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Biochemistry

Cathepsin D-mediated processing of procollagen: Lysosomal enzyme involvement in secretory processing of procollagen

(carboxyl propeptide cleavage/lysosomotropic agents)

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ABSTRACT **The proteolytic removal of the extension COOH-terminal propeptide from procollagen has been examined *in vitro*. A crude enzyme activity was identified in a whole-chicken-embryo extract that acted at acid pH and appeared to be similar to one identified previously [Davidson, J. M., McEneany, L. S. G. & Bornstein, P. (1979) *Eur. J. Biochem.* 100, 551–558]. This activity was inhibitable by pepstatin but not by leupeptin, suggesting that it might be cathepsin D. Cathepsin D was purified 907-fold from chicken livers by affinity chromatography on pepstatin-aminohexyl-Sepharose 4B and was found to remove the COOH propeptides from procollagen. At pH 6.0, the site of cleavage appeared to shift from the COOH telopeptide to the COOH telopeptide/propeptide junction, based upon the difference in electrophoretic migration of the cleavage products, although determining the actual cleavage site will require end-group analysis. A model for the involvement of cathepsin D in the *in vivo* processing of procollagen is presented.**

The enzymes responsible for the sequential removal of the extension propeptides from procollagen, procollagen NH_2-terminal proteinase and procollagen COOH-terminal proteinase, have been actively studied, but only the former has been well characterized (1). *In vivo* it appears that the COOH-terminal propeptide (COOH propeptide) is removed first, with the NH_2-terminal propeptide (NH_2 propeptide) removed later, possibly after the intermediate has assembled into extracellular fibrils (2–4). The site of COOH propeptide removal has not been well defined. In cell culture experiments, the apparent *in vivo* pathway is reversed. The COOH propeptide is not cleaved from the product that accumulates in cell culture media, yet conversion is complete in organ culture (5), suggesting that an intact extracellular matrix is required for conversion.

In searching for a neutral metalloproteinase that is a COOH-terminal proteinase (6), we observed a cathepsin D-like activity, which removed the COOH propeptide from procollagen at acid pH. We report here that the cleavage of procollagen with purified cathepsin D is specific for the COOH propeptide and that cleavage occurs at or near the authentic cleavage site when digestion is carried out near pH 6.0. We postulate a role for cathepsin D in the *in vivo* conversion of procollagen to collagen.

MATERIALS AND METHODS

Procollagen was isolated from 17-day chicken-embryo tendon fibroblasts (7, 8). After tendon dissection and dissociation with collagenase and trypsin, fibroblasts were incubated (2×10^7 cells per ml) for 6 hr in modified Krebs medium II in the presence of 1 μCi (1 Ci = 37 GBq) of a mixture of 15 ^{14}C-labeled amino acids (New England Nuclear) per ml. Procollagen and partially processed collagen from which the NH_2 propeptide was removed (pC-collagen) were precipitated from the medium with 30% $(NH_4)_2SO_4$ plus protease inhibitors (9), reprecipitated with 2.5 M NaCl in the presence of carrier type I collagen (1), and then separated on DEAE-cellulose (Whatman DE-53) in 50 mM Tris, pH 8.6/2 M urea (8). Peak fractions were pooled, dialyzed, precipitated with $(NH_4)_2SO_4$, and stored at −20°C in 0.4 M NaCl/0.1 M Tris/0.02% NaN_3, pH 7.50 (20°C).

The COOH propeptide was isolated from culture media of 17-day chicken-embryo leg tendons by chromatography on DEAE-cellulose and CM-cellulose (Whatman CM-52) (8). Purified peptide was desalted on Bio-Gel P-6 (Bio-Rad) in 0.2 M NH_4HCO_3 (pH 8.0), lyophilized, and stored at −20°C.

Cathepsin D Isolation. Cathepsin D was purified from frozen adult chicken livers (10). All work was done at 4°C. Thawed livers (1.5 kg) were homogenized in 1% NaCl/2% 1-butanol/10 mM Na_2EDTA, pH 6.5 (11), precipitated at pH 3.7, and then chromatographed on DEAE-cellulose in 50 mM sodium phosphate (pH 7.4) (10). The breakthrough peak on DEAE-cellulose was adjusted to pH 3.5 with HCl and stirred overnight with 15 g of pepstatin-Sepharose 4B, prepared from aminohexyl-Sepharose 4B by the method of Huang *et al.* (12). The settled bed was packed into a column and eluted with 50 mM NaOAc, pH 3.5/0.2 M NaCl until A_{280} returned to baseline. Cathepsin D was eluted with 50 mM Tris, pH 8.5/0.2 M NaCl. The peak of activity was dialyzed against distilled water and lyophilized.

Enzyme Assays. Cathepsin D activity was measured by the release of trichloroacetic acid-soluble peptides from hemoglobin at 45°C (13); 0.25 ml of 1 M sodium formate (pH 3.0) (20°C), 0.25 ml of 8% (wt/vol) hemoglobin (Sigma), and 0.5 ml of sample were mixed in 1.5-ml Microfuge tubes. Samples were incubated for 60 min at 45°C, and then 0.2 ml of cold 18% trichloroacetic acid was added. After incubation on ice for 5 min, samples were centrifuged for 5 min at 13,000 × *g*. One milliliter of supernatant was diluted with 5.0 ml of 3% trichloroacetic acid, and A_{280} was measured. Blanks were prepared by adding trichloroacetic acid immediately after adding the enzyme and also including 1 μg of pepstatin A (Sigma) per ml in the incubation.

The pH dependence of cathepsin D activity was determined by using a stock buffer of 0.6 M formic acid/0.6 M acetic acid/0.6 M KH_2PO_4, titrated to the final pH at 20°C. Substrates tested were 4% (wt/vol) acid-denatured hemoglobin dialyzed versus distilled water (14) and native procollagen in 0.4 M NaCl/0.1 M Tris, pH 7.5/0.02% NaN_3.

For assays with procollagen, the reaction was stopped by

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Abbreviations: kDa, kilodalton(s); Man-6-*P*, mannose 6-phosphate; NH_2 and COOH propeptides, extension propeptides at the NH_2 and COOH ends of procollagen; pC-collagen, partially processed procollagen from which the NH_2 propeptide has been removed; pN-collagen, partially processed procollagen from which the COOH propeptide has been removed; C-1 and C-2, subunits of the COOH propeptide arising from the pro-α1- and pro-α2-chains, respectively, of type I procollagen.

cooling to 4°C, adding pepstatin A (5 μg/ml), neutralizing with 3 M Tris (pH 7.4), adding 10× $NaDodSo_4$ sample buffer (15) and 5% 2-mercaptoethanol, and then boiling for 5 min. Reaction products were visualized by electrophoresis on either 6% or 12.5% polyacrylamide slab gels containing $NaDodSO_4$ (15), followed by fluorography. Gels were prepared for fluorography by using either 2,5-diphenyloxazole in glacial acetic acid (16) or sodium salicylate (17) and were exposed to preflashed (18) Kodak X-Omat AR film at −70°C.

RESULTS

A search for tissues and extraction conditions that would yield a neutral metalloproteinase capable of removing the COOH propeptide from procollagen proved unsuccessful, although appropriate levels of procollagen NH_2-terminal proteinase could be demonstrated in most cases. Homogenates of whole chicken embryo, extracts of chicken embryo calvaria and tendon, and tissue culture media from calvaria and tendon organ culture were tested. All of these represent sources where active enzyme should be abundant, based upon the rapid collagen processing observed (5, 19).

When the conditions of Weeks *et al.* (20) for extracting active collagenase were applied to the whole-chicken-embryo homogenate (0.1 M $CaCl_2$/50 mM Tris, pH 7.5/1 mM phenylmethylsulfonyl fluoride/0.02% NaN_3; extracted at 60°C for 5 min), activity was found that quantitatively converted procollagen to pN-collagen (procollagen minus the COOH propeptide) during an 18-hr *in vitro* assay at 37°C. This activity was seen only when EDTA was added and resulted from a pH shift upon Ca–EDTA complex formation, with the pH decreasing from 7.5 to 4.4 as observed by Davidson *et al.* (21). Acidification of the $CaCl_2$-containing mixture without EDTA produced the same effect, and titration of EDTA-acidified samples to pH 7.5 abolished activity. The activity at pH 4.4 could be completely inhibited with 1 μg of pepstatin A but not with 1 μg of leupeptin per ml. When the chicken-embryo extract was assayed for 12 hr at 37°C at either pH 4.4 or pH 6.0 in the presence of 1 mM phenylmethylsulfonyl fluoride, ≈95% conversion of procollagen to pN-collagen was seen (Fig. 1). Davidson *et al.* (21) isolated a similar acid proteinase activity from chicken tendon and fibroblast culture media.

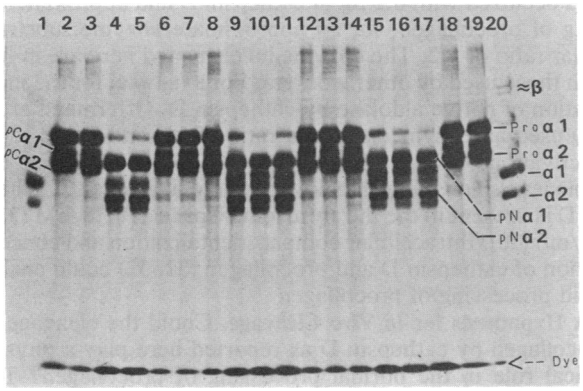

FIG. 1. *In vitro* assay of whole-chicken-embryo homogenate extracted with 0.1 M $CaCl_2$/50 mM Tris, pH 7.5, using procollagen uniformly labeled with a ^{14}C-labeled amino acid mixture (New England Nuclear) as substrate. Assay was for 12 hr at 37°C in the presence of 1 mM phenylmethylsulfonyl fluoride, followed by electrophoresis on a 6% acrylamide slab gel containing $NaDodSO_4$ and visualization with sodium salicylate fluorography (17). The reaction products formed are pN-collagen (pN) α1 and pNα2 chains, as well as some α1 and α2 chains arising from pC-collagen (pC) α1 and pCα2 in the substrate. All samples (except buffer blanks) contained 20 μl of extract and 3 μg of procollagen per 100-μl assay. Lanes: 1, type I collagen standard; 2, pH 7.5 buffer blank (0.15 M NaCl/50 mM Tris/5 mM $CaCl_2$); 3, extract assayed at pH 7.5; 4, extract in pH 7.5 buffer with $CaCl_2$ at 23 mM and 25 mM EDTA (pH 7.5), resulting in a pH shift to 4.4; 5, extract with EDTA and leupeptin (1 μg/ml); 6, extract with EDTA and pepstatin (1 μg/ml); 7, extract with EDTA and leupeptin and pepstatin (1 μg/ml); 8, pH 4.4 buffer blank (30 mM sodium acetate/1.4 mM dithioerythritol/0.7 mM EDTA); 9, extract assayed at pH 4.4; 10, extract at pH 4.4 with 25 mM EDTA (pH 7.5); 11, extract at pH 4.4 with leupeptin (1 μg/ml); 12, extract at pH 4.4 with pepstatin (1 μg/ml); 13, extract at pH 4.4 with leupeptin and pepstatin (1 μg/ml); 14, pH 6.0 buffer blank (30 mM sodium phosphate/1.4 mM dithioerythritol/0.7 mM EDTA); 15, extract assayed at pH 6.0; 16, extract at pH 6.0 with 25 mM EDTA (pH 7.5); 17, extract at pH 6.0 with leupeptin (1 μg/ml); 18, extract at pH 6.0 with pepstatin (1 μg/ml); 19, extract at pH 6.0 with leupeptin and pepstatin (1 μg/ml); 20, type I collagen standard.

The likelihood that this activity was cathepsin D, based upon its inhibition with pepstatin (Fig. 1), led us to purify large amounts of this enzyme from chicken livers. After affinity chromatography on pepstatin-aminohexyl Sepharose 4B (12), the overall purification was 907-fold with 8% recovery. The purified cathepsin D and ovalbumin [43 kilodaltons (kDa)] were eluted from Sepharose G-100 at the same position. $NaDodSO_4$/polyacrylamide gel electrophoresis showed two subunits with relative molecular sizes of ≈30 and 13 kDa (13). The pH optimum against acid-denatured bovine hemoglobin was ≈3.0 (14). Activity could not be accurately detected above pH 5 because of precipitation of the hemoglobin substrate (14).

When affinity-purified cathepsin D was assayed with native procollagen, two cleavage patterns were observed (Figs. 2 and 3). After 1 hr at 37°C, the major activity occurred between pHs 2.5 and 5.0 (Fig. 2) as observed (21). However, this activity also produced some degradation of the collagen α chains (Fig. 2, lanes 3–6, and Fig. 3, lanes 3–6). Upon incubation for 4 hr, conversion was also seen at pHs 5.0 and 6.0 (Fig. 2, lanes 15 and 16). Analysis of the cleavage products using a 12.5% acrylamide gel concentration revealed a remarkable cleavage pattern (Fig. 3). The product from cleavage at pH 5.0 and below was a major band that migrated slightly slower than the C-1 subunit for purified chicken tendon COOH propeptide, while the C-2 subunit product migrated in the same position as the authentic subunit. (The C-1 and C-2 subunits of COOH propeptide arise from the pro-α1- and pro-α2-chains of type I procollagen.) A minor band

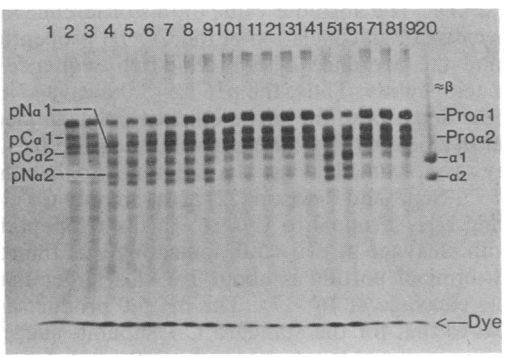

FIG. 2. *In vitro* assay of the cleavage of procollagen by 0.1 μg of affinity-purified cathepsin D. Samples were buffered with a composite buffer (0.15 M formate/0.15 M acetate/0.15 M phosphate) (14). Reactions were for 1 hr (lanes 2–14) or 4 hr (lanes 15–19) at 37°C, followed by reduction with 5% 2-mercaptoethanol and boiling. Samples were split equally for electrophoresis on a 6% acrylamide slab gel (seen here) and a 12.5% acrylamide gel (see Fig. 3). Samples were visualized by using acetic acid/2,5-diphenyloxazole fluorography (16) with preflashed X-Omat AR film (18), exposed for 24 hr at −70°C. Lanes: 1, (empty); 2, cathepsin D at pH 3.0 with pepstatin (1 μg/ml); 3, cathepsin D at pH 2.0; 4, pH 2.5; 5, pH 3.0; 6, pH 3.5; 7, pH 4.0; 8, pH 4.5; 9, pH 5.0; 10, pH 5.5; 11, pH 6.0; 12, pH 6.5; 13, pH 7.0; 14, pH 7.5; 15, pH 5.0 for 4 hr; 16, pH 6.0 for 4 hr; 17, pH 6.5 for 4 hr; 18, pH 7.0 for 4 hr; 19, pH 7.5 for 4 hr; 20, type I collagen standard.

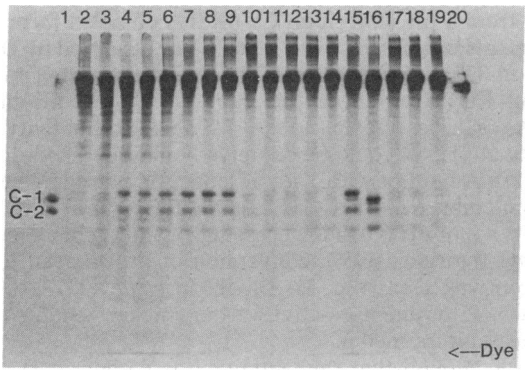

FIG. 3. *In vitro* assay of the cleavage of procollagen by affinity-purified cathepsin D. Samples were the second half of the assay shown in Fig. 2, with electrophoresis being run on a 12.5% acrylamide slab gel. Exposure to preflashed X-Omat AR film was for 92 hr at −70°C. Lanes are as identified in Fig. 2, except that lane 1 contained purified COOH propeptide isolated from chicken tendon organ culture.

was also observed below C-2 at pH 2.5–3.5. In contrast, a 4-hr incubation at pH 6.0 resulted in a C-1 cleavage product that comigrated with the authentic tendon culture medium product. The position of the C-2 cleavage product was unchanged. The difference in molecular mass between the C-1 cleavage products at pHs 5.0 and 6.0 (Fig. 3, lanes 15 and 16) is ≈1.2 kDa by comparison with globular protein standards.

DISCUSSION

Cleavage at the Telopeptide/Propeptide Junction. Cathepsin D has been considered previously as a participant in procollagen processing, but there have been no reports of cleavage occurring at or near the COOH telopeptide/propeptide junction. Davidson *et al.* (21) observed multiple cleavage products when they assayed at pH 4.2 (30°C). Scott and Pearson (22, 23) found that the cleavage of crosslinked bovine collagen by cathepsin D at pH 4.0 and 45°C occurred at position 6^{C}-7^{C} (Leu-Ser in calf) in the COOH telopeptide.* Consistent with this, we observed that cleavage of native procollagen in the pH range 2.5–5.0 at 37°C resulted in a cleavage product larger than the authentic C-1 subunit by at least 1.2 kDa. The absolute value of this difference is uncertain, because of glycosylation of the COOH propeptide subunits and the presence of a proline-rich sequence in the COOH telopeptide. Thus, the pH 2.5–5.0 cleavage may occur at the same position in the chicken collagen [sequence Phe-Ser in chicken (25)]. Alternatively, the pH 5.0 C-1 product may result from cleavage at a different site than the position 6^{C}-7^{C}. Scott and Pearson (22) assayed only for cleavage occurring NH_2-terminal to Lys 16^{C} in the telopeptide. Our data with cleavage at pH <5.0, indicating that the released COOH-terminal portion is about 1.2 kDa larger than C-1, suggests cleavage at 16^{C}-17^{C}. The pH 6.0 product as an R_f identical to that for the standard C-1 subunit, suggesting a shift in cleavage site to, at or near the authentic cleavage site (26^{C}-$1^{C\text{-}1}$; Ala-Asp in chicken). There is still the possibility that the pH 6.0 C-1 product represents multiple cleavages because we have not determined whether the COOH telopeptides on the resultant collagen α1 chains are intact. Whatever the cleavage mechanism, it is completely inhibitable by pepstatin but not by phenylmethylsulfonyl fluoride, *o*-phenanthroline, or leupeptin (data not shown), showing that it is not due to a contaminating second proteinase or activation of a latent enzyme.

The ability of cathepsin D to produce the authentic C-1 propeptide product only at a specific pH may be related to a change in conformation of the cleavage site or the enzyme active site, or both. Studies with pepstatin (26) show a rapid increase in the K_d for the enzyme–inhibitor complex above pH 5.4. This may reflect a change in the enzyme's active site conformation. Also, the sequence Asp-Asp located COOH-terminal to the cleavage site should approach complete ionization near pH 6.0.

Attempts to characterize the reaction kinetics have been hampered by the scarcity of radiolabeled procollagen and limitations to its solubility. By using a rapid assay (6, 27) to measure radioactive COOH propeptide liberated at pH 6.0, cpm measurements were linear with added enzyme (data not shown). The time course of release showed an apparent lag phase. Figs. 2 and 3 show that no cleavage occurred in 1 hr at pH 5.5 or 6.0 but that 4 hr at pH 6.0 gave appreciable conversion. This is similar to the observation by Irvine *et al.* (28). The lag phase was most pronounced at low concentrations of substrate. They attributed this to cathepsin D self-association in stock solutions, with dissociation upon dilution being accelerated by interaction with substrate. Consistent with this, complete conversion of procollagen was seen in longer assays (12–15 hr).

The degree of conversion at pH 6.0 was estimated to be on the order of 1–5%, based upon the radioactivity released in a 4-hr rapid assay (6, 27) with uniformly labeled procollagen. This occurred with 0.1 μg of cathepsin D and approximately 2 μg of procollagen, for an approximate enzyme:substrate molar ratio of 1:2. The enzyme levels tested here are in line with those used by other workers. For example, for the inactivation of native aldolase by cathepsin D, Offermann *et al.* (29) used an enzyme:substrate ratio of 1:8 and found rapid inactivation between pHs 4.2 and 5.2. However, these enzyme levels are still well below physiological levels. Cathepsin D is present in the intralysosomal space at 0.78 mM (33.5 mg/ml) (30). Intracellular compartmentalization and concentration of cathepsin D and procollagen (31, 32) could enable rapid processing of procollagen.

A Hypothesis for *in Vivo* Cleavage. Could the cleavage of procollagen by cathepsin D as reported here play a physiological role in the normal processing of procollagen? The main physiological role of cathepsin D is believed to involve intracellular degradation of proteins in a lysosomal compartment (13, 30). We recently extended the observations of Ryhänen *et al.* (33), showing that amines and other lysosomotropic agents such as chloroquine could selectively block the removal of the COOH propeptide from procollagen in organ culture (34). These agents might act not as direct inhibitors of a "COOH-terminal proteinase" (33) but as lysosomotropic agents. This would imply an intracellular site for COOH propeptide removal as illustrated in Fig. 4. Proteins destined for the lysosome, such as cathepsin D, move through the cell by a pathway distinct from that of secretory proteins, such as procollagen (35, 36). The "branch point" is the Golgi complex, and the sorting mechanism appears to involve mannose-6-phosphorylation of proteins destined for the lysosome. We propose that a portion of the "prelysosomes" containing cathepsin D fuse with procollagen-containing secretory granules prior to secretion. Then, either inside the cell or at the time of secretion, cathepsin D specifically removes the COOH propeptides from procollagen molecules contained in the secretory granule. Cathepsin D would presumably remain attached to the Man-6-*P* receptor since it requires pH <5.5 for release (37) and would be reinternalized into the lysosomal compartment. Activation of procathepsin D (38–40) is illustrated as possibly occurring after

*The telopeptides are short, nontriple helix-forming sequences found at either end of the collagen molecule. In the precursor form, procollagen, the telopeptides connect the triple helical domain of collagen to the precursor-specific propeptide extensions. Sequences in the carboxyl telopeptide are numbered as in ref. 24, with the modification that residue 1 of the COOH propeptide, C-1 subunit, is designated $1^{C\text{-}1}$.

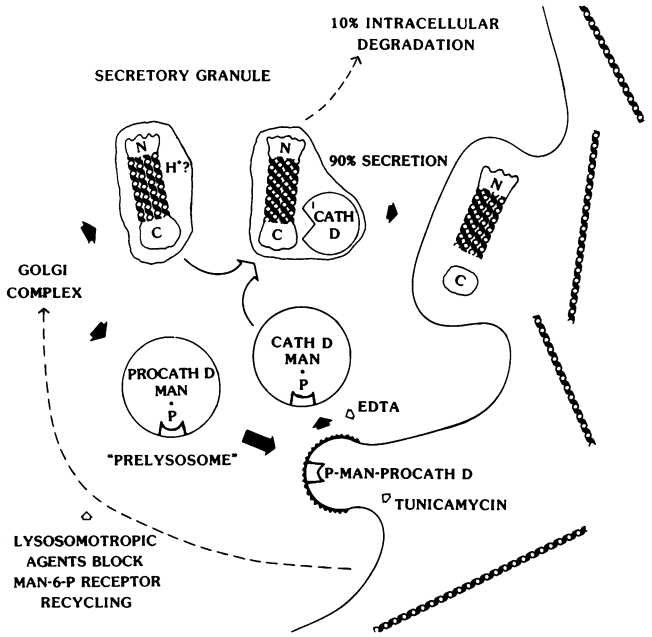

FIG. 4. A model for the role of cathepsin D in the *in vivo* processing of procollagen. Cathepsin D (Cath D) is illustrated as being bound via the mannose 6-phosphate (Man-6-*P*) receptor during exocytosis and reuptake at the cell surface, prior to fusion with a secretory granule containing procollagen. Lysosomotropic agents (open arrowhead) prevent reinternalization of cathepsin D by preventing the recycling of Man-6-*P* receptors necessary for normal targeting of lysosomal enzymes. Tunicamycin (open arrowhead) prevents reinternalization by preventing the formation of the mannose core, which would normally be phosphorylated. EDTA (open arrowhead) prevents reinternalization by preventing the influx of extracellular Ca^{2+} ions necessary for the calmodulin-mediated clathrin-coated vesicle internalization. Any of these would result in secretion of procathepsin D (ProCath D). Cathepsin D may act intracellularly or at the site of secretion. See text for discussion.

exocytosis and reinternalization, prior to fusing with the secretory granule.

This model explains several observations. First, tunicamycin has been reported (41, 42) to inhibit the processing of procollagen in organ culture, apparently because it affects the enzyme involved in COOH propeptide removal. Hasilik and Neufeld (38, 43) showed that cells with abnormal glycosylation mechanisms lose procathepsin D into their tissue culture media, and others (39, 40) have shown that tunicamycin causes the secretion of procathepsin D by interfering with the incorporation of the mannose core which must be phosphorylated for normal targeting and/or reuptake. Similarly, lysosomotropic agents such as chloroquine cause the secretion of procathepsin D (38, 39) by preventing the recycling of the Man-6-*P* receptors (44). Also, EDTA in the extracellular space inhibits the processing of procollagen in organ culture (5, 34), a result usually interpreted as supporting an extracellular inhibition of neutral metalloproteinases. However, as Davidson *et al.* (21) also pointed out, this is consistent with the known requirement for an influx of millimolar Ca^{2+} from the extracellular space during calmodulin-mediated clathrin-coated vesicle receptor-mediated endocytosis (45). Finally, the hypothesis that a prelysosomal vesicle might fuse with a procollagen secretory granule shows how normal secretory mechanisms may connect to the intracellular degradation of "defective" procollagen (46–50). This possibility was raised by Neblock and Berg (48), who showed that NH_4Cl and chloroquine interfered with synthesis and secretion of procollagen, and by Curran *et al.* (50), who reported kinetic evidence suggesting "a branch point in the secretory pathway at which intracellular degradation occurs in parallel to the secretion of intact procollagen molecules." Conceivably, if cathepsin D and other lysosomal enzymes were to fuse with a secretory granule whose pH was too low (<6.0) or which contained procollagen with a defective triple helix (47, 49), degradation of procollagen would become the main routing. Cathepsin D can specifically cleave within the triple helical sequence of denatured type I collagen (refs. 22 and 23; see also Figs. 2 and 3, lanes 3–6).

Bruns and co-workers (31, 32) have isolated procollagen segment-long-spacing-like aggregates from cell homogenates and culture media, which appear similar to those seen within cells (51). Another effect of lysosomotropic amines could be to dissociate the procollagen within these secretory granules, either by causing osmotic swelling or by directly interacting with the segment-long-spacing-like aggregates. Woessner (52) reported that amine compounds, including several tested by Ryhänen *et al.* (33), dissociate proteoglycan and prevent *in vitro* cleavage by cathepsin D. Similarly, arginine is known to prevent *in vitro* collagen fibril formation (53). This has been used to show that procollagen aggregates were the required substrate for neutral metalloproteinases isolated from fibroblast culture, since arginine prevented COOH-propeptide removal *in vitro* (54).

There is ample evidence for the presence of an acid proteinase in the culture media of connective tissue cells (9, 21). Yet, studies of the site of processing have been confused by tissue culture experiments, either the "matrix-free" cell suspension (7) or monolayer culture, where virtually all of the procollagen is secreted intact. Frequently, the NH_2 propeptide is subsequently removed from the secreted procollagen by the neutral metalloproteinase, procollagen NH_2-terminal proteinase (55). The excision of the COOH propeptide has rarely been observed unless the medium has been acidified or allowed to incubate for a long period (56). Yet, COOH propeptide excision is quite rapid in the intact tissue (5, 33), and milligram quantities of COOH propeptide can be recovered from spent media of tendon organ culture (57). COOH propeptide removal appears to require the presence of an intact matrix around the procollagen-secreting cells (5).

The finding that purified cathepsin D specifically removes the COOH propeptides from procollagen and the hypothesis that cathepsin D is involved *in vivo* by the mechanism diagramed in Fig. 4 appear to be in disagreement with two lines of earlier work. Several workers have proposed that the procollagen COOH-terminal proteinase is a neutral metalloproteinase (6, 27, 54, 58, 59). Most of these studies were performed with enzymes isolated from tendon fibroblast culture media. These cells do not have a normal matrix, which may be important for normal enzyme reuptake or for critical pericellular conditions at secretion. The only paper showing the isolation of a neutral metalloproteinase from intact tissue (6) actually describes the isolation of a multifunctional proteinase. This enzyme, even when purified over 5,000-fold (60), still possesses a higher NH_2-terminal proteinase activity than COOH-terminal proteinase, and these were not separable under conditions with which others have successfully separated the activities from tissue culture media (54). Furthermore, cleavage of procollagen with this enzyme produces large tactoidal collagen fibrils (61), reminiscent of the fibrils observed upon reconstituting proteinase-treated collagen lacking intact COOH telopeptides (62–64). It is possible that Njieha *et al.* (6) isolated "proteoglycanase," a neutral metalloproteinase purified by Galloway *et al.* (65) from calvaria organ culture, which also removes both propeptides from purified procollagen to yield α chains.

It is more difficult to reconcile our data with that of the Fesslers (54, 66), where in both organ culture and cell culture, well-documented pulse–chase experiments showed cleavage of the NH_2-propeptide before the COOH propeptide. pC-collagen accumulated in their system and was proposed as a distinct intermediate in the processing pathway to

collagen. Veis *et al.* (67), on the other hand, had shown the presence of pN-collagen-like molecules in normal skin (and no pC-collagen) and proposed that these might be in the processing pathway. Fleischmajer *et al.* (2, 3), using specific anti-pN-collagen antibodies, have shown that newly formed thin fibrils possess pN-collagen but not pC-collagen. Several workers have shown that pN-collagen can assemble *in vitro* to give thin fibrils with the characteristic collagen banding pattern (68, 69), while pC-collagen will not (61). Hulmes (4) recently proposed that biological fibril assembly requires pN-collagen as an intermediate, followed by the trimming of NH_2 propeptides from the fibrils by procollagen NH_2-terminal proteinase. Although it is possible that alternate pathways might exist at different stages of development and in different tissues, the culture conditions used in the pulse–chase experiments must be reexamined in the light of possible lysosomotropic effects.

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