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Antibodies to a Precursor of Human Collagen

Abstract. *Antiserum prepared against serum-free medium from human fibroblast cultures reacted specifically with protropocollagen, an assembled, soluble precursor of tropocollagen. The antibodies were directed to antigenic determinants in the nonhelical, amino terminal peptide extensions (propeptides). Amino acid sequences in the precursor molecule corresponding to the telopeptides and helical alpha chains of tropocollagen were not recognized by the antiserum.*

Tropocollagen, the molecule extracted from extracellular collagen fibers, is generally composed of two identical $\alpha 1$ chains and one $\alpha 2$ chain. The chains are in triple helical array except for regions about 15 amino acids long (telopeptides) at the NH_2- and COOH-terminal ends of the three chains. Antibodies to tropocollagen have been prepared in many laboratories (*1*), but conventional protocols for immunization generally fail to elicit high-titer antiserums. In most cases, the antibodies against collagen are directed against determinants in the telopeptide segments, and the helical portions of the three chains are less immunogenic.

Precursor forms of the collagen chains (procollagen; pro $\alpha 1$ and pro $\alpha 2$ chains) have been reported (*2, 3*); these chains have nonhelical, NH_2-terminal peptide extensions (propeptides) of approximately 200 amino acid residues. In human diploid fibroblasts in culture (*3*), procollagen chains are assembled intracellularly to form a molecule (protropocollagen) composed of two pro $\alpha 1$ chains and one pro $\alpha 2$ chain. This molecule is the soluble, secreted form of collagen, and is stabilized by interchain disulfide bonds between cysteine residues in the propeptides and by noncovalent interactions between the helical α chain segments. The propeptides are sequentially excised by an extracellular enzyme (or enzymes), initially generating a family of soluble intermediates and ultimately producing tropocollagen with its abbreviated NH_2-terminal telopeptides. As the latter have some immunogenic potential, we anticipated that protropocollagen with its longer nonhelical propeptides would prove to be a more potent immunogen.

Rabbits were immunized with concentrated, serum-free culture medium containing protropocollagen (*4*). When the antiserum was titrated against radioactive medium, 45 percent of the labeled protein was specifically precipitated at antibody excess (Fig. 1). To characterize the radioactive protein in the immune precipitates, washed precipitates were solubilized and subjected to electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate (SDS) (*3*). Figure 2a shows the electrophoretic patterns both for medium after 24 hours of labeling with [^{3}H]-glycine and [^{3}H]proline, and for a solubilized immune precipitate from that medium run on a parallel gel. In earlier studies (*3*) peak A was identified as intact protropocollagen, and the other lettered peaks were found to be shortened pro α chains released by SDS and urea from digestion intermediates of protropocollagen. Only intact protropocollagen was precipitated, and digestion intermediates with partially excised propeptides were not recognized by the antiserum. From the electrophoretic pattern of the control medium, it can be calculated that peak A contains 45 percent of the total radioactivity in procollagen species, confirming the titration data of Fig. 1.

To demonstrate further that the antiserum was directed against protropocollagen molecules, the radioactive immune precipitates were subjected to three procedures used earlier (*3*) to characterize protropocollagen (Fig. 2b). (i) Immune precipitates were digested with purified collagenase. After digestion, 70 percent of the radioactivity was dialyzable. When the remainder was applied to gels, the protropocollagen peak was absent; this confirmed that the material in the peak is a collagenous protein. (ii) Immune precipitates were solubilized in 0.5*M* acetic acid and incubated with pepsin under

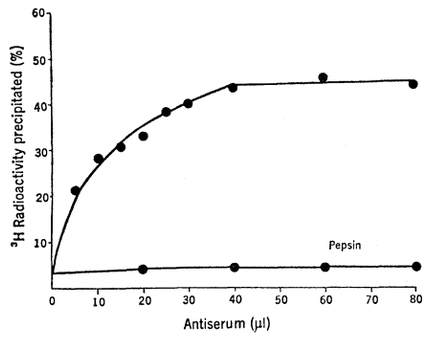

Fig. 1. Titration of antiserum against untreated and pepsin-treated radioactive medium. Test antigen was prepared by labeling confluent plates of human diploid fibroblasts with 50 μc each of [^{3}H]glycine and [2,3-^{3}H]proline (New England Nuclear) for 24 hours. The medium was made 0.5*M* in acetic acid and dialyzed at 4°C against phosphate-buffered saline (PBS) consisting of 0.05*M* phosphate buffer, *p*H 7.1, containing 0.15*M* NaCl. Indicated amounts of antiserum were added to tubes containing 1.0 ml of ^{3}H-labeled medium (124,000 count/min per tube). Normal rabbit serum was added to give a final volume of 80 μl of serum in each tube. After incubation at 37°C for 30 minutes, an excess of goat antiserum against rabbit gamma globulin was added, and incubation was continued for 30 minutes at 37°C and then for 16 hours at 4°C. Precipitates were centrifuged at 3000*g* for 15 minutes, washed four times with PBS, and solubilized in 0.5*M* acetic acid. Absorbance measurements at 280 nm revealed that equal amounts of protein were precipitated in each tube. Portions were counted in Aquasol (New England Nuclear). For prior treatment with pepsin, medium was made 0.5*M* in acetic acid and digested for 5 hours at 15°C with pepsin (Worthington, twice crystallized) (100 μg/ml). The pepsin was inactivated by bringing the *p*H to 8.2 with NaOH, and the medium was dialyzed against PBS and titrated as above with 50,000 count/min per tube.

conditions in which all nonhelical peptides are excised and triple helical portions of protropocollagen are not digested (*3*). Electrophoresis of the pepsin-treated sample on an SDS-polyacrylamide gel showed that $\alpha 1$ and $\alpha 2$ chains were generated in a 2/1 ratio, as expected. (iii) Immune precipitates were solubilized and then reduced and alkylated. Electrophoresis of this material showed that pro $\alpha 1$ and pro $\alpha 2$ chains were obtained in the 2/1 ratio expected for intact protropocollagen. These data confirmed that protropocollagen was specifically precipitated by the antiserum.

Experiments were performed to determine more precisely the distribution of antigenic sites on the protropocollagen molecule. To distinguish between helical and nonhelical determinants, labeled medium was digested with pepsin. Electrophoresis of the treated medium showed that nonhelical peptides had been excised from all procollagen species, inasmuch as only $\alpha 1$ and $\alpha 2$ chains were recovered in a 2/1 ratio (not shown). Titration of the antiserum against the pepsin-treated medium showed that no radioactivity could now be specifically precipitated (Fig. 1), a result indicating that all the immunogenic determinants had been removed. This experiment indicates that under our conditions of immunization, the helical (pepsin-resistant) portion of the protropocollagen molecule is not immunogenic and that the immunogenic regions are in the nonhelical propeptides or telopeptides.

To determine whether the antigenic determinants resided in the propeptides or telopeptides, the antiserum was reacted under the same conditions as for Fig. 1 with labeled tropocollagen extracted from the cell layers of the cultures (*3*). No radioactivity above control levels (3 percent) was precipitated. In another experiment, 0.5 ml of antiserum was absorbed with 7 mg of purified human skin collagen and then titrated against the labeled medium of Fig. 1. The absorption did not change the amount of antiserum required for maximal (45 percent) precipitation. These experiments indicate that the antibodies produced with our immunization protocol were directed against sequences in the propeptides of protropocollagen and did not have detectable specificity for the helical regions or their immediately adjacent telopeptides. Since only intact protropocollagen is precipitated by antibody, whereas digestion intermediates lacking some portion of the propeptides are not recognized (Fig. 2a), our results further suggest that the antibodies are directed against the propeptide sequences nearest the NH_2-terminal ends.

Although we have not quantitated the antibody in our antiserum, the relative ease in obtaining these antibodies suggests that protropocollagen is a more potent immunogen than tropocollagen. The data also indicate that major interspecies differences between protropocollagen molecules are likely to be in sequences near the NH_2-terminal ends of the intact pro α chains. Antibodies protropocollagen will facilitate studies on the synthesis, assembly, and intracellular transport of the pro α chains and should provide a means of rapid purification of protropocollagen and its propeptides. Whether such antibodies are produced in vivo in the course of pathologic reactions involving collagenous structures is an area for further study.

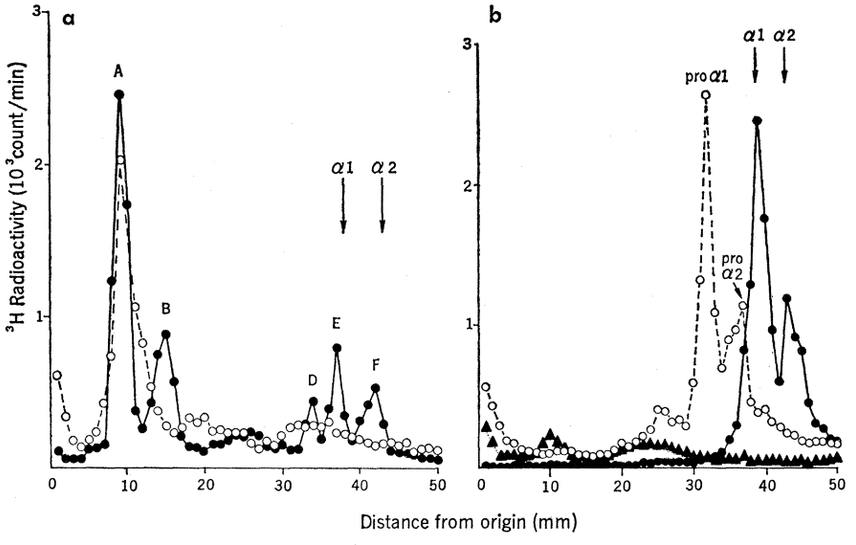

Fig. 2. Electrophoresis on SDS-polyacrylamide gels. (a) Medium labeled with [^{3}H]glycine and [^{3}H]proline was processed and run on gels as described (*3*). Peak A is protropocollagen. Denatured digestion intermediates are designed as follows: peak B, disulfide-stabilized dimer of pro $\alpha 1$; peak D, pro $\alpha 2$; and peaks E and F, shorter forms of pro $\alpha 1$ and pro $\alpha 2$, respectively (*3*). Antibody precipitations of labeled medium were performed with 40 μl of antiserum per milliliter of medium as described for Fig. 1, except that normal rabbit serum was eliminated. Washed precipitates were solubilized in 0.1*M* phosphate buffer, *p*H 7.1, containing 1 percent SDS and 0.5*M* urea and were run on gels in the same buffer (75 ma-hour per gel); ●, ^{3}H-labeled medium; ○, ^{3}H-labeled immune precipitate. Arrows indicate positions of ^{14}C-labeled α chain markers run as internal standards on the same gels. (b) (i) Immune precipitates were suspended in 0.05*M* tris-HCl buffer, *p*H 7.5, containing 0.14*M* NaCl and 0.001*M* $CaCl_2$ and treated with 100 μg of purified collagenase (Worthington, CLSPA grade) at 37°C for 16 hours (total protein, 2.6 mg in 1.5 ml). Digests were dialyzed against the buffer used for gel electrophoresis; ▲, nondialyzable ^{3}H radioactivity. (ii) Immune precipitates were solubilized in 0.5*M* acetic acid and digested with pepsin as described for Fig. 1. After pepsin was inactivated, digests were dialyzed against the buffer used for gel electrophoresis; ●, ^{3}H radioactivity. (iii) Immune precipitates were dissolved in 0.5*M* tris-HCl buffer, *p*H 8.4, containing 1 percent SDS and 0.5*M* urea, and were reduced with 0.025*M* dithiothreitol (Calbiochem) at 22°C for 60 minutes. Iodoacetamide (twice recrystallized from petroleum ether) was added to a concentration of 0.06*M*. After 60 minutes, the sample was dialyzed against electrophoresis buffer; ○, ^{3}H radioactivity.

Although we have not quantitated the antibody in our antiserum, the relative ease in obtaining these antibodies suggests that protropocollagen is a more potent immunogen than tropocollagen. The data also indicate that major interspecies differences between protropocollagen molecules are likely to be in sequences near the NH_2-terminal ends of the intact pro α chains. Antibodies protropocollagen will facilitate studies on the synthesis, assembly, and intracellular transport of the pro α chains and should provide a means of rapid purification of protropocollagen and its propeptides. Whether such antibodies are produced in vivo in the course of pathologic reactions involving collagenous structures is an area for further study.

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4. Seventeen confluent plates (3×10^6 cells per plate) of human diploid fibroblasts (CRL-1121, American Type Culture Collection) were placed on serum-free medium for 2 days with ascorbate supplement (75 μg/ml). The combined medium (136 ml) was made 0.5*M* in acetic acid, centrifuged at 20,000*g* for 20 minutes, dialyzed against distilled water, and lyophilized. The product was suspended in 0.05*M* tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl) buffer, *p*H 7.5, and stored at −20°C until used. Individual rabbits received approximately 0.5 mg of this material in complete Freund's adjuvant (Difco), divided among the four footpads. After 3 and 6 weeks, animals were given booster injections with the same amount of antigen without adjuvant, and pooled serums from two rabbits were prepared 1 week after the second booster.

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Hormonal Control of Sexual Morphogenesis in Achlya: Dependence on Protein and Ribonucleic Acid Syntheses

Abstract. *The induction of the male sexual organ primordia (antheridial hyphae) by the steroid hormone antheridiol in the water mold* Achlya ambisexualis *requires both transcription and translation. Inhibition of either of these processes eliminates the expected increase in the production and release of the enzyme cellulase, which accompanies the formation of the antheridial hyphae.*

The hormonal regulation of sexual morphogenesis in the water molds, especially in the genus *Achlya*, was revealed by Raper's experimental demonstrations of this system (*1*). The first morphological phase is initiated by the secretion of hormone (or hormones) by the vegetative female thallus and results in the induction of numerous lateral branches, called antheridial hyphae, on the male thallus. In a regular mating this sexually induced male secretes a hormone which induces the female thallus to produce oogonial initials. The antheridial hyphae then grow to the oogonial initials where they make physical contact and delimit an antheridial cell. As originally conceived, a complex of four hormones controlled the initiation of antheridial hyphae. The major hormone of this complex was called hormone A, and a highly concentrated extract from the culture medium was used for some chemical and physical characterization (*2*). The preparation of a crystalline compound has been described and named antheridiol (*2*), and a chemical structure has been proposed (*2*). Two possible isomers were synthesized, one with the same range of activity and physical properties as the natural extract (*2*). It is a normal tetracyclic steroid nucleus with a hydroxyl at C-3, a carbonyl at C-7, and a side chain at C-17 consisting of a ten-carbon chain in an α,β-unsaturated γ-lactone ring with three oxygen atoms. The chemical structure of sexual hormones in lower plants has been reviewed (*2*).

Thomas and Mullins (*3*) proposed that a prerequisite for the hormonal induction of these antheridial hyphae is a localized softening of the wall by the enzyme cellulase, which leads to the formation of lateral pegs by turgor pressure.

Although the structural formulas of sex hormones in lower plants have been studied (*2*), little is known about the synthesis of proteins and nucleic acids in terms of their relation to sexual differentiation. The requirements for the formation of antheridial hyphae should give direct information on processes required for this initial phase of sexual morphogenesis in a eukaryote. We now report on the question of whether protein or RNA synthesis, or both, are required for the hormonal induction of antheridial hyphae, and on their relation to the production of cellulase.

The strain E87 (male) of *Achlya ambisexualis* Raper was grown on a chemically defined medium (*4*). Mycelia in the early log phase of growth were obtained by inoculating zoospores into a nutrient medium and allowing these to grow for 48 hours at 24°C on a reciprocating shaker. The mycelia were then harvested, divided into equal lots, resuspended in the spent culture media (*3*), and equilibrated for 1 hour on a shaker at 24°C.

For experiments with inhibitors the mycelia were subjected to preliminary treatment with cycloheximide (final concentration, 1 μg/ml) for 1 hour or to preliminary treatment with actinomycin D (final concentration, 15 μg/ml) for 20 minutes. These inhibitors and the concentrations were chosen after tests were made on normal matings to find those concentrations which were fungistatic and would prevent antheridial hyphae production, but would not be fungicidal (*5*). Radioactive labeling was done by incorporation of a mixture of amino acids uniformly labeled with ^{14}C (specific activity, 1 mc/mg) into proteins (*6*), and by incorporation of uridine uniformly labeled with ^{14}C (specific activity, 160 mc/mmole) into RNA (*6*). The hormone antheridiol was a purified extract from culture media and was used at 5 or 15 unit/ml (*6*).

Protein was separated by a trichloroacetic acid method (*7*), dissolved in 2.0 ml of 0.1*N* NaOH, and analyzed by the Folin method and liquid scintillation counting (*7*).

For extraction of RNA, frozen mycelia were processed according to the method of Holdgate and Goodwin (*8*). The resulting powder was hydrolyzed in 0.5*N* KOH at 30°C for 12 to 15 hours. Protein, DNA, cell wall material, and $KClO_4$ were precipitated by acidifying the KOH digest to *p*H 2 with 1*M*

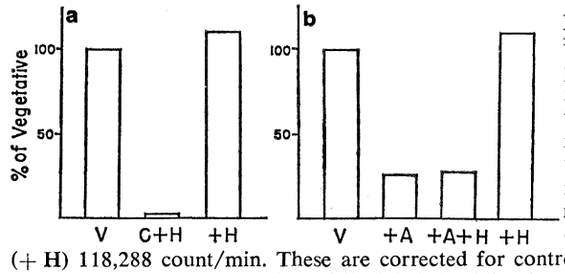

Fig. 1. (a) Incorporation of ^{14}C-labeled amino acids into protein (measured as counts per minute per microgram of protein from three separate experiments). The averages were: vegetative (V) 105,954 count/min; cycloheximide and hormone added (+ C + H) 1,025 count/min; and hormone added (+ H) 118,288 count/min. These are corrected for controls; the counting efficiency was 65.1 percent. (b) Incorporation of ^{14}C-labeled uridine into RNA (measured as counts per minute per microgram of RNA per minute of exposure to ^{14}C). The averages were: vegetative (V) 4.73; actinomycin added (+ A) 1.25; actinomycin and hormone added (+ A + H) 1.29; hormone added (+ H) 5.27. The V and + H values were from six separate determinations, and the + A + H values were from three separate determinations. The results were corrected for control counts, with a counting efficiency of 60 percent.