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Formation of Recombinant Human Procollagen I Heterotrimers in a Baculovirus Expression System

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The present study describes the production of human procollagen I in a baculovirus expression system. Recombinant baculovirus carrying proα1(I) or proα2(I) cDNA was constructed and infected to Sf9 cells. Full-length proα1(I) or proα2(I) chains were synthesized by the cells infected with either of the recombinant viruses. The proα1(I) chains formed pepsin-resistant homotrimers stabilized by interchain disulfide bonds, a small proportion of which was secreted into the culture medium. The proα2(I) chains were not linked into trimers by disulfide bonds and failed to form stable triple helices, although some chains were suggested to exist as dimers or unstable trimers in which only two chains were linked by disulfide bonds. In spite of their non-helicity, the proα2(I) chains were secreted at a higher rate than the proα1(I) chains. Sf9 cells simultaneously synthesized both proα1(I) and proα2(I) chains when the cells were co-infected with the two recombinant viruses. Pepsin-treatment of the product clearly demonstrated the production of procollagen I heterotrimers composed of two proα1(I) chains and one proα2(I) chain, homotrimers of the proα1(I) chains being negligible. This expression system appears to offer a unique means of studying the mechanism of chain association and secretion during procollagen biosynthesis.

Key words: baculovirus vector, collagen, heterotrimer, molecular assembly, recombinant protein.

Type I collagen is a major structural protein of the extracellular matrix and exists as cross-striated fibrils in a variety of tissues such as the dermis and bone. This molecule has the triple helical structure comprised of two α1(I) chains and one α2(I) chain. These chains are synthesized as precursors, proα1(I) and proα2(I) chains, respectively, and then assembled into trimers called procollagen I which have globular propeptide domains at the amino (N) and carboxyl (C) terminals (*1*). Procollagen I generally occurs as the [proα1(I)]$_2$[proα2(I)] heterotrimer. It is known that proα1(I) chains can also form a [proα1(I)]$_3$ homotrimer (*2, 3*), while the existence of a [proα2(I)]$_3$ homotrimer has not been reported. Association of procollagen chains is initiated by noncovalent interactions among C-propeptide domains and is stabilized by the formation of interchain disulfide bonds. However, the mechanism of this process is not yet fully understood (for review, see Refs. *4* and *5*).

Analyses of naturally occurring mutations in collagen genes provided information on chain assembly process. For example, mutations in C-propeptides of proα1(I) or proα2(I) chains revealed their critical role in the process (*6-8*). Studies on procollagen synthesis using recombinant expression systems may contribute to understanding the mechanism of chain assembly. Two different experiments have been reported which produced hybrid procollagens I consisting of recombinant human chains and endogenous murine chains. Schnieke *et al.* (*9*) introduced human proα1(I) genes into proα1(I)-deficient Mov-13 mouse cells in which proα2(I) chains were undetectable due to rapid degradation. Expression of recombinant proα1(I) chains in the cells rescued proα2(I) chains from degradation, resulting in the production of hybrid heterotrimers. Smith and colleagues (*10-12*) used proα2(I)-deficient W8 rat cells for the introduction of human proα2(I)-cDNA, and the transfected cells synthesized both hybrid heterotrimers and endogenous proα1(I)-homotrimers. Homotrimers of human proα1(I) chains were also synthesized by introducing proα1(I)-cDNA to HT1080 human cells (*13*). However, the production of recombinant procollagen I heterotrimers composed of human chains has not been reported.

Recently, we demonstrated the possibility of producing recombinant human procollagen using a baculovirus expression system. Host cells, an insect cell line Sf9, possessed activity of prolyl 4-hydroxylase, an enzyme required for the formation of stable triple helix. Infection of a recombinant baculovirus carrying proα1(III)-cDNA to these cells produced the triple helical procollagen III homotrimers (*14*). This finding encouraged us to construct an expression model which can produce procollagen I heterotrimers composed of recombinant human proα1(I) and proα2(I) chains. In the present study, the cDNA for either of the two procollagen chains was introduced into Sf9

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and its expression was analyzed. Then, both cDNAs were simultaneously introduced into the cells to discover whether procollagen I heterotrimers could be produced.

MATERIALS AND METHODS

Cell Culture—Sf9 cells were obtained from the Riken Cell Bank and maintained in Grace's insect medium (Gibco/BRL) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco/BRL) at 28°C. The cells were cultured in serum-free Sf-900 II medium (Gibco/BRL) when they were subjected to transfection of DNAs. Human dermal fibroblasts were cultured as described previously (*14*).

Construction of Recombinant Baculoviruses—Plasmids carrying cDNAs for human proα1(I) and proα2(I) chains were described previously (*15, 16*) and were kindly provided by Dr. Ramirez (Mount Sinai School of Medicine, New York). Proα1(I)-cDNA inserts were excised from one of the plasmids with *Xba*I and *Sal*I, blunt-ended with T4 DNA polymerase, and ligated into the transfer vector pAcYM1 (*17*) which had been digested with *Bam*HI and blunt-ended. The resultant plasmid (pAc1A1/Pol) contained 6 bp of 5′-untranslated region (UTR), the full-length proα1(I)-coding region, and 223 bp of 3′-UTR. The proα1(I)-cDNA was also introduced into the blunt-ended *Bam*HI site of pAcMP3 (Pharmingen), downstream of baculovirus basic protein promoter. The 3′-UTR was deleted as a 262 bp fragment from the *Eco*RI site at nucleotide (nt) 4476 of the coding region to the *Bgl*II site of pAcMP3, and the sequence of the missing coding region (38 bp) from the *Eco*RI site to the stop codon was synthesized and ligated with the deleted cDNA. The final construct thus obtained was designated pAc1A1/MP.

The proα2(I)-cDNA in the other plasmid provided by Dr. Ramirez coded for the proα2(I) chain polypeptide, but lacked the first 129 amino acids. To construct the cDNA coding for the entire proα2(I) chain, a fragment corresponding to the missing sequence from nt130 to nt521 of proα2(I)-cDNA was generated from human placental mRNA (Clontech) by reverse transcriptase polymerase chain reaction, and ligated into the *Pst*I site at nt521 in the original proα2(I)-plasmid. From the resultant plasmid, a cDNA fragment including 5 bp of 5′-UTR, the entire proα2(I) coding region, and 42 bp of 3′-UTR was released and introduced downstream of the basic protein promoter in pAcMP3. The 3′-UTR of proα2(I)-cDNA was deleted from this transfer vector by removing a sequence from the *Eco*RI site at nt4197 to the *Bgl*II site of pAcMP3 and supplying the synthetic DNA for the missing coding region (38 bp) as above, which generated pAc1A2/MP.

DNAs of the recombinant transfer vectors and wild-type AcNPV (Pharmingen) were co-transfected into Sf9 cells by lipofection (*18*) to obtain recombinant baculoviruses (Ac1A1/Pol, Ac1A1/MP, and Ac1A2/MP), which were isolated, purified, and amplified as described previously (*19*).

Expression and Radiolabeling of Recombinant Procollagen Chains—Monolayers of Sf9 cells seeded at a density of 10^6 cells/35-mm-diameter dish were infected at a multiplicity of 10 plaque-forming units/cell with wild-type or recombinant viruses, and incubated in Grace's medium supplemented with 10% (v/v) FBS and 50 μg/ml ascorbic acid (Gibco/BRL) at 28°C. To generate radiolabeled proteins, cells were preincubated for 30 min in proline-free Grace's medium (Sanko Junyaku) containing 10% dialyzed FBS and 50 μg/ml ascorbic acid, and incubated for 4 h in the presence of 370 kBq/ml of L-[2,3-^{3}H]proline (Amersham) or 37 kBq/ml of [*U*-^{14}C]proline (Amersham). For pulse-chase experiment, cells were labeled with 74 kBq/ml of [^{14}C]proline for 10 min, and chased up to 120 min in the presence of 50 mM proline and 10 μg/ml cycloheximide (Sigma). The labeled cells were harvested, washed with phosphate buffered saline (PBS), and lysed in 50 mM Tris/HCl, pH 7.5, by three cycles of freezing and thawing. Proteins in the culture medium were precipitated with 33% saturated $(NH_4)_2SO_4$ for 16 h at 4°C and collected by centrifugation.

Determination of Collagen Synthesis and Extent of Prolyl Hydroxylation—Proteins in the lysates of labeled cells were precipitated with 5% (w/v) trichloroacetic acid (TCA) and digested with 100 units/ml of highly purified bacterial collagenase (Advance Biofactures). Digested samples were treated again with 5% TCA to separate collagenase-sensitive (supernatant) and insensitive (precipitate) materials, and radioactivities of both fractions were measured to calculate the ratio of collagen synthesis (*20*). The supernatants were further hydrolysed in 6 N HCl for 24 h at 110°C, and the extent of prolyl hydroxylation of synthesized procollagens was determined by measuring the distribution of radioactivity between proline and hydroxyproline in the hydrolysates as described previously (*14*).

Enzymatic Digestion of Secreted Material—Proteins in the medium were precipitated with 33% $(NH_4)_2SO_4$, dissolved in 2 mM $CaCl_2$ and 120 mM Hepes, pH 7.2, and treated with bacterial collagenase as above. For pepsin digestion, the precipitates were suspended in 0.5 M acetic acid and incubated with pepsin (Sigma) at 4°C for 16 h at the concentrations indicated in the text. The reaction was stopped by neutralization and addition of the sample buffer for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS/PAGE). In some cases, the neutralized samples were additionally incubated at 4°C for 16 h to inactivate pepsin completely, then treated with bacterial collagenase.

Determination of Thermal Stability—Pepsin-treated collagens were precipitated with 20% (w/v) NaCl at neutral pH, and dissolved in and dialyzed against 0.5 M acetic acid (*21*). They were then lyophilized and again dissolved in 0.4 M NaCl and 0.1 M Tris/HCl, pH 7.4. The collagen solutions were heated at different temperatures for 5 min in a DNA thermal cycler (Perkin Elmer Cetus), and digested with a mixture of 100 μg/ml trypsin (Sigma) and 250 μg/ml chymotrypsin (Sigma) for 2 min at 20°C. The digests were mixed immediately with an excess amount of soybean trypsin inhibitor (0.5 mg/ml, Sigma) and an equal volume of two times concentrated boiling SDS/PAGE sample buffer, then subjected to SDS/PAGE as described below.

SDS/PAGE—Electrophoresis of recombinant and standard protein samples was performed in 0.1% SDS/6% polyacrylamide gels according to Laemmli (*22*). Unlabeled proteins were visualized by staining with Coomassie Brilliant Blue R-250. Immunoblot analysis was performed as described previously (*14*) using polyclonal antibody raised against a mixture of human and bovine type I collagen (LSL). In this analysis, human type I collagen purchased from CHEMICON was used as a standard. For

detection of radiolabeled proteins, the gels were fixed, treated with EN³HANCE™ (NEN/Du Pont), dried under vacuum, and exposed to a Kodak X-Omat AR film following the manufacturer's protocol. Relative amounts of radioactivity among bands of SDS/PAGE were quantified as follows. Dried gels were exposed to BAS2000 imaging plates (Fuji Film) and the density of bands was quantified using a BIO-IMAGE ANALYZER BAS2000 (Fuji Film). Standard procollagens for the analysis of labeled proteins were prepared as follows. Confluent human fibroblasts were cultured for 24 h in the presence of [³H]proline (370 kBq/ml) and procollagens were obtained from the culture medium as 5% TCA-precipitates. Pepsinized collagen standard was also prepared from human fibroblasts as described previously (*21*).

RESULTS

Effect of Promoters on Expression and Prolyl Hydroxylation of proα1(I) Chains—Two recombinant viruses, Ac1A1/Pol and Ac1A1/MP, were generated, in which proα1(I)-cDNA was placed downstream of the polyhedrin promoter and the basic protein promoter, respectively. The latter promoter becomes active after infection earlier than the former promoter (*24*). Sf9 cells infected with wild-type or recombinant viruses were labeled with [³H]proline for 4 h from 20 to 24 h post-infection (p.i.) (1 day p.i.), and for another 4 h from 44 to 48 h p.i. (2 day p.i.). Cells were harvested and the ratio of procollagen synthesis to total protein synthesis was determined by collagenase assay (Table I). In wild-type-infected cells, collagenase-sensitive protein was not detected, indicating that no endogenous collagen was synthesized in the infected Sf9 cells. The synthesis of recombinant proα1(I) chains was detected in Ac1A1/MP-infected cells at 1 day p.i. (6.7% of total protein synthesis). In contrast, Ac1A1/Pol-infected cells started to synthesize proα1(I) chains (1.2%) at 2 day p.i., when the rate of collagen synthesis by Ac1A1/MP-infected cells had decreased to 4.4%. The extent of prolyl hydroxylation of collagenase-sensitive products was measured in Ac1A1/MP-infected cells at 1 day p.i. and Ac1A1/Pol-infected cells at 2 day p.i. (Table I): 13.4% hydroxylation of proline residues for Ac1A1/MP and 5.1% for Ac1A1/Pol. Apparently, the basic protein promoter became active earlier than the polyhedrin promoter in procollagen synthesis and supported a higher prolyl hydroxylation. Therefore, this promoter was used for the expression of both proα1(I) and proα2(I) chains in the following experiments.

Expression and Secretion of Recombinant proα1(I) and proα2(I) Chains—Sf9 cells were infected with Ac1A1/MP or Ac1A2/MP virus and harvested after 28 h. The cell pellets were dissolved in SDS/PAGE sample buffer and analyzed by electrophoresis under reducing conditions. Coomassie-stained polypeptides, which were not observed in wild-type-infected cells, were detected in Ac1A1/MP- and Ac1A2/MP-infected cells at migration positions corresponding to globular proteins of *ca.* 170 kDa and *ca.* 135 kDa, respectively (Fig. 1, lanes 2-4). These polypeptides were reactive with polyclonal antibody against type I collagen (Fig. 1, lanes 6-8). This result indicates that these polypeptides were recombinant products encoded by introduced proα1(I) and proα2(I) cDNA, respectively. Proα1(I) chains were stained more weakly than proα2(I) chains, probably because the antibody was more immunoreactive with α2(I) chains than with α1(I) chains of standard human type I collagen (Fig. 1, lane 5). To visualize the recombinant products clearly and analyze them quantitatively, Sf9 cells were infected with Ac1A1/MP or Ac1A2/MP at the multiplicity of 10, and labeled with [¹⁴C]proline for 4 h from 20 to 24 h p.i. Proteins precipitated from cell lysates and culture medium were electrophoresed under reducing conditions and visualized by fluorography. Since collagenous proteins behave anomalously in SDS/PAGE as compared to typical globular proteins (*23*), [³H]proline-labeled procollagens prepared from human fibroblasts were also electrophoresed as a molecular weight marker. Recombinant chains of proα1(I) and proα2(I) were detected in infected Sf9 cells, and comparison of their migration with

TABLE I. **Synthesis and prolyl hydroxylation of recombinant proα1(I) chains.** Sf9 cells were infected with wild-type virus (w.t.), Ac1A1/MP (MP), or Ac1A1/Pol (Pol), and labeled with [³H]proline for 4 h from 20 to 24 h p.i. and for another 4 h from 44 to 48 h p.i. Procollagen synthesis in each type of infected cells was determined by the collagenase assay (*20*) and is expressed as the percentage of total protein synthesis. The extent of prolyl hydroxylation of collagenase-sensitive products was determined by measuring the distribution of radioactivity between proline and hydroxyproline, and is shown as the percentage of Pro + Hyp radioactivity.

	p.i.	Cells infected with		
		w.t.	MP	Pol
Collagen synthesis	24 h	0.1%	6.7%	0.0%
	48	0.0	4.4	1.2
Extent of prolyl hydroxylation	24	n.d.	13.4	n.d.
	48	n.d.	n.d.	5.1

n.d., not determined.

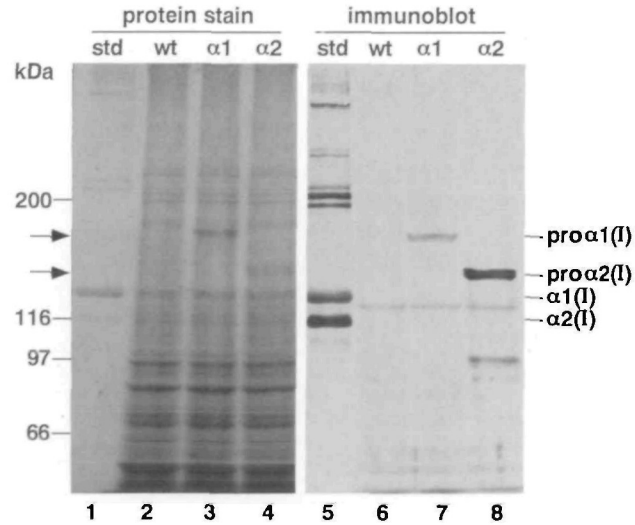

Fig. 1. **Expression of recombinant proα1(I) and proα2(I) chains.** Sf9 cells were infected with wild-type virus, Ac1A1/MP, or Ac1A2/MP, and harvested after 28 h. The cell pellets were dissolved in SDS/PAGE sample buffer and analyzed by SDS/PAGE under reducing conditions, followed either by staining with Coomassie Blue (lanes 1-4) or by immunoblot analysis with anti-type I collagen antibody (lanes 5-8). Arrows indicate *ca.* 170-kDa and *ca.* 135-kDa polypeptides, respectively. A band of *ca.* 120-kDa in immunoblot was a cross-reactive protein (lanes 6-8). std, human type I collagen; wt, wild type virus-infected cells; α1, Ac1A1/MP-infected cells; α2, Ac1A2/MP-infected cells.

that of the procollagen standard indicated that both were of full length (Fig. 2, lanes 1-4). Measuring relative radioactivities of these chains using an image analyzer revealed that the expression of recombinant proα1(I) and proα2(I) chains was at a similar level when recombinant viruses were infected at the same multiplicity of infection (data not shown).

Most proα1(I) chains remained within the cells, while a small fraction was secreted into the medium (Fig. 2, lanes 3 and 6). In contrast, a relatively large fraction of proα2(I) chains was detected in the medium (Fig. 2, lanes 4 and 7). To compare the secretion rates of these procollagen chains, virus-infected cells were pulse-labeled with [^{14}C]proline and chased up to 120 min in the presence of excess unlabeled proline. The ratio of secreted procollagen to total procollagen was determined by measuring the relative radioactivity of procollagen chains in the cell and medium fractions. As shown in Fig. 3, recombinant proα2(I) chains were secreted at a rate approximately four times higher than the proα1(I) chains. At 120 min after synthesis, the proportion of proα2(I) chains secreted into the medium reached 44.7%, while that of proα1(I) chains was 11.1%.

Conformation of proα1(I) and proα2(I) Chains—Prior to co-expression experiments of proα1(I) and proα2(I) chains, the biochemical nature of products synthesized by cells infected with either of the two recombinant viruses was analyzed by SDS/PAGE. To detect the formation of interchain disulfide bonds, the secreted proteins were subjected to the gel electrophoresis under reducing or non-reducing conditions. Monomeric proα1(I) chains were observed under reducing conditions, but hardly detected under non-reducing conditions (Fig. 4, *cf.* lanes 2 and 7). Instead, a higher molecular weight material appeared. This protein was shown to be a homotrimer of proα1(I) because it co-migrated with trimers of standard procollagens (Fig. 4, lanes 6 and 7) and it was degraded by highly purified bacterial collagenase (Fig. 4, lane 8). Therefore, it was concluded that the secreted proα1(I) chains formed homotrimers linked by disulfide bonds.

When the proα2(I) chains were electrophoresed under

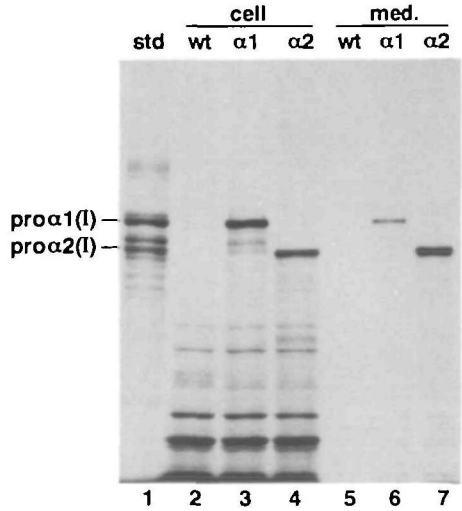

Fig. 2. **Synthesis and secretion of recombinant proα1(I) and proα2(I) chains.** Sf9 cells were infected with wild-type virus, Ac1A1/MP, or Ac1A2/MP, and labeled with 37 kBq/ml of [^{14}C]proline for 4 h at 1 day p.i. Proteins precipitated from cell lysates (lanes 2-4) and culture medium (lanes 5-7) were analyzed on a 6% SDS-polyacrylamide gel under reducing conditions. std, human fibroblasts-derived procollagens; wt, wild type virus-infected cells; α1, Ac1A1/MP-infected cells; α2, Ac1A2/MP-infected cells.

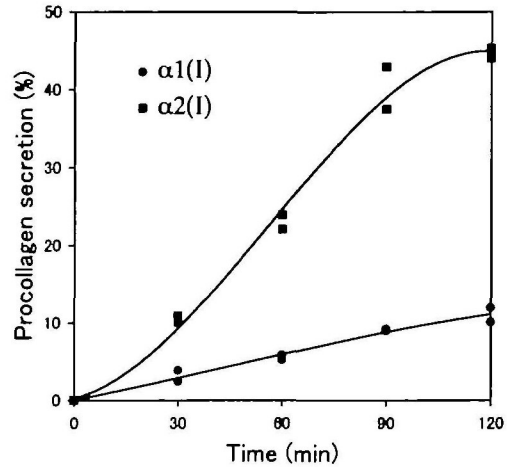

Fig. 3. **Pulse-chase analysis of the secretion of recombinant proα1(I) and proα2(I) chains.** Sf9 cells were infected with Ac1A1/MP (α1) or Ac1A2/MP (α2). The infected cells were labeled with 74 kBq/ml of [^{14}C]proline for 10 min and chased in the presence of excess amounts of non-radioactive proline. Proteins in cell lysates and culture medium were electrophoresed under reducing conditions, and the relative radioactivity of the bands corresponding to recombinant procollagen chains was estimated using a BAS2000 image analyzer. Procollagen secretion represents the ratio (%) of the procollagen chains found in the medium compared to the sum of the cell and medium fractions. Values from duplicate determinations were plotted and regression curves were drawn.

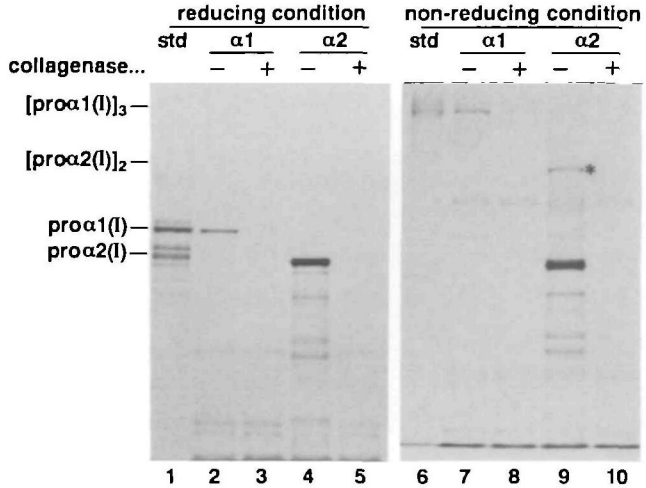

Fig. 4. **Formation of interchain disulfide bonds.** Sf9 cells were infected with Ac1A1/MP (α1) or Ac1A2/MP (α2) and labeled with 37 kBq/ml [^{14}C]proline for 4 h at 1 day p.i. Procollagen chains in the medium were precipitated with 33% $(NH_4)_2SO_4$ at 4°C and collected by centrifugation. The procollagen chains thus obtained were treated with (+) or without (−) 100 units/ml of highly purified bacterial collagenase, and analyzed by SDS/PAGE under reducing (lanes 1-5) or non-reducing (lanes 6-10) conditions. The asterisk indicates dimers of proα2(I) chains. std, human fibroblasts-derived procollagens; α1, proteins from Ac1A1/MP-infected cells; α2, proteins from Ac1A2/MP-infected cells.

non-reducing conditions, disulfide-linked trimers were not detected and the major product was found to migrate to the monomer position (Fig. 4, *cf.* lanes 4 and 9). In addition, a collagenase-sensitive material was observed at a position between monomer and trimer (Fig. 4, asterisk). This material was estimated to be dimers of procollagen chains by comparing its migration with that of standard procollagens. This result indicates the formation of disulfide bonds between two proα2(I) chains.

To ascertain whether the recombinant procollagen chains take a triple helical conformation, they were treated with pepsin before electrophoresis under reducing conditions (Fig. 5). When the proα1(I) chains were treated with pepsin at the standard concentration (100 μg/ml), a collagenase-sensitive product co-migrating with α1(I) chains of standard collagens was detected, although its intensity was weaker than that of the proα1(I) chains, indicating that some of the proα1(I) chains formed stable triple helices (Fig. 5a, lanes 2-4). In contrast, the proα2(I) chains were completely digested by pepsin at the same concentration, showing the absence of stable triple helices (Fig. 5a, lanes 5-7). Nevertheless, disulfide bonds between two proα2(I) chains were detected as described above. This implies that a portion of the chains might form dimers. Alternatively, the proα2(I) chains could form unstable homotrimers in which only two chains were disulfide-linked, although such molecules were undetectable by SDS/PAGE under non-reducing conditions. To examine whether unstable homotrimers of the proα2(I) chains were formed, the samples were treated with pepsin at decreased concentrations (Fig. 5b). Pepsin treatment of the proα2(I) chains at concentrations below 10 μg/ml produced α2(I)-sized and lower molecular weight materials, both of which were collagenase-sensitive (Fig. 5b, panel N). On the other hand, proα2(I) chains which had been denatured at 65°C for 10 min before pepsin treatment were completely degraded even at such decreased concentrations (Fig. 5b, panel D). These results suggest that recombinant proα2(I) chains might form a homotrimer, although this structure is unstable compared to the helical structure of recombinant proα1(I)-homotrimers and normal procollagen I heterotrimers.

Production of Procollagen I Heterotrimers—Sf9 cells were co-infected with Ac1A1/MP and Ac1A2/MP in a ratio of 1:1. Both proα1(I) and proα2(I) chains were synthesized by the infected Sf9 cells at a similar level and secreted into the medium as they were in individual infections (Fig. 6a). Their rates of secretion were unaltered by the co-expression. Namely, most proα1(I) chains remained in the cells and considerable amounts of the proα2(I) chains were secreted in the culture medium, as in their single expressions (Fig. 6a, lanes 5 and 9). Essentially the same result was obtained when the ratio of infected virus (Ac1A1/MP: Ac1A2/MP) was changed to 2:1, in which case the ratio of expression level of proα1(I) chains to that of proα2(I)

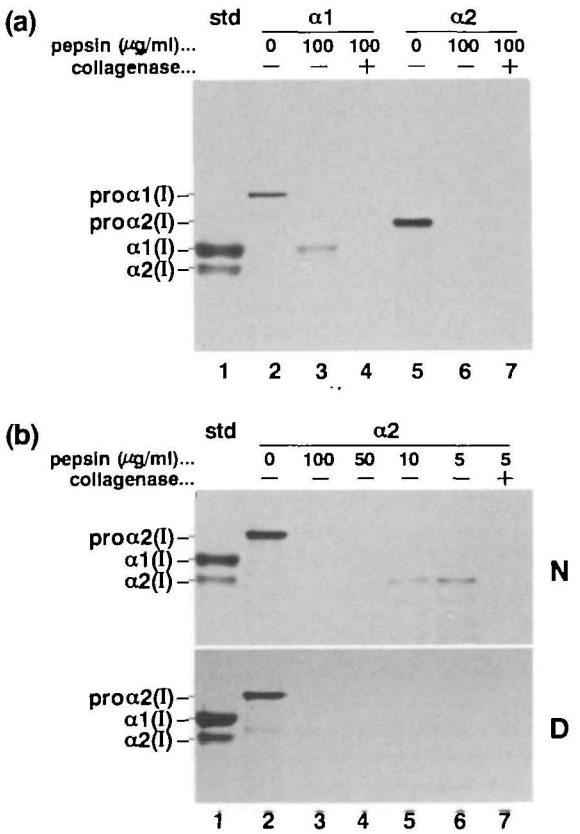

Fig. 5. **Formation of pepsin-resistant homotrimers.** (a) [^{14}C]-Proline-labeled procollagen chains were obtained as in Fig. 4 and treated with 100 μg/ml of pepsin. After the pepsin had been inactivated by neutralization, proteins were treated with (+) or without (−) 100 units/ml of bacterial collagenase. The samples were analyzed by SDS/PAGE under reducing conditions. (b) Labeled proα2(I) chains were treated with varied amounts of pepsin, then with bacterial collagenase (panel N). The experiment shown in panel D was identical to that in panel N except that the samples were denatured at 65°C for 10 min prior to the enzyme treatments. std, human pepsinized collagens; α1, proteins from Ac1A1/MP-infected cells; α2, proteins from Ac1A2/MP-infected cells.

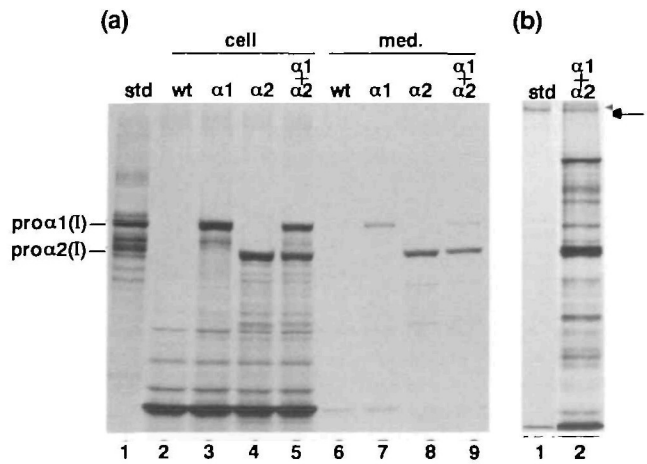

Fig. 6. **Co-expression of the proα1(I) and proα2(I) chains.** (a) Sf9 cells were infected with wild-type virus, Ac1A1/MP, Ac1A2/MP, or both Ac1A1/MP and Ac1A2/MP. [^{14}C]Proline-labeled proteins from cell lysates (lanes 2-5) and culture medium (lanes 6-9) were obtained as in Fig. 2 and analyzed by SDS/PAGE under reducing conditions. (b) The proteins secreted from cells co-infected with Ac1A1/MP and Ac1A2/MP were also analyzed under non-reducing conditions. Exposure to X-ray films was 10 times longer than in the case of (a). The arrow indicates disulfide-linked trimers. std, human fibroblast-derived procollagens; wt, wild type virus-infected cells; α1, Ac1A1/MP-infected cells; α2, Ac1A2-infected cells; α1+α2, cells infected with both Ac1A1/MP and Ac1A2/MP.

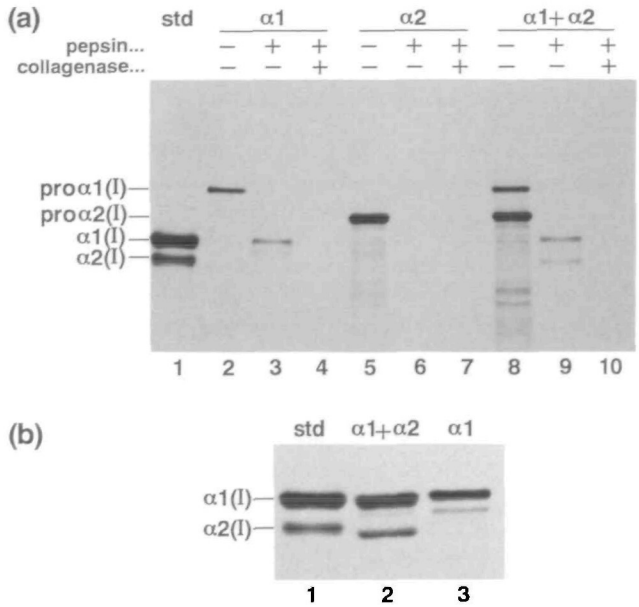

Fig. 7. **Formation of pepsin-resistant procollagen I heterotrimers.** Sf9 cells were infected with Ac1A1/MP, Ac1A2/MP, or both, and labeled with [^{14}C]proline as in Fig. 4. (a) The secreted proteins were treated with (+) or without (−) 100 μg/ml of pepsin. After neutralization, the samples were treated with (+) or without (−) 100 units/ml of bacterial collagenase and analyzed by SDS/PAGE under reducing conditions. (b) Pepsin-resistant proteins from cells co-infected with Ac1A1/MP and Ac1A2/MP and from cells infected with Ac1A1/MP alone were concentrated by salt precipitation, and analyzed by SDS/PAGE under reducing conditions. std, human fibroblast-derived pepsinized collagens; α1, proteins from Ac1A1/MP-infected cells; α2, proteins from Ac1A2/MP-infected cells; $\alpha 1+\alpha 2$, proteins from cells infected with both Ac1A1/MP and Ac1A2/MP.

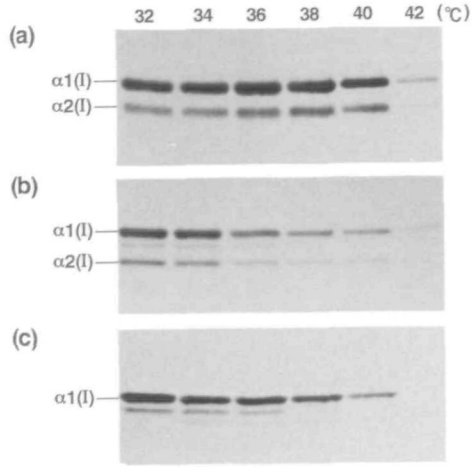

Fig. 8. **Thermal stability of pepsinized recombinant collagen I.** Human fibroblast collagen I (a), pepsinized recombinant heterotrimers (b), and homotrimers (c) were assessed for their thermal stability by examining their resistance to a mixture of trypsin and chymotrypsin at 20°C. Before the enzyme treatment, collagens were heated for 5 min at the temperatures indicated. Recombinant heterotrimers and homotrimers were prepared as in Fig. 7b.

chains was approximately 2:1 (data not shown). When the proteins secreted from co-infected cells were electrophoresed under non-reducing conditions, disulfide-linked trimers were detected (Fig. 6b). Due to their very slow migration on the polyacrylamide gel, it could not be determined from their electrophoretic mobilities whether these trimers were [proα1(I)]$_3$, [proα1(I)]$_2$[proα2(I)], or both.

To examine the composition of the trimer molecules, the secreted proteins were subjected to pepsin treatment (Fig. 7a). We confirmed again that some proα1(I) chains synthesized by single expression were converted to α1(I) chains upon the treatment with 100 μg/ml pepsin, whereas the singly expressed proα2(I) chains were completely degraded by the enzyme (Fig. 7a, 2 through 7). The treatment of chains secreted from the co-infected cells produced α1(I) and α2(I)-sized chains which were sensitive to collagenase (Fig. 7a, lanes 8-10). The existence of pepsin-resistant α2(I) chains in the medium from the co-infected cells, but not from the cells infected with Ac1A2/MP alone, was indicative of the formation of heterotrimeric stable helices in the former cells (Fig. 7a, *cf.* lanes 6 and 9). The ratio of α1(I) to α2(I) chains resistant to pepsin was estimated to be 2.2 by measuring radioactivity of corresponding bands. From these results, we concluded that [proα1(I)]$_2$[proα2(I)] heterotrimers were produced in substantial amount in Sf9 cells co-infected with Ac1A1/MP and Ac1A2/MP, while [proα1(I)]$_3$ homotrimers were produced in a negligible amount. Accordingly, disulfide-linked trimers detected in the proteins from co-infected cells as shown in Fig. 6b were most likely to be heterotrimers. Disulfide-linkages might occur between proα1(I) and proα2(I) chains of recombinant heterotrimers.

The pepsin-resistant heterotrimers and α1(I)-homotrimers were concentrated by salt precipitation, and their properties were further analyzed. Close comparison of the electrophoretic mobility showed that α1(I) and α2(I) chains of recombinant heterotrimers migrated slightly faster than those of corresponding chains from fibroblasts (Fig. 7b, lanes 1 and 2), whereas α1(I) chains of homotrimers showed similar migration to the latter (Fig. 7b, lanes 1 and 3). These differences in electrophoretic mobility may reflect different extents of prolyl hydroxylation or other post-translational modifications. To test this possibility, the thermal stability of pepsinized collagens was assessed (Fig. 8). Human fibroblast collagen I, recombinant heterotrimers, and α1(I)-homotrimers were heated at different temperatures, then treated with a mixture of trypsin and chymotrypsin at 20°C. Fibroblast collagen I was insensitive to the enzymes up to approximately 40°C, whereas the majority of recombinant heterotrimers became sensitive after incubation at temperatures above 36°C for 5 min (Fig. 8, a and b). This indicates that prolyl hydroxylation of recombinant heterotrimers is still insufficient. On the other hand, α1(I)-homotrimers persisted to approximately 38°C, suggesting that they were prolyl-hydroxylated to a higher extent than recombinant heterotrimers (Fig. 8c).

DISCUSSION

Many foreign genes have been successfully expressed in the baculovirus system under the transcriptional control of the *very late* polyhedrin promoter (*19, 25*). This promoter, however, functions at a time when the synthesis of host proteins (*26*), probably including post-translational modi-

fication enzymes, is declining. Accordingly, this promoter seems to be unsuitable to produce proteins requiring extensive post-translational modifications, such as highly glycosylated proteins (*27-29*). Fibrillar collagens also require post-translational modifications during their biosynthesis (*4, 5*). In particular, prolyl hydroxylation of susceptible residues within the triple helical region is critical for the formation of a stable triple helix (*30, 31*). In the previous study, we noticed that such modification is insufficient when proα1(III) chains are expressed under the control of the polyhedrin promoter, although the Sf9 host cells possess a certain level of prolyl hydroxylase activity (*14*). Recent reports showed that proper modification (glycosylation) of some proteins occurs post-translationally under the control of the *late* basic protein promoter, which becomes active earlier than the polyhedrin promoter (*32, 33*). Therefore, the present study tested the effect of the basic protein promoter on the procollagen production, and it was found that the proα1(I) chains were hydroxylated at an extent higher than the product by the polyhedrin promoter. This result showed that the use of the basic protein promoter is suitable for the production of procollagens.

Full-length proα1(I) chains were synthesized by Sf9 cells infected with proα1(I)-virus, and a small fraction of them was secreted into the culture medium. The secreted proα1(I) chains formed disulfide-linked and pepsin-resistant homotrimers, although all chains were not resistant to pepsin probably due to their insufficient prolyl hydroxylation. However, the melting temperature of pepsin-resistant α1(I)-homotrimers was significantly higher than that of recombinant heterotrimers, suggesting a higher rate of prolyl hydroxylation in the homotrimers than heterotrimers. This is probably due to delayed helix formation of proα1(I)-homotrimers. Similar observations were reported for proα1(I)-homotrimers from patients with osteogenesis imperfecta (*34*) and for homotrimers expressed by a recombinant means (*13*).

Full-length proα2(I) chains were also synthesized, and they were secreted at a higher rate than the proα1(I) chains. In contrast to the proα1(I) chains, the proα2(I) chains did not form stable triple helices. However, two proα2(I) chains linked by disulfide bonds were detected. The formation of such structures in mammalian cells has not been reported. Since it is generally accepted that noncovalent association of three chains among C-propeptides precedes the formation of interchain disulfide bonds, it is unlikely that two proα2(I) chains associate and form dimer molecules. We assume that recombinant proα2(I) chains form unstable trimers in which only two chains are linked by disulfide bonds. This assumption is supported by the result of pepsin digestion under a lower stringent condition. The previous report showed that isolated α2(I) chains are capable of forming a triple helix with a melting temperature of 20–24°C (*35*). Trimers of recombinant proα2(I) chains may be helical in part at the temperature used for pepsin digestion (4°C), but non-helical under the culture conditions for Sf9 (28°C), although we have not yet obtained direct evidence showing non-helicity of recombinant proα2(I) chains in the cells.

The proα1(I) chain has eight cysteine residues in the C-propeptide domain (*36*), while the proα2(I) chain has seven (*37*). The missing cysteine in the proα2(I) chain corresponds to the second cysteine in the proα1(I) C-propeptide, which participates in the formation of interchain disulfide bonds (*38*). Therefore, the absence of this residue has been suggested to be responsible for the inability of proα2(I) chains to form disulfide-linked homotrimers (*39*). As predicted from this suggestion, disulfide bonds failed to link three recombinant proα2(I) chains together. However, it was clearly demonstrated that proα2(I) chains have the potential to form disulfide bonds that link only two chains together. Previous studies indicated that interchain disulfide bonds form between the first and second cysteine residues and between the third and fourth cysteine residues of the C-propeptide of procollagen I heterotrimers (*38*). Therefore, two proα2(I) chains of the present study could be linked by disulfide bonds between the third and fourth cysteines.

When proα1(I) and proα2(I) chains were co-expressed in Sf9 cells, disulfide-linked and pepsin-resistant heterotrimers of two proα1(I) chains and one proα2(I) chain were produced. Since the proα2(I) chains were over-expressed as compared to the proα1(I) chains, excess proα2(I) chains might exist as monomers or unstable homotrimers. On the other hand, there is no evidence for the existence of proα1(I)-homotrimers, showing that almost all proα1(I) chains take part in heterotrimer formation. It is thought that the interaction between proα1(I) and proα2(I) chains precedes that between proα1(I) and proα1(I) chains, or that the dimers of proα1(I) chains preferentially bind to proα2(I) rather than to proα1(I) chains. Co-expression of both chains did not alter the secretion rate of either chain. That is, heterotrimers, as in the case of proα1(I)-homotrimers, are secreted at a slower rate than the proα2(I) chains. This observation is inconsistent with published observations in mammalian cells. Analyses of proα1(I)-deficient Mov-13 cells demonstrated that proα2(I) chains are not secreted in the absence of proα1(I) chains (*9*). It was also observed that mutant proα2(I) chains which cannot be assembled into heterotrimers are not secreted into the medium (*8*). From these observations, it has been suggested that proα2(I) chains that cannot be incorporated into stable heterotrimers would not be secreted but would be intracellularly degraded. A pulse-chase experiment revealed that recombinant proα2(I) chains are almost undegraded in insect cells (data not shown), but this result does not explain the high secretion rate of the chains. In vertebrate fibroblasts, helix formation is a prerequisite for the secretion of procollagen at the maximum rate (*40, 41*). However, insect cells secreted non-helical proα2(I) chains at a higher rate than triple helical proα1(I)-homotrimers and heterotrimers.

The mechanism of procollagen secretion has been poorly understood. Recently, evidence is accumulating that suggests the involvement of ER-resident stress proteins in procollagen secretion. HSP47 is associated with procollagen in ER, and this association is increased when helix formation of procollagen is inhibited, suggesting that this protein might have a role in the conformation-dependent procollagen secretion (*42, 43*). Another stress protein, BiP/GRP78, is also suggested to be involved in procollagen secretion from the observation that it is associated with mutant proα1(I) chains that are incapable of chain association (*44, 45*). In insect cells, these proteins might function insufficiently for human procollagen chains, and consequently, the mechanism of conformation-dependent secre-

tion might be disturbed.

The present report demonstrates for the first time the production of a recombinant procollagen I heterotrimer in which all three chains are derived from exogenous genes. This system will offer a tool for investigating the mechanism of chain association and folding during biosynthesis of procollagen. Moreover, a possible role of stress proteins in the secretion pathway of procollagen can be studied with the present system.

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