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Detection of Cell-Associated Aminoterminal Procollagen Peptidase Activity in Cultured Fibroblasts (41068)

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Abstract. Aminoterminal procollagen peptidase activity was assayed in cultures of human fibroblasts derived from fetal, neonatal, and adult skin. Tritium-labeled, acid-extracted pN collagen from chick calvarial bones was used as substrate. Peptidase activity was detected in both the culture medium and the cell homogenate of fetal and neonatal fibroblast strains. The cell layer and medium peptidase enzymes were inhibited by 20 m*M* EDTA and acidic pH. In contrast, procollagen peptidase activity was detected only in the culture medium of adult fibroblasts as previously reported (8). These results suggest that the aminoterminal procollagen peptidase enzyme in fetal and neonatal fibroblasts is either activated prior to secretion from the cell, or that the activated enzyme is bound to the cell surface for a limited time before its release into the culture medium.

Collagen is synthesized as a procollagen precursor containing propeptide extensions at both the amino- and carboxyterminal ends of the molecule (1, 2). These propeptides are relatively large and it has been supposed that they must be removed before stable collagen fibers can be formed (3). A procollagen peptidase that cleaves the aminoterminal propeptides from procollagen has been found in bone, skin, tendon, and the medium of cultured fibroblasts (4–9). Evidence for the presence of a separate carboxyterminal procollagen peptidase has been indicated by observations that both pN collagen (containing only the amino propeptides) and pC collagen (containing only the carboxypropeptides) could be isolated from cell and organ cultures (10–12), and that tunicamycin inhibited the conversion of pC collagen, but not pN collagen, to collagen (13). The propeptides are removed in a sequential manner; the aminopropeptides are cleaved first, at or near the cell surface, followed by cleavage of the carboxypropeptides at some later time (10, 14). Layman and Ross (8) previously reported that essentially all of aminoterminal procollagen peptidase activity was secreted into medium of cultured adult fibroblasts and that little or no activity was associated with the cell layer. The enzyme was inhibited by 20 m*M* EDTA and acidic pH of 4.0. In the present study significant aminoterminal procollagen peptidase activity was associated with the cell layer, as well as the medium of confluent cultures of fetal and neonatal skin fibroblasts.

Materials and Methods. *Cell culture.* Fibroblasts were obtained from explants of fetal (second trimester), neonatal, and adult (35 years) human skin taken at routine autopsy. Explants were grown in McCoy's modified medium (Flow Laboratories) containing 20% human serum, 200 units/ml of penicillin and 200 μg/ml of streptomycin. Cells that grew out from the explants were subcultured by trypsin–EDTA dissociation in 250-ml tissue culture flasks containing McCoy's medium with 10% human serum. Confluent cultures of fibroblasts from the third to eighth subculture and containing 5 $\times$ 10^6 cell/flask were assayed for aminoterminal procollagen peptidase activity.

Assay of aminoterminal procollagen peptidase activity. The aminoterminal procollagen peptidase activity in the culture medium was concentrated by adding an equal volume of cold saturated $(NH_4)_2SO_4$ solution, pH 7.5, to medium which had been exposed for 48 hr to confluent fibroblasts. The precipitate was collected by centrifugation at 10,000*g* for 10 min, dissolved in 2 ml of 0.15 *M* NaCl, 0.05 *M* Tris

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buffer, pH 7.5, and dialyzed against the same buffer overnight at 4°. These medium preparations were assayed for enzyme activity.

Cells (5×10^6 cells/flask) were washed twice with the neutral salt buffer, and then scraped from the flasks with 2 ml of fresh neutral salt buffer. Cell suspensions were homogenized in a glass homogenizer and then dialyzed against cold neutral salt solution overnight at 4°. These cell homogenate suspensions were assayed for procollagen peptidase activity.

Labeled procollagen substrate was prepared from calvaria bones of 17-day chick embryos as described by Bornstein *et al.* (4). Following an 18-min incubation with 20 μCi/ml each of L-[^{3}H]proline and [^{3}H]-glycine, the labeled procollagen was extracted from the calvaria bones with 20 ml of cold 0.5 *M* acetic acid containing 50 m*M* EDTA by a brief homogenization using a glass homogenizer and vigorous stirring for 2 days at 4°. This homogenate was centrifuged at 12,000*g* for 15 min and the supernatant obtained was used as substrate after overnight dialysis against the neutral salt buffer. This procollagen substrate was judged to be pN collagen since it did not contain disulfide bonds and was composed of alpha chains and having a molecular weight of 125,000 daltons by gel filtration on 6% agarose. [^{3}H]pN α1 chains were isolated by carboxymethyl cellulose (CMC) chromatography of heat-denatured [^{3}H]pN collagen. CMC chromatography was performed on heat-denatured procollagen as described by Piez *et al.* (15), except that the starting buffer contained 0.02 ionic strength sodium acetate and 1.0 *M* urea, pH 4.8. Radioactivity of the column eluant was assayed using aquasol and a liquid scintillation spectrometer.

The standard enzyme assay consisted of 2.0 ml of the [^{3}H]pN collagen substrate containing $2-3 \times 10^5$ cpm and 2.0 ml of the procollagen peptidase preparation from medium or cell layer at pH 7.4. Incubations were performed overnight at 18–20°. The reaction was stopped by dialyzing the incubation mixtures against the starting buffer used for chromatography. After the addition of 15 mg of lathyritic rat skin collagen to mark the position of the α1 and α2 chains, each sample was heat denatured at 60° and then chromatographed on CM-cellulose. Aminoterminal procollagen peptidase activity was qualitatively determined by the relative conversion of pN α1 chains of native pN collagen to α1 chains of collagen because these chains were readily separated by CMC chromatograpy.

Results. *Specificity of aminoterminal procollagen peptidase activity.* Previous studies by Layman and Ross (8) have shown that aminoterminal procollagen peptidase activity was detectable only in the medium of cultured adult fibroblasts; the cell layer contained little or no activity. However, in extending our studies to fetal and neonatal fibroblasts, enzyme activity was detected in both the medium and cell layer of these fibroblasts.

In order to determine several parameters of the aminoterminal procollagen peptidase from cultured fibroblasts, enzyme preparations from the culture medium or cell layer were incubated under various conditions and in the presence of native [^{3}H]pN collagen. The results of these incubations are shown in Fig. 1 and Table I.

As shown by the chromatogram illustrated in Fig. 1A, when native [^{3}H]pN collagen was incubated with medium buffer alone little or no conversion of pN collagen occurred. In contrast, native [^{3}H]pN collagen was converted to collagen by the medium aminoterminal procollagen peptidase (Fig. 1B). Similar chromatograms were obtained for the cell layer enzyme. These results are tabulated in Table I. Control and experimental α1/pN α1 ratios were obtained by incubating [^{3}H]pN collagen with medium buffer alone or with medium or cell layer enzyme preparations, respectively, and then dividing the area under the radioactive α1 peak by the area of the pN α1 peak. Aminoterminal procollagen peptidase activity is quantitatively expressed as a ratio of the experimental to control α1/pro α1 ratios. In the presence of procollagen peptidase activity this ratio will be greater than 1, in the absence of conversion this ratio will be approximately 1.

As shown in Table I, both the medium and cell layer enzyme were inhibited by

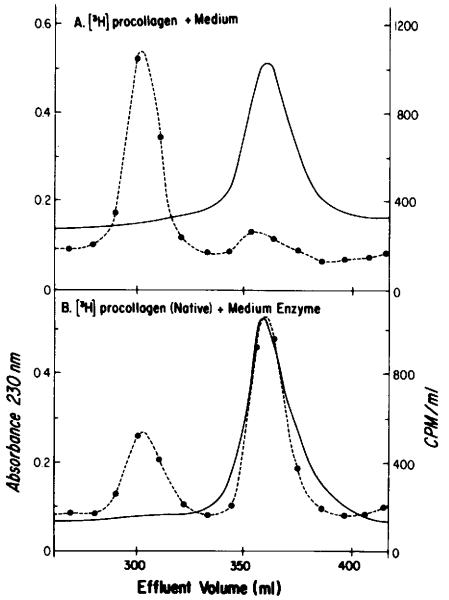

FIG. 1. Carboxymethyl cellulose chromatograms showing only the elution region of [^{3}H]pN α1 chains (dotted line, cpm) and α1 chains of the carrier collagen (solid line, absorbance) (A) [^{3}H]pN collagen incubated with the medium buffer, pH 7.5, alone (B) [^{3}H]pN collagen incubated with enzyme prepared from medium after exposure to cells for 48 hr. Incubations carried out at 20°, pH 7.5 for 12 hr. Chromatography was performed at 43° with 0.02 ionic strength sodium acetate buffer containing 1 *M* urea, pH 4.8. Separation of the collagen chains was achieved using a linear 0.0 to 0.11 *M* NaCl gradient over total volume of 600 ml.

acidic pH and 20 m*M* EDTA. No conversion of [^{3}H]pN collagen occurred when human serum was incubated with the substrate, indicating that the fibroblasts, and not the serum, were the source of the enzyme activity (not shown). These results are similar to what has been described for aminoterminal procollagen peptidase from other sources (2).

Aminoterminal procollagen peptidase activity in cultures of confluent fibroblasts. The conversion of [^{3}H]pN collagen to collagen by confluent cultures of fetal, neonatal, and adult fibroblasts is shown in Table II. Although variable in amount, enzyme activity was detected in both the medium and cell homogenates of only the fetal and neonatal fibroblast strains. Quantitatively, aminoterminal procollagen peptidase activity appeared to be greater in cell homogenates than in the culture medium. Moreover, peptidase activity appeared to be greater in cultures of fetal and neonatal fibroblasts than adult fibroblasts as judged by the total conversion of pN collagen to collagen.

Previous studies have shown that lysyl oxidase (17) and aminoterminal procollagen peptidase activities (8) were detected only in cultures of adult fibroblasts at the end of the logarithmic phase of cell proliferation. To determine when aminoterminal procollagen peptidase appeared during the growth of fetal fibroblasts, medium and cells were assayed for activity at various times after subculture. Peptidase activity was not significant in the medium during the early log phase of fetal cell proliferation, but as the fibroblasts became confluent enzymatic activity accumulated rapidly. These results

TABLE I. SUBSTRATE SPECIFICITY OF AMINOTERMINAL PROCOLLAGEN PEPTIDASE FROM MEDIUM AND CELLS OF CULTURED FETAL FIBROBLASTS

Incubation mixture	Control (α1/pro α1)	Experimental (α1/pro α1)	Experimental/control (α1/pro α1)
Medium			
[^{3}H]Procollagen + buffer, pH 7.4	0.22	—	—
[^{3}H]Procollagen (native) + enzyme	—	1.04	4.7
[^{3}H]Procollagen + enzyme, pH 4.0	—	0.26	1.2
[^{3}H]Procollagen + enzyme, 20 mM EDTA	—	0.20	1.0
Cells			
[^{3}H]Procollagen (native) + buffer, pH 7.4	0.21	—	—
[^{3}H]Procollagen (native) + enzyme	—	2.39	11.4
[^{3}H]Procollagen + enzyme, pH 4.0	—	0.23	1.1
[^{3}H]Procollagen + enzyme, 20 m*M* EDTA	—	0.24	1.1

Note. For incubation conditions see Materials and Methods.

TABLE II. AMINOTERMINAL PROCOLLAGEN PEPTIDASE ACTIVITY IN CULTURES OF CONFLUENT HUMAN FIBROBLASTS

Enzyme source	Control (α1/pro α1)	Experimental (α1/pro α1)	Experimental/control (α1/pro α1)
Fetal			
Cell layer	0.52	4.05	7.8
Medium	0.52	1.57	3.0
Cell layer	0.20	1.16	5.8
Medium	0.20	0.93	4.7
Neonate			
Cell layer	0.91	4.63	5.1
Medium	0.91	1.41	1.6
Cell layer	0.52	4.70	9.0
Medium	0.52	1.37	2.6
Adult			
Cell layer	0.33	0.36	1.1
Medium	0.33	1.39	4.2
Cell layer	0.60	0.65	1.1
Medium	0.60	1.42	2.4

Note. Aminoterminal procollagen peptidase activity in cultures of confluent fetal, neonatal, and adult fibroblasts.

are similar to those previously reported for cultures of adult fibroblasts (8). Because peptidase activity was not detected in the medium and since the number of cells was low, an assay for cell layer peptidase activity was not performed during the early logarithmic phase of cell proliferation. Thus, the possibility remains that a low level of peptidase enzyme may have been bound to the cells and was not released into the medium during rapid cell proliferation.

Discussion. The results shown here demonstrate the presence of aminoterminal procollagen peptidase activity in the medium and cell layer of cultured fetal and neonatal skin fibroblasts. Presumably, the cell layer and medium peptidase activities represent the activity of one peptidase enzyme, because both enzyme activities were inhibited by acidic pH of 4.0 and 20 m*M* EDTA. Moreover, the conversion of pN collagen to collagen was directly proportional to the amount of enzyme added to a constant amount of substrate, which further indicated that a specific aminoterminal procollagen peptidase was measured (not shown).

These observations suggest that fetal and neonatal cells either contain an active intracellular aminoterminal procollagen peptidase enzyme, or retain the active enzyme on their cell surface for a limited time before releasing it into the culture medium. It appears unlikely that fetal and neonatal cells contain an active intracellular peptidase enzyme because of the observation

TABLE III. AMINOTERMINAL PROCOLLAGEN PEPTIDASE ACTIVITY DURING CELL PROLIFERATION OF FETAL FIBROBLASTS

Enzyme source	Days after plating	Control (α1/pro α1)	Experimental (α1/pro α1)	Experimental/control
Medium	3	0.58	0.41	0.71
Medium	7	0.17	0.23	1.15
Medium	14	0.36	0.93	2.58
Cell Layer	14	0.17	1.16	6.81

Note. The appearance of aminoterminal procollagen peptidase activity during cell proliferation of fetal fibroblasts.

of segment-long-spacing (SLS) crystallites of procollagen within secretory vacuoles of collagen-synthesizing embryonic fibroblasts (9), and because of the proposed function of the propeptides to inhibit intracellular fiber formation. What appears to be more likely is that the enzyme is synthesized as an inactive precursor, which upon secretion from the cell is activated. Instead of secreting the enzyme directly into the medium as do adult cells, fetal and neonatal cells retain the active peptidase enzyme on their cell surface for a limited time before releasing it into the culture medium. This supposition is supported by observations which have shown that procollagen is converted to collagen on or at the surface of embryonic chick calvaria bone cells (16), and by Bruns *et al.* (9), who have shown SLS crystallites of procollagen directly associated with membraneous components of embryonic chick fibroblasts in culture.

It is unclear why aminoterminal procollagen peptidase activity should be present in the cell layer of fetal and neonatal fibroblasts and absent in the cell layer of adult fibroblasts. A possible explanation may be that in rapidly growing embryos where collagen synthesis and remodeling are high, an efficient mechanism for collagen fiber orientation and deposition may be required. This could be accomplished directly by cells provided they had the ability to control the conversion of procollagen to collagen on, or at the cell surface. As the procollagen molecules and, presumably, the inactive peptidase enzymes were secreted from vacuoles into the extracellular space, they would be momentarily bound to the cell surface. Activation of the aminoterminal peptidase, conversion of procollagen, and the subsequent spontaneous fibrillogenesis would take place at the cell surface along an orientation dictated by the cell. In mature animals where collagen turnover is relatively low, such an efficient cellular control of fiber formation may not be required and the newly secreted procollagen molecules would more readily diffuse away from the cell before being converted to collagen on or near existing fibrils.

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