Characterization of Human Type III Collagen Expressed in a Baculovirus System

PRODUCTION OF A PROTEIN WITH A STABLE TRIPLE HELIX REQUIRES COEXPRESSION WITH THE TWO TYPES OF RECOMBINANT PROLYL 4-HYDROXYLASE SUBUNIT*

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An efficient expression system for recombinant collagens would have numerous scientific and practical applications. Nevertheless, most recombinant systems are not suitable for this purpose, as they do not have sufficient amounts of prolyl 4-hydroxylation activity. Pro-α1 chains of human type III collagen expressed in insect cells by a baculovirus vector are reported here to contain significant amounts of 4-hydroxyproline and to form triple-helical molecules, although the Tₘ of the triple helices was only about 32–34 °C. Coexpression of the pro-α1(III) chains with the α and β subunits of human prolyl 4-hydroxylase increased the Tₘ to about 40 °C, provided that ascorbate was added to the culture medium. The level of expression of type III procollagen was also increased in the presence of the recombinant prolyl 4-hydroxylase, and the pro-α1(III) and α1(III) chains were found to be present in disulfide-bonded molecules. Most of the triple-helical collagen produced was retained within the insect cells and could be extracted from the cell pellet. The highest expression levels were obtained in High Five cells, which produced up to about 80 μg of cellular type III collagen (120 μg of procollagen) per 5 × 10⁶ cells in monolayer culture and up to 40 mg/liter of cellular type III collagen (60 mg/liter procollagen) in suspension. The 4-hydroxyproline content and Tₘ of the purified recombinant type III collagen were very similar to those of the nonrecombinant protein, but the hydroxylysine content was slightly lower, being about 3 residues/1000 in the former and 5/1000 in the latter.

The collagens are a family of closely related but distinct extracellular matrix proteins. At least 19 proteins in insect cells by coinfection with two recombinant baculoviruses, one coding for the α subunit and the other for the β subunit (18), suggested that it might be possible to produce collagens with stable triple helices by using three baculoviruses, one of them coding for the recombinant collagen polypeptide chain and two coding for the α and β subunits of prolyl 4-hydroxylase. The expression levels were obtained in High Five cells, which produced up to about 80 μg of cellular type III collagen (120 μg of procollagen) per 5 × 10⁶ cells in monolayer culture and up to 40 mg/liter of cellular type III collagen (60 mg/liter procollagen) in suspension. The 4-hydroxyproline content and Tₘ of the purified recombinant type III collagen were very similar to those of the nonrecombinant protein, but the hydroxylysine content was slightly lower, being about 3 residues/1000 in the former and 5/1000 in the latter.

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4-hydroxylase. The present paper reports on the special features of such a system with human type III collagen as the test collagen and on the characterization of the recombinant collagen produced.

MATERIALS AND METHODS

Construction of the Baculovirus Transfer Vector and Generation of the Recombinant Virus—A BglII site was created 16 base pairs upstream of the translation initiation codon to a full-length cDNA for the pro-α1 chain of human type III procollagen (19) by polymerase chain reaction, and the CDNA was digested with the BglII and XbaI restriction enzymes. The BglII-XbaI fragment was then ligated to pVL1392 (Invitrogen). The recombinant pVL construct was cotransfected into Spodoptera frugiperda Sf9 insect cells with a modified Autographa californica nucleus polyhedrosis virus DNA using the BaculoGold transfection kit (Pharmingen), and the resultant viral pool was collected, amplified, and plaque-purified (17). The recombinant virus termed rhproCIII, was checked by a polymerase chain reaction-based method (20).

Analysis of Recombinant Proteins in Insect Cell Cultures—Insect cells (Sf9 or High Five insect) were cultured in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear) or in a serum-free HyQ CCM3 medium (HyClone), either as monolayer suspension in spinner or shaker flasks at 27 °C. To produce recombinant proteins, insect cells seeded at a density of 5–6×10⁶/ml were infected with the rhproCIII virus and with the viruses for the α subunit (virus α9) and β subunit of human prolyl 4-hydroxylase (18), the rhproCIII virus being used in a 5–10-fold excess over the other two. The cells were incubated for 20 h at 22 °C (22), and the sample was digested with a final concentration of 150 μg/ml of pepsin for 1 h at 22 °C. The sample was then chromatographed on a Sephacryl HR-500 gel filtration column (Pharmacia Biotech Inc.), eluted with a solution of 0.2 M NaCl and 0.05 M Tris, pH 7.4, dialyzed against 0.1 M acetic acid, and lyophilized.

Other Assays—The amount of purified type III collagen obtained was determined by Sircol collagen assay (Biorad). Amino acid analysis of the purified type III collagen was performed in an Applied Biosystems 421 amino acid analyzer. The melting curve was determined in a Jasco J-500 spectropolarimeter, equipped with a temperature-controlled quartz cell of 1-cm path length (Giford). The collagen concentration for the analysis shown was 104 μg/ml in 0.05% acetic acid. Thermal transition curves were recorded at a fixed wavelength (221 nm) by raising the temperature linearly at a rate of 30 °C/h using a Gilford temperature programmer.

RESULTS

Expression of Recombinant Human Type III Procollagen in Sf9 and High Five Cells—In order to ascertain whether it is possible to produce full-length pro-α1 chains of human type III procollagen in insect cells and whether the pro-α1(III) chains produced in these cells can form triple-helical molecules, a recombinant baculovirus coding for the pro-α1(III) chains was generated and used to infect Sf9 and High Five cells. The cells were cultured in TNM-FH medium supplemented with 10% fetal bovine serum or in a serum-free medium if the cell culture medium was to be analyzed, harvested 72 h after infection, homogenized in a buffer containing 0.2% Triton X-100, and centrifuged. Samples of the Triton X-100 soluble protein fraction and the concentrated cell culture medium were then analyzed by SDS-PAGE under reducing conditions, followed either by Coomasie staining (Fig. 1A) or Western blotting with an antibody to the N-propeptide of human type III procollagen. Other aliquots were studied by a radioimmunoassay for the trimeric subunit of human prolyl 4-hydroxylase. The cells infected with the three viruses were then cultured, harvested, and plaque-purified (17).

The triple-helix collagen is resistant to proteolytic enzymes, whereas nontriple-helical pro-α1(III) chains and the propeptides of triple-helical procollagen molecules are digested.

The level of pro-α1(III) chain expression was too low for these to be detected in the Coomassie-stained SDS-PAGE (Fig. 1A, lanes 2, 4, 6, and 8), but they could be seen by Western blotting in samples of the Triton X-100 soluble proteins (Fig. 1B, lanes 2 and 6) and cell culture media (Fig. 1B, lanes 4 and 8) in the case of both the Sf9 and High Five cells. After the pepsin digestion, α1 chains of type III collagen were seen in the High Five cells in the Coomassie-stained gel (Fig. 1A, lane 7). The peptide analysis for the α1(III) chains were not detected in the Western blot (Fig. 1B, lanes 3, 5, 7, and 9), since the antibody used reacts only with the N-propeptides of pro-α1(III) chains, which were apparently digested by the pepsin.

One possible explanation for the low level of expression of pepsin-resistant type III collagen could be that insect cells have insufficient amounts of prolyl 4-hydroxylase activity. In order to study this possibility, insect cells were coinfected with three recombinant baculoviruses, one of them coding for the pro-α1(III) chain as above, and the other two coding for the α and β subunits of human prolyl 4-hydroxylase. The cells infected with the three viruses were then cultured, harvested, and
Prolyl 4-hydroxylase-coding viruses increased the amount of type III procollagen in Sf9 and High Five cells by SDS-PAGE under reducing conditions. Sf9 and High Five cells were infected with a recombinant baculovirus coding for the pro-α1(III) chains, harvested 72 h after infection, homogenized in a buffer containing 0.2% Triton X-100, and centrifuged. Aliquots of the Triton X-100 soluble protein fraction and the concentrated cell culture medium were then analyzed either without pepsin treatment or after treatment with pepsin for 1 h at 22 °C. The samples were electrophoresed on 8% SDS-PAGE under reducing conditions and analyzed by Coomassie staining in panel A and by Western blotting using an antibody to the N-propeptide of human type III procollagen in panel B. Lane 1, molecular weight markers; lanes 2 and 3, cell extracts; lanes 4 and 5, media from Sf9 cell cultures; lanes 6 and 7, cell extracts; lanes 8 and 9, media from High Five cell cultures. The samples in the odd numbered lanes were digested with pepsin. Because the antibody used in the Western blotting reacts only with the N-propeptide of type III procollagen, it does not recognize pepsin-digested samples. The arrows indicate the pro-α1(III) and α1(III) chains. The intense band of about 64 kDa seen on lanes 4 and 8 in panel A probably represents bovine serum albumin. The bands with mobilities higher than that of the pro-α1(III) chains in panel B probably represent degradation products of these chains.

The efficiency of multiple baculovirus infection was assessed by immunocytochemical staining of the insect cells. Sf9 cells were coinfected with two recombinant viruses coding for the α and β subunits of prolyl 4-hydroxylase and immunostained with antibodies to these two subunits (Fig. 3). When the analysis was performed 48 h after infection, 87% of the cells were found to express at least one of the two types of subunit, with 90% of the cells expressing one type of subunit also expressing the other.

Prolyl 4-Hydroxylase Activity in the Insect Cells—The 0.2% Triton X-100 extracts of the cell homogenates were analyzed for prolyl 4-hydroxylase activity with an assay based on the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate (23). As reported previously (25), a significant level of prolyl 4-hydroxylase activity was found in both the Sf9 and High Five cells, that in the High Five cells being distinctly greater than that in the Sf9 cells (Table I). Infection of the cells with a virus coding for the pro-α1(III) chains had only minor effects on this activity, whereas the activity in the cells infected with the virus coding for the pro-α1(III) chains together with viruses coding for the two types of subunit of human prolyl 4-hydroxylase was markedly higher (Table I).

Effect of Recombinant Prolyl 4-Hydroxylase on the Level of Type III Procollagen Expression—Sf9 and High Five cells were infected with the virus coding for the pro-α1(III) chains either with or without viruses coding for the two types of subunit of prolyl 4-hydroxylase (Table II). The level of expression of total type III procollagen was measured with a radioimmunoassay for the trimeric N-propeptide, and the amount of 4-hydroxyproline formed in the cells was determined by a colorimetric assay. Both values were used to calculate the amount of type III collagen produced, assuming that all the pro-α1(III) chains formed triple-helical molecules and that all the hydroxylatable proline residues in the pro-α1(III) chains had been converted to 4-hydroxyproline. Starting out from the known structure of type III procollagen and the amount of 4-hydroxyproline in type III collagen, the amount of type III collagen in the samples was calculated by multiplying the N-propeptide values by 7 and the 4-hydroxyproline values by 8. If all the pro-α1(III) chains are present in fully hydroxylated, triple-helical molecules, both assays should give identical values, whereas if some of the type III procollagen is converted to type III collagen and the propeptides are subsequently degraded, then the values calculated from the 4-hydroxyproline assay should be higher. All these measurements were made 72 h after infection.

A considerable variation was found in the values obtained in different experiments, as shown in Table II, but a number of conclusions can still be made from the results. First, the amount of 4-hydroxyproline formed was distinctly higher in the cells infected with the prolyl 4-hydroxylase-coding viruses than in their absence in all the experiments. Second, the level of expression obtained in the High Five cells was consistently higher than that obtained in the Sf9 cells. Third, the level of type III collagen produced in the cells coinfected with the prolyl 4-hydroxylase-coding viruses was always higher when calculated from the 4-hydroxyproline values than from the radioimmunoassay values, suggesting that some of the N-propeptides of type III procollagen had been degraded or that some of the fully hydroxylated pro-α1(III) chains had remained nontriple-helical. The highest type III collagen expression values were seen in the High Five cells that also expressed prolyl 4-hydroxylase, the amount of cellular type III collagen in these cells being about 41–81 μg/5 × 10⁶ cells (Table II). The amount of type III collagen found in the culture medium, as measured with the radioimmunoassay, was about 25–50% of the total in the Sf9 cells and about 10–30% that in the High Five cells.

Experiments were also performed in which High Five cells were grown in suspension in spinner or shaker flasks. A similar effect of the prolyl 4-hydroxylase-coding viruses was seen as above. The highest expression levels found in such experiments were approximately 40 mg of cellular type III collagen/liter of culture in 72 h, about 80–90% of the total collagen produced being found in the cell pellet and 10–20% in the medium (details not shown).

Time Course for the Synthesis and Secretion of Type III Procollagen in High Five Cells—Since the level of expression of type III collagen in the High Five cells was found to be about 3-10-fold relative to that in the Sf9 cells, High Five cells were selected for the subsequent experiments. The time course for
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Conformational Integrity of the Recombinant Type III Collagen—Association of the pro-α(I) chains into trimers was studied by SDS-PAGE analysis under nonreducing conditions. Essentially all the pro-α(I) chains synthesized were found as disulfide-bonded trimers, judging from the disappearance of the band corresponding to monomeric pro-α(I) chains and the appearance of a protein band of high molecular weight (Fig. 4, lane 2). After pepsin digestion, the band corresponding to the recombinant type III procollagen was converted to one corresponding to type III collagen, and the protein remained in the form of the trimer, thus indicating the existence of disulfide bonds between the α(I) chains (Fig. 4, lane 3). Virtually all the type III procollagen expressed was soluble in the homogenization buffer containing Triton X-100, as no band corresponding to type III procollagen was seen in the Triton X-100-insoluble, SDS-soluble fraction (Fig. 4, lane 4).

The thermal stability of the type III collagen expressed under different cell culture conditions was studied using digestion with a mixture of trypsin and chymotrypsin after heating to various temperatures (22), ascorbate being either added to the cell culture medium daily as usual or omitted during infection. The Triton X-100-soluble proteins were first digested with pepsin for 1 h at 22 °C to convert the type III procollagen to type III collagen (22), and the trypsin/chymotrypsin digestion was then performed for aliquots of the pepsin-treated samples. When the pro-α(I) chains were expressed in the absence of prolyl 4-hydroxylase and ascorbate, the Tm of the type III collagen was found to be about 32–34 °C (Fig. 5A). The presence of either ascorbate or prolyl 4-hydroxylase without the other caused virtually no increase in thermal stability (Fig. 5, B and C), but

FIG. 2. Analysis of the expression of recombinant human type III procollagen by SDS-PAGE under reducing conditions in insect cells also expressing the α and β subunits of recombinant human prolyl 4-hydroxylase. SF9 and High Five cells were coinfected with three recombinant baculoviruses, one of them coding for the pro-α(I) chains and the other two coding for the α and β subunits of human prolyl 4-hydroxylase. The cells were harvested 72 h after infection and analyzed as described in the legend to Fig. 1. The samples were electrophoresed on 8% SDS-PAGE and analyzed by Coomassie staining in panel A and by Western blotting in panel B. The antibody used in this experiment was the same as in Fig. 1. Lane 1, molecular weight markers; lanes 2 and 3, cell extracts; lanes 4 and 5, media from SF9 cell cultures; lanes 6 and 7, cell extracts; lanes 8 and 9, media from High Five cell cultures.

FIG. 3. Analysis of the efficiency of expression of the α and β subunits of recombinant human prolyl 4-hydroxylase in SF9 cells. SF9 cells were coinfected with two recombinant viruses coding for the α and β subunits of human prolyl 4-hydroxylase, and analyzed 48 h after infection. The cells were double-immunostained with a rabbit polyclonal antibody to the α subunit and a mouse monoclonal antibody to the β subunit of prolyl 4-hydroxylase. Fluorescein-conjugated sheep antibody to rabbit IgG and rhodamine-conjugated sheep antibody to mouse IgG were used as secondary antibodies. Cells expressing both the α and β subunits are yellow. Cells expressing the β subunit only are indicated by closed arrows, while cells expressing the α subunit only are indicated by open arrows. Negative cells are labeled with asterisks.

TABLE I

| Cells and recombinant polypeptides expressed* | Prolyl 4-hydroxylase activity (dpm/10 μl) |
|---------------------------------------------|------------------------------------------|
| High Five cells                             |                                         |
| None                                        | 480                                      |
| Pro-α(I) chains                             | 500                                      |
| Pro-α(I) chains and α and β subunits        | 4810                                     |
| SF9 cells                                   |                                         |
| None                                        | 150                                      |
| Pro-α(I) chains                             | 60                                       |
| Pro-α(I) chains and α and β subunits        | 3360                                     |

*The cells expressed either no recombinant polypeptide, only the pro-α(I) chains, or the latter plus the α and β subunits of prolyl 4-hydroxylase. The analysis was performed 72 h after infection.

**The values are given in dpm/10 μl of the Triton extract, as means of duplicate values obtained in each of three experiments for High Five cells, and as means of duplicate values in one experiment for SF9 cells.
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| Cells and recombinant polypeptides expressed | Type III collagen in cells | Type III collagen in medium |
|---------------------------------------------|---------------------------|-----------------------------|
|                                             | Amount by radioimmunoassay| By 4-hydroxyproline         | Amount by radioimmunoassay| Percentage of total |
|                                             | μg/5 × 10^6 cells         | μg/5 × 10^6 cells           | μg/5 × 10^6 cells         |                     |
| Experiment I                                |                           |                             |                           |                     |
| Sf9 cells                                   |                           |                             |                           |                     |
| Pro-a(III)                                  | 26.0                      | 3.8                         | ND'                        | ND                  |
| Pro-a(III) + α + β                         | 6.8                       | 12.0                        | ND                         | ND                  |
| High Five cells                             |                           |                             |                           |                     |
| Pro-a(III)                                  | 38.2                      | 16.3                        | ND                         | ND                  |
| Pro-a(III) + α + β                         | 69.1                      | 81.1                        | ND                         | ND                  |
| Experiment II                               |                           |                             |                           |                     |
| Sf9 cells                                   |                           |                             |                           |                     |
| Pro-a(III)                                  | 3.9                       | 2.7                         | 4.9                        | 56                  |
| Pro-a(III) + α + β                         | 2.8                       | 9.0                         | 1.2                        | 30                  |
| High Five cells                             |                           |                             |                           |                     |
| Pro-a(III)                                  | 22.9                      | 46.0                        | 6.0                        | 21                  |
| Pro-a(III) + α + β                         | 28.7                      | 64.1                        | 3.2                        | 10                  |
| Experiment III                              |                           |                             |                           |                     |
| Sf9 cells                                   |                           |                             |                           |                     |
| Pro-a(III)                                  | 10.6                      | ND                          | 4.1                        | 28                  |
| Pro-a(III) + α + β                         | 10.9                      | ND                          | 3.3                        | 23                  |
| High Five cells                             |                           |                             |                           |                     |
| Pro-a(III)                                  | 18.6                      | 20.3                        | 9.1                        | 33                  |
| Pro-a(III) + α + β                         | 32.8                      | 40.7                        | 8.4                        | 20                  |

* The cells that expressed only the pro-a1(III) chains or these chains together with the α and β subunits of human prolyl 4-hydroxylase were analyzed 72 h after infection.
* The amount of type III collagen was calculated by multiplying the amount of the trimeric N-propeptide of type III procollagen determined by radioimmunoassay by 7, or the amount of 4-hydroxyproline by 8, as described under “Results.”
* ND, not determined.

![Fig. 4. Nonreducing SDS-PAGE analysis of trimer formation of the pro-a(III) chains expressed in High Five insect cells.](image)

The purified type III collagen was analyzed by 5% SDS-PAGE under reducing (Fig. 6, lane 2) and nonreducing (Fig. 6, lane 3) conditions. No contaminants were seen in the Coomassie-stained gel, and the α1(III) chains were disulfide-bonded. Amino acid and CD spectrum analyses were performed on the purified type III collagen. The amino acid composition corresponded well with that reported for the human protein (Table I), although the 4-hydroxyproline content was slightly lower.

A distinct difference was found in the amount of hydroxlysine, which was about 3 residues/1000 amino acids in the recombinant type III collagen rather than 5/1000 amino acids in the authentic human type III collagen. The T_m of the recombinant type III collagen was 40.8 ± 1.3 °C (± S.D., n = 4), and virtually all of the recombinant protein was stable at 37 °C (Fig. 7).

DISCUSSION

The data reported here indicate that it is possible to achieve large scale expression of native-type triple-helical human collagens in insect cells. The High Five cells gave consistently higher production rates than the Sf9 cells, the highest rates seen in High Five cells when cultured in monolayers being about 80 μg of cellular recombinant human type III collagen/5 × 10^6 cells, which corresponds to about 120 μg of type III procollagen. The largest amount of cellular type III collagen produced when the High Five cells were cultured in suspension in spinner or shaker flasks was about 40 mg/liter, corresponding to about 60 mg/liter of type III procollagen.

Prolyl 4-hydroxylase plays a central role in the biosynthesis of all collagens, as 4-hydroxyproline residues are essential for the folding of the newly synthesized polypeptide chains into triple-helical molecules (6, 12, 26). When the pro-a1 chains of type III procollagen were expressed in insect cells alone, without recombinant prolyl 4-hydroxylase, considerable amounts of 4-hydroxyproline were generated in the cells and the pro-a1 chains formed triple-helical molecules, as indicated by the resistance of the collagenous domains of these chains to pepsin digestion at 22 °C. However, the T_m of the triple helices of such
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FIG. 5. Analysis of the thermal stability of the recombinant human type III collagen produced in insect cells by a brief protease digestion. High Five cells were infected with viruses coding for the pro-α1(III) chains and the α and β subunits of human prolyl 4-hydroxylase. The cells were harvested 72 h after infection, homogenized in a buffer containing 0.2% Triton X-100, and centrifuged. The Triton-soluble proteins were digested with pepsin for 1 h at 22 °C, and the samples were subsequently treated with a mixture of trypsin and chymotrypsin at temperatures between 27 and 42 °C as described (22), the digestion being terminated by the addition of soybean trypsin inhibitor. The samples were electrophoresed on 8% SDS-PAGE and analyzed by Coomassie staining. Panel A, cells infected only with the virus coding for the pro-α1(III) chains, ascorbate omitted from the culture medium; panel B, cells infected only with the virus coding for the pro-α1(III) chains, ascorbate present in the culture medium as usual; panel C, cells infected with viruses coding for the pro-α1(III) chains and the α and β subunits of prolyl 4-hydroxylase, ascorbate was omitted from the culture medium; panel D, cells infected with the three viruses, ascorbate present in the culture medium. Lane P shows a sample digested with pepsin without subsequent trypsin/chymotrypsin digestion, lanes 27–42 show samples treated with the trypsin/chymotrypsin mixture at the temperatures indicated. The arrows show the positions of the α1(III) chains.

FIG. 6. SDS-PAGE analysis of purified type III collagen under reducing and nonreducing conditions. The reduced type III collagen sample is shown in lane 2, and the nonreduced sample is shown in lane 3. Molecular weight markers were run in lane 1. The gel was stained with Coomassie Brilliant Blue. The positions of the trimeric α1(III) chains and the monomeric α1(III) chains are shown by arrows.

TABLE III

| Amino acid | Recombinant human type III collagen | Nonrecombiant human type III collagen |
|------------|------------------------------------|--------------------------------------|
| Aspartic acid | 45.9 ± 8.3 | 42 |
| Glutamic acid | 78.9 ± 4.2 | 71 |
| 4-Hydroxyproline | 111.1 ± 4.2 | 125 |
| Serine | 45.9 ± 1.9 | 39 |
| Glycine | 339.7 ± 1.8 | 350 |
| Histidine | 12.5 ± 0.4 | 6 |
| Arginine | 37.0 ± 1.5 | 46 |
| Threonine | 18.6 ± 0.6 | 13 |
| Alanine | 96.3 ± 1.5 | 96 |
| Proline | 109.9 ± 4.3 | 107 |
| Valine | 17.3 ± 1.0 | 14 |
| Methionine | ND | 8 |
| Tyrosine | ND | 3 |
| Isoleucine | 16.4 ± 0.5 | 13 |
| Leucine | 20.4 ± 0.5 | 22 |
| Hydroxylysine | 2.8 ± 0.5 | 5 |
| Phenylalanine | 9.5 ± 0.3 | 8 |
| Lysine | 32.7 ± 0.6 | 30 |

* The values are given as mean ± S.D., n = 4.
* Ref. 37.
* Our analysis of a commercial preparation of purified human type III collagen (Chemicon) gave a 4-hydroxyproline content of 119.6 ± 4.9 residues/1000.
* ND, not determined.

were rapidly degraded. The insect cell system was found to resemble human fibroblasts (27, 28) in that the presence of ascorbate in the culture medium was necessary to produce collagen molecules with stable triple helices.

Previous experiments had demonstrated that a fully active human prolyl 4-hydroxylase tetramer can be produced in insect cells by infecting them with two recombinant baculoviruses, one of them coding for the α subunit of human prolyl 4-hydroxylase and the other the β subunit (18). Nevertheless, the recombinant enzyme had on all previous occasions been extracted from the insect cell homogenates, and the assays had been performed in the presence of the polypeptide substrate and the various cosubstrates in vitro (18, 25, 29). Although the findings suggested that the enzyme may also be active in insect cells in vivo, the present data constitute the first demonstration that this is indeed the case. Double-immunostaining experiments demonstrated that about 90% of the insect cells expressing one of the two types of subunit of human prolyl 4-hydroxylase also expressed the other.

The β subunit of prolyl 4-hydroxylase is a highly unusual multifunctional polypeptide (6, 12, 26, 30), being identical to the enzyme protein-disulfide isomerase (31, 32), which is regarded as the in vivo catalyst of disulfide bond formation in the biosynthesis of various secretory and cell surface proteins, including collagens (6, 12, 30). Although insect cells have a small amount of endogenous protein-disulfide isomerase activity, this activity is markedly increased when the cells are infected with a recombinant baculovirus coding for the human protein-disulfide isomerase/β subunit polypeptide (33). In agreement with this, the recombinant pro-α1(III) chains produced in insect cells expressing the two types of subunit of recombinant prolyl 4-hydroxylase were found to be properly disulfide-bonded when studied by SDS-PAGE under nonreducing conditions.

A major difference between the insect cell system studied here and human fibroblasts is that most of the triple-helical collagens produced by the insect cells were found to be retained within the cells, whereas collagenous molecules are rapidly secreted from human fibroblasts after formation of their triple helices (27). The low rate of secretion of triple-helical recombinant collagens from insect cells may be related to the finding...
duced in insect cells were found to be very similar to those of type III collagen extracted from various tissues (1–6). In particular, the 4-hydroxyproline content and the $T_m$ of the triple helices, when determined by CD analysis, were found to be very similar to those of nonrecombinant type III collagen. Interestingly, the hydroylsine content of the recombinant collagen was found to be about 60% of that of type III collagen extracted from various mammalian tissues even though no recombinant lysyl hydroxylase was coexpressed. This indicates that insect cells must have a considerable level of lysyl hydroxylase activity. We did not study the level of glycosylation of the 3 hydroxylysine residues formed per $\alpha$ chain, as even the $\alpha$ chains of type III collagen extracted from various tissues contain only about 0.1 residue of galactosylhydroxylsine and 0.8 of glycosylgalactosylhydroxylsine (36). However, as insect cells appear to have relatively high levels of prolyl 4-hydroxylase and lysyl hydroxylase activity, they may well have relatively high levels of collagen glycosyltransferase activities so that some of the hydroxylysine present in the recombinant $\alpha_{III}$ chains may be glycosylated.

The insect cell system studied here should allow various recombinant human collagens to be produced for use in medical applications. Furthermore, the insect cell system should make it possible to produce large quantities of various collagens that are present in tissues in amounts too small to be characterized at the protein level.

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