Journal of Cellular Biochemistry 67:338–352 (1997)

# Differential Effects of Ascorbate Depletion and α,α′-Dipyridyl Treatment on the Stability, But Not on the Secretion, of Type IV Collagen in Differentiated F9 Cells

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**Abstract** Ascorbic acid stimulates secretion of type I collagen because of its role in 4-hydroxyproline synthesis, but there is some controversy as to whether secretion of type IV collagen is similarly affected. This question was examined in differentiated F9 cells, which produce only type IV collagen, by labeling proteins with [$^{14}$C]proline and measuring collagen synthesis and secretion. Hydroxylation of proline residues in collagen was inhibited to a greater extent in cells treated with the iron chelator α,α′-dipyridyl (97.7%) than in cells incubated without ascorbate (63.1%), but both conditions completely inhibited the rate of collagen secretion after 2–4 h, respectively. Neither treatment affected laminin secretion. Collagen synthesis was not stimulated by ascorbate even after treatment for 2 days. On SDS polyacrylamide gels, collagen produced by α,α′-dipyridyl-treated cells consisted mainly of a single band that migrated faster than either fully (+ ascorbate) or partially (− ascorbate) hydroxylated α1(IV) or α2(IV) chains. It did not contain interchain disulfide bonds or asn-linked glycosyl groups, and was completely digested by pepsin at 15°C. These results suggested that it was a degraded product lacking the 7 S domain and that it could not form a triple helical structure. In contrast, the partially hydroxylated molecule contained interchain disulfide bonds and it was cleaved by pepsin to collagenous fragments similar in size to those obtained from the fully hydroxylated molecule, but at a faster rate. Kinetic experiments and monensin treatment suggested that completely unhydroxylated type IV collagen was degraded intracellularly in the endoplasmic reticulum or cis Golgi. These studies indicate that partial hydroxylation of type IV collagen confers sufficient helical structure to allow interchain disulfide bond formation and resistance to pepsin and intracellular degradation, but not sufficient for optimal secretion. J Cell. Biochem. 67:338–352, 1997.
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**Key words:** basement membrane; vitamin C; degradation; proline hydroxylation; teratocarcinoma cells

Type IV collagen is one of the major protein constituents of basement membranes, where it exists as a supramolecular structure that interacts with other glycoproteins including laminin, entactin, and heparan sulfate proteoglycan [Timpl and Martin, 1982; Yurchenko and O'Rear, 1994]. Assembly of the type IV collagen supramolecular structure involves tetramerization of the 7 S domain at the N-terminal through disulfide bonds and lysine-derived cross-links, dimerization of the NC1 domain, and noncovalent interactions between dimerized NC1 domains and the triple helical domain [Kühn, 1994]. There is a family of type IV collagens composed of six genetically distinct α-chains [Hudson et al., 1993], but the most prevalent type is a trimer composed of two α1(IV) and one α2(IV) chains.

F9 teratocarcinoma stem cells can be differentiated into parietal endoderm-like cells in the presence of retinoic acid plus dibutyryl cAMP or isobutyl methylxanthine [Strickland et al., 1980]. Differentiation is associated with a striking change in cell morphology and increased gene expression of basement membrane proteins, including type IV collagen, laminin, and entactin [Strickland et al., 1980; Carlin et al., 1983]. Thus F9 teratocarcinoma cells provide a model system for studying the synthesis and secretion of type IV collagen. Type IV collagen has a characteristically high content of 4-hydroxyproline compared to other collagen types and some 3-hydroxyproline, and two distinct prolyl hydroxylases catalyze the synthesis of these imino acids [Kivirriko and Myllylä, 1982].

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Received 12 May 1997; Accepted 30 June 1997

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The requirements for 4-hydroxyproline formation are $O_2$, $Fe^{2+}$, α-ketoglutarate, ascorbate, and a substrate containing an X-pro-gly sequence [Kivirikko et al., 1989]. Therefore, in the Gly-X-Y repeating tripeptide of collagen, 4-hypro is confined to the Y position while 3-hypro is found in the X position. In type I procollagen, 4-hypro stabilizes the triple helix [Berg and Prockop, 1973; Rosenbloom and Harsch, 1973]. Inhibition of hypro formation in cells, either by omission of ascorbate [Blanck and Peterkofsky, 1975; Peterkofsky, 1991] or by the addition of the iron chelator α,α′-dipyridyl [Blanck and Peterkofsky, 1975] for relatively short periods results in slower secretion of type I procollagen. Longer treatment of human fibroblasts (1–3 days) with ascorbate also induces types I and III procollagen mRNA expression, and synthesis of the proteins [Geesin et al., 1988], but this effect has been attributed to lipid peroxidation induced by ascorbate [Chojkier et al., 1989].

The helical domain of type IV collagen differs from type I in that it contains many interuptions of the typical Gly-X-Y structure [Hudson et al., 1993; Yurchenko and O'Rear, 1994], and there is some controversy as to whether hydroxylation affects its secretion. Studies of both human amniotic fluid cells [Crouch and Bornstein, 1979] and rat parietal yolk sacs [Maragoudakis et al., 1978] showed that the rate of secretion of type IV procollagen was unaffected by treatment of the cells with α,α′-dipyridyl. In contrast to these results, other studies with rat parietal yolk sacs [Karakashian and Kefalides, 1982] showed that the addition of α,α′-dipyridyl inhibited the secretion of type IV collagen, while in F9 cells, omission of ascorbate led to decreased accumulation of type IV collagen in the medium, although it was not clear if this was due to decreased synthesis or secretion [Prehm et al., 1982].

In this study, we examined the question of whether the secretion or synthesis of type IV collagen in differentiated F9 cells was affected when proline hydroxylation was inhibited, either by omitting ascorbate or by adding α,α′-dipyridyl to cultures. It was found that although both treatments led to inhibition of secretion, the extent of inhibition of proline hydroxylation differed considerably, so we also examined the properties of the type IV collagen produced under these conditions.

## MATERIALS AND METHODS

L-[U-$^{14}$C]Proline was purchased from Moravek Biochemicals (Brea, CA). Partially purified bacterial collagenase was purchased from Worthington Enzymes (Freehold, NJ) and passed through a Sephacryl S-200 column to remove nonspecific protease activity [Peterkofsky, 1982]. Powdered cell culture medium was purchased from ICN (Costa Mesa, CA) and fetal calf serum was from Hyclone (Logan, UT). Monensin and dibutyryl cAMP (dbcAMP) were purchased from Sigma and isobutylmethylxanthine (IBMX) was from Aldrich Chemical Company (St. Louis, MO). AG 50W-X8 (100–200 mesh) ion-exchange resin was purchased from BioRad Laboratories (Hercules, CA).

### Cell Culture and Radioactive Labeling

The F9 mouse embryonal carcinoma cell line (ATCC No. CRL-1720) was obtained from the American Type Culture Collection (Rockville, MD). Undifferentiated cells were cultured and maintained in MEM-5-PIE growth medium [Peterkofsky and Prather, 1992] containing 5% fetal calf serum (FCS), 1 mM pyruvate, 2 μg/ml insulin, 2 ng/ml epidermal growth factor, 50 μg/ml gentamicin, and 1 μg/ml fungizone, and the medium was changed at 2- or 3-day intervals. To differentiate cells to parietal endoderm-like cells, cultures were initiated on day zero at $1 \times 10^4$ cells per cm$^2$ in 60-mm culture dishes in 6 ml of MEM-5-PIE containing $10^{-7}$ M all-*trans*-retinoic acid (RA). On days 3 and 4, the cells were refed with 6 ml of MEM-5-PIE containing RA plus dbcAMP ($2 \times 10^{-4}$ M) and IBMX ($10^{-4}$ M). Ascorbate was not added to the medium used for growth or differentiation.

Radiolabeling experiments were performed on day 5, when most of the cells acquired a neural-like morphology, an indicator for the differentiation of F9 cells [Kuff and Fewell, 1980]. Growth medium was removed from cultures, cell layers were rinsed twice with serum-free medium containing only the gentamicin supplement (MEM-0); 1 ml of MEM-0 containing 0.1 mM β-aminopropionitrile alone or with either 0.1 mM sodium ascorbate or 0.5 mM α,α′-dipyridyl was then added. In some experiments, 0.2 μM monensin was added together with ascorbate. After a 15-min preincubation at 36.5°C, [$^{14}$C]proline was added to each dish and incubation was continued for time intervals specified in legends to figures and tables. For

quantitative assay of collagen synthesis by collagenase digestion, 2.5 μCi per dish was used and duplicate dishes were analyzed. For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis, 5 μCi per dish was used and three dishes were pooled.

### Collagen and Noncollagen Protein Synthesis

The procedure for quantitating collagen and noncollagen protein (NCP) synthesis has been described in detail elsewhere [Peterkofsky et al., 1995] and is briefly summarized here. After incubation of cells with radioactive proline, the dishes were placed on ice and the culture medium was removed. The cells were scraped off in 0.11 M NaCl/0.05 M Tris-HCl, pH 7.4 (TBS) containing 10 mM proline, collected by centrifugation, and the supernate was pooled with the medium. The cell pellet was suspended in 0.05 M Tris–HCl, pH 7.6/10 mM proline, and sonicated. Proteins in the cell and medium fractions were precipitated with trichloroacetic acid (TCA), incubated with or without purified bacterial collagenase (20 μg/ml), and the collagenase-resistant proteins (NCP) were separated from the released collagen peptides by acid precipitation. Radioactivity in the digest was measured in a liquid scintillation counter. The NCP fraction was hydrolyzed in 6 N HCl at 150°C for 30 min prior to measuring radioactivity. The relative rate of collagen production (%) was calculated by the formula; collagen dpm $\times$ 100/collagen dpm + (NCP dpm $\times$ 5.4) as described previously [Peterkofsky et al., 1995]. Protein concentrations were measured by a dye-binding technique [Bradford, 1976], using Bio-Rad reagent and immunoglobulin as a standard.

### Isolation of Collagenous Proteins

Labeling of cells and separation of the medium and cell fractions were carried out as described above, except that protease inhibitors (N-ethylmaleimide, 1 mM; EDTA, 1 mM; phenylmethylsulfonylfluoride [PMSF], 0.25 mM) and 10 mM proline were added to the medium and to the cell suspension prior to sonication. Proteins were precipitated at 33% saturated ammonium sulfate as described previously [Peterkofsky and Prather, 1992]. The precipitate (P33) was dissolved in 0.2 M NaCl/0.05 M Hepes buffer, pH 7.2, and stored at −20°C. Quantitation of radioactive collagen in the P33 fractions was carried out without prior TCA precipitation, using purified bacterial collagenase as described above.

### Gel Electrophoresis and Pepsin Digestion

SDS–PAGE was performed as described previously [Peterkofsky and Prather, 1992]. In some cases, the P33 suspension was concentrated on a microdialysis apparatus against 0.2 M NaCl/0.05 M Hepes buffer, pH 7.2. In order to identify collagenous polypeptides on gels, duplicate samples were incubated for 20 min at 37°C in a reaction solution either with or without purified bacterial collagenase (80 μg/ml) as described previously [Peterkofsky et al., 1995]. An equal volume of twice concentrated sample buffer with or without 20 mM dithiothreitol was added, and the samples were heated at 100°C for 5 min, cooled, and electrophoresed on 5% SDS-polyacrylamide gels. Gels were treated with Fluoro-Hance (Research Products International, Mount Prospect, IL) for 20 min, dried under vacuum, and the fluorogram was exposed to Kodak X-OMAT film. Collagen and laminin on gels were quantitated by densitometry using the NIH Image program. Limited pepsin digestion of proteins was carried out at a pepsin:protein ratio of 1:20 based on total protein concentration. Portions of P33 fractions were incubated with or without pepsin at 15°C for the time intervals indicated in figure legends. The reaction was stopped by the addition of 0.5 volume of 1 M ammonium bicarbonate with vigorous mixing, the samples were frozen in dry ice, and lyophilized. The dry samples were dissolved in 30 μl of reaction mix with or without bacterial collagenase and incubated and electrophoresed as described above.

### Western Blotting

Proteins on gels were blotted to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were incubated with antibody to mouse laminin and a mouse laminin standard was used, both of which were from Collaborative Biomedical Products (Bedford, MA). A Vectastain kit and DAB peroxidase substrate (Vector Laboratories, Burlingame, CA) were used for detection of immunoreactive bands. To detect N-linked glycosyl groups, membranes were blocked with TBS containing 0.1% Tween-20 (TBS-Tween) and then incubated in TBS containing 10 μg/ml of concanavalin A (Sigma), and 1 mM each of $CaCl_2$ and $MnCl_2$. The membrane was rinsed twice with TBS-Tween and then

incubated with biotinylated anti-concanavalin A antiserum (Vector Laboratories). Immunoreactive bands were detected as indicated above.

### Analysis of Proline Hydroxylation

[$^{14}$C]Proline-labeled P33 fractions derived from the cell and medium compartments were analyzed separately. The acid-soluble supernates from collagenase digests and minus enzyme controls were passed through Bio-Gel P-2 columns equilibrated with 0.1 N acetic acid to remove tannic acid. The samples were collected in screw capped tubes and dried under vacuum. The dried samples were dissolved in 6 N HCl and hydrolyzed at 150°C for 30 min. The hydrolyzates were evaporated with heat to remove HCl, and the residue was dissolved in $H_2O$. This process was repeated twice. The samples were dissolved in column starting buffer (0.2 M sodium citrate buffer, pH 3.0) and applied to a 1 × 15-cm AG 50X8 anion-exchange column as previously described [Chojkier et al., 1982]. Radioactive material was eluted by a linear gradient from 0.2 M to 0.4 M sodium citrate. L-[U-$^{14}$C]Proline and DL-hydroxy-[2-$^{14}$C]proline standards were chromatographed under identical conditions to serve as markers. Radioactivity in fractions was measured in a liquid scintillation counter. The total radioactivity in peaks corresponding to proline and hydroxyproline was determined for each sample, and values for cell and medium fractions were added together. Hydroxylation was calculated as the percentage of radioactivity in hypro compared to the sum of radioactivity in pro + hypro.

## RESULTS

### Collagen Synthesis and Secretion

The effect of conditions that cause inhibition of proline hydroxylation; i.e., omission of ascorbate or addition of α,α′-dipyridyl, on type IV collagen production and secretion in differentiated F9 cells was examined. Cells were labeled with [$^{14}$C]proline over a period of 2–8 h, and incorporation into collagen was measured by the collagenase digestion procedure. The results represent effects on type IV collagen, since this is the only type produced by the differentiated cells [Strickland et al., 1980; Prehm et al., 1982]. The rate of collagen production relative to total protein synthesis was not significantly different under any of the experimental conditions over the entire time course (Fig. 1B). In contrast, the rate of collagen secretion (Fig. 1A) was almost completely inhibited after 2–4 h in the presence of α,α′-dipyridyl (DP) or in the absence of ascorbate (NA), respectively.

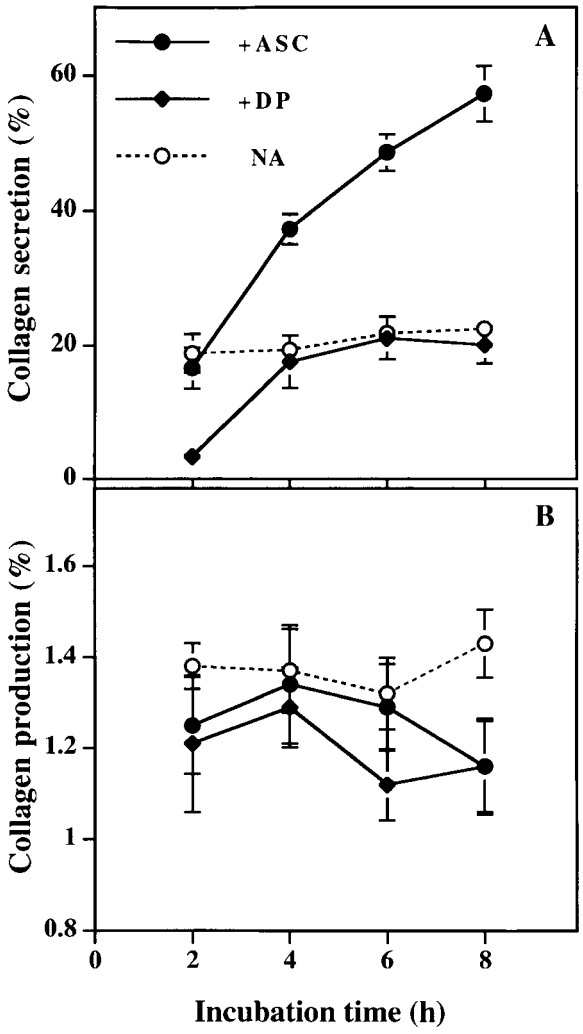


**Fig. 1.** Time course of collagen secretion and production in F9 cells treated with ascorbate or α,α′-dipyridyl. Cells were differentiated as described in Materials and Methods. On day 5, cells were refed with MEM-O with no additions (○, NA), 0.1 mM ascorbate (●, +ASC), or 0.5 mM α,α′-dipyridyl (◆, + DP) and incubated with [$^{14}$C]proline for up to 8 h. At 2 h intervals, incorporation into collagen and noncollagen proteins in the cell and medium fractions was determined using purified bacterial collagenase. Secretion **(A)** represents the percentage of total radioactive collagen in the medium. Production **(B)** is the percentage of radioactivity incorporated into collagen relative to total protein. Results are the mean values ± SE from separate experiments (control, n = 5; + ascorbate, n = 5; + α,α′-dipyridyl, n = 3), each with duplicate samples at each time point.

In addition to the short-term effect of ascorbate on the secretion of type I procollagen, treatment of fibroblasts with ascorbate for 1–3

days induces the level of mRNAs for types I and III collagen and synthesis of the proteins [Geesin et al., 1988]. As relatively little is known about the long-term effects of ascorbate on the synthesis or secretion of type IV collagen, this question was examined. After exposure to ascorbate in growth medium for 1, 4, 16, and 24 h, the medium was removed, cells were labeled in serum-free medium for 6 h in the presence or absence of ascorbate, and type IV collagen production and secretion were measured. The results (Table I) confirmed the conclusions from the initial experiment that ascorbate stimulates secretion when present solely during the 15-min preincubation and the 6-h labeling period. In addition, it was observed that when cells were preincubated for as little as 1 h, sufficient ascorbate was taken up and retained in cells so that removal of exogenous ascorbate did not inhibit secretion. Further preincubation for up to 24 h led to a slightly higher percentage of collagen secreted. Whether collagen production was calculated in relative (column 4) or absolute (column 5) terms, there was no significant stimulation of this function by long-term exposure to ascorbate. In other experiments, even a 48-h preincubation with ascorbate had no effect on collagen production (data not shown).

### SDS-PAGE Analysis of Proteins

The radioactive proteins in the medium of F9 cells incubated with or without ascorbate or with α,α′-dipyridyl were analyzed by SDS–PAGE under reducing conditions. Collagen was identified by its sensitivity to purified bacterial collagenase. After collagenase digestion of samples from cells treated with or without ascorbate, a doublet band remained (Fig. 2A, C'ase +). Since it is known that laminin B (β and γ) chains migrate similarly to type IV α-chains [Strickland et al., 1980; Prehm et al., 1982], the position of mouse laminin was determined with an anti-laminin antibody (Fig. 2B). The results confirmed the similar migration of laminin B and type IV collagen α-chains. Therefore, in samples from cells labeled in the absence or presence of ascorbate, most of the radioactivity in the co-migrating bands represents collagen and the radioactivity remaining after collagenase digestion represents mainly laminin B chains. In this experiment, collagenase digestion of one of the samples (Fig. 2A, ASC + c'ase +, lane 4) was not complete. This appeared to be a random problem that did not occur in subsequent digestions (see Figs. 4–7). The type IV collagen chains produced in the absence of ascorbate migrated only slightly faster than the chains produced in the presence of ascorbate (Fig. 2B), which would be expected for underhydroxylated collagen. The most striking observation was that the medium from cultures treated with α,α′-dipyridyl contained a single collagenous polypeptide (Fig. 2B, DP +) that migrated much faster than expected, based on previous studies using α,α′-dipyridyl [Crouch and Bornstein, 1979]. This result suggested that the polypeptide represented degraded collagen (DC).

**TABLE I. Effect of Long-Term Treatment With Ascorbate on the Synthesis and Secretion of Type IV Collagen**

| Preincubation with ascorbate* (h) | Ascorbate during labeling | Collagen secretion (%) | Collagen synthesis | | NCP synthesis |
|---|---|---|---|---|---|
| | | | Relative (%) | Absolute (dpm/μg) | Absolute (dpm/μg) |
| 0 | − | 29.3 ± 3.4 | 1.4 ± 0.1 | 35.9 ± 4.7 | 2,545 ± 230 |
| | + | 53.5 ± 6.1 | 1.2 ± 0.1 | 33.3 ± 5.1 | 2,715 ± 198 |
| 1 | − | 57.7 ± 5.5 | 1.3 ± 0.1 | 34.7 ± 5.2 | 2,718 ± 206 |
| | + | 52.0 ± 6.0 | 1.3 ± 0.2 | 36.8 ± 6.5 | 2,724 ± 311 |
| 4 | − | 55.6 ± 1.3 | 1.3 ± 0.2 | 40.2 ± 4.2 | 3,122 ± 288 |
| | + | 51.4 ± 2.5 | 1.2 ± 0.1 | 35.3 ± 4.6 | 2,900 ± 301 |
| 16 | − | 58.4 ± 5.9 | 1.3 ± 0.1 | 38.5 ± 7.5 | 2,988 ± 233 |
| | + | 61.6 ± 3.3 | 1.2 ± 0.2 | 33.2 ± 4.4 | 2,784 ± 226 |
| 24 | − | 65.9 ± 4.2 | 1.2 ± 0.1 | 38.6 ± 6.9 | 3,206 ± 333 |
| | + | 62.3 ± 2.8 | 1.2 ± 0.1 | 38.5 ± 6.6 | 3,251 ± 341 |

*Ascorbate was added to the growth medium and cells were incubated for the time intervals indicated. The medium was removed, cells were refed with serum-free medium with or without ascorbate, and after 15 min [$^{14}$C]proline was added and incubation continued for an additional 6 h, as described under Materials and Methods. Results are means ±SD (n = 3).

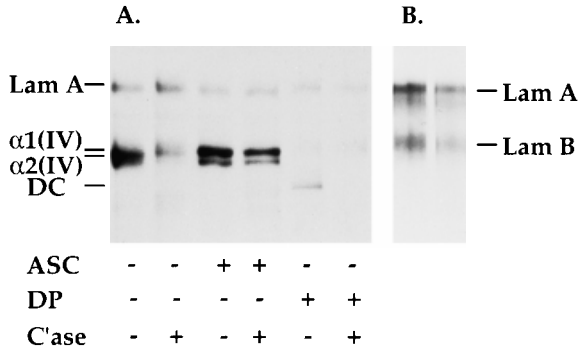


**Fig. 2.** SDS-PAGE analysis of collagen synthesized by F9 cells and identification of laminin chains by Western blotting. **A:** Differentiated F9 cells were incubated with and without ascorbate (ASC) or with α,α′-dipyridyl (DP) and labeled with [$^{14}$C]proline for 6 h as described in the legend to Fig. 1. Radioactive P33 ammonium sulfate fractions were incubated with (+) or without (−) purified bacterial collagenase (C'ase) prior to denaturation in the presence of DTT and analysis on SDS-PAGE. **B:** Purified mouse laminin was electrophoresed on a 5% SDS-polyacrylamide gel under reducing conditions and a Western blot with anti-laminin was prepared. The positions of laminin (LAM), type IV collagen chains and an apparently degraded collagen (DC) chain are indicated.

Because of the different properties of the type IV collagen produced when ascorbate was omitted or when α,α′-dipyridyl was added, the extent of proline hydroxylation in newly synthesized collagen was examined. [$^{14}$C]Proline-labeled, acid-soluble peptides derived from collagen by collagenase digestion were hydrolyzed with acid, and the radioactivity in proline and hydroxyproline was determined. Almost 70% of the proline in type IV collagen chains synthesized by the F9 cells in the presence of ascorbate was hydroxylated (Table II). This level is much higher than that found in type I collagen and is in agreement with the reported amino acid composition of type IV collagen chains [Crouch and Bornstein, 1979]. While both experimental conditions resulted in inhibition of hydroxylation, as expected, the degree of inhibition differed. Treatment with α,α′-dipyridyl resulted in 98% inhibition while in the absence of ascorbate there was a 63% inhibition (Table II).

The properties of the unhydroxylated type IV collagen were studied further by SDS–PAGE analysis of cell and medium fractions from F9 cells treated with ascorbate or α,α′-dipyridyl and radiolabeled for 2, 4, and 6 h (Fig. 3). After 2 h, most type IV collagen chains synthesized in the presence of ascorbate remained intracellular (Fig. 3B, 2 h, C'ase −), but the proportion

**TABLE II. Extent of Hydroxylation of Proline in Newly Synthesized Type IV Collagen**

| Additions | Hydroxylation (%) | Inhibition (%) |
|---|---|---|
| Ascorbate | 69.9 | 0 |
| None | 25.8 | 63.1 |
| α,α′-dipyridyl | 1.6 | 97.7 |

secreted increased for up to 6 h (Fig. 3A), in agreement with the results shown in Fig. 1. In cultures that had been incubated with α,α′-dipyridyl (Fig. 3A and B, DP +), intact type IV collagen chains were not observed in the cell or medium fractions. The putative degraded collagen (DC) already was present in cells by 2 h (Fig. 3B, 2 h, DP +, C'ase −) with very little in the medium (Fig. 3A, 2 h, DP +, C'ase −), but as synthesis continued, slightly more accumulated in the medium. The size of this polypeptide was approximately 145 kDa, based on comparison to the fully and partially hydroxylated type IV α-chains (185 and 170 kDa) and to type I collagen α-chains (95 kDa). These results suggested that degradation of unhydroxylated type IV collagen occurred intracellularly. At 6 h, another collagenous polypeptide that migrated slightly slower than the DC band was secreted into the medium, but it did not appear in the cell fraction. Two noncollagenous (collagenase resistant) polypeptides were observed only in the medium when fully hydroxylated type IV collagen was digested with collagenase prior to SDS–PAGE (Fig. 3B, 6 h, ASC +, C'ase +). Since they did not appear when collagen in the cell fraction was digested, the results suggested that these bands did not result from incomplete collagenase digestion and that they may represent proteins that interacted with collagen after secretion and were dissociated when the collagen was digested. These bands were observed in several experiments although their intensity varied. Densitometric scanning of laminin A and B chains in samples that were collagenase digested revealed that their secretion was not affected by inhibition of proline hydroxylation (data not shown).

### Subcellular Localization of Degradation Using Monensin

The kinetic experiment suggested that completely unhydroxylated collagen was degraded intracellularly, and in order to determine the subcellular compartment where this occurred,

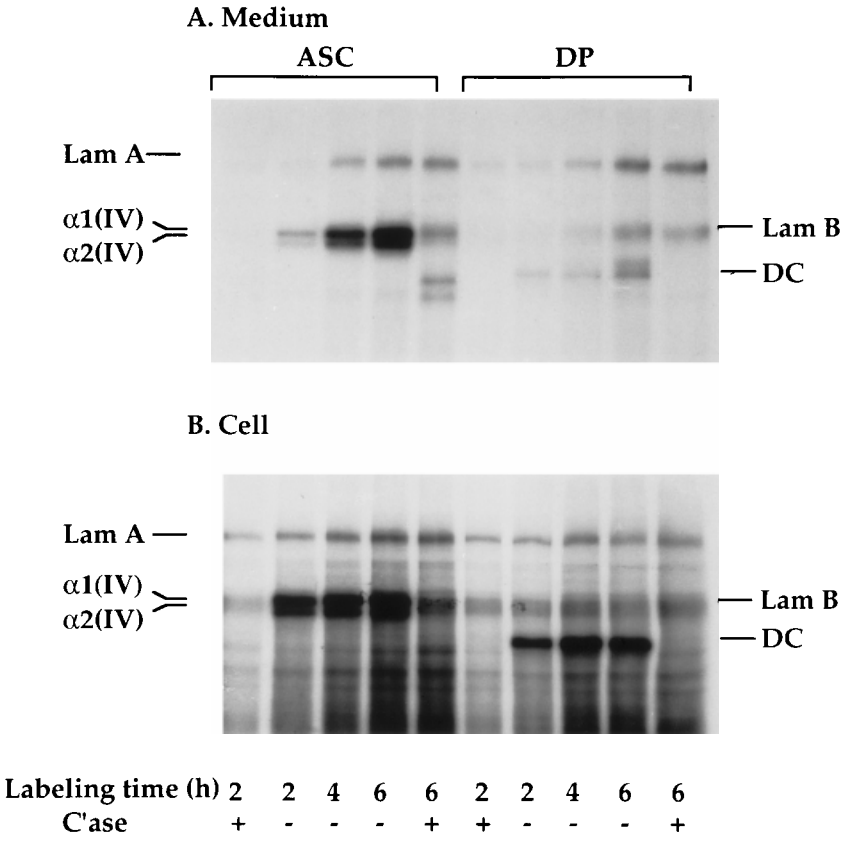


**Fig. 3.** Time-course of degradation of type IV collagen produced in the presence of α,α′-dipyridyl. Procedures were similar to those described in the legend to Figure 2 except that cells were radiolabeled for the times indicated and the cell and medium fractions were analyzed.

cells were treated with monensin. This compound blocks secretion between the cis and medial Golgi compartments [Tartakoff, 1983]. Initial dose response experiments with ascorbate-treated cells, using the quantitative collagenase digestion method, showed that collagen secretion was inhibited maximally (approximately 80%) at 0.2 μM monensin while the relative rate of its production was not greatly affected. Cells then were incubated with or without ascorbate, or with α,α′-dipyridyl, in the presence or absence of 0.2 μM monensin. Proteins were radiolabeled for 6 h, and the P33 fraction was analyzed by SDS–PAGE under reducing conditions (Fig. 4). In cells treated with ascorbate and monensin, secretion of type IV collagen chains was markedly inhibited (Fig. 4A, ASC +, Mon +), as expected. It accumulated in the cells (Fig. 4B, ASC +, Mon +), although not to as great an extent as expected, based on the decrease in the medium. Monensin also inhibited laminin secretion, and in this case also accumulation in the cell was less than expected. In α,α′-dipyridyl-treated cells, degraded collagen (DC) was observed in the cell fraction (Fig. 4B, DP +) and in the medium (Fig. 4A, DP +), but the medium also contained the more slowly migrating collagenous polypeptide, as observed in the previous experiment. In cells that were treated with α,α′-dipyridyl and monensin, degraded collagen accumulated intracellularly (Fig. 4B, DP +, Mon +) and its already low rate of secretion into the medium was inhibited still further (Fig. 4A, DP +, Mon +). Partially hydroxylated collagen chains produced in the absence of ascorbate also accumulated intracellularly in the presence of monensin, but there was no evidence of degradation (Fig. 4B, ASC −, Mon +).

### Differences in Disulfide Bonding in Partially and Completely Unhydroxylated Type IV Collagen

It has been reported that interchain disulfide bonds do not occur in intact type IV collagen secreted by human amniotic fluid cells [Crouch and Bornstein, 1979] and a teratocarcinoma

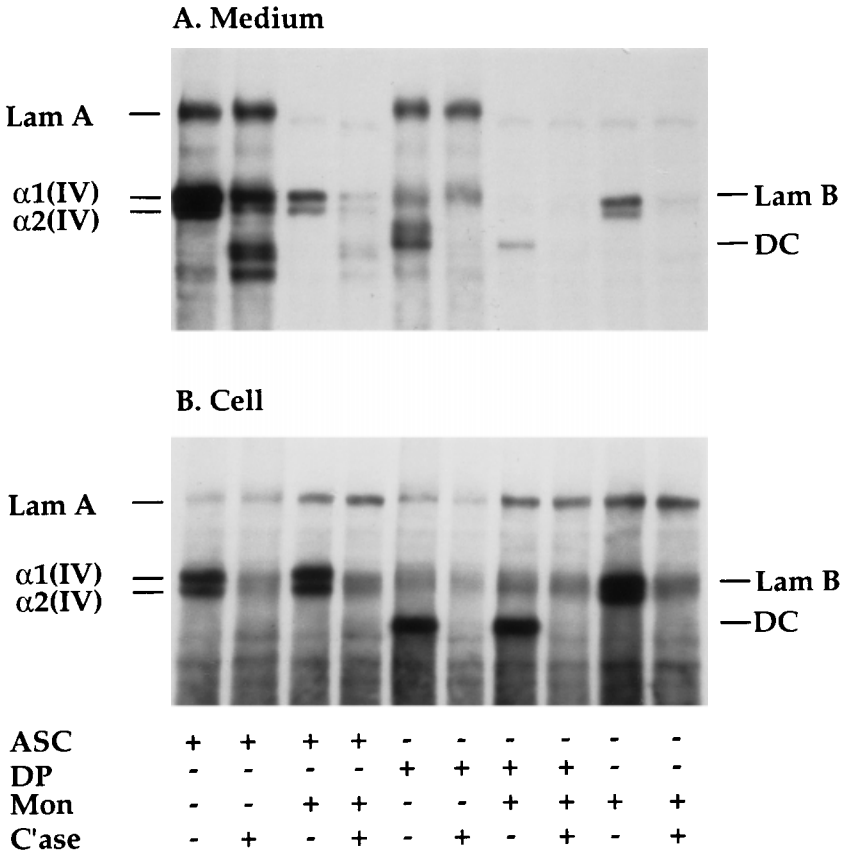


**Fig. 4.** Localization of type IV collagen degradation in α,α′-dipyridyl-treated cells. The procedures used were similar to those described in the legend to Figure 2 except that 2 μM monensin (Mon) was present (+) in some samples during the 15 min preincubation and the 6 h radiolabeling period.

cell line [Fessler and Fessler, 1982] treated with α,α′-dipyridyl. Interchain disulfide bonds in type IV collagen can be formed from several cysteine residues in the nonhelical N-terminal telopeptide, one in the 7 S collagenous domain at the N-terminal region of the molecule and two in the central triple helical domain [Hudson et al., 1993; Kühn, 1994]. The cysteine in the 7 S domain precedes an asparagine residue that is glycosylated [Langeveld et al., 1991; Nayak and Spiro, 1991]. Two cysteines in an interrupted region of the α2(IV) gly-X-Y sequence form an intrachain disulfide-bonded loop, and cysteines in the C-terminal noncollagenous NC1 domain also form intrachain disulfide bonds [Kühn, 1994]. Collagen chains were analyzed for the presence of disulfide bonds and asn-linked glycosyl groups in order to gain some information on which domains might be missing in the degraded chains. Although lysine-derived cross-links can occur in the 7S domain, β-aminopropionitrile was added to cultures to prevent their formation. When F9 cells were labeled with [$^{14}$C]proline in the presence of ascorbate, both intracellular and secreted type IV collagen chains were disulfide bonded since in the absence of reducing agents (Fig. 5A,B, ASC +, DTT −) α1(IV) and α2(IV) subunits were not observed, and they appeared only after reduction (Fig. 5A,B, ASC +, DTT +). Most of the partially hydroxylated collagen chains produced in the absence of ascorbate behaved similarly (Fig. 5A,B, ASC −, DTT −, and +), indicating that they also contained interchain disulfide bonds. The major degraded type IV collagen chain (DC) appeared in the cell and medium fractions even without reduction (Fig. 5A,B, DP +, DTT −), indicating that it did not participate in interchain disulfide bonding. Its migration was slightly slower after reduction (Fig. 5A,B, DP +, DTT +), which is indicative of intrachain disulfide bonding. The minor collagenous band migrating just above DC in Figures 3 and 4 also was observed in the medium in this experiment, but only after reduction (Fig. 5A, DP +, DTT +).

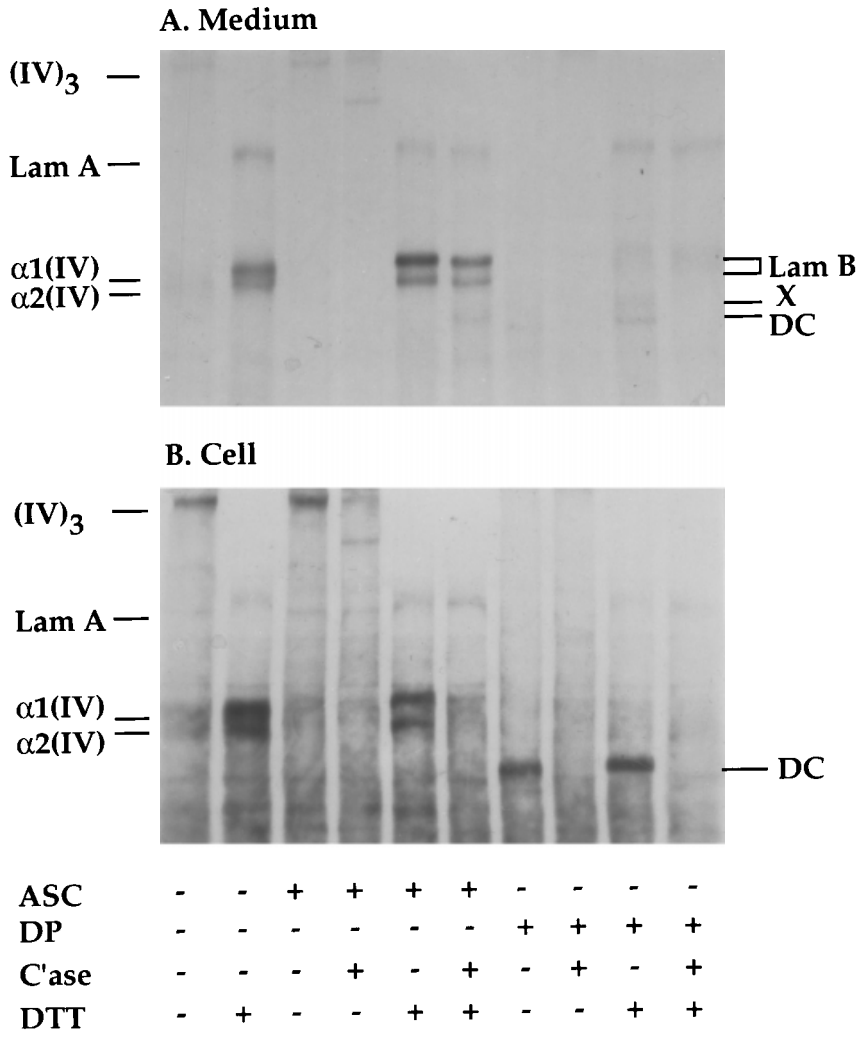


**Fig. 5.** Analysis of disulfide bonding in hydroxylated and unhydroxylated type IV collagen. Procedures were similar to those described in the legend to Figure 2 except that P33 fractions from the medium **(A)** and cells **(B)** were analyzed, and some samples were unreduced (DTT−) or reduced (DTT+) prior to SDS-PAGE. Positions of laminin and hydroxylated type IV collagen chains are indicated on the sides. DC refers to degraded collagen, while X refers to a polypeptide that appears only in the medium after reduction, and that migrates slightly slower than DC.

### Absence of N-Glycosylation in Completely Unhydroxylated Type IV Collagen

Reduced and unreduced proteins also were probed for asn-linked glycosylation with concanavalin A lectin (Fig. 6). Fully hydroxylated α1(IV) and α2(IV) subunits, as well as laminin A and B chains, in the medium and cell fractions bound the lectin (Fig. 6A,B, ASC +, DTT +). The medium and cell fractions from cultures treated with α,α′-dipyridyl contained nonradioactive, partially hydroxylated collagen subunits that contained interchain disulfide bonds and also were glycosylated (Fig. 6A,B, DP +, DTT +). Their presence can be explained by the fact that the F9 cells were differentiated in the absence of ascorbate and thus accumulated partially hydroxylated collagen prior to radiolabeling the cells in the presence of α,α′-dipyridyl. During the 6-h labeling period, the unlabeled collagen was secreted along with the radioactive newly synthesized unhydroxylated collagen, which was observed in a fluorogram run in parallel (data not shown). There were no concanavalin A-reactive bands in the positions corresponding to reduced or unreduced DC (Fig. 6A,B, DP +, DTT − and +). A minor glycosylated, collagenase-sensitive polypeptide (X) was observed in the medium only after reduction (Fig. 5B right panel; DP +, DTT + compared to −). It appeared to correspond to the

minor radioactive collagenous band that migrated above DC, as observed in Figures 3–5 and in a fluorogram run in parallel with this gel (data not shown).

### Differential Resistance of Partially and Completely Hydroxylated Type IV Collagen to Pepsin Digestion

The triple helical structure of type I collagen is resistant to pepsin cleavage; defects in this structure caused by underhydroxylation [Rosenbloom and Harsch, 1973] or mutations [Bonadio et al., 1985] can be detected as an increased sensitivity of the collagenous domain to proteolysis. Type IV collagen contains noncollagenous sequences that are sensitive to proteolysis, so that limited pepsin digestion produces distinct fragments consisting of the resistant collagenous domains [Timpl and Martin, 1982]. Since secretion of the partially hydroxylated

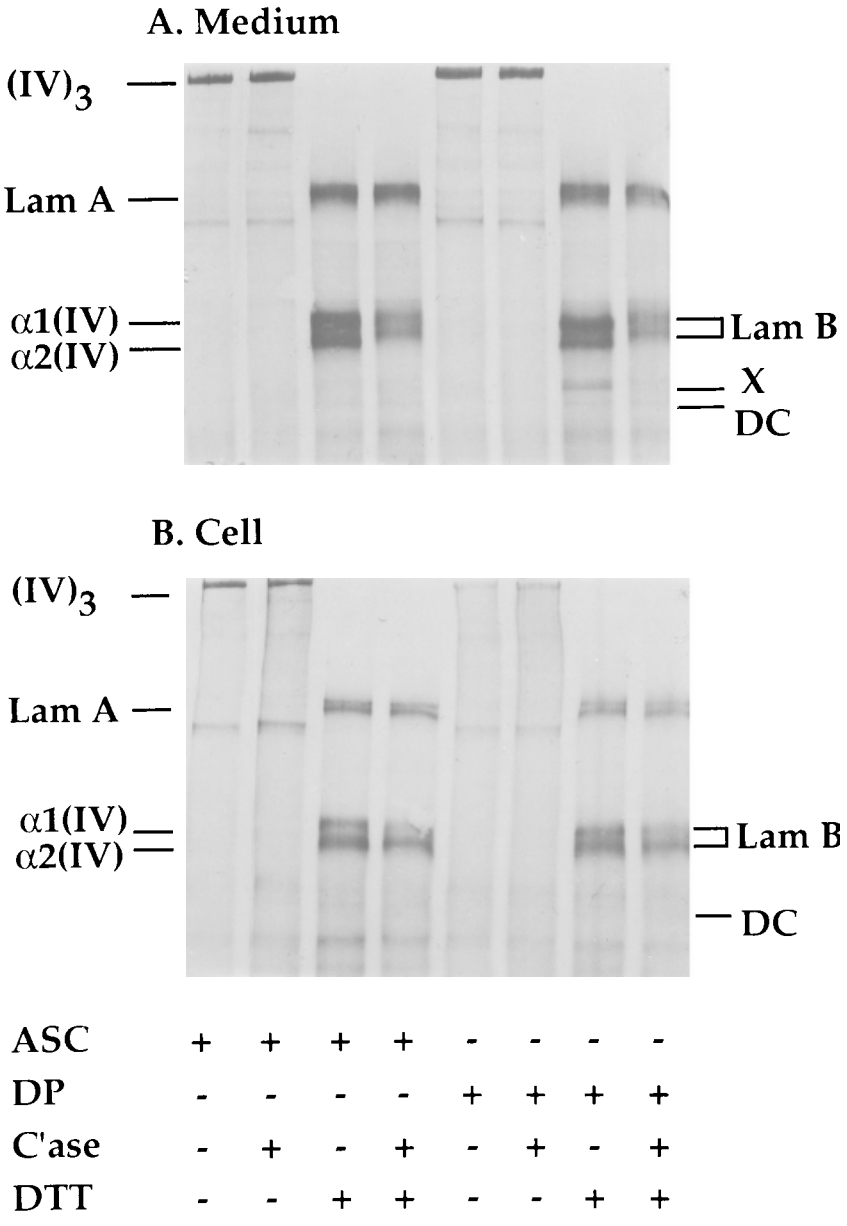


**Fig. 6.** Analysis of N-glycosylation in hydroxylated and unhydroxylated type IV collagen. Procedures were similar to those described in the legend to Figure 5 except that proteins were transferred to a membrane and concanavalin A binding was measured as described in Materials and Methods. The positions of DC and X were determined on a fluorogram of duplicate samples prepared from cells treated with α,α′-dipyridyl.

collagen was inhibited to about the same extent as completely unhydroxylated collagen, yet it contained disulfide bonds and was not degraded, its susceptibility to proteolysis was examined. Under relatively mild conditions, the partially hydroxylated molecule (Fig. 7, lanes 3, 5, and 7) was cleaved more rapidly than fully hydroxylated type IV collagen (Fig. 7, lanes 4, 6, and 9). Quantitation by densitometric scanning showed that after 2 h, 60% of the partially hydroxylated α-chains were already cleaved into multiple large fragments of approximately 95 kDa (lane 3), while only 10% of the hydroxylated chains were cleaved (lane 4). After 6 h, there was further cleavage of the partially hydroxylated large fragments into characteristic collagenous fragments (lane 5) that were relatively resistant to further proteolysis. The amount of radioactivity in the P50-95 fragments remained approximately the same even after 24 h of treatment (lane 7). After 24 h, 40% of the completely hydroxylated chains had been cleaved (lane 9). As in the case of the intact molecules, the fragments obtained from the partially hydroxylated molecule (lanes 5 and 7) migrated slightly faster than those from the fully hydroxylated molecule (lanes 6 and 9). Pepsin digestion for 2 h at 15°C cleaved the chains produced in the presence of α,α′-dipyridyl into small peptides not visible on gels, and this occurred even when the digestion temperature was reduced to 4°C (data not shown), indicating that they were completely in a denatured form.

## DISCUSSION

This study demonstrates that, like other collagens, type IV collagen must be fully hydroxylated in order to attain a maximum rate of secretion. Surprisingly, secretion was inhibited similarly by omitting ascorbate, which only partially inhibited proline hydroxylation, or by adding α,α′-dipyridyl, which completely inhibited hydroxylation. The partially and completely unhydroxylated molecules differed in their structure with respect to interchain disulfide bonding, and sensitivity to proteolysis, and only the collagen produced in the presence of α,α′-dipyridyl appeared to be degraded intracellularly. These results confirm previous studies

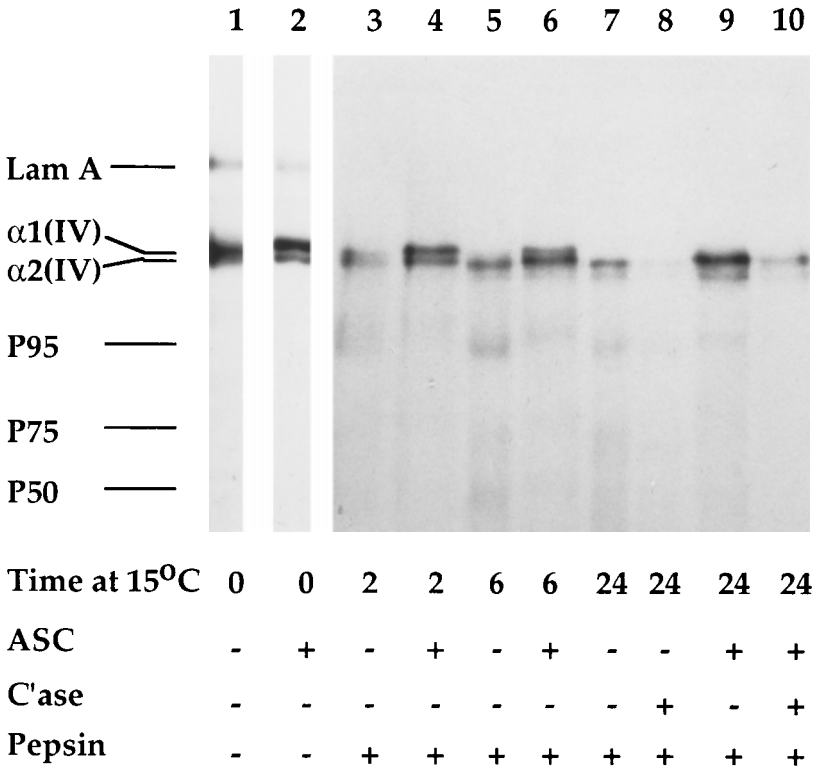


**Fig. 7.** Pepsin digestion of collagen synthesized in the absence or presence of ascorbate. Procedures were similar to those described in the legend to Figure 2 except that the ammonium sulfate fractions were treated with pepsin at 15°C for the times indicated. P95, P75, and P50 indicate the fragments formed by pepsin digestion.

showing that type IV collagen secretion in rat parietal yolk sacs is dependent on proline hydroxylation [Karakashian and Kefalides, 1982] but do not agree with studies in amniotic fluid cells [Crouch and Bornstein, 1979] and other studies in rat parietal yolk sacs [Maragoudakis et al., 1978], which concluded that secretion was not affected by α,α′-dipyridyl.

In most cell cultures, omission of ascorbate leads to production of type I collagen that is completely unhydroxylated, but some cells exhibit varying extents of ascorbate-independent hydroxylation [Peterkofsky, 1991]. This phenomenon is not due to either synthesis of ascorbate by the cells or to exogenous ascorbate, but rather to a protein in the RER with a terminal cysteinyl-cysteine sequence that can replace ascorbate as a reducing cofactor for prolyl hydroxylase [Chauhan and Peterkofsky, 1985]. The ability of differentiated F9 cells to hydroxylate proline in type IV collagen to 27% of the normal level in the absence of ascorbate, may be due to the presence of this protein. Ascorbate in culture medium is very unstable and completely disappears by 24 h after its addition [Peterkofsky, 1972]. Our current results indicate that despite this instability, once ascorbate was taken up by cells it was stored and functioned as a reducing factor for as long as 24 h. This finding may be related to the persistence of ascorbate in the tissues of guinea pigs fed an ascorbate-free diet for as long as 3 weeks [Chojkier et al., 1983]. In cells and in vivo, ascorbate is probably protected from oxidation once it is transported into the RER (Peterkofsky, 1991), where prolyl hydroxylase resides.

While short exposures of fibroblasts to ascorbate stimulate secretion of type I procollagen, longer exposures (1–3 days) lead to induction of its gene expression [Geesin et al., 1988]. Relatively few studies have been carried out on the long term effects of ascorbate on the expression of type IV collagen. When 3T3-L1 cells are differentiated into adipocytes in the presence of ascorbate, type IV collagen synthesis is stimulated after 6–15 days [Ono et al., 1990]. Since ascorbate also accelerates conversion to adipocytes, it is not clear whether the increased synthesis of type IV collagen occurred because its expression is greater in adipocytes or through some other mechanism. Our studies showed that the synthesis of type IV collagen was not stimulated in differentiated F9 cells, even after 48 h of treatment with ascorbate. Failure to see stimulation may be due to differences in regulation between types I and IV or to a difference in the metabolism of ascorbate in F9 cells compared to fibroblasts and adipocytes. Results of the quantitative assay of collagen production (Fig. 1) also showed that there was no significant effect of α,α′-dipyridyl on the relative rate of collagen production. On SDS–PAGE, however, total radioactivity in type IV collagen in the cell and medium fractions from α,α′-dipyridyl-treated cells was considerably lower than in samples from ascorbate-treated cells. This did not appear to be due to a generalized inhibition of protein synthesis, since laminin was not affected. This discrepancy may be due to the different procedures used to precipitate proteins for the two assays. In the quantitative collagenase digestion procedure, proteins were precipitated with trichloroacetic acid prior to digestion, while for gels they were precipitated with ammonium sulfate. Trichloroacetic acid may precipitate collagen fragments smaller than the DC molecule formed in dipyridyl-treated cells that are not precipitated by ammonium sulfate.

Type IV collagen differs from type I and other fibrillar collagens in that it contains segments of collagenous sequence interrupted by noncollagenous sequences [Kühn, 1994; Hudson et al., 1993]. These noncollagenous sequences are targets of proteolytic enzymes, whereas the triple helical regions are relatively resistant at temperatures below the $T_m$ of the molecule. Based on SDS–PAGE, the partially hydroxylated collagen produced in the absence of ascorbate was intact. Both α-chains were observed and they migrated only slightly faster than the corresponding, fully hydroxylated α-chains, as would be expected for underhydroxylated collagen. The molecule also contained interchain disulfide bonds. The structure was sufficiently defective, however, to allow more rapid cleavage by pepsin compared to the fully hydroxylated molecule, even though digestion was carried out at a relatively low temperature (15°C). Surprisingly, the collagenous fragments obtained after digestion of the partially hydroxylated molecule were similar in size to those eventually obtained from the fully hydroxylated molecule, and relatively stable. These results suggest that pepsin cleaved the same noncollagenous sequences in the partially and fully hydroxylated molecules, but the helical structures adjoining the interrupting sequences must be denatured

enough to allow pepsin easier access. At 37°C, the partially unhydroxylated helices would be even more denatured, which must be a sufficient defect to prevent secretion at the maximal rate.

The completely unhydroxylated chain produced in the presence of α,α′-dipyridyl could not form a helical structure even at 4°C, since the collagenous domains were completely digested by pepsin. In the case of completely unhydroxylated type I heterotrimer, enough helical structure forms at temperatures below its $T_m$, so that a large proportion of the molecule resists proteolysis [Rosenbloom and Harsch, 1973]. By analogy, it might be expected that if both type IV α-chains were present, then at 4°C they should form a structure that would confer at least partial resistance of the collagenous domains to pepsin cleavage. Therefore, our results imply that the completely unhydroxylated product represents only one of the type IV α-chains.

There were several reasons for concluding that the unhydroxylated type IV collagen produced in the presence of α,α′-dipyridyl was degraded. It consisted mainly of a single chain on SDS–PAGE, whereas both of the partially hydroxylated α-chains were observed. In addition, the partially hydroxylated chains produced by F9 cells migrated only slightly ahead of the corresponding hydroxylated chain, while the completely unhydroxylated polypeptide migrated much faster than either of them to a position corresponding to a collagen chain of approximately 145 kDa. By contrast, type IV collagen chains produced by amniotic fluid [Crouch and Bornstein, 1979] and B16 melanoma [Tokimitsu and Tajima, 1994] cells in the presence of α,α′-dipyridyl migrate just slightly ahead of the corresponding normal chains. Finally, the completely unhydroxylated chain in both the cell and medium fractions did not contain the N-glycosylation site in the 7 S domain, while it was present in both of the partially unhydroxylated type IV α-chains. Loss of the N-terminal 7 S domain by intracellular degradation could account for the decreased size of the major unhydroxylated product.

Inhibition of proline hydroxylation does not prevent formation of interchain disulfide bonds in the noncollagenous C-propeptide of type I procollagen chains (Fessler and Fessler, 1974). By contrast, formation of interchain disulfide bonds in the N-terminal propeptide of type III procollagen [Bächinger et al., 1981], and in the collagenous (Col 1) and C-terminal noncollagenous (NC1) domains of type XII collagen [Mazzorana et al., 1993], requires prior hydroxylation. It also has been reported that intact type IV collagen produced in the presence of α,α′-dipyridyl does not form interchain disulfide bonds [Crouch and Bornstein, 1979; Fessler and Fessler, 1982]. Our data suggest that the major unhydroxylated, degraded chain does not participate in the interchain disulfide bonding that normally occurs in the central region of type IV collagen [Kühn, 1994]. If these bonds had formed soon after synthesis, then a disulfide-bonded product should have been observed, even if the N-terminal region was missing.

Newly synthesized, fully hydroxylated helical type I collagen is degraded intracellularly at a basal level, while abnormal chains that are unhydroxylated, mutated, or contain proline analogs are degraded at an enhanced rate [Ripley et al., 1993]. Most studies on the degradation of type IV collagen have focused on extracellular degradation involving matrix metalloproteinases 2 and 9 [Matrisian, 1992], although the intracellular degradation of type IV collagen in mammary gland myoepithelial cells, as measured by the release of free hydroxyproline, has been reported [Warburton et al., 1986]. In F9 cells, kinetic experiments and those using monensin to inhibit secretion, indicate that degradation of the unhydroxylated chains synthesized in the presence of α,α′-dipyridyl occurs rapidly, and most likely in the RER. By analogy with the behavior of unhydroxylated type I collagen, newly synthesized, unhydroxylated type IV collagen chains would be retained in the RER and only slowly transported from this compartment. We suggest that while in the RER, these chains are rapidly degraded by removal of the 7S domain from at least one of the chains, while the other subunit may be completely degraded. The partially degraded product would then be secreted at a much reduced rate. Enhanced degradation of abnormal type I collagen has been reported to occur in the TGN [Ripley et al., 1993], lysosomes [Andersson and Warburton, 1995], or the RER [Lamandè et al., 1995]. In addition, degradation of other malfolded proteins in the RER has been described (Klausner, 1990).

In summary, our studies not only establish that proline hydroxylation is required for the secretion of type IV collagen, but they also

demonstrate that interchain disulfide bond formation can occur when relatively few hypro residues and an imperfect helical structure is present, and that the extent of hydroxylation also determines whether the molecule will be degraded intracellularly. It does not appear that the initiation of differentiation of F9 cells requires type IV collagen since it proceeds in the absence of ascorbate, unless the partially hydroxylated molecule is able to be incorporated into the extracellular matrix. A differentiation-defective F9 mutant that contains a defective prolyl hydroxylase has been described [Wang et al., 1989]. In the absence of ascorbate, type IV collagen produced by the mutant was almost completely unhydroxylated while, as in our study, the wild type F9 produced partially hydroxylated collagen. The authors suggested that defective hydroxylation caused delayed terminal differentiation, but a number of other changes that occurred, such as decreased expression of early response genes and laminin, might explain the defect. Induction of type IV collagen and laminin chains occur in parallel during differentiation of F9 cells in the absence of ascorbate [Kleinman et al., 1987; Prehm et al., 1982]; our results indicate that ascorbate is not required for laminin secretion. Another murine teratocarcinoma cell line, M1536-B3, when differentiated, produces an extracellular matrix lacking type IV collagen but containing laminin and entactin, and it forms a structure resembling naturally occuring basement membrane [Brauer and Keller, 1989]. It has been suggested that the vascular defects observed in vitamin C-deficiency are related to defects in the basement membrane [Chatterjee, 1967]. Our results suggest that defective type IV collagen may be involved, especially in light of the fact that proline hydroxylation is only partially inhibited in several tissues of vitamin C-deficient guinea pigs [Peterkofsky, 1991].

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