Lysosomal Function in the Degradation of Defective Collagen in Cultured Lung Fibroblasts†

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ABSTRACT: Human fibroblasts when induced to make nonhelical, defective collagen have mechanisms for degrading up to 30% of their newly synthesized collagen intracellularly prior to secretion. To determine if at least a portion of the degradation of defective collagen occurs by lysosomes, extracts of cultured HFL-1 fibroblasts were examined for proteinases capable of degrading denatured type I [^{3}H]procollagen. The majority of the proteolytic activity against denatured [^{3}H]-procollagen had a pH optimum of 3.5–4; it was stimulated by dithiothreitol and inhibited 95% by leupeptin, 10% by pepstatin, and 98% by leupeptin and pepstatin together. Extracts of purified lysosomes from the fibroblasts were active in degrading denatured [^{3}H]procollagen and were completely inhibited by leupeptin and pepstatin. To demonstrate directly that human lung fibroblasts can translocate a portion of their defective collagen to lysosomes, cultured cells were incubated with *cis*-4-hydroxyproline and labeled with [^{14}C]proline to cause the cells to make nonhelical [^{14}C]procollagen. About 3% of the total intracellular hydroxy[^{14}C]proline was found in lysosomes. If, however, the cells were also treated with NH_4Cl, an inhibitor of lysosomal function, 18% of the intracellular hydroxy[^{14}C]proline was found in lysosomes. These results demonstrate that cultured human lung fibroblasts induced to make defective collagen are capable of shunting a portion of such collagen to their lysosomes for intracellular degradation.

Collagen is a well-characterized extracellular protein that traverses a defined intracellular pathway from its site of synthesis on the rough endoplasmic reticulum to its secretion through the Golgi system and secretory vesicles (Fessler & Fessler, 1978; Prockop et al., 1979a,b; Davidson & Berg, 1981). Interestingly, a significant fraction of newly synthesized collagen[1] is degraded to small peptides by cultured cells prior to secretion. The proportion of newly synthesized collagen that undergoes intracellular degradation seems to be modulated, in part, by the conformation of the collagen molecule. In this regard, studies have indicated that if fibroblasts are induced to synthesize nonhelical collagen, by causing cells to synthesize either collagen deficient in hydroxyproline or collagen containing an analogue of proline such as azetidine or *cis*-4-hydroxyproline, such collagen is susceptible to intracellular degradation [for review, see Bienkowski (1983)]. In addition, recent studies have indicated that the degradation of this newly synthesized, nonhelical collagen can be inhibited by N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK),[2] chloroquine, NH_4Cl, and leupeptin, all of which are capable of inhibiting lysosomal proteinases (Berg et al., 1980). Thus, the concept has arisen that fibroblasts "monitor" the helicity of collagen they produce and degrade a fraction of their nonhelical, defective collagen by a process that likely involves lysosomal proteinases.

To evaluate directly whether human lung fibroblasts utilize lysosomes to degrade a fraction of their nonhelical, newly synthesized collagen, we have examined these cells for lysosomal proteinase activity directed against denatured collagen, and we have examined cells induced to make defective collagen to show that their lysosomes can accumulate collagen fragments containing hydroxyproline.

Experimental Procedures

Cell Cultures. Normal diploid human fetal lung fibroblasts, HFL-1 (ATCC CCL 153), between the 10th and the 20th passage were used for all studies. The cells were maintained and subcultured at 37 °C in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.06% glutamine) as previously described (Bienkowski et al., 1978; Berg et al., 1980; Breul et al., 1980). These cells were plated at a density of 2×10^6 cells/150-mm culture plate and were grown for 5 days until they became confluent.

Extraction of HFL-1 Proteinases. To examine proteolytic activity in HFL-1 cells, the growth medium was removed from a culture plate containing confluent cells. The cells were removed from the plate by trypsinization, the trypsin was inactivated by the addition of growth medium containing 10% fetal calf serum, and a cell pellet was obtained by centrifugation at 2000*g* for 5 min. The cells were suspended in 1 mL of 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.5% Nonidet P-40. The cells were homogenized in a Teflon and glass homogenizer (10 strokes) and centrifuged at 1200*g* for 20 min. The resulting supernatant was used as the source of HFL-1 proteinases.

Preparation of Lysosomes. To prepare cells for subcellular fractionation on colloidal silica gradients, the method of Rome et al. (1980) was used. The cells from one to five culture plates were trypsinized by using buffered trypsin (Roth & White,

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[1] Current data suggest that collagen is synthesized in a precursor form termed "preprocollagen" (Palmiter et al., 1979). Since the form (i.e., prepro, pro, Pn, Pc, etc.) of collagen chains undergoing intracellular degradation is not known, and since the "marker" for intracellular degradation of collagen is hydroxyproline (Bienkowski et al., 1978; Berg et al., 1980), a modified amino acid found predominantly in the α-chain region, we will use the terms "collagen" and "procollagen" interchangeably when referring to the macromolecules undergoing degradation.

[2] Abbreviations: BAPN, β-aminopropionitrile fumarate; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid.

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1972). The cells were subjected to nitrogen cavitation followed by homogenization by hand in a glass and Teflon homogenizer and were processed exactly ass a previously described (Rome et al., 1980). The postnuclear supernatants of cells were then centrifuged on a self-generating colloidal silica gradient (Rome et al., 1980) at 18 000 rpm. For small samples of 1 mL derived from a single culture plate, the silica gradient consisted of 3.7 mL of a 1.065 g/mL silica mixture over a 0.5-mL saturated sucrose cushion in a 0.5 × 2 in. Beckman cellulose nitrate tube, and centrifugation was for 1 h in an SV80 rotor (Sorvall). For larger samples of 5 mL derived from up to five culture plates, the gradient consisted of 25 mL of a 1.065 g/mL silica mixture over a 4-mL saturated sucrose cushion in a 1 × 3.5 in. polyallomer tube, and the centrifugation was for 2.5 h in an SV288 rotor (Sorvall). Fractions (0.22 mL for small gradients; 1.0 mL for large gradients) were collected as described (Rome et al., 1980).

The gradient was assayed for α-mannosidase activity, a marker for lysosomes, by using 10 μL of each fraction and 1 mM 4-methylumbelliferyl α-D-mannoside as substrate (Rome et al., 1980). The product (4-methylumbelliferone) was measured in a Farrand-2 ratio fluorometer using a narrow-band interference filter at 367 nm for excitation and a 3-73 sharp-cut yellow filter for emission. The plasma membrane marker was leucyl-β-naphthylamidase. Aliquots (10 μL) of each fraction were incubated with 0.75 mM leucyl-β-naphthylamine, and the liberated product (β-naphthylamine) was measured as described above, except that a narow-band interference filter of 405 nm was used for emission. Endoplasmic reticulum was located by assaying fractions for prolyl hydroxylase (Berg & Prockop, 1973a) with nonhydroxylated [^{14}C]proline-labeled procollagen as the substrate (Berg & Prockop, 1973b). After 30 min at 37 °C, the reaction was stopped with the addition of 1 mL of 12 N HCl, and the reaction mixtures were hydrolyzed at 110 °C for 16 h. The hydroxy[^{14}C]proline was determined by a radiochemical assay (Juva & Prockop, 1966).

Measurement of Proteinase Activity against Denatured Collagen. Procollagen substrate was prepared by labeling 100 culture plates (100 mm) of confluent HFL-1 fibroblasts with 50 μCi/mL [^{3}H]proline in 4 mL per plate of Dulbecco's modified Eagle's medium for 24 h. The medium was dialyzed against 10 mM Tris, pH 7.4, containing 2 M urea, prior to chromatography on DEAE-cellulose using 0.2 M NaCl gradient (Davidson et al., 1977). The peak containing type I procollagen was pooled and stored frozen at −20 °C. The purification of this substrate was verified by polyacrylamide slab gel electrophoresis (King & Laemmli, 1971).

To assay HFL-1 proteolytic activity against the procollagen substrate, up to 40 μL of cell extract was added to a 400-μL reaction mixture containing 30 000 dpm of procollagen, denatured at 56 °C for 15 min prior to its addition to the reaction mixture, in 375 mM NaCl, 12 mM EDTA, 10 mM dithiothreitol, and 250 mM sodium citrate buffer, pH 4.2. To determine the pH dependence of the reaction, the following buffers were used: pH 2, 3, and 5, 250 mM citrate buffer; pH 6 and 7, 250 mM phosphate buffer; pH 7.4 and 8.0, 250 mM Tris-HCl buffer. The reactions were carried out for 4 h at 30 °C in 1.5-mL plastic tubes that had been coated with 1 mg/mL bovine serum albumin for 16 h prior to utilization for assays. At the end of incubation, 5 μl of 10% bovine serum albumin was added to each tube followed by 1 mL of 15% trichloroacetic acid. The tubes were kept on ice for 30 min and then centrifuged at 10000*g* for 10 min. The supernatant was removed and mixed with 10 mL of Aquasure (New England Nuclear) and counted in a scintillation counter.

To test the extracts of HFL-1 for the presence of specific classes of proteinases, the proteinase assay was carried out in the presence of leupeptin (Peninsula Laboratories, San Carlos, CA), an inhibitor of cysteine proteinases such as cathepsins b1 and L (Huisman et al., 1974; Kirschke et al., 1975) as well as some proteinases having a trypsin-like mechanism (Umezawa & Aoyagi, 1977). We also carried out the proteinase assay in the presence of pepstatin (Peninsula Laboratories, San Carlos, CA), an inhibitor of aspartate proteinases such as cathepsin D (Dean, 1975; Umezawa & Aoyagi, 1977). We examined these agents both individually and together. The concentrations for each inhibitor tested were 10 or 50 μg/mL. Also, TLCK (Sigma Chemical Co.), an inhibitor of certain serine and cysteine proteinases such as cathepsin B and cathepsin L (Kirschke et al., 1975; Barrett, 1977), was examined at concentrations of 20 and 100 μM.

Direct Evaluation of Lysosomes for the Presence of Collagenous Peptides. To demonstrate that newly synthesized defective collagen could be found in fibroblast lysosomes, HFL-1 cultures were rinsed 3 times with phosphate-buffered saline, pH 7.4, and 6 mL of fresh labeling medium was added to each plate. The labeling medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.06% glutamine, 40 μg/mL ascorbic acid, 50 μg/mL BAPN, and 10 μCi/mL [^{14}C]proline (Amersham Corp.) and 1.5 mM *cis*-4-hydroxyproline. The *cis*-4-hydroxyproline was included in the incubation mixtures to cause the cells to synthesize collagen which was unable to assume the normal triple-helical conformation (Berg et al., 1980; Neblock & Berg, 1982). Since the steady-state level of collagen undergoing degradation in the lysosomal system in fibroblasts may be quite low, an additional experiment was carried out where cells were incubated as described above with *cis*-4-hydroxyproline and [^{14}C]proline but in the presence of 20 mM ammonium chloride, an inhibitor of lysosomal proteolysis (Berg et al., 1980; Amenta et al., 1978).

The cultures were labeled for 4 h, the medium was removed, and the cells were trypsinized for 30 min in 10 mL of buffered trypsin (Roth & White, 1972) for each 150-mm culture plate. Subcellular organelles from the labeled fibroblasts were then isolated by using colloidal silica gradients as described above. Gradient fractions (0.22 mL) were then hydrolyzed (110 °C, 16 h) and examined for the presence of hydroxy[^{14}C]proline (Juva & Prockop, 1966).

Results and Discussion

Degradation of Collagen by Proteinases from HFL-1 Cells. Extracts of confluent HFL-1 fibroblasts were able to degrade denatured type I procollagen to Cl_3CCOOH-soluble peptides. The reaction was markedly dependent on the pH of the reaction mixture with an optimum occurring in the acid region of the pH profile (Figure 1). The reaction was linearly dependent on the amount of cell extract used and the time of incubation and was stimulated by dithiothreitol (data not shown). Since it is known that acid proteases including cathepsins B (Etherington, 1974) and L (Kirschke et al., 1975) are capable of degrading denatured collagen, we tested for their presence in HFL-1 fibroblasts by using selective inhibitors. Leupeptin (10 μg/mL) inhibited the degradation of denatured procollagen at pH 4.2 by 95%; increasing the concentration to 50 μg/mL had little additional effect (Table I). Pepstatin (10 μg/mL) was able to inhibit the degradation of denatured procollagen by 10%; raising the concentration to 50 μg/mL caused the digestion of procollagen to be inhibited by 20%.

Table I: Inhibition of Proteinases in Extracts of HFL-1 Fibroblasts[a]

additions	concn	pH 4.2 procollagen degraded (dpm × 10^{-3})	pH 4.2 inhibition	pH 7.4 procollagen degraded (dpm × 10^{-3})	pH 7.4 inhibition
none		18.0	0	7.2	0
leupeptin	10 μg/mL	0.9	95	3.5	51
	50 μg/mL	0.8	95	3.1	62
pepstatin	10 μg/mL	16.2	10	6.7	8
	50 μg/mL	14.3	20	6.4	12
leupeptin + pepstatin	50 μg/mL (each)	0.3	98	2.0	72
TLCK	20 μM	1.8	89	7.4	0
	100 μM	2.2	87	7.4	0

[a] HFL-1 fibroblasts were grown to confluency as described under Experimental Procedures. The cell layer of confluent 150-mm culture plates was homogenized in 1 mL of 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.5% Nonidet P-40 and was centrifuged at 1200*g* for 20 min, and the supernatant was used as the source of enzyme. The incubation mixture contained 40 μL of cell extract, 26 × 10^3 dpm of [^{3}H]proline-labeled procollagen, 375 mM NaCl, 12 mM EDTA, and 10 mM dithiothreitol in 250 mM citrate buffer, pH 4.2, or in 250 mM Tris-HCl buffer, pH 7.4. The reactions were carried out at 37 °C for 4 h and were stopped by the addition of 15% Cl_3CCOOH as described under Experimental Procedures. Less than 380 dpm of procollagen was solubilized in the absence of cell extract. This background was subtracted from each value in the table. Each value is the average of duplicate determinations.

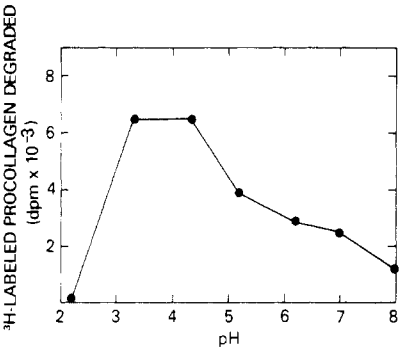

FIGURE 1: pH dependency of proteolytic activity in extracts of HFL-1 fibroblasts directed against denatured type I procollagen. The cell extracts were incubated with denatured [^{3}H]procollagen in various buffers at the pHs indicated. The reaction was stopped by adding 15% Cl_3CCOOH and the proteolytic activity quantified by determining Cl_3CCOOH-soluble radioactivity.

Since leupeptin is a relatively specific inhibitor for the thiol-dependent cathepsins B and L, and pepstatin is a relatively specific inhibitor for aspartate proteinases such as cathepsin D, these results suggest that a large proportion of the acid proteinases capable of degrading denatured procollagen in HFL-1 cells were both thiol and aspartate proteinases. In fact, both inhibitors together were able to inhibit the ability of HFL-1 extracts to degrade procollagen at pH 4.2 by 98% (Table I). Since the major known intracellular thiol and aspartate proteinases are cathepsins B, L, and D, these three proteinases are most likely involved. In addition, TLCK, an inhibitor of cathepsins B and L, was tested for its ability to inhibit the acid proteinases contained in extracts of HFL-1 cells. It was found that 20 μM TLCK at pH 4.2 was able to inhibit the degradation of procollagen by HFL-1 extracts by 89% (Table I). Since leupeptin and TLCK have also been shown to inhibit the degradation of defective collagen in living cells (Berg et al., 1980), it is reasonable to conclude that the degradation of a fraction of newly synthesized collagen occurs because it is shunted into the lysosomal system.

In contrast to the effect of the inhibitors on the activity of HFL-1 extracts to degrade denatured collagen at acid pH, the inhibitors were less effective in inhibiting the degradation of collagen by these extracts when assayed at pH 7.4 (Table I). Leupeptin inhibited a neutral proteolytic activity against denatured collagen by 62%, and pepstatin inhibited an activity by 12%. TLCK, at concentrations up to 100 mM, failed to inhibit the neutral proteolytic activity at all. These results suggested that the majority of proteolytic activity found in HFL-1 extracts against denatured collagen that was inhibited by leupeptin, pepstatin, and TLCK consisted of proteinases having acid pH optima. However, since TLCK inhibited proteolytic activity at pH 4.2 but not at pH 7.4, it is possible that the neutral proteolytic activity in extracts of HFL-1 cells represented, at least in part, different proteinases than those with acid proteolytic activity. Further evidence to support such a possibility is that leupeptin but not TLCK inhibited neutral proteolytic activity. This phenomenon argues against the residual activity of cathepsins B and L at neutral pH and argues for the presence of a neutral proteinase capable of degrading collagen in extracts of HFL-1 cells. Although the identity of such a proteinase in HFL-1 cells is unknown, at least one proteinase (calcium-dependent proteinase II) is inhibited by leupeptin but not by TLCK and is known to exist in certain mammalian cells (Barrett & McDonald, 1980). Since pepstatin does not bind to cathepsin D at neutral pH (Knight & Barrett, 1972), an aspartate proteinase other than cathepsin D may be responsible for the pepstatin-inhibitable proteolytic activity at neutral pH. An example of such a proteinase is renin, a nonlysosomal proteinase from kidney, which is active in plasma at neutral pH and is inhibited by pepstatin (Aoyagi et al., 1972). Although specific neutral proteinases which degrade collagen in cells have not been identified, neutral proteinases have been found to be associated with intracellular protein degradation. An ATP-dependent, nonlysosomal system for degrading proteins such as abnormal hemoglobin has been described in rabbit reticulocytes and shown to have a pH optimum of 7.9 (Etlinger & Goldberg, 1977; Ciehanover et al., 1978; DeMartino & Goldberg, 1979). Whether or not such a system is operative in fibroblasts on collagen is unknown, but the possible involvement of both acidic and neutral proteinases in the intracellular degradation of newly synthesized collagen should not be overlooked.

Degradation of Collagen by Proteinases from Purified Lysosomes. To demonstrate directly that HFL-1 lysosomes contained proteinases capable of digesting denatured procollagen at acid pH, lysosomes were prepared from HFL-1 cells and tested for proteolytic activity. To accomplish this, a postnuclear supernatant of cultured HFL-1 cells was centrifuged on a gradient of colloidal silica to prepare a lysosomal fraction. The lysosomal enzyme, α-mannosidase, was contained in two regions of the gradient (Figure 2A). The region of greatest density that contains α-mannosidase on these gradients has previously been shown to contain highly purified

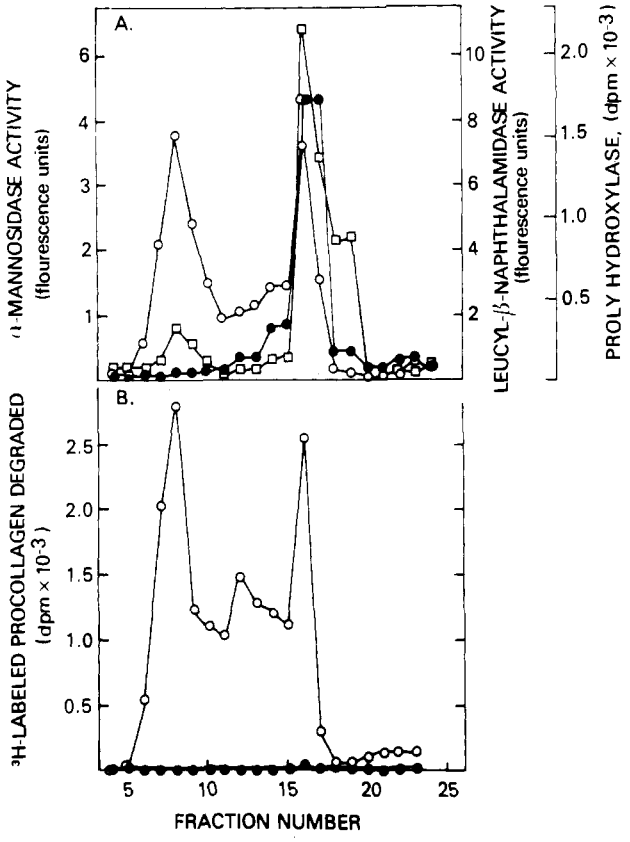

FIGURE 2: Subcellular fractionation of cultured HFL-1 fibroblasts. A postnuclear supernatant of cultured HFL-1 cells was prepared as described under Experimental Procedures and fractionated on a 25-mL gradient of colloidal silica (Rome et al., 1980). α-Mannosidase and leucyl-β-naphthylamidase were assayed as described under Experimental Procedures. Prolyl hydroxylase was assayed with [^{14}C]proline-labeled nonhydroxylated procollagen (Berg & Prockop, 1973). Acid proteolytic acitvity directed against denatured type I procollagen was assayed as described under Experimental Procedures. (A) α-Mannosidase activity (○); leucyl-β-naphthylamidase activity (□); prolyl hydroxylase activity (●). (B) Acid proteinase activity against [^{3}H]procollagen (○); proteinase activity assayed in the presence of 50 μg/mL leupeptin + 50 μg/mL pepstatin (●).

lysosomes (Rome et al., 1980), while a region of lighter density contains various subcellular organelles such as endoplasmic reticulum, smooth vesicles, plasma membrane (Rome et al., 1980), and endosomes (Helenius et al., 1983). This is verified by the fact that leucyl-β-naphthylamidase (a plasma membrane enzyme marker) was primarily contained in the α-mannosidase peak of lighter density. Also, the enzyme prolyl hydroxylase (an endoplasmic reticulum marker) was contained only in the peak of α-mannosidase of lighter density and was not present in the dense lysosomal fraction. Interestingly, acid proteinases capable of degrading denatured collagen were found in both peaks that contained the lysosomal marker (Figure 2B). In addition, leupeptin and pepstatin together were capable of completely inhibiting all of the acid proteolytic activity against denatured collagen found in the lysosomes of HFL-1 cells (Figure 2B).

Presence of Collagen Polypeptides in Lysosomes. To determine if nonhelical, defective collagen can be translocated to the lysosomes of fibroblasts, cultures of HFL-1 cells were incubated in the presence of *cis*-4-hydroxyproline to produce nonhelical procollagen. The cells were labeled with [^{14}C]-proline, and the lysosomes were isolated on a colloidal silica gradient (Figure 3). Examination of the gradient indicated that approximately 3% of the intracellular hydroxy[^{14}C]proline was present with the lysosomal marker (fractions 6–10). In contrast, when the cells were incubated with 20 mM NH_4Cl,

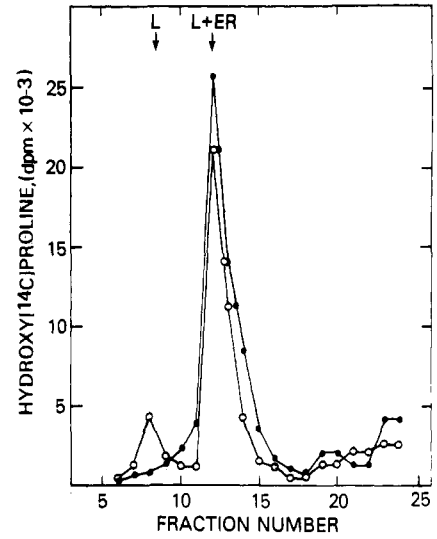

FIGURE 3: Distribution of intracellular hydroxy[^{14}C]proline in extracts of HFL-1 cells fractionated on a colloidal silica gradient. Cells were labeled with [^{14}C]proline in the presence of *cis*-4-hydroxyproline for 4 h prior to fractionation as described under Experimental Procedures. The arrows indicate the positions of lysosomes (L) and lysosomes + endoplasmic reticulum (L + ER) determined by using methods similar to those described in Figure 2. The direction of centrifugation is from right to left. Labeling was carried out in the absence (●) or presence (○) of NH_4Cl, an inhibitor of lysosomal function. Each fraction was hydrolyzed with HCl (16 h, 10 °C) prior to analysis for hydroxy-[^{14}C]proline. If the fractions were not hydrolyzed, no hydroxy-[^{14}C]proline was detected.

an inhibitor of lysosomal function, 18% of the intracellular hydroxy[^{14}C]proline was found in the lysosomes (Figure 3). The results indicated that a small fraction of the total intracellular hydroxy[^{14}C]proline can be found within lysosomes when cells were induced to make nonhelical, defective collagen. The steady-state level of cllagen in lysosomes may be quite small and may represent a small although metabolically active pool of intracellular collagenous peptides. To block the exit of collagen from such a pool, cells were incubated with NH_4Cl, an inhibitor of the lysosomal function. When this was done, the accumulation of hydroxy[^{14}C]proline in lysosomes was much greater, thus providing evidence for a role for the lysosome in the process by which cells degrade a fraction of their newly synthesized defective collagen.

Basal vs. Enhanced Intracellular Degradation. In the experiments described here, cells were induced to synthesize defective collagen by incorporating *cis*-4-hydroxyproline into their collagen. Cells not so induced still degrade 10–20% of their newly synthesized collagen (Berg et al., 1980; Bienkowski & Engels, 1981; Bienkowski, 1983). It is unknown whether this basal degradation represents defective collagen synthesized under normal conditions or whether there are additional mechanisms for degrading newly synthesized collagen. The latter must be considered since TLCK and leupeptin were not completely effective in inhibiting the basal degradation in confluent cells (Berg et al., 1980). Furthermore, in additional experiments, it was determined that native triple-helical procollagen was not degraded by the acid proteinases in extracts of cultured HFL-1 cells (data not shown). As would be expected for cathepsin B, only the propeptides were removed from the molecule, and the helical portion was resistant to proteolysis. If the basal level of degradation in fibroblasts represented helical collagen which is being degraded, the lack of inhibition by leupeptin argues for a nonlysosmal pathway for the degradation of helical procollagen. Clearly, the mechanism for the basal level of degradation of newly synthesized collagen in fibroblasts requires further study because

it may or may not share features of the degradation of defective collagen described in the present study.

The involvement of lysosomes in fibroblasts may not be limited to degrading defective collagen but may play another role in collagen metabolism such as processing propeptide fragments of collagen. In support of such a suggestion is the observation that antibodies to procollagen have been shown to stain lysosomes in odontoblasts (Karin et al., 1979). In this case, however, it is unknown whether or not the procollagen fragments in lysosomes were newly synthesized.

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Registry No. Proteinase, 9001-92-7; cathepsin B, 9047-22-7; cathepsin L, 60616-82-2; cathepsin D, 9025-26-7.

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