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CORRELATION OF THE STEADY-STATE RNA LEVELS AMONG THE α-SUBUNIT OF PROLYL 4-HYDROXYLASE AND THE α1 AND α2 CHAINS OF TYPE I COLLAGEN DURING GROWTH OF CHICKEN EMBRYO TENDON FIBROBLASTS

James A. Bassuk and Richard A. Berg

Department of Biochemistry, Robert Wood Johnson Medical School
University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854

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SUMMARY: The relative steady-state levels of RNAs encoding type I collagen and prolyl 4-hydroxylase were examined in exponentially growing primary cultures of chicken embryo tendon fibroblasts. The RNA levels of the α1 and α2 chains of type I collagen were maximal when the fibroblasts reached the confluent state. The RNA levels of the α-subunit of prolyl 4-hydroxylase were also maximal at confluency and rose and fell with the RNA levels of the two collagen chains. The RNA levels of the β-subunit of prolyl 4-hydroxylase did not correlate with the changes observed for the α-subunit or for either chain of type I collagen. The RNA levels of the β-subunit were slightly higher than the RNA levels of the α-subunit. These results support our hypothesis that the synthesis of the α-subunit and thus the association of newly synthesized α-subunits with pre-existing β-subunits is the rate-limiting factor in determining prolyl 4-hydroxylase activity in cultured cells.

Primary cultures of chicken embryo tendon fibroblasts reach confluency after 4-6 days when grown in the presence of fetal calf serum. During growth, these fibroblasts synthesize and secrete large quantities of type I collagen. Collagen thus forms a substratum to which the fibroblasts attach, grow and divide. Prior to secretion from the cell, procollagens undergo a series of post-translational modifications within the lumen of the endoplasmic reticulum. One such modification is the formation of 4-hydroxyproline in nascent procollagen polypeptides. This modification is catalyzed by the prolyl 4-hydroxylase (EC 1.14.11.2) (1). This enzyme plays a central role in collagen synthesis, as the 4-hydroxyproline residues formed from proline residues are essential for the folding of the newly synthesized procollagen polypeptide chains into stable triple helical molecules (2).

Prolyl 4-hydroxylase is a tetramer comprised of two pairs of nonidentical subunits ($\alpha_2\beta_2$) (3). Peptide mapping has demonstrated that the α and β subunits are products of different genes (3). Complete cDNA-derived amino acid sequences have recently been determined for both subunits of the human (4,5) and the chick enzyme (6,7,8). The calculated molecular size for the tetramer is 232,312 daltons. The β-subunit is identical with the enzyme protein disulphide isomerase (PDI) and prolyl 4-hydroxylase possesses disulphide isomerase activity (9,4).

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The activity of prolyl 4-hydroxylase increases in parallel with the synthesis and secretion of collagen in cultures of chicken tendon fibroblasts (10,11). We hypothesized that prolyl 4-hydroxylase and collagen is coordinately regulated at the level of transcription and we tested this hypothesis by measuring the steady-state mRNA levels for prolyl 4-hydroxylase and type I collagen. We report that the relative RNA steady-state levels of the α-subunit of prolyl 4-hydroxylase rise and fall in parallel with the relative RNA steady-state levels of both the α1 and α2 chains of type I collagen, suggesting that these three genes are coordinately regulated.

MATERIALS AND METHODS

Cell Culture. Leg tendons were dissected from 17-day chicken embryos. Matrix-free cells were prepared by digestion with trypsin and crude bacterial collagenase according to Kao et al. (10) and Quinones et al. (12) and grown in culture for 8 days. Matrix-free cells were seeded at 4.1 x 10^5 cells per 10 cm petri dish on day 0 and grown in the presence of Dulbeccos's Modified Eagle Medium supplemented with 5% fetal calf serum, ascorbate (10 μg/ml), penicillin (100 units/ml), and streptomycin (100 μg/ml). The cells were grown at 37 °C in an atmosphere containing 5% CO_2. Aliquots were removed for the determination of cell numbers based on the measured amount of DNA (13). The remaining cells were dissolved in 4 M guanidine isothiocyanate (see below). When RNA was to be prepared for Northern analyses (see below), primary cultures were divided and maintained as secondary cultures for up to 5 passages.

Preparation of labeled cDNA probes. Five different cDNAs were used to analyze the mRNA from tendon fibroblasts. The following cDNA probes were used: A1, a 3.1 kbp clone that codes for the α-subunit of chicken prolyl 4-hydroxylase (6); ppdi100, a 2.3 kbp clone that codes for rat liver PDI (14); Hf667, a 1.8 kbp clone encoding human α1(I) procollagen (15); Hf32, a 2.2 kbp clone encoding human α2(I) procollagen (16); and p60, a 0.83 kbp clone encoding mouse ribosomal protein L18 (17). For northern and slot blot hybridizations, recombinant plasmids were restricted with EcoRI for ppdi100 (PDI), A1 (α-subunit), and Hf677 α1(I). p60 was restricted with HincII and Hf32 with PstI. Linearized recombinant plasmids were extracted with phenol, precipitated with ethanol, dried, and dissolved in water at 15-20 ng/ul. One ul was used in an oligolabelling reaction using ^{32}P-dCTP and the Klenow fragment of E.coli DNA polymerase. Labeled probes were adjusted to the same specific activity by either dilution or concentration.

Analysis of RNA. Total RNA was prepared from cultured fibroblasts by acid guanidinium thiocyanate-phenol-chloroform extraction as described (18). For northern blot analysis, RNA samples were fractionated on 0.8 or 1% agarose gels containing 2.2 M formaldehyde, 0.05 M morpholinopropanesulfonic acid and transferred to nitocellulose membranes. Denatured Hind III fragments of λ DNA were included as size markers on every gel. The filters were air dried and baked at 80 °C for 30-90 min. For slot-blot hybridizations, RNA was applied to nylon membranes using vacuum manifolds. Aliquots were removed from each day's RNA which corresonded to the number of cells present on day 2. The number of cells present on day 2 was 1.26 x 10^6 ($\log_{10}$ = 6.1). RNA from days 2 through 7 were analyzed. Filters were incubated in a solution containing 45% (v/v) formamide, 5 X SSPE (0.18 M NaCl, 10 mM $NaPO_4$, pH 7.7, 1 mM EDTA), denatured salmon sperm DNA (0.1 mg/ml), 0.1 % SDS and 5X Denhardt's solution (0.02% ficoll; 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) for 16 hr at 40 °C. Nitrocellulose filters were washed twice in 2X SSPE/0.1% SDS for 10 min at room temperature, twice in 0.1X SSPE/0.1% SDS at room temperature, and once in 0.1X SSPE/0.1% SDS at 65 C for 45 min. Nylon membranes were washed twice in 6X SSPE/0.2% SDS for 10 min at room temperature, twice in 1X SSPE/0.2% SDS at 37 °C, and once in 0.1X SSPE/0.2% SDS at 65 C for 30 min. Blots were exposed at -80 °C to Kodak XAR-5 films overnight or for 16 hr for slot-blot analyses using intensifying screens. Serial dilutions (1:1, 1:10 and 1:100) of each day's RNA was analyzed to ensure the linearity of hybridization with the RNA concentration. The autoradiograms were scanned with a P1000 Optronics microdensitometer and a DEC MicroVaxII interfaced with a Lexidata Lex-90 display monitor.

RESULTS

Experimental Design. The tendon is histologically a homogeneous tissue, comprised almost exclusively of fibroblasts which synthesize and secrete large amounts of type I collagen (19). Tendon fibroblasts isolated from 16-day chicken embryos grown at high density in medium containing 10μg/ml ascorbate and 0.5% serum may devote as much as 50% of their protein synthesis of type I collagen (20,21). Tendon fibroblasts grown in a medium containing 10% serum and no ascorbate synthesize as much as 35% of total protein as type I collagen (22). Since our conditions used 5% serum and 10μg/ml ascorbate, type I collagen comprised the predominant polypeptides synthesized and secreted from the fibroblast (10,11).

Northern Blot Analysis of mRNAs. The tendon fibroblasts were of embryonic chicken origin, whereas the cDNA probes used in this study were made from chicken, mouse, rat or human mRNAs. The specificity of the hybridization with these probes was therefore established using Northern blot analyses of total cellular RNA from chicken embryo tendon fibroblast. Figure 1 displays the specificity of the cDNA probes used in this study. The human cDNA probe encoding the α1 chain of type I collagen hybridized to a major mRNA species of 5 kb and to a minor mRNA species of 7.5 kb (Fig 1, lane 1). The human cDNA probe encoding the α2 chain of type I collagen hybridized to a major mRNA species of 4.7 and a minor species of 5.2 kb in length (Fig 1, lane 2). The observed appar-

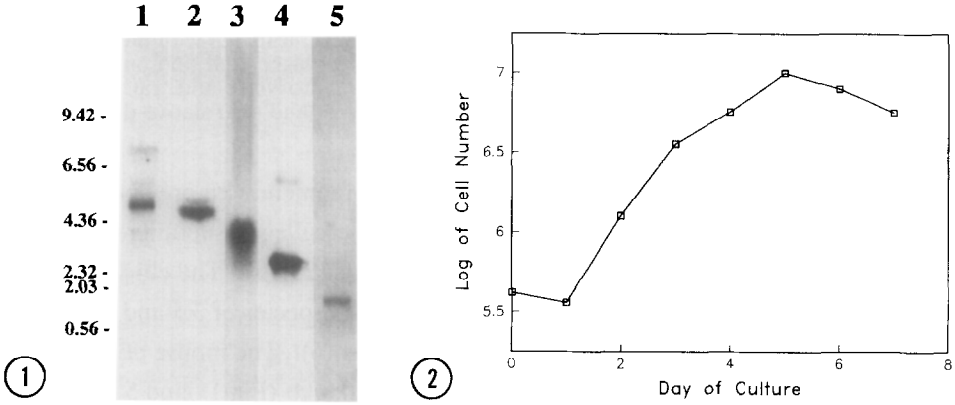

Fig. 1. Specificity of cDNA probes encoding the α1 and α2 chains of type I collagen, the α- and β-subunits of prolyl 4-hydroxylase, and ribosomal protein L18. Total RNA was prepared from tendon fibroblasts and analyzed by Northern analyses as described in the Methods section. cDNAs were labelled and hybridized to RNA bound to a nitrocellulose filter for 16 hr. Shown are autoradiograms from overnight exposures. The following cDNA probes were used: Lane 1, human fibroblast α1(I) procollagen; Lane 2, human fibroblast α2(I) procollagen; Lane 3: α-subunit of chicken embryo prolyl 4-hydroxylase; Lane 4, rat liver PDI; Lane 5, mouse ribosomal protein L18. The molecular sizes of Hind III digests of bacteriophage lambda, denatured and run on the same gel as the RNA samples, are given to the left of the figure.

Fig. 2. Growth curve of primary cultures of 17-day chicken embryo tendon fibroblasts. Tendons were dissected and incubated with trypsin and collagenase. Matrix-free cells were seeded on day 0 in multiple 10 cm dishes. On each day of culture, cells were harvested and a cell count was performed. Remaining cells were used for the preparation of RNA.

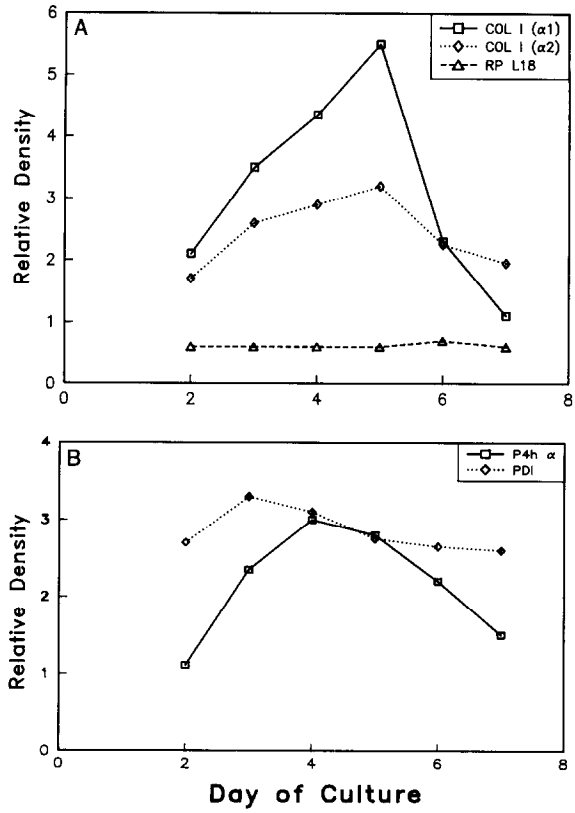

Fig. 3. Time course of the change in relative steady-state levels for the mRNAs encoding type I collagen and prolyl 4-hydroxylase. RNA was prepared each day from tendon cells grown in primary culture (Figure 2) and applied in parallel to 3 different nylon membranes as described in Materials and Methods. Each membrane was incubated with radiolabelled cDNA clones encoding (A) the α1 and α2 chains of type I collagen, and ribosomal protein L18; and (B) the α-subunit of chicken embryo prolyl 4-hydroxylase and rat liver PDI. Autoradiograms were scanned using a video image analyzer and the relative density was determined.

ent molecular sizes for the two collagen chains are in agreement with previously published values (22). The presence of multiple transcripts for the collagen subunits is presumably due to the utilization of multiple polyadenylation signals (23,16). The chicken α-subunit probe and the rat liver PDI cDNA hybridized with mRNA species of 3.5 and 2.5 kb (Fig 1, lanes 3 and 4), values in agreement with a previous report (6). The mouse cDNA encoding ribosomal protein L18 reacted with a mRNA species of 0.8 kb (Fig 1, land 5), a value also in accord with earlier reports (17,24).

Relative steady-state RNA levels. Chicken tendon fibroblasts were grown in primary culture under conditions that approach confluency (Figure 2). RNA was prepared daily from equalivent numbers of cells and hybridized to the specific cDNA probes described in Figure 1. Figure 3 is a plot of relative signal density vs day in culture of the relative levels of mRNAs encoding type I collagen and prolyl 4-hydroxylase. The results demonstrate that (i) the levels of each type I α chain increased during early phase of growth into confluency, day 5, and then declined. (ii) The levels of the α1 chain were higher during cellular growth and division (days 3-5) than the levels of the α2 chain, a result which

agrees with previous observations on these cells (12). This higher level of the α1 chain correlated with two copies of the α1 chain polypeptide to 1 copy of the α2 chain polypeptide in the heterotrimeric type I collagen molecule (25). (iii) The relative steady-state level of ribosomal protein L18 remained constant over the 6 days examined. This ribosomal protein thus appears to be expressed constitutively and the RNA levels are unaltered during cellular growth and division. (iv) The levels of PDI mRNA increased 1.3 fold at day 3 and then declined. (v) The RNA levels of PDI were slightly higher than the RNA levels of the α-subunit. (vi) The levels of the α-subunit increase by 2.5 fold from day 2 to day 4 and followed the changes in the levels of the α chains of type I collagen.

DISCUSSION

The cellular level of active prolyl 4-hydroxylase reflects the rate of collagen production (26,27). Previously, our laboratory reported that the activity of prolyl 4-hydroxylase in L-929 cells increased as cells approached confluency and the activity of prolyl 4-hydroxylase is dependent on *de novo* protein synthesis (28). However, the mechanism by which the level of prolyl 4-hydroxylase is regulated is presently unknown. The achievement of the molecular cloning of cDNAs encoding the collagens and prolyl 4-hydroxylase has allowed us to investigate aspects of the control mechanisms which fibroblasts use to regulate the production of collagen in cells.

The level of prolyl 4-hydroxylase activity changes when cells approach confluency in culture (29). This state of confluency is indicated by a plateau level when cell number is plotted against days in culture (Figure 2). The transcriptional activity of types I and III collagen is maximal when the cultures approach confluency. However, there is a decline in transcription as cell proliferation is decreased. Many hypotheses have been offered to explain this increased activity: cell-cell interaction, cell-extracellular matrix interaction, induction of the formation of cell-cell channels, and interactions between cells and the substratum (e.g. plastic, collagen gel, or collagen sponge).

The β-subunit polypeptide is present in large excess with respect to the α-subunit polypeptide (30,31). This observation is based on immunological studies using antibodies raised against the purified β-subunit with the immunoreactive protein species designated as cross-reacting protein (CRP). CRP is found in numerous non-collagen producing tissues. The discovery that PDI is equivalent to the β-subunit was interpreted to explain this large excess of β over α, and thus solved the CRP paradox (4). Thus, we were surprised to observe only slightly greater relative steady-state levels of β-subunit mRNA than α-subunit mRNA. One possible explanation is when maintained as primary cultures, the fibroblast has already been programmed to synthesize and secrete collagen, and there is enough PDI present within the lumen of the endoplasmic reticulum to satisfy the cell's needs. However, we favor an alternative explanation for excess CRP. We have recently found that the CRP isolated from chicken is heterogeneous. Anti-PDI immunoglobulins react with three dif-

ferent chicken proteins: PDI, glycosylation site binding protein (32,33) and a novel 53-kDa PDI-like protein which contains both unique and β-endorphin sequences (Bassuk et al., manuscript submitted). Thus, in western immunoblots analyses, an immunoreactive band at 55 kDa is actually a composite of 3 different proteins. The quantification of each of these chicken 53-55 kDa proteins has yet to be determined.

Mouse F9 teratocarcinoma stem cells is a model system to study the regulation of prolyl 4-hydroxylase and type IV collagen. These cells differentiate in the presence of retinoic acid into primitive endoderm cells. The differentiation process is accompanied by 40 fold increases in the levels of prolyl 4-hydroxylase enzyme protein and activity (34). A recent study reported that when these teratocarcinoma cells differentiate in the presence of retinoic acid, dibutyryl cAMP and isobutyl methylxanthine, there is a marked increase in the RNA levels for both subunits of prolyl 4-hydroxylase and of α1(IV) collagen (35). These results are consistent with our observations here for coordinate regulation of prolyl 4-hydroxylase and collagen in developmental growth.

We have presented the cultured tendon fibroblast as a valuable model for the study of the regulation of prolyl 4-hydroxylase gene expression. The production of collagen is a major *in vivo* function of this cell type. The observation that α-subunit mRNA levels correlate with collagen mRNA levels is in support of the hypothesis that the synthesis of the α-subunit and thus the association of newly synthesized α-subunits with pre-existing β-subunits is the rate-limiting factor in determining prolyl 4-hydroxylase levels in fibroblasts. Our observations that the relative steady-state RNA levels of the α-subunits rise and fall with the levels of the α1 and α2 chains of type I collagen raise the possibilty that these three genes may be coordinately regulated. As soon as we obtain clones for the 5'-regulatory region of the α-subunit gene, then nuclear transcription run-off assays can be performed to directly examine the concept of coordinate regulation.

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