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IN VITRO TRANSLATION OF CHICKEN TYPE X COLLAGEN IN THE PRESENCE OF PANCREAS MICROSOMES

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Total RNA from epiphysis of 17-day-old chick embryo tibiae was used to direct protein synthesis in a wheat germ cell free system. The type X collagen chain, identified on the basis of its electrophoretic migration and of peptides obtained by S. aureus V8 protease digestion, was the major translation product. The newly synthesized chain included a signal sequence that was removed when dog pancreas membranes were added at the time of the protein synthesis.

After the first detection of type X collagen in cultures of chick embryo tibial chondrocytes (1,2) and of sternal chondrocytes grown within collagen gel (3) an increasing number of reports have focused on this particular molecule. Although its role is still under debate, it is widely accepted that type X collagen is a specific marker of endochondral chondrocytes at late stages of differentiation; it is infact specifically synthesized by hypertrophic chondrocytes in the regions where cartilage is removed and replaced by bone (4,5). In culture of chondrocytes type X collagen is found in a form composed of three identical chains with an apparent size of 64,000 daltons ; after limited pepsin digestion it is converted to a form composed of chains of 45,000 daltons.(6,7) Type X collagen has been purified in quantities sufficient for biophysical and biochemical analysis

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from hypertrophic cartilage by limited pepsin digestion in the 45,000 form (8,9) and from chondrocytes culture medium in the 64,000 form (6). The 64,000 form has both a collagenous and a non collagenous domain (10).The non collagenous domain, located at one end of the molecule, is absent after limited pepsin digestion. Since the type X chain is approximately half the length of known interstitial collagens, it has been questioned whether the 64,000 form was a breakdown product of a larger molecule. Pulse-chase labeling experiments performed in our laboratory strongly suggested that the type X collagen was synthesized directly as 64,000 form (1,8). Here we report that the primary translation product of the messenger RNA for type X collagen is indeed slightly larger than the corresponding molecule recovered from the culture medium. The polypeptide synthesized in vitro has, most probably at the amino terminal, a signal sequence which is cleaved when the translation is made in the presence of dog pancreas membranes.

MATERIALS AND METHODS

Total RNA from the epiphysis of 17-day-old chick embryo tibiae was isolated by a guanidine extraction method (11).Preparation and properties of the wheat germ translation system (12) and the composition of the incubation mixture (13) have been already described. When indicated, dog pancreas membranes (14) at 3.3 A_{280} / ml were added to the incubation mixture at the beginning or during the incubation. In the same experiments, at the end of the incubation, collagenase (type VII, Sigma) was added at a final concentration of 12 µg/ml and the incubation continued at 37° C for 2 hours either in the presence or in the absence of 2% Triton X 100.

Chondrocytes were cultured according to (1). Labeled collagen from culture medium was prepared by preincubation of the cells for 2 hours in methionine-free medium containing 1% fetal calf serum and subsequent addition of [^{35}S] methionine at a concentration of 100 µCi/ml. After 2 hours the medium was collected and clarified by low speed centrifugation. Where indicated α-α' dipyridyl at a final concentration of 5 mM was added to the medium during starvation and labeling periods.
SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (15) and modified by Bonatti and Descalzi-Cancedda (16) under reducing conditions. Gel concentration was 9%.
To compare S. aureus V8 proteolytic peptides, [^{35}S] labeled collagen synthesized in vitro and from cell culture medium was

purified from a preparative SDS polyacrylamide gel; the unfixed dried gel was autoradiographed and the gel portion corresponding to type X collagen was electroeluted in the presence of 100 µg of type I collagen as carrier. The digestion of collagen with S. aureus V8 protease was performed as described (17) except that the enzyme substrate ratio was 1:10. Peptides were analysed on a 10-20 % gradient polyacrylamide gel.

RESULTS AND DISCUSSION

We have previously shown that mRNAs from epiphyses of 17-day-old chick embryo tibiae direct the translation in a wheat germ cell free system in the presence of [^{3}H] proline of a major collagenase sensitive protein identified as type X collagen (13). The same experiment was repeated in the presence of [^{35}S] methionine (not shown), and the identity of the type X collagen synthesized in vitro was definitively proven by electrophoretic comparison of the peptides obtained by S. aureus V8 protease digestion of the collagen synthesized in vitro and that purified from culture medium (Fig.1). The two patterns were clearly related, but we constantly observed that the bands in the lane of collagen

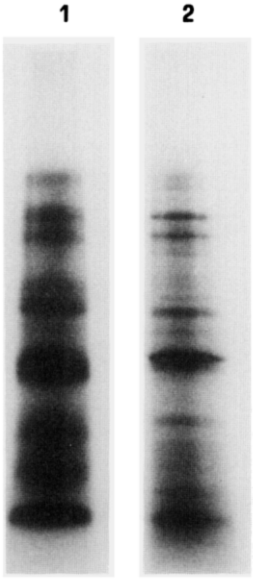

Figure 1). Electrophoretic analysis of the V8 protease peptides from the type X collagen purified from culture medium (lane 1) and the type X collagen synthesized in vitro (lane 2). For details see text.

purified from the medium were less sharp and had some trail. This is most probably due to the fact that the chains of the collagen released by the cells are partially hydroxylated while the chains synthesized in vitro are not.

In order to compare the migrations of type X collagens without interference of post-translational hydroxylations, we loaded onto the gel the proteins synthesized in vitro together with an

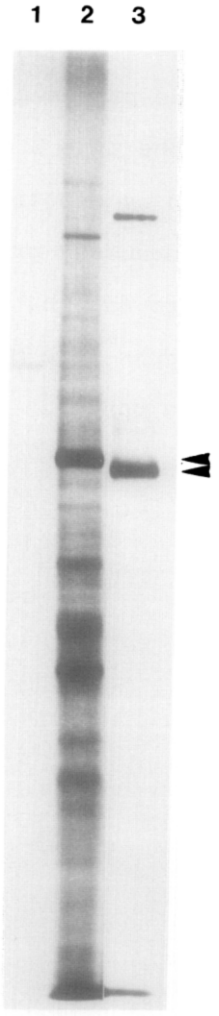

Figure 2). Gel electrophoresis of proteins synthesized in vitro and from culture medium. Lane 1) In vitro translation mixture with no RNA added. Lane 2) In vitro translation mixture supplemented with RNA extracted fom epiphysis. Lane 3) Culture medium of cell labeled for 2 hours in the presence of $\alpha - \alpha'$ dipyridyl. Arrowheads refer to type X collagen.

aliquot of medium from cells labeled in the presence of α-α' dipyridyl, a known inhibitor of collagen hydroxylation (18-19) (Fig.2). Once again the result was in agreement with the absence of a large precursor from which the 64,000 chain could be cleaved. Furthermore we observed that type X collagen from the medium moved slightly ahead of the collagen synthesized in vitro. The difference in the migration was compatible with a difference in the apparent size of about 1,500-2,000 daltons equivalent to 15-20 aminoacid residues.

It is known that the secretion of proteins involves the interaction of a 15-30 residue long amino acid signal sequence with the cell membranes. This sequence is usually located at the amino terminus of the protein and is cleaved in the rough endoplasmic reticulum during the secretion process (20). The difference in the migration between the type X collagen synthesized in vitro and the type X collagen from the culture medium could be explained if a signal sequence was present in the polypeptide chain synthesized in vitro, that did not pass the cell membrane barrier, and absent in the collagen secreted by the cell. To prove our hypothesis we performed the in vitro translation either in the absence or presence of dog pancreas membranes (14).

The results are shown in fig. 3. As predicted, when we added membranes in the cell-free system we observed a decreased molecular weight of the type X collagen synthesized (lane 5).The cleavage of the sequence occurred in the process of secretion. Digestion with collagenase of the incubation mixture containing membranes resulted in the protection of the type X collagen (lane 6); there was no protection when the digestion was carried out in the presence of Triton X 100 (lane 7) or when the incubation mixture did not contain membranes (lane 4). The sequence was not

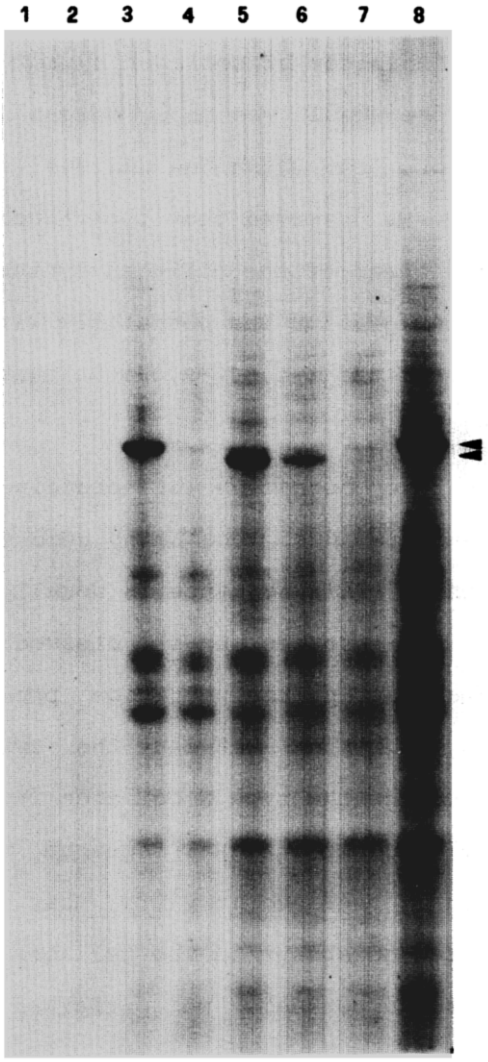

Figure 3). In vitro translation in the presence of dog pancreas membranes. No RNA added (lanes 1-2). RNA from epiphysis added (lanes 3-8). Dog pancreas membranes were added at the beginning (lanes 2,5-7) or after 1 hour incubation (lane 8). At the end of the incubation same samples were further incubated for 90 min. at 37° with a final concentration of 12 ug/ml collagenase in the absence (lanes 4,6) or in the presence (lane 7) of 1% triton X 100. Arrowheads refer to type X collagen.

cleaved if the membranes were added 1 hour after the initiation of the translation, when the synthesis of all the chains was already stopped (lane 8).

The results of this report are in excellent agreement with recent data obtained in the laboratory of B. Olsen (Harvard Medical

School, Boston). Sequencing analysis of a genomic clone of chicken type X collagen revealed the presence of a hydrophobic sequence resembling a typical signal sequence located at the amino terminus of the molecule (21).

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