Biosynthesis of recombinant human pro-α1(III) chains in a baculovirus expression system: production of disulphide-bonded and non-disulphide-bonded species containing full-length triple helices

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We have investigated the expression of human procollagen III by insect cells infected with a recombinant baculovirus carrying cDNA for the pro-α1(III) chain of type-III collagen. A high level of expression was obtained, and a small proportion of the heterologously expressed pro-α1(III) chains formed normally disulphide-bonded procollagen III, which was secreted into the culture medium. This species displayed a melting temperature (T_m) of approx. 38 °C as assessed by its resistance to digestion by a mixture of trypsin and chymotrypsin, slightly lower than that of 39.5 °C for procollagen III synthesized by cultured human dermal fibroblasts, and reflected a slight degree of under-hydroxylation of prolyl residues. This is possibly a consequence of the lower incubation temperature of insect cells, or of an insufficiency of prolyl hydroxylase activity within them. A significant proportion of the expressed chains formed trimeric molecules of similar thermal stability containing an apparently full-length triple-helical region, but were not disulphide-bonded and not secreted. In addition to providing a source of recombinant human procollagen III, the system promises to be useful in the study of procollagen chain association and subsequent folding.

INTRODUCTION

Biosynthesis of the fibrillar collagens requires the activity of some eight, or more, intracellular enzymes, at least five of which are specific [1,2]. Critical among these is prolyl 4-hydroxylase which catalyses the formation of 4-hydroxyproline from susceptible residues within the repeating Xaa-Pro-Gly sequences of the pro-α chains, without which a triple-helical conformation stable at physiological temperature cannot be achieved [3,4]. Although 'rescue' experiments with human genomic or cDNAs in rodent cells, synthesizing only one of the two chains of type-I procollagen [5,6], or one chain of type-V procollagen [7], have resulted in the production of hybrid heterotrimeric recombinant procollagen molecules, such complexity of post-translational modification has hindered the production of a fully recombinant procollagen, and rendered necessary the use of mammalian host cell lines. Following initial success in expressing human procollagen II in stably transfected mouse NIH 3T3 cells [8], stably transfected HT1080 cells (a human kidney tumour cell line that synthesizes type-IV collagen but no fibrillar collagens), have been utilized for the production of thermostable, recombinant pro-α1(I) homotrimeric human type-I procollagen [9] and human type-II procollagen, from which it was possible to generate collagen II fibrils [10,11].

The yields obtainable from such mammalian expression systems are not great; to obtain 1 mg of procollagen II requires about 100 175-cm² flasks of HT1080 cells [10], for example, and we have been interested to explore the potential of the baculovirus expression system which utilizes Lepidopteran insect host cells [12]. This system is capable of performing many of the post-translational modifications that occur in mammalian cells [12–14]. The study of insect connective tissues has been relatively neglected, but it is clear that the class contains both fibrous and basement-membrane triple-helical collagens resembling their mammalian counterparts, with comparable, if not greater, levels of prolyl and lysyl hydroxylation [15–18].

Our results demonstrate the ability of the system to express at a high level human pro-α1(III) chains which are associated in molecules with apparently full-length triple-helical regions, with a thermal stability only slightly lower than normal. A small proportion of these are stabilized by interchain disulphide bonding and secreted.

MATERIALS AND METHODS

Materials

The *Autographa californica* multinuclear polyhedrosis virus (AcMNPV), and the Sf9 host cell line, derived from pupal ovarian tissue of *Spodoptera frugiperda*, have been described previously [19]. Both the baculovirus and plasmid pAcYM1 [20] were gifts from Dr. Y. Matsuura (National Institute of Health, Tokyo, Japan). Sf9 cells were obtained from the Riken Cell Bank (Tsukuba, Japan) and cultured at 28 °C in Grace's insect medium (GIBCO-BRL, MD, U.S.A.) supplemented with 10% (v/v) foetal-calf serum (Biocell, Carson, CA, U.S.A.). Serum-free Sf-900 II medium was obtained from GIBCO-BRL. Human dermal fibroblast cultures were established from a skin biopsy of a normal 6-year-old male by standard means, and grown in Eagle's Minimum Essential medium (GIBCO-BRL) containing 10% (v/v) foetal-calf serum. Chromatographically purified collagenase, form III, from *Clostridium histolyticum*, was from Advance Biofactures (Lynbrook, NY, U.S.A). Pepsin, trypsin and chymotrypsin were obtained from Sigma (St. Louis, MO, U.S.A). Standard type-III collagen for use as an electrophoretic marker was extracted by pepsin digestion from the skins of lathyritic rats

Abbreviations used: βAPN, β-aminopropionitrile fumarate; AcMNPV, *Autographa californica* multinuclear polyhedrosis virus; SLS, segment-long-spacing; T_m, melting temperature.

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from which soluble type-I collagen had previously been extracted with 1 M NaCl at neutral pH and purified by differential salt precipitation at acidic and neutral pH values [21,22]. Pepsin-treated, human placental collagen III, for the formation of standard SLS (segment-long-spacing) aggregates, was purchased from Fuji Chemical (Takaoka, Japan), and rabbit anti-(human collagen III) and anti-(bovine collagen III) sera from LSL (Tokyo, Japan). L-[2,3-^{3}H]Proline and L-[2-^{3}H]glycine were obtained from Amersham International (Tokyo, Japan) and L-[4-^{3}H]proline was from NEN–DuPont (Tokyo, Japan).

Characterization of Sf9 cells

The extent of collagen synthesis by Sf9 cells as a proportion of total protein production, and the distribution of collagenous peptides between the cell layer and the medium, were determined using highly purified bacterial collagenase [23]. Cells at early confluence in Grace's medium supplemented with foetal-calf serum were preincubated overnight with ascorbic acid (50 μg/ml), and then labelled for 24 h with L-[2,3-^{3}H]proline (10 μCi/ml) in fresh medium containing ascorbic acid (50 μg/ml) and β-aminopropionitrile fumarate (βAPN; 50 μg/ml) prior to processing for collagenase digestion. The production of any pepsin-resistant collagen by Sf9 cells was examined essentially as described previously for human dermal fibroblasts [24], except that labelling with [^{3}H]proline and [^{3}H]glycine was performed in Sf-900 II medium. The activity of prolyl hydroxylase (procollagen-proline, 2-oxoglutarate 4-dioxygenase, EC 1.14.11.2) in extracts of Sf9 cells was determined using an L-[4-^{3}H]proline-labelled protocollagen (unhydroxylated procollagen) substrate prepared from the calvariae of 16-day-old chick embryos, as described previously [25]. In each case, human dermal fibroblasts were examined in parallel to provide a positive control.

Construction of recombinant baculovirus

A full-length cDNA for the human collagen pro-α1(III) chain was isolated from a human placental cDNA library in λgt11 (Clontech, Palo Alto, CA, U.S.A.) using a PCR-generated probe derived from human dermal fibroblast mRNA, comprising nucleotides 460–1049 (numbering according to Ala-Kokko et al. [26]), and subcloned into the *Eco*RI site of the plasmid pUC118 (Takara Shuzo, Shiga, Japan). A *Bgl*II site was generated at the junction of nucleotide sequences for the signal peptide and N-propeptide by site-directed mutagenesis [27] using the oligonucleotide 5′-ATTATTTTGGCACAAGATCTTCAGGAAG-CTGTTG-3′ (underlining indicating the introduced sequence) as mutagenesis primer, and most of the 3′ untranslated region, from the *Nde*I site in the non-coding sequence to the *Kpn*I site in the pUC118 sequence, was deleted. From the resultant plasmid, a 4.4 kb cDNA fragment from the introduced *Bgl*II site to the *Bam*HI site in the pUC118 sequence was isolated, and introduced into the *Bam*HI site of transfer vector pAcYM1-Mel. This vector had been created by inserting the nucleotide sequence encoding the honeybee melittin signal peptide, which facilitates the secretion of recombinant protein [14], just downstream of the polyhedrin promoter in pAcYM1. The recombinant plasmid containing cDNA inserted in the correct transcriptional orientation was designated pAc3A1. DNAs of pAc3A1 and wild-type AcMNPV were co-transfected into Sf9 cells by lipofection [28] to allow homologous recombination. The resultant pro-α1(III) recombinant virus Ac3A1 was plaque-purified, and amplified by repeated infection of Sf9 cells.

Analysis of recombinant human pro-α1(III) chains

Monolayer cultures of Sf9 cells (1×10^6 cells/3.5-cm-diam. dish) were infected with Ac3A1 virus or wild-type AcMNPV at a multiplicity of about 10 plaque-forming units/cell. After 42 h the medium was removed, and proteins were precipitated by the addition of solid $(NH_4)_2SO_4$ to 30% satn. at 4 °C; after 3 h they were collected by centrifugation and resuspended in, and dialysed against, 0.5 M acetic acid. Cells were detached by sluicing with PBS, washed and lysed in PBS by three cycles of freezing and thawing. Both cell layer and medium samples were lyophilized and analysed by SDS/PAGE either directly, or following digestion with bacterial collagenase or pepsin. For collagenase digestion, lyophilized samples were resuspended in 2 mM $CaCl_2$, 50 mM Tris/HCl, pH 7.2, at 4 °C, containing 5 mM PMSF and 10 mM *N*-ethylmaleimide, and treated with 2 units of enzyme for 2 h at 37 °C, according to the manufacturer's protocol. The digestion was stopped by the addition of an equal volume of double-strength SDS/PAGE sample buffer. For pepsin digestion, the lyophilized samples were dissolved in 0.5 M acetic acid containing pepsin (100 μg/ml), and incubated at 4 °C for 16 h. The digests were subsequently lyophilized and dissolved in SDS/PAGE sample buffer.

SDS/PAGE

Electrophoresis was performed in SDS/5%-polyacrylamide gels, with 3% stacking gels, using the buffer system of Laemmli [29]. Radiolabelled proteins were visualized by fluorography [30] using Kodak X-Omat AR film. Unlabelled proteins were visualized either by staining gels with Coomassie Brilliant Blue R250, or after transfer to nitrocellulose membrane (BA85; Schleicher and Schuell, Dassell, Germany) [31] and reaction with anti-(collagen III) serum, in which case visualization was achieved with a VectaStain ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.).

Determination of thermal stability

The thermal stability of recombinant proteins was determined by assessing their resistance to a mixture of trypsin (100 μg/ml) and chymotrypsin (250 μg/ml) for 2 min at 20 °C, after having been heated to different temperatures for 5 min [24]. Digestions were stopped by the addition of excess soybean trypsin inhibitor (0.5 mg/ml; Sigma) and an equal volume of boiling double-strength SDS/PAGE sample buffer.

Determination of extent of prolyl hydroxylation

Ac3A1-infected cells and control human dermal fibroblasts preincubated for 24 h in the presence of ascorbic acid were labelled with [^{3}H]proline as described above, except that βAPN was omitted. Collagenous proteins were precipitated from the culture medium and cell lysate by the addition of solid $(NH_4)_2SO_4$ to 30% satn. at 4 °C, and treated with bacterial collagenase either directly or after prior pepsin digestion, both as described above. The extent of prolyl hydroxylation in such proteins was determined by measuring the distribution of radioactivity between proline and hydroxyproline in acid hydrolysates of collagenase-digestible material as described previously [32], except that the column eluant was 0.2 M sodium citrate, pH 3.1.

Purification of recombinant procollagen III

Ac3A1-infected cells were cultured for 72 h in the presence of ascorbic acid (50 μg/ml), after which time the medium was harvested. EDTA and PMSF were added to 2.5 mM and 0.2 mM

respectively, and proteins were precipitated over 16 h following the addition of solid $(NH_4)_2SO_4$ to 20% satn. at 4 °C, subsequently being collected by centrifugation. The precipitate was resuspended in, and dialysed against, 2 M urea/20 mM NaCl/50 mM Tris/HCl (pH 8.0) at 4 °C. The retentate was clarified by centrifugation at 10000 *g* for 1 h and subjected to HPLC on a column (0.7 cm × 10 cm) of DEAE-Toyopearl (Tosoh, Tokyo, Japan) with a linear gradient of NaCl from 0.02 to 0.2 M in the above urea/Tris solution. The eluate was monitored at 220 nm, and fractions corresponding to disulphide-bonded procollagen III, as confirmed by SDS/PAGE, were pooled, dialysed against 0.1 M acetic acid and lyophilized. The amino acid composition of the purified procollagen III was determined after acid hydrolysis using a Hitachi L835 automated analyser.

Formation of SLS aggregates

Cell-associated recombinant procollagen III was purified by salt precipitation at neutral and acidic pH values and treated with pepsin, which was subsequently inactivated by pH neutralization. SLS aggregates of these molecules, and of pepsin-treated human placental collagen III, were formed by incubating collagen solutions in 0.05 M acetic acid containing 0.7% ATP at 4 °C for 1 week, and examined by electron microscopy after staining with phosphotungstic acid and uranyl acetate [33].

RESULTS

Endogenous collagen-synthesizing ability of Sf9 cells

The proportion of total protein production dedicated to collagenase-sensitive material by Sf9 cells was found to be low by comparison with human dermal fibroblasts, being only a fraction of 1% compared with approx. 5% (results not shown). The very great majority (88%) of the collagenase-sensitive material synthesized by Sf9 cells was found in the cell layer, despite the presence of the lysyl oxidase inhibitor, βAPN, during labelling, and was presumably therefore intracellular. The comparable figure for human dermal fibroblasts was 11%. Analysis by SDS/PAGE of labelled material synthesized by Sf9 cells, following pepsin digestion, revealed the existence of no resistant macromolecular species (results not shown). Despite their low level of endogenous collagen synthesis, the Sf9 cells did appear to possess significant prolyl hydroxylase activity, although at a level only about one-fifth of that in human dermal fibroblasts (results not shown). These results indicated that the Sf9 cell might be an appropriate host for the expression of recombinant collagen.

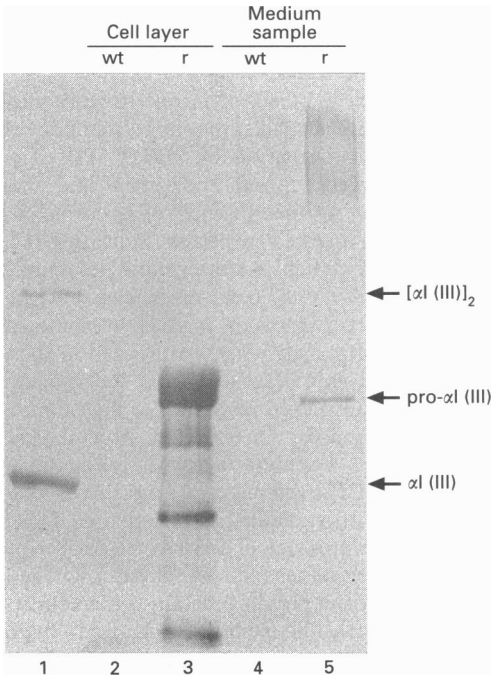

Figure 1 Synthesis of recombinant human pro-α1(III) chains

Proteins produced by wild-type and recombinant virus-infected cells were analysed by SDS/PAGE under reducing conditions, and visualized by immunostaining. Lane 1, standard rat skin collagen III; lanes 2 and 3, cell layer samples; lanes 4 and 5, medium samples. Abbreviations: wt, wild-type virus-infected Sf9 cells; r, recombinant virus-infected Sf9 cells.

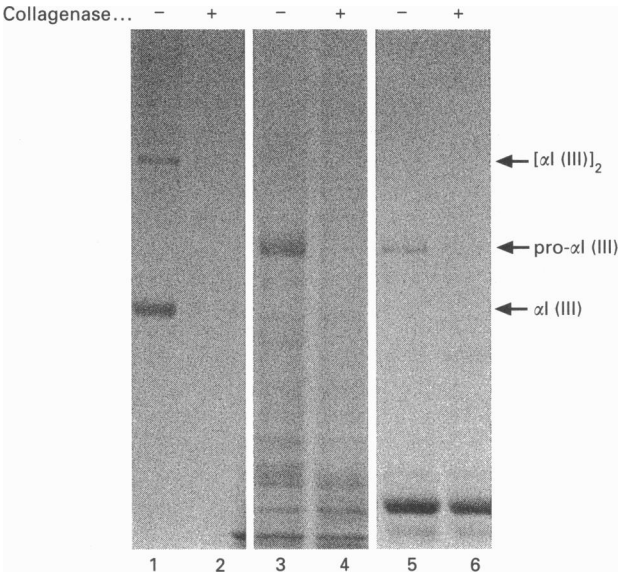

Figure 2 Sensitivity of recombinant pro-α1(III) chains to collagenase

Recombinant proteins were analysed by SDS/PAGE under reducing conditions, both without (−) and with (+) prior digestion by highly purified bacterial collagenase, and visualized by staining with Coomassie Brilliant Blue. Lanes 1 and 2, standard rat skin collagen III; lanes 3 and 4, cell lysate sample; lanes 5 and 6, medium sample.

Expression of recombinant human pro-α1(III) chains

In initial experiments, cell lysates and culture medium precipitates of wild-type and Ac3A1-infected cells were analysed by SDS/PAGE under reducing conditions (Figure 1). A major anti-(collagen III) immunoreactive product, not produced by wild type-infected cells, was apparent in the cell layer of Ac3A1-infected cells (Figure 1, lane 3), and was estimated to be equivalent in size to full-length pro-α1(III) chains (approx. 150 kDa) by comparison with the migration position of standard collagen III. A small proportion of this product was secreted into the medium (Figure 1, lane 5). The chains were sensitive to highly purified bacterial collagenase (Figure 2). The yield of immunoreactive recombinant products was estimated to be approx. 40 mg/l of culture medium (10^9 cells) by ELISA. These observations indicated that the recombinant product of approx. 150 kDa was encoded by the introduced human pro-α1(III) cDNA, and that this expression system might allow the high-level production of human collagen. Lower-molecular-mass immunoreactive bands

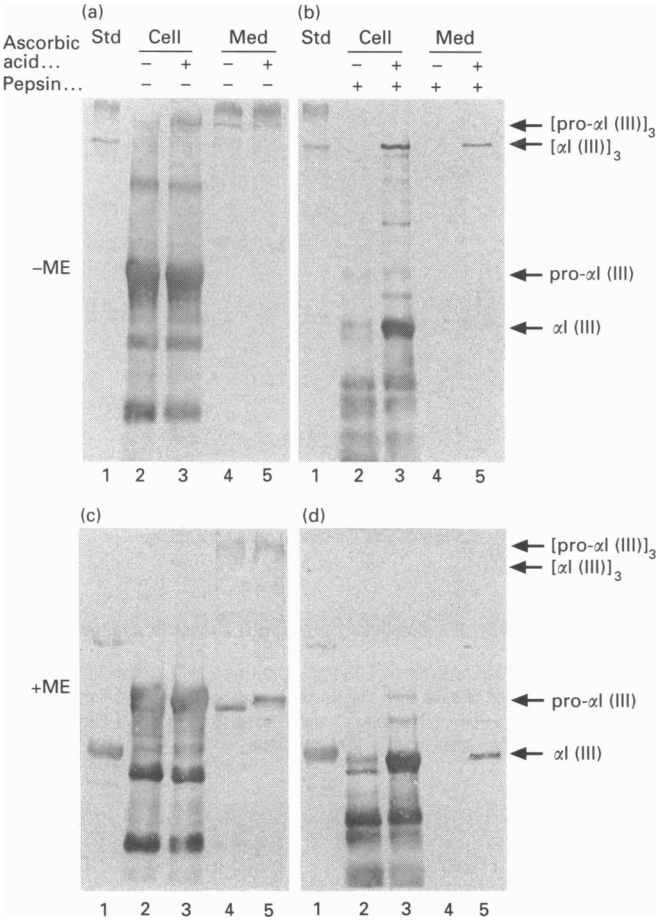

Figure 3 The effect of ascorbic acid on triple-helix formation

Anti-(collagen III) immunoreactive proteins synthesized by recombinant virus-infected cells in the absence (−) or presence (+) of ascorbic acid were analysed by SDS/PAGE under non-reducing (**a**, **b**) and reducing (**c**, **d**) conditions prior to (**a**, **c**) and following (**b**, **d**) digestion with pepsin. Abbreviations: ME, mercaptoethanol; std, standard rat skin collagen III; cell, cell lysate sample; med, medium sample.

(Figure 1, lane 3) may represent degradation products, or partially completed chains.

The effect of ascorbic acid on triple-helix formation

Ascorbic acid is a cofactor of prolyl hydroxylase in mammalian cells [1,34], and an adequate content of hydroxyproline within collagen is an obligatory requirement for the formation of a triple helix stable at physiological temperature [1,3]. Consequently, ascorbic acid was included in the culture medium of recombinant virus-infected Sf9 cells to determine whether it would induce the production of triple-helical procollagen III. When analysed by SDS/PAGE under non-reducing conditions (Figure 3a), the major product again migrated in the expected position for pro-α1(III) chains, whether the cells had been incubated in the absence or presence of ascorbic acid (Figure 3a, lanes 2 and 3). A minor product, slightly larger than standard $[\alpha1(III)]_3$ trimers, was also noted in the lysate of cells incubated both with and without ascorbic acid, its proportion appearing to be slightly increased in the former circumstance (Figure 3a, lanes 2 and 3). Regardless of incubation conditions, the monomer-sized product remained within the cell layer, while the trimer-

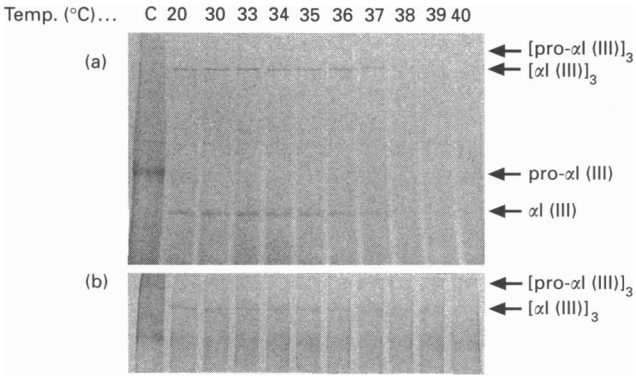

Figure 4 Thermal stability of triple-helical recombinant products

The thermal stability of species in the cell layer (**a**) and medium (**b**) presumed to be triple helical was assessed by examining their resistance to a mixture of trypsin and chymotrypsin at 20 °C after having been heated to the temperatures indicated. SDS/PAGE was performed under non-reducing conditions, and visualization was achieved by staining with Coomassie Brilliant Blue. Abbreviation: C, starting material.

sized product was secreted into the culture medium (Figure 3a, lanes 4 and 5). Reduction eliminated this latter material, which subsequently migrated as pro-α1(III) chains, thereby allowing its interpretation as procollagen III (Figure 3c, lanes 4 and 5). Pro-α1(III) chains synthesized in the presence of ascorbic acid migrated more slowly than those synthesized in its absence, indicative of the vitamin having induced prolyl hydroxylation (Figure 3c, cf. lanes 4 and 5). The migration position of the monomer-sized and lower-molecular-mass products was not altered by reduction, although a dimer-sized product which did disappear on reduction was also noted (cf. lanes 2 and 3 in Figures 3a and 3c).

To assess the possible existence of a triple-helical conformation within the recombinant products, they were treated with pepsin before being subjected to analysis by SDS/PAGE. The trimer-sized molecules, as well as both monomer- and dimer-sized products, synthesized in the absence of ascorbic acid were essentially completely digested by pepsin, indicating the absence within them of any triple-helical conformation (Figure 3b, lanes 2 and 4), although lower-molecular-mass fragments in the cell lysate suggested the existence of regions of some resistance (Figure 3b, lane 2). However, when synthesized in the presence of ascorbic acid, the procollagen III and the monomer- and dimer-sized products displayed resistance to digestion by pepsin (Figure 3b, lanes 3 and 5). As expected, procollagen III was cleaved to a product co-migrating with authentic $[\alpha1(III)]_3$ collagen, and pro-α1(III)-sized material was cleaved to a product which co-migrated with standard α1(III) chains. This suggests the production, in the presence of ascorbic acid, of triple-helical species. When analysed under reducing conditions, the majority of the procollagen-derived pepsin-resistant triple-helical material in both the cell layer and the medium appeared to migrate with authentic α1(III) chains (Figure 3d, lanes 3 and 5), while the migration of the monomer-sized material was unaltered (cf. lanes 3, Figures 3b and 3d), indicating an absence of disulphide bonding. The migration position of the pepsin-resistant lower-molecular-mass products was also unaltered by reduction, although the dimer-sized material was no longer apparent (cf. lanes 3, Figures 3b and 3d). This latter may derive from trimeric material in which only two chains were disulphide-bonded.

Table 1 Extent of prolyl hydroxylation of recombinant products

The extent of prolyl hydroxylation of recombinant products retained within the cell layer and secreted into the culture medium of Ac3A1-infected Sf9 cells was determined by measuring the distribution of radioactivity between proline and hydroxyproline following labelling of the cells with [^{3}H]proline and collagenase digestion of the two compartments, both without and with prior pepsin digestion. Comparably treated human dermal fibroblast cultures served to provide control samples.

	Prolyl hydroxylation (%)*	
Sample	Sf9/Ac3A1	Human fibroblasts
Without pepsin		
Cell lysate	12.0	44.6
Medium	28.4	49.8
With pepsin		
Cell lysate	25.1	47.8
Medium	40.0	47.5

* Results are expressed as the percentage of Pro+Hyp radioactivity accounted for by hydroxyproline.

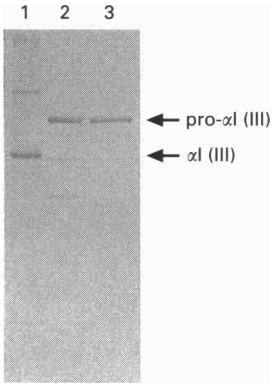

Figure 5 Purification of recombinant procollagen III

Proteins were analysed by SDS/PAGE (7.5% gel) under reducing conditions with Coomassie Brilliant Blue staining. Lane 1, standard rat skin collagen III; lane 2, $(NH_4)_2SO_4$-precipitated proteins from culture medium; lane 3, HPLC-purified recombinant procollagen III.

Thermal stability of triple-helical recombinant products

The thermal stability of recombinant procollagen and monomer-sized species synthesized in the presence of ascorbic acid, and adjudged to be triple helical on the basis of their resistance to pepsin, was assessed by examining their susceptibility to a mixture of trypsin and chymotrypsin at 20 °C after having been heated to different temperatures within the range 20–41 °C. As would be expected, both procollagen and monomer-sized pro-α1(III) chains were cleaved by the enzyme treatment at 20 °C to $[\alpha1(III)]_3$- and α1(III)-sized species respectively (Figures 4a and 4b). These species were subsequently resistant to enzymic digestion until over 37 °C, showing a T_m of approx. 38 °C.

Extent of prolyl hydroxylation of recombinant products

The extent of prolyl hydroxylation of recombinant products synthesized in the presence of ascorbic acid was determined both before and after digestion of cell layer and medium samples with pepsin, and the results are shown in Table 1. Without prior pepsin digestion, the overall extent of prolyl hydroxylation of the largely unsecreted products within the cell layer was considerably lower in relation to the value obtained for material within control human dermal fibroblasts. About 70% of the radioactively labelled intracellular recombinant material was sensitive to pepsin digestion, and the extent of hydroxylation of the resistant proteins was now elevated to 25%, although this remained, however, rather lower than the 48% hydroxylation of comparable material within human fibroblasts.

In contrast to the cell layer material, the extent of prolyl hydroxylation of recombinant procollagen III secreted into the culture medium was rather closer to values obtained with control human fibroblasts, both before and after pepsin digestion.

Purification of recombinant procollagen III

Secreted procollagen was concentrated by $(NH_4)_2SO_4$ precipitation and purified by HPLC. As shown in Figure 5 (lane 2), the $(NH_4)_2SO_4$ precipitate was relatively free of accompanying species, so that a single chromatographic step was sufficient to purify the recombinant procollagen III to homogeneity (Figure 5, lane 3). The yield of purified procollagen III from 1 litre of culture was 2.4 mg as dry weight, and its amino acid composition is shown in Table 2. This latter analysis demonstrated an extent of prolyl hydroxylation of 42%, in agreement with the value previously obtained for secreted pepsin-resistant material shown in Table 1. It also showed that the recombinant procollagen was hydroxylated only very poorly at lysyl residues.

Table 2 Amino acid composition of purified recombinant human procollagen III

Results are expressed as residues/1000 residues.

	As predicted from cDNA (residues)	As determined (residues)
4-Hyp	105*	72
Asx	67	73
Thr	21	26
Ser	49	57
Glx	80	93
Pro	89	100
Gly	286	263
Ala	78	79
Cys	15	14
Val	24	27
Met	10	11
Ile	24	25
Leu	29	35
Tyr	10	12
Phe	15	17
Hyl	18*	0.3
Lys	24	43
His	9	12
Arg	42	42

* Considering Y-position Pro and Lys residues as being fully hydroxylated.

SLS aggregates

SLS aggregates of pepsinized recombinant human collagen III were essentially identical to those formed from standard pepsinized human placental collagen III (Figure 6), except for the absence of two minor bands, which are arrowed in the Figure. This may be a consequence of regional alterations in the extent of post-translational modification.

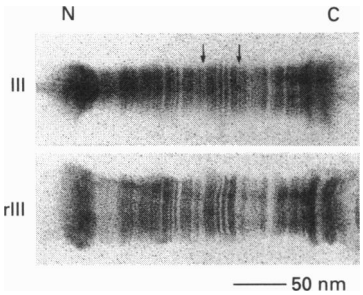

Figure 6 SLS aggregates

SLS aggregates were prepared from pepsinized human placental collagen III (upper panel, III), and pepsinized recombinant human procollagen III (lower panel, rIII) and examined by electron microscopy. The arrows indicate the location of two bands present in aggregates prepared from the collagen standard, but absent in those prepared from the recombinant product.

DISCUSSION

The establishment of a system capable of the production of recombinant human pro-α1(III) chains, and, particularly, of recombinant human procollagen III, is valuable because of the difficulty of obtaining by standard biochemical means pure, undegraded procollagen or collagen III in useful quantities from human tissues, which may themselves not be readily obtainable.

Using standard culture conditions, with no added ascorbic acid, the predominant anti-(collagen III) immunoreactive product of the expression of the pro-α1(III) cDNA in Sf9 cells migrated upon SDS/PAGE to the approximate position expected for pro-α1(III) chains, although a minor proportion did appear to form non-triple-helical, disulphide-bonded pro-α1(III) trimers, which were secreted into the medium, as is known to occur also in mammalian cells [35,36]. When synthesized in the presence of the prolyl hydroxylase cofactor, ascorbic acid, both the monomer- and the trimer-sized species were resistant to digestion by pepsin, indicative of their possessing a triple-helical conformation, the existence of which was demonstrated by their resistance to a mixture of trypsin and chymotrypsin. That this was a consequence of post-translational prolyl hydroxylation was suggested by the observation that the electrophoretic mobility of secreted disulphide-bonded trimer-derived pro-α1(III) chains synthesized in the presence of ascorbic acid was less than that of similar chains synthesized in its absence [24]. However, the fact that the T_m of the recombinant products was slightly lower than the 39.5 °C reported for collagen III produced by cultured human dermal fibroblasts [37], suggested the extent of prolyl hydroxylation to have been less than maximal, with consequent regions of triple-helical instability. Subsequent determination showed overall the intracellular material to be considerably underhydroxylated, which would explain the proportion lost upon pepsin digestion and serve as a barrier to secretion. Nevertheless, within this pool a population of molecules existed with an extent of prolyl hydroxylation sufficient to allow triple helicity and secretion, although it was less than that noted with control human fibroblasts. Such underhydroxylation may be a result of the insect cells having been incubated, of necessity, at 28 °C, or it may be that the available prolyl hydroxylase activity was insufficient to cope with the overproduction of the heterologous gene product, being only about one-fifth of that in human dermal fibroblasts, as determined using an assay temperature of 37 °C, which may, however, not be optimal for the insect enzyme.

The high-molecular-mass trimeric material synthesized in the presence of ascorbic acid apparently represents normal type-III procollagen molecules, as indicated by their ability to be secreted, and their conversion into $[\alpha1(III)]_3$-sized molecules upon pepsin digestion. In addition to the interchain disulphide bonds between the C-propeptide regions common to the procollagens of the major fibril-forming collagens, type-III procollagen is also stabilized by such bonding at sites within the triple-helical region [1]. Disulphide bonding in each of these regions appeared to be normal, reduction leading to migration as pro-α1(III) chains, or as α1(III) chains following pepsin digestion of the parent molecules.

The major proportion of the expressed products, however, was not disulphide-bonded, its electrophoretic migration corresponding to that of monomeric pro-α1(III) chains under both non-reducing and reducing conditions. From their studies of C-propeptide interactions in the product of a type-III procollagen minigene (lacking some 80% of the triple-helical region), expressed in a microsomally supplemented cell-free system, Lees and Bulleid [38] concluded that interchain disulphide bonding was a prerequisite for trimer formation. However, the conformation of the triple-helical region of the minigene product was not examined in that study, and our results would indicate the ability of trimer formation to occur in the absence of disulphide bonds, the molecules in question being clearly triple helical when synthesized in the presence of ascorbic acid, their constituent chains displaying resistance to both pepsin and a mixture of trypsin and chymotrypsin. The co-migration of the chains following pepsin digestion with standard α1(III) chains would further suggest the triple-helical region to have been essentially full-length.

It is not clear why disulphide bond formation should have failed to occur, but one possible explanation may be that there was insufficient available protein disulphide isomerase activity to cope with the level of heterologous gene expression, this enzyme, which also constitutes the β-subunit of prolyl 4-hydroxylase [39], having been postulated to play a direct role in procollagen chain assembly [40,41]. Alternatively, a misalignment might have occurred during chain association, since the system also produced species which migrated faster than monomers, but which were derived, as judged by their resistance to pepsin when synthesized in the presence of ascorbic acid, from triple-helical molecules. These species are likely to represent products derived from chains which have formed triple helices in a misaligned manner, and subsequently been partially degraded. Such species have been observed by others in cell-free translation systems [42–44].

There is increasing evidence that protein assembly and folding are assisted by molecular chaperones (for a review see [45]), and although the procollagen chains themselves seem to contain sufficient information to direct chain association and subsequent triple-helix formation, it may be that the mediation of a chaperone is required for the absolute accuracy of the procedures. Hsp 47/colligin has been implicated, in that it binds to non-triple-helical intracellular procollagen chains [46] and its antisense oligonucleotide depletion in mouse 3T6 cells results in a reduction in the production of procollagen I [47]. However, if one or more chaperones is involved, it cannot be excluded that insect proteins might function inefficiently with human procollagen chains, or their availability might have been insufficient to cope with the high level of expression of the introduced gene product.

In conclusion, we have developed a non-mammalian expression system for the high-level production of human procollagen III, a small proportion of which appears to be correctly disulphide-bonded and secreted, and which may be obtained in a purified form at a yield of > 2 mg/l of culture. The system may

provide a convenient means whereby the processes of chain association and folding, and the possible involvement of chaperones in these processes, may be studied.

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