level of secreted enzyme between 12-24 h after PTH treatment (15).

The Northern blot in Figure 5b was also hybridized with UMR A+ a probe specific for the rat transglutamase. The transglutamase cDNA, which was not present in UMR 106-01 cells (Figure 5b). The 1.6 kb rat transglutamase is present at lower levels in B77 cells and was detected in the RNA from those cells upon overexposure of the autoradiogram in Figure 5b. The transglutamase cDNA probe did not cross-hybridize. Therefore we concluded that our probe was specific for the rat collagenase gene and transglutamase is not expressed in UMR A+ cells.

We also performed nuclear run-on experiments using nuclei isolated from PTH treated UMR 106-01 cells. These data (Figure 6) demonstrated a 4-fold increase in the rate of transcription 24 h after PTH treatment (15). The nuclear run-on data, together with the steady state mRNA measurements, led us to conclude that the inductive effect of PTH on collagenase mRNA is, in part, at the transcriptional level.

Figure 8. Northern blot analysis of UMR 106-01 cell lines using a rat collagenase cDNA probe. A duplicate blot of Figure 3, showing the total (15 μg. of PTH) and poly [A] RNA (no PTH), 4 and 24 h after PTH treatment (5 μg. of PTH). (a) A 2.5 kb fragment of the rat transglutamase gene, B77 (15) total shows an overexpression of the 1.8 kb fragment. 1.6 and 4.8 kb are the ribosomal bands. The 2.5 kb band is identical to that in Figure 1 and is seen present in the 4 h sample (overexposure). (b) Probed with [32P]-labeled PTH, the rat transglutamase probe, a message of 1.5 kb is present only in the PTH A+ cells.

Figure 9. Nuclear run-on. Slot blot of a nuclear run-on study with samples at 2 and 4 h obtained by incubation with PTH alone with or without PTH (15 μg). The RNAs are: (1) control, (2) collagenase, (3) pBR322, (4) plasmid plasmid.