PROCOLLAGEN SYNTHESIS IN CELL CULTURE: NASCENT CHAIN POPULATION CONSISTENT WITH POLYCISTRONIC mRNA

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SUMMARY

The majority of the collagen-synthesizing polyribosomes have been isolated from homogenates of a clonal strain of dermatosparactic cells by means of differential centrifugation. These polyribosomes contained nascent procollagen chains approximately the size of pro-α1, pro-α2, θ chain (155,000 Daltons) and, most importantly, larger molecular species (greater than 300,000 Daltons). By using antibodies directed against the NH_2-terminal procollagen extension, the same population of nascent chains was specifically immunoprecipitated with the polyribosomes. The relative abundance of the largest nascent chains remained the same as observed in total polysomes. These results suggest that procollagen may be synthesized as a large molecular weight polypeptide and that there may be discrete steps in the formation of this large polypeptide.

INTRODUCTION

The heritable connective tissue disease in cattle, called dermatosparaxis, is probably caused by the lack of an active processing enzyme, procollagen peptidase (1, 2). Fibroblasts from the skin of a dermatosparactic calf have been isolated and established as clonal strains in culture (3). These cells secrete procollagen into the medium in the form of two distinct molecules, which are also present in extracellular fibers (4). One of the molecules is composed of pro α1 and pro α2 chains, as well as a larger polypeptide, θ chain, while the other molecule is composed only of pro α1 chains and the θ chain (4, 5). Both procollagen molecules also contain non-collagen peptide extensions at the NH_2 and COOH terminii (5, and unpublished), consistent with a model having intermediate forms which have been processed from a single large polypeptide

containing all three collagen chains linked together. The original model also predicted that such a polypeptide would contain alternate collagen and non-collagen regions (6). The present report describes a further test of this model, by examining the size of the procollagen nascent chains, using specific antibodies directed against the NH_2-terminal procollagen extension. The results suggest that procollagen may first be synthesized as a high molecular weight species.

MATERIALS AND METHODS

Preparation of Polysomes. Clonal cell strains (CD) were maintained as previously described and were grown in Dulbecco's Modified Eagle Medium (3). Cells were incubated with ^{3}H-pro for 30 min (20 µCi/ml). They were harvested, homogenized and the homogenate was fractionated as described (7). The polysome pellet (P-15) was resuspended in 10 ml of homogenizing buffer (0.25 M sucrose, 5 mM $MgCl_2$, 2 mM DTT, 0.1 mM EDTA, pH 7.4) and was dispersed by adding 1 ml of 5% Triton-X-100 and 2% sodium deoxycholate. The released polysomes were partially purified by discontinuous sucrose gradient centrifugation as described by Shafritz (8) with a slight modification in that centrifugation was performed in an SW 27.1 rotor (Beckman) at 95,400 x g for 18 hrs. The polysomes were pelleted at the bottom of the centrifuge tube, as determined by resuspending them and measuring the ratio of absorbance at 280 and 260 nm (9).

Analysis of Polysomes for Radioactive Proteins. A portion of the resuspended polysomes was reduced and alkylated in 8 M urea by standard methods (10). The reduced and alkylated polysome products were extensively dialyzed vs. H_2O and lyophilized. Following lyophilization, the material was dissolved in 50 µl of 0.2 N NaOH, neutralized and digested with purified protease-free bacterial collagenase under optimal conditions as described by Peterkofsky et al. (11).

Antisera. Antisera to purified procollagen isolated from dermatosparactic

calf skin were produced in 6 adult rabbits. They received a subcutaneous injection of 0.25 mg dermatosparactic procollagen dissolved in 0.05% acetic acid and mixed with an equal volume of Freund's complete adjuvant. Two weeks later a subcutaneous injection of 0.25 mg antigen mixed with Freund's incomplete adjuvant was given. The antisera contained a monospecific antibody to procollagen, as judged by double immunodiffusion. The antibody titers were determined by a quantitative precipitation technique (12). The antibodies cross-reacted with both procollagens isolated from the culture medium of dermatosparactic cells (5, and unpublished). Immune γ-globulin was isolated from the antisera by standard methods (12).

Immunoprecipitation of Polysomes. Polysome preparations at concentrations of 12 A_{260} units per ml in 0.1 M NaCl, 0.5% Triton-X-100 and 0.2% sodium deoxycholate were used. 250 µl samples were incubated with specific antibody, antigen and specific antibody in a molar ratio of 1:1.2:3.4, respectively, as described by Palmiter et al. (9). The reactions were carried out at 0-4° C and additions were made sequentially.

Determination of Nascent Procollagen in Polysomes and Immunoprecipitates. The reduced and alkylated samples were redissolved as noted above. Collagenase digestion was done on a portion of each sample. Control and enzyme-treated samples were subjected to electrophoresis in a 4% polyacrylamide gel in the presence of SDS (4). Reference gels containing ^{3}H-proline labeled procollagen, which had previously been purified from the culture medium, were run in parallel with the samples. All gels were fractionated by means of a Gilson Gel Fractionator. Only the top 5 cm of the 10 cm gel were analyzed.

RESULTS

Short-term incorporation of ^{3}H-proline into collagenous proteins in CD cells demonstrated a progressive increase with the number of days in culture (Fig. 1); as shown, the number of cells in these cultures paralleled this increase. The polysome

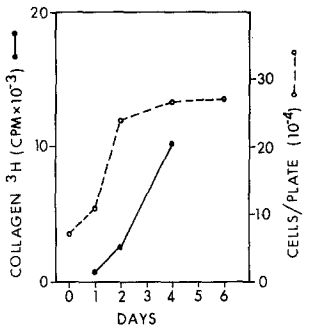

Fig. 1 The incorporation of ^{3}H-proline into collagenase-digestible cellular proteins as a function of days in culture. Procedure was that of Peterkofsky (1972).

Table 1. Distribution of Collagenase-Digestible Proteins Following Centrifugation at 15,000 x g.

Fraction	Radioactivity[+] CPM	Distribution
Supernatant (S-15)	183	16.4%
Precipitate (P-15)	934	83.6%

[+]Values are corrected for parallel control incubation (no enzyme).

Table 2. Collagenase-Digestible Proteins of Membrane-Bound Polysomes.

	^{3}H-Pro content of polysomes cpm	Collagenase digestible[+] cpm	Procollagen content %
Exp. 1	14410	4675	32.4
Exp. 2	47080	16995	36.1

[+]Values are corrected for parallel control incubation (no enzyme).

experiments were carried out using cells harvested at day 4 or day 6 when the number of cells was constant. The majority of the collagen-synthesizing polyribosomes was found to sediment at 15,000 x g (Table 1). Approximately 84% of the total collagenase-

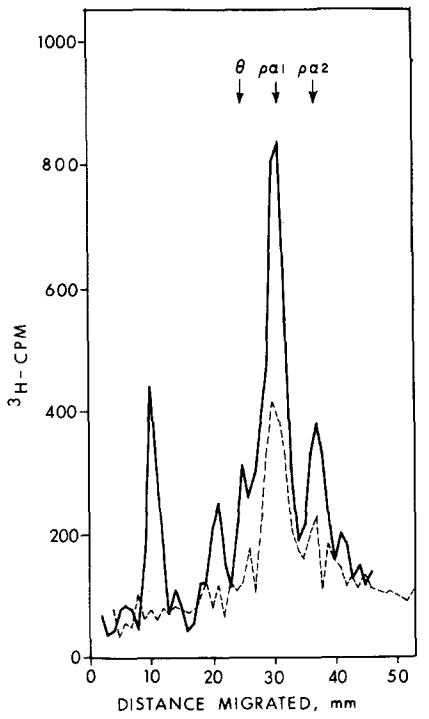

Fig. 2 SDS-polyacrylamide gel electrophoresis of the large ^{3}H-proline labeled proteins present in the membrane-bound polyribosomes (P-15 fraction, see text). ____, intact proteins, _ _ _ _, treated with collagenase. Molecular weight markers were the pro α1 and pro α2 chains (120,000 Daltons) and θ chains (155,000 Daltons) of purified calf dermatosparactic procollagen isolated from cell cultures.

digestible radioactivity in the cytoplasmic fraction was present in the pelleted polysome-containing vesicles, consistent with the results of Diegelman et al. (7). Collagenase-digestible proteins comprised at least 34% of the total ^{3}H-proline labeled material in these polyribosomes (Table 2). Examination of the larger polypeptide chains by SDS-polyacrylamide gel electrophoresis, following reduction and alkylation, showed that some of them were about the size of the reference procollagen polypeptides (Fig. 2); these are the peaks denoted as pro α1, pro α2 (both about 120,000 Daltons) and θ chain (about 155,000 Daltons). Prior digestion with collagenase (Fig. 2) showed that: /1/ about 50% of the radioactivity migrating at the pro α1 position was resistant to collagenase, /2/ pro α2 and θ were destroyed by the enzyme, and /3/ a radioactive

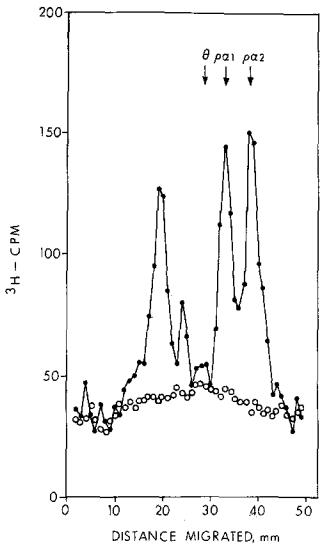

Fig. 3 SDS-polyacrylamide gel electrophoresis of ^{3}H-proline labeled proteins from polysomes which had been immunoprecipitated with specific procollagen antiserum. Similar results obtained with immune γ-globulin. Results are from 6 day old cultures. Molecular weight markers were identical to those in Fig. 2. ____, intact proteins, _ _ _ _, treated with collagenase.

peak considerably larger than θ was destroyed by collagenase digestion.

The polyribosomes were released from the membrane vesicles by detergents and were then partially purified by centrifugation on a discontinuous sucrose gradient. In experiments similar to those of Table 2, approximately 35% of the nascent chains of these polysomes were digested by collagenase. Immunoprecipitation of the polysomes, using antiserum to the NH_2-terminal extension of procollagen, yielded about 38% of the radioactivity in the pellet, while about 5-10% of the radioactivity was precipitated by preimmunization serum. Purified immune γ-globulin, obtained from the antiserum, precipitated about 26% of the radioactivity. The population of large collagenase-sensitive chains in the SDS-polyacrylamide gels (Fig. 3) was similar to that in the membrane-bound polyribosomes (Fig. 2). The major difference seemed to be the relative amount of radioactivity in the pro α1 region was reduced in Fig. 3, consistent with the elimination of a non-collagen nascent chain (Fig. 2), by use of the

specific antiserum. As seen in Fig. 3, all of the peaks were destroyed by prior incubation with the purified collagenase. Of particular interest in Figs. 2 and 3 are the collagenase-sensitive polypeptides larger than θ chains, especially the prominent peak at 2 cm (Fig. 3).

DISCUSSION

The results in Fig. 1 indicate that the older cultures, which contain more cells at confluence, incorporate a much greater amount of ^{3}H-proline into collagen-related proteins than the young cultures. Thus, CD cells appear to differ from other cultured cells in this regard (13).

Diegelman and coworkers (7) have previously shown that the fractionation of membrane-bound polyribosomes by differential centrifugation pelleted most of the procollagen nascent chains at relatively low centrifugal forces; similar results are reported in the present studies, using CD cells as the source of polyribosomes. Of interest is the finding that a considerable proportion of the large, proline-labeled nascent chains in this fraction are collagen-related, including a specific class of polypeptides larger than 300,000 Daltons.

Theoretically, procollagen nascent chains of all sizes should occur on the polyribosomes and it is not apparent why only a limited number of smaller distinct sizes corresponding to pro α1, pro α2 and θ as well as the very large polypeptide are present. The results of the immunoprecipitation studies strongly reinforce this observation and indicate that the procollagen nascent chains all contain similar, if not identical, antigenic sites. This is consistent with the known specificity of the antiserum, which is primarily directed against the NH_2-terminal procollagen extension (14) and which cross-reacts with both procollagens produced by dermatosparactic cells in culture (5, and unpublished). Thus, in CD cells, all procollagen nascent chains containing intact NH_2-terminal extensions would be

expected to bind the antiserum. A possibility for the observation of pro $\alpha 1$, pro $\alpha 2$, and θ chains associated with polysomes is that intracellular non-nascent procollagen is binding to the RNA in the polysome complex as an artifact of the homogenization and fractionation procedure (15, 16).

We interpret the present results to indicate that: /1/ the initial translational product of procollagen mRNA is a polypeptide larger than 300,000 Daltons; /2/ there may be discrete steps in the formation of this polypeptide (possibly accounting for the presence of pro α and θ chains associated with the polysome complex); /3/ the secreted procollagen contains polypeptides which are smaller than the initial translational product (4, 5). This scheme is consistent with the translation of a polycistronic mRNA for procollagen and it is possible that there may be rate-limiting steps during translation, possibly accounting for the specific intermediate-size polypeptides observed. That the very large (greater than 300,000 Daltons) collagenase-sensitive polypeptide is an aggregate of pro α chains or caused by intramolecular crosslinks is ruled out by the use of BAPN in the medium and by reducing and alkylating the protein before analysis. This last point was checked by control studies in which the times of reduction and alkylation were each doubled and the incubation temperature was raised to 37°; no change in the nascent chain population was detected, either qualitatively or quantitatively.

This model of procollagen biosynthesis is consistent with recent data which indicate that larger forms of the protein can be isolated when proteolysis is minimized (17-20). The present study was designed to circumvent the problem of proteolysis and to detect the initial size of the procollagen translational product; we believe that the data support a polycistronic procollagen mRNA.

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