Journal of Cellular Biochemistry 71:233–242 (1998)

Role of the Pro-α2(I) COOH-terminal Region in Assembly of Type I Collagen: Disruption of Two Intramolecular Disulfide Bonds in Pro-α2(I) Blocks Assembly of Type I Collagen

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Abstract Collagen biosynthesis is a complex process that begins with the association of three procollagen chains. A series of conserved intra- and interchain disulfide bonds in the carboxyl-terminal region of the procollagen chains, or C-propeptide, has been hypothesized to play an important role in the nucleation and alignment of the chains. We tested this hypothesis by analyzing the ability of normal and cysteine-mutated pro-α2(I) chains to assemble into type I collagen heterotrimers when expressed in a cell line (D2) that produces only endogenous pro-α1(I). Pro-α2(I) chains containing single or double cysteine mutations that disrupted individual intra- or interchain disulfide bonds were able to form pepsin resistant type I collagen with pro-α1(I), indicating that individual disulfide bonds were not critical for assembly of the pro-α2(I) chain with pro-α1(I). Pro-α2(I) chains containing a triple cysteine mutation that disrupted both intrachain disulfide bonds were not able to form pepsin resistant type I collagen with pro-α1(I). Therefore, disruption of both pro-α2(I) intrachain disulfide bonds prevented the production and secretion of type I collagen heterotrimers. Although none of the individual disulfide bonds is essential for assembly of the procollagen chains, the presence of at least one intrachain disulfide bond may be necessary as a structural requirement for chain association or to stabilize the protein to prevent intracellular degradation. J.Cell. Biochem. 71:233–242, 1998. © 1998 Wiley-Liss, Inc.

Key words: assembly of type I collagen; COOH-terminal propeptide; pesin-resistant heterotrimers; disulfide bonds

The complex process of collagen biosynthesis is not completely understood. Signals governing procollagen chain associations and collagen trimer maturation may be contained in the collagen amino acid sequences. Alternatively, the newly formed protein chains may form interactions with cellular components such as molecular chaperones and proteolytic machinery, which may affect the assembly, maturation, degradation, or secretion of the collagen molecules from the cell. Current research of collagen-specific [Kurkinen, 1984; Jain et al., 1994a] and other molecular chaperones [Gething et al., 1992; Chessler et al., 1993] and degradation pathways [Ripley et al., 1993; Jain et al., 1994b] that affect the synthesis, maturation, and secretion of collagen molecules has expanded our knowledge of collagen biosynthesis at the cellular level. This information has led to the idea that the collagen chains may contain sequence signals necessary for their assembly and processing into mature collagen molecules.

Previous studies have shown that the carboxyl-terminal end of procollagen chains (C-propeptide) is necessary for formation of the type I collagen heterotrimer [Schofield et al., 1974; Rosenbloom et al., 1976; Uitto et al., 1981]. Specific chain nucleation and alignment are thought to occur between the C-propeptide domains, although the exact sequences or structures involved have not been identified. A series of conserved disulfide bonds is present in the C-propeptide domains of all the fibrillar collagens. In type I collagen, the pro-α1(I) C-propeptide contains eight cysteine residues, and the pro-α2(I) C-propeptide contains seven cysteine residues. The last four cysteine residues in both chains are believed to form intrachain disulfide bonds, based on data obtained from pro-α1(I) chains [Showalter et al., 1980]. The remaining cysteines, four in the pro-α1(I) and three in the pro-α2(I), form interchain disulfide bonds that

Contract grant sponsor: Veterans Administration; Contract grant sponsor: National Cancer Institute; Contract grant number: CA23540; Contract grant sponsor: NIH; Contract grant number: HD31419.

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Received 7 January 1998; Accepted 2 June 1998

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link the procollagen chains of the heterotrimer together.

Due to their conservation, the C-propeptide disulfide bonds have been considered important for procollagen assembly. As support for this hypothesis, a frameshift mutation in a patient with osteogenesis imperfecta that changed the sequence of the last 33 amino acids of pro-α2(I) chain and removed the last cysteine prevented heterotrimer formation [Pihlajaneimi et al., 1984]. Initially, it was reasoned that the role of the disulfide bonds may be to align and tether the procollagen chains during the initial stages of assembly, or to create secondary structure necessary for chain recognition. Based on the new wealth of information concerning the role of collagen specific chaperones and proteolytic systems that specifically target collagens and other secreted proteins, new possibilities for the role of the disulfide bonds may be envisioned. The disulfide bonds of procollagen chains may provide stability of the folded conformation of the C-propeptide to protect it from intracellular degradation, possibly mediated by the binding of specific intracellular proteins. Alternatively, the disulfide bonds may contribute to motifs recognized by endoplasmic reticulum (ER) resident chaperones that may assist in the recognition and nucleation of the procollagen chains. For these reasons, we have chosen to study collagen assembly in an in vivo cell system.

We began investigating the role of C-propeptide sequences in collagen assembly by analyzing the ability of pro-α2(I) chains truncated by 10 amino acids (which removes the last cysteine residue) to assemble into heterotrimers when expressed in several cell lines [Lim et al., 1998]. Our results indicated that the last 10 amino acids were critical for heterotrimer assembly, which further suggested that the intrachain disulfide bond formed by the last cysteine may be important for assembly. In addition, we established an intraspecies cell system for the analysis of pro-α2(I) sequences important for assembly, composed of a mouse bone marrow cell line, called D2, that expresses only endogenous pro-α1(I) procollagen chains [Diduch et al., 1993], and a mouse pro-α2(I) cDNA [Gunning et al., 1987]. Type I collagen heterotrimers secreted from the D2 cells stably transfected with wild-type pro-α2(I) cDNA were pepsin-resistant and displayed normal thermal denaturation characteristics.

In this study, we focused our attention on the functional role of the pro-α2(I) C-propeptide disulfide bonds in type I collagen assembly using the mouse D2 cell line and mouse pro-α2(I) cDNA. We used site-directed mutagenesis to replace the C-propeptide cysteine residues with alanines and analysed the ability of single, double, and triple cysteine mutant pro-α2(I) chains transfected into D2 cells to assemble with endogenous pro-α1(I) chains and produce secreted stable type I collagen heterotrimers.

EXPERIMENTAL METHODS

Materials

All tissue culture media, trypsin, most supplements, and Lipofectin Transfection System were purchased from Gibco Life Technologies (Gaithersburg, MD). Fetal bovine serum was purchased from Hyclone Serum, (Logan, UT). Radioisotopes were from New England Nuclear (Boston, MA). All nucleic acid modifying enzymes were purchased from New England Biolabs (Beverly, MA). The mutagenesis kit used to mutate cysteines to alanines was a Mutagene Phagemid In Vitro Mutagenesis Kit Version 2 (BIO-RAD, Hercules, CA). The expression vector, pcDNA3 from Invitrogen (San Diego, CA) was used in all transfection studies. All sequencing was performed using a Sequenase 7-deazadGTP Sequencing kit (United States Biochemicals, Cleveland, OH). RNA extraction was performed using TRIzol reagent (Gibco Life Technologies). and mRNA content was analysed by using the 3' RACE System (Gibco Life Technologies). following the manufacturers' protocols. In vitro transcription reactions were performed using the T7 mMESSAGE mMachine kit (Ambion, Austin, TX). PCR was performed on a programmable thermal controller (MJ Research, Watertown, MA).

Cell Types and Maintenance

D1 and D2 cells, previously cloned from a mouse bone marrow culture [Diduch et al., 1993], were kindly provided by Dr. Gary Balian. The cells were grown as previously described [Diduch et al., 1993; Lim et al., 1998].

Cloning and Mutagenesis

Subcloning wild type mouse pro-α2(I). The mouse pro-α2(I) cDNA encoding plasmids were generated by isolating the mouse pro-α2(I) cDNA from pHBAPr-1-neo-mα2(I) [Gunn-

ing et al., 1987] by digestion with Hind III and Bam HI. The 4.3 kb mouse pro-α2(I) fragment was ligated with the 5.4 kb Hind III-Bam HI fragment of pcDNA3, creating the expression plasmid referred to as pc3mα2. The same fragment was also cloned into pTZ18U to create pTZ18Umα2 used for subcloning as described below.

Site-directed mutagenesis. Mutagenesis reaction was performed using the Mutagene Phagemid In Vitro Mutagenesis Kit Version 2. The last 710 bp of the mouse pro-α2(I) cDNA was cloned into pTZ18U following digestion with Xba I and Bam HI creating the plasmid pTZ18UCterm. This plasmid was used to produce the mutagenesis template, because addition of the full-length cDNA prevented efficient production of the single stranded mutagenesis template. Mutations were confirmed by double-stranded DNA sequencing using the Sequenase 7-deaza-dGTP Sequencing kit. Mutated cysteine residues are named for the amino acid change and the position of the residue in the C-propeptide. For example, C82A is a cysteine to alanine change in the 82nd amino acid from the amino terminus of the C-propeptide. The names of the resultant plasmids, mutated residue, and the oligonucleotides used in the mutagenesis reactions are listed in Table 1.

Subcloning of single mutants. The cloning of the mutated sequences back into the full-length mouse pro-α2(I) sequence in the transfection plasmid pcDNA3 involved multiple steps due to the formation of deletion products when the mutagenic sequences were cloned directly into pc3mα2. The mouse pro-α2(I) sequence containing the desired mutation was removed from pTZ18UCterm by digestion with Afl II and Bam HI and ligated with the Afl II - Bam HI fragment of pTZ18Umα2. The resulting plasmids were then digested with Hind III and Bam HI, and the 4.3 kb fragments, containing the full-length mutant mouse pro-α2(I) cDNA sequences, were ligated with the Hind III - Bam HI fragment of pcDNA3.

Subcloning of double and triple mutants. To create pc3mα2/C82A:243A, the plasmids pTZ18UCterm/C82A and pTZ18UCterm/C243A were digested with Bst XI and Bam HI. The 423 bp fragment containing the α2(I) cDNA from the C243A mutant plasmid and the 3.1 kb fragment from the C82A mutant plasmid were ligated. The double mutant fragment was then subcloned into pcDNA3 using the same strategy described for the single mutants. To create pc3mα2/C151A:196A, the plasmids pc3mα2/C151A and pc3mα2/C196A were digested with Hind III and Bsm BI. The 3.9 kb fragment from pc3mα2/C151A and the 5.7 kb fragment from pc3mα2/C196A were then ligated.

The triple mutant pc3mα2/C82A:151A:196A was constructed from pc3mα2/C151A:196A and pc3mα2/C82A. Both plasmids were digested with Hind III and Nhe I. The 3.9 kb fragment containing the C82A mutation site from pc2mα2/C82A and the 5.7 kb fragment from pc3mα2/C151A:196A were ligated.

Transfection of Plasmid DNA

Transfections were performed using the Lipofectin Transfection System following the manufacturers' protocol. D2 cells were plated in 100 mm^2 dishes at ~800,000 cells per dish and incubated for 16 h in a 5% CO_2 atmosphere at 37°C prior to transfection. Plasmids were linearized with Pvu I before addition to the lipofectin reagent. Transfected cells were replated in T75 flasks after 48 h, and Geneticin was added

TABLE I. Plasmids and Mutations

Plasmid	Mutagenic oligonucleotides sequence (5′-NNN-3′)	Mutation
pc3mα2	N/A	0
pc3mα2/C243A	CCCGTC**GC**TTTCAAATAAGTG	C243A
pc3mα2/C196A	GTCGATGGC**GC**CTCCAAAAAG	C196A
pc3mα2/C151A	ACCTACCAC**GC**CAAGAACAGC	C151A
pc3mα2/C82A	TGAAACC**GC**CATCCAGG	C82A
pc3mα2/C74A	CAAAGTGTAC**GC**TGATTTCTC	C74A
pc3mα2/C65A	CAAGGA**GC**TACTATGGATGCC	C65A
Pc3mα2/C 82A:243A	Subcloned from pc3mα2/C82A and pc3mα2/C243A	C82A, C243A
Pc3mα2/C151A:196A	Subcloned from pc3mα2/C151A and pc3mα2/C196A	C151A, C196A
Pc3mα2/C82A:151A:196A	Subcloned from pc3mα2/C82A and pc3mα2/C151A: 243A	C82A, C196A, C243A

immediately to obtain a final concentration of 1,000 µg/ml. The cells were grown in the presence of Geneticin for ~2 weeks, or until all negative control cells were dead.

RT-PCR

RNA extraction was performed using TRIzol reagent and mRNA content was analysed by using the 3' RACE System following the manufacturers' protocols. Two µl from the cDNA synthesis reaction was used for subsequent amplification of the target DNA, following the manufacturers' protocol. The forward primer (5'- CATCCAGGCCCAACCTGTAAACACCCC-AGC-3') annealing to the mouse pro-α2(I) sequence and the reverse primer (5'- GCATTTAG-GTGACACTATAGAATAGGGCA-3') annealing to the vector sequence in pcDNA3 at 59°C were used for PCR. The reverse primer was also used in the cDNA synthesis reaction. Control RNA was created by in vitro transcription of the mouse pro-α2(I) sequence in pc3mα2 using the T7 mMESSAGE Machine kit following the manufacturer's protocol. The plasmid was linearized with Avr II prior to cDNA synthesis.

Protein Analysis

Biosynthetic radiolabelling of collagen. Confluent cells in culture were preincubated for 2 h in a 37°C, 5% CO_2 atmosphere in DME medium without L-glutamine containing 0.4% fetal calf serum and 50 µg/ml ascorbate. For 16–24 h radiolabelling, 2 µCi/ml [14C]proline was added to the medium, and the cells were incubated in the conditions described above. To harvest secreted proteins, the medium was removed and protease inhibitors were added as described elsewhere [Lim et al., 1998]. To recover the cell layer proteins, cell lysis buffer (1% NP-40, 150 mM NaCl, 5mM EDTA, 1 mM PMSF, 1 mM NEM, 1 µg/ml leupeptin, 1 ug/ml pepstatin) was added and the lysed cells were scraped off the tissue culture dish. To harvest intracellular protein, the cells were lifted off the dish by incubation with trypsin and pelleted by centrifugation at 2,000 x g for 5 min. The resulting cell pellet was washed with PBS and resuspended in 500 µl cell lysis buffer.

Limited pepsin digestion. Proteins secreted into the medium were precipitated by addition of 3 volumes of 100% ethanol and 10 µg BSA for a 300 µl medium sample, followed by incubation at - 70°C for 1 h. The samples were centrifuged at 13,000 x g for 15 min at 4°C to collect the precipitated protein. The pellet was resuspended in 200 µl 0.5N acetic acid, and pepsin was added to obtain a final concentration of 0.1 mg/ml. The sample was gently stirred at 4°C for 16 - 24 h, diluted with 1 ml H_20, frozen at - 70°C, and lyophilized overnight. Cell layer and intracellular protein samples were homogenized for 30 sec with a polytron, and insoluble debris was removed by centrifugation (13,000 x g for 15 min at 4°C). The clarified sample was then treated as described above.

SDS-PAGE analysis. Polyacrylamide gels (6%) were used to separate pepsin- digested collagen fragments under nonreducing conditions. Forty milliamps per gel were applied to the system, and electrophoresis continued until the sample dye was at the bottom of the gel. The gels were fixed in a solution containing 50% methanol, 40% H_20, and 10% glacial acetic acid for 20 min, enhanced for 20 min in Enlightening solution (NEN Research Products, Boston, MA). The dried gel was either exposed to Kodak Xomat or BioMax film.

RESULTS

Single Cysteine to Alanine Mutations in the Mouse Pro-α2(I) C-propeptide

The C-propeptides of type I collagen chains contain a set of conserved cysteine residues that form intra- and interchain disulfide bonds during trimerization. The C-propeptide residues, C42, C65, and C74, are believed to be involved in interchain disulfide bonds [Olsen, 1982; Dion and Myers, 1987], whereas residues C82, C151, C196, and C243 form intrachain disulfide bonds as shown in Figure 1.

These disulfide bonds have been considered essential to the assembly process. To test this hypothesis, site-directed mutagenesis was used to mutate the six cysteines closest to the C-terminus in mouse pro-α2(I) to alanines both singly (C65A, C74A, C82A, C151A, C196A, C243A) and in various combinations. These mutations disrupted both intrachain disulfide bonds and two of three interchain disulfide bonds. The ability of these mutants to assemble with pro-α1(I) into stable type I collagen was examined to determine the necessity of these bonds for assembly.

Wild-type and the six single cysteine mutant pro-α2(I) cDNAs were subcloned into the vector pcDNA3 and stably transfected into D2 cells. Neomycin-resistant cells transfected with mutated pro-α2(I) cDNA were compared to cells

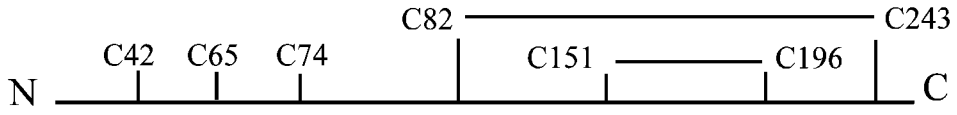

Fig. 1. The disulfide bonds of type I C-propeptide. The cysteines residues are numbered from the beginning of the C-propeptide.

transfected with the wild-type pro-α2(I) cDNA, D2mα2 cells, or to nontransfected D1 and D2 cells. Total RNA was harvested from the selected cells and the presence of pro-α2(I) mRNA was confirmed by RT-PCR (Fig. 2A). Media samples harvested from metabolically labeled stable cell lines and control cells were pepsin-treated and proteins separated by SDS-PAGE. Pepsin-resistant α2(I) chains were detected in the media of all single cysteine mutant proteins and wild-type cell lines except D2 cells (Fig. 2B). The α2(I) protein from all the transfected cell lines migrated as broad bands on these gels, rather than a sharp, α2(I) protein band possibly because of overmodification of the protein as demonstrated in osteogenesis imperfecta [Willing et al., 1988]. Most important, all the individual cysteine to alanine pro-α2(I) mutants could assemble with endogenous pro-α1(I) chains into a stable helix. Thus individually none of the single disulfide bonds of the mouse pro-α2(I) C-propeptide appeared to be necessary for trimerization.

Double and Triple Cysteine to Alanine Mutations in the Mouse Pro-α2(I) C-propeptide

When single cysteines of the pro-α2(I) C-propeptide were replaced by site-directed mutagenesis, disulfide bonds could have been rearranged. To prevent this possibility, double and triple cysteine mutants were created and subcloned into the vector pcDNA3. Based on the previous bonding assignments [Olsen, 1982], the mutations, C82A:243A, should disrupt the disulfide bond that forms the larger loop in the pro-α2(I) C-propeptide (Fig. 1). The mutations, C151A:196A, should disrupt the disulfide bond that forms the smaller internal loop of the C-propeptide. Hence, the triple mutant C82A: 151A:196A disrupts both intrachain disulfide bonds.

The mutated constructs were stably transfected into D2 cells. Total RNA was harvested from the selected cells and the presence of pro-α2(I) mRNA was confirmed by RT-PCR (Fig. 3A). Media samples harvested from metabolically labeled transfected and control cells were pepsin-treated and separated by SDS-PAGE. Pepsin-resistant α1(I) and α2(I) chains were detected in the media of both double mutant cell lines. However, no α2(I) band was detected in the triple mutant samples after repeated experiments (Fig. 3B). Thus the single, individual intrachain disulfide bonds of mouse pro-α2(I) were not critical for the formation of a stable pepsin-resistant type I collagen heterotrimer. The presence of at least one of these disulfide bonds, however, appeared to be necessary for the assembly process.

DISCUSSION

In order to test the hypothesis that disulfide bonds are necessary for the initial stages of procollagen chain trimerization [Olsen, 1982; Pihlajaniemi et al., 1984; Dion and Myers, 1987; Koivu and Myllyla, 1987;Tsipouras and Ramirez, 1987; Prockop, 1990; Dion and Myers, 1987], cysteine residues were replaced with alanines in the mouse pro-α2(I) cDNA by site-directed mutagenesis. Mouse pro-α2(I) chains lacking each single intra- or interchain disulfide bond retained the ability to assemble into stable type I collagen (Fig. 2). However, loss of both intrachain disulfide bonds (C82A:243A, C151A:196A) prevented the formation of stable type I molecules (Fig. 3). These results are summarized in Figure 4.

Whereas several recent studies have analyzed the importance of interchain disulfide bonds, this study is the first that examines the necessity of procollagen intrachain disulfide bonds.

Our finding that the specific intrachain disulfide bond formed by C82:C243 is not required for assembly was interesting since the importance of this intrachain disulfide bond (C82: C243) has been extensively analyzed and discussed in the literature [Dion and Myers, 1987]. Previous evidence indicated that a frameshift mutation in pro-α2(I) changing the last 33 amino acids including C243 prevented assembly of type I collagen [Pihlajaneimi et al., 1984].

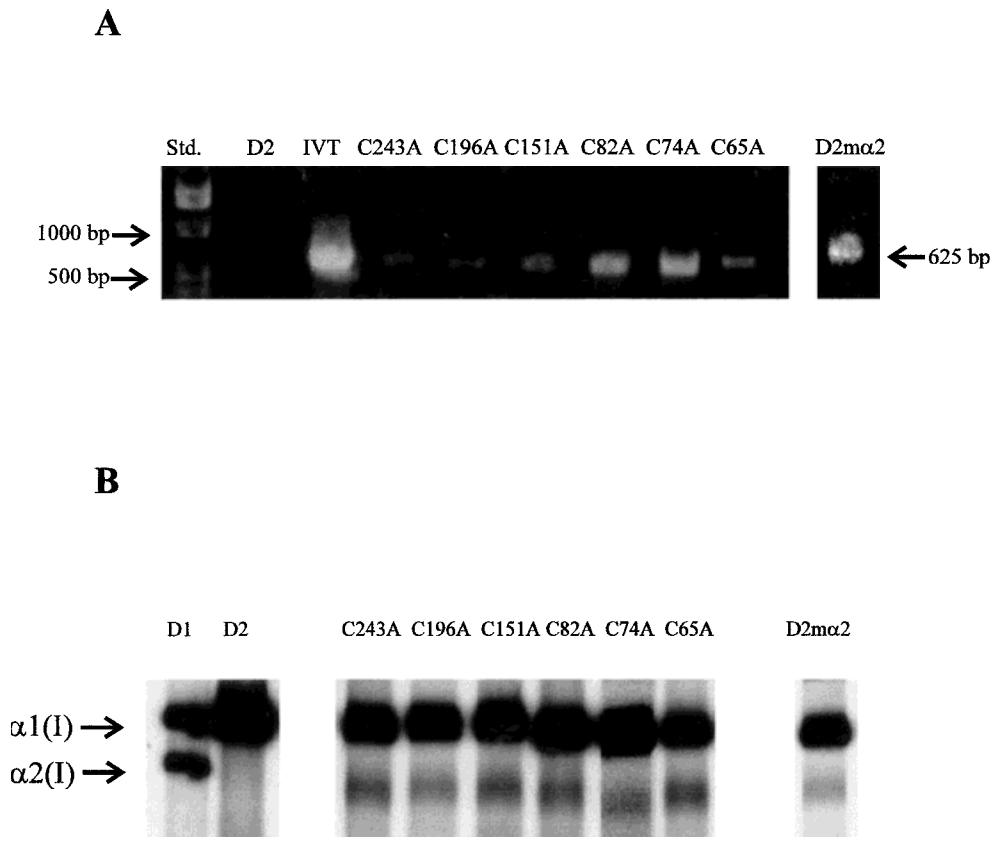

Fig. 2. Individual cysteine residues of mouse pro-α2(I) are not essential for assembly of stable type I collagen. **A.** RT-PCR was performed on RNA from D2 cells, D2 cells transfected with cysteine to alanine mutant pro-α2(I) cDNA, D2 cells transfected with wild-type pro-α2(I) cDNA, D2mα2, and in vitro transcription of pc3mα2 (lane IVT) using the reverse primer for cDNA synthesis. The forward and reverse primers used for amplification of the cDNA are described in Experimental Methods. **B.** D1, D2, D2mα2, and single cysteine mutant cells were metabolically radiolabeled with [14C] proline for 6 h. Media proteins were harvested, precipitated with ethanol, digested with pepsin, and lyophilized as described in Experimental Methods. Pepsin-resistant proteins were separated on 6% polyacrylamide gels, which were enhanced, dried, and exposed to X-ray film.

Although the change in the distribution of charged residues and the addition of a new cysteine in the last 33 amino acids were proposed as reasons for inhibition of assembly, the loss of the cysteine-forming disulfide bond seemed most plausible reason for the blockage of assembly.

Since all the previous studies examined larger disruptions of the polypeptide, it was impossible to distinguish between effects of the loss of the cysteine and the other surrounding amino acid changes. A study performed in our laboratory suggested that C243 was important for assembly. In the first study in this series [Lim et al., 1998], we demonstrate that truncation of the last 10 amino acids of the human and mouse pro-α2(I) chain (including C243) prevents assembly of stable type I collagen. The data reported here clearly demonstrate that the importance of the C-terminal region is not due to the disulfide bond formed by C243 alone. Therefore, the C-terminal amino acids must perform another function in collagen assembly.

The necessity of one intrachain disulfide bond for the assembly of pro-α2(I) into stable type I collagen can be explained several ways. The specific three-dimensional structure of the C-propeptide domain formed by the presence of either intrachain disulfide bond in pro-α2(I) could be essential for recognition or associations of the chains. Supportive of this idea, a study by Lees et al. [1997] using pro-α1(III) / pro-α2(I) chimeric procollagen chains identified that a discontinuous sequence in the pro-α2(I) chain is necessary to direct procollagen self-association. The fact that this sequence is not continuous suggests that a secondary or tertiary structure may be required to position the region for assembly with specific chains.

Alternatively, the presence of at least one intrachain disulfide bond could be necessary for stability of the folded procollagen chain. In gen-

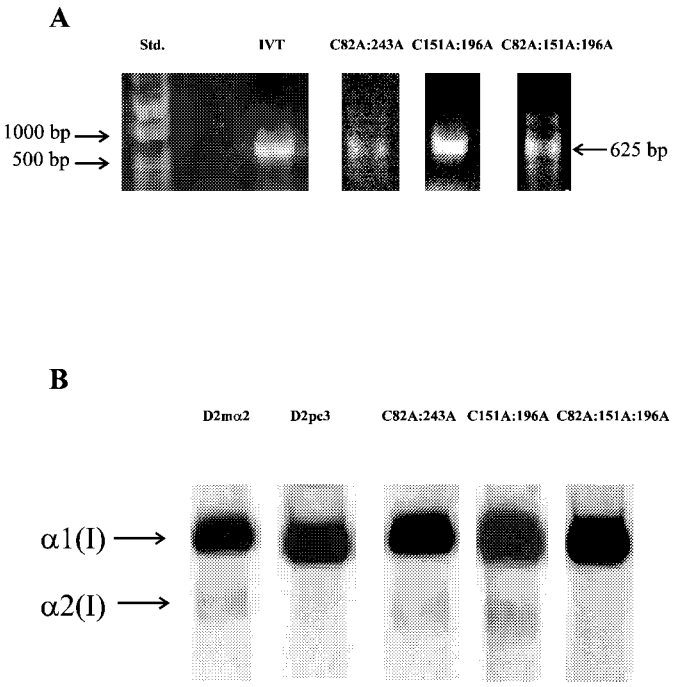

Fig. 3. The presence of either intrachain disulfide bond of mouse pro-α2(I) is essential for assembly of stable type I collagen. A. RT-PCR was performed on RNA from D2 cells transfected with cysteine to alanine mutant pro-α2(I) cDNA and in vitro transcription of pc3mα2 (lane IVT) using the reverse primer for cDNA synthesis. The forward and reverse primers used for amplification of the cDNA are described in Experimental Methods. B. D2pc3, D2mα2, and cells transfected with double or triple cysteine mutant pro-α2(I) cDNA were metabolically radiolabeled with [14C]proline for 6 h. Media proteins were harvested, precipitated with ethanol, digested with pepsin, and lyophilized as described in Methods. Pepsin-resistant proteins were separated on 6% polyacrylamide gels, which were enhanced, dried, and exposed to X-ray film.

eral, disulfide bonds are thought to increase the stability of protein structure by decreasing the conformational entropy of the unfolded chain [Alber, 1989]. Several common characteristics of disulfide bonds that increase protein stability have been determined from studies on T4 lysozyme: a large loop size formed by the disulfide, and a location in a flexible part of the molecule, such as the COOH-terminus [Matsumara et al., 1989]. These criteria for stabilization of the folded protein structure by disulfide bond are directly applicable to the pro-α2(I) intrachain disulfides, especially the C82:C243 bond. The C82:C243 bond forms a large loop structure (161 amino acids), that is present at the C-terminus of the C-propeptide, which is likely to be a flexible part of the molecule. The C151:C196 disulfide bond, which forms a smaller loop (44 amino acids) within the loop formed by C82:C243, would provide less of an entropic effect, but may still provide a stabilizing effect for the procollagen chain. The presence of both intrachain disulfide bonds in the wild-type chain may provide for maximal stabilization and serve as extra protection against destabilization caused by mutation of the cysteine residues. Thus the loss of either bond alone may not destabilize the protein structure, whereas the loss of both may destabilize structure.

The resultant destabilized, unfolded protein could then be more susceptible to intracellular degradation [Branden and Tooze, 1991]. Intracellular processes such as protein degradation may play an important part in how collagen sequences and/or structures exert their role in the assembly process. This is especially relevant since new cell-based controls of collagen synthesis and degradation are continuing to be discovered.

Our data also show that individual interchain disulfide bonds of mouse pro-α2(I) are not critical for assembly of stable type I collagen. This concept was first proposed by Doege and Fessler [1986] based on the reassembly of trimeric C-propeptides in vitro. However, definitive data was not available at that time, and reports on collagen synthesis still suggested that the interchain disulfide bonds were important for collagen assembly [Tsipouras and Ramirez, 1987; Ramirez and de Wet, 1992].

Additionally, Beck et al. [1996] demonstrated that assembly of triple helical type I collagen can occur in the absence of interchain disulfide bonding. In this study, the C-propeptide domains were shown to interact with the ER membrane during folding and helix formation (without interchain disulfide bonds). This process could facilitate the nucleation and alignment of the procollagen chains. It is, therefore, possible that the association of the procollagen chains with the membrane may play the role previously ascribed to the interchain disulfide bonds, namely, to hold and align the three pro-

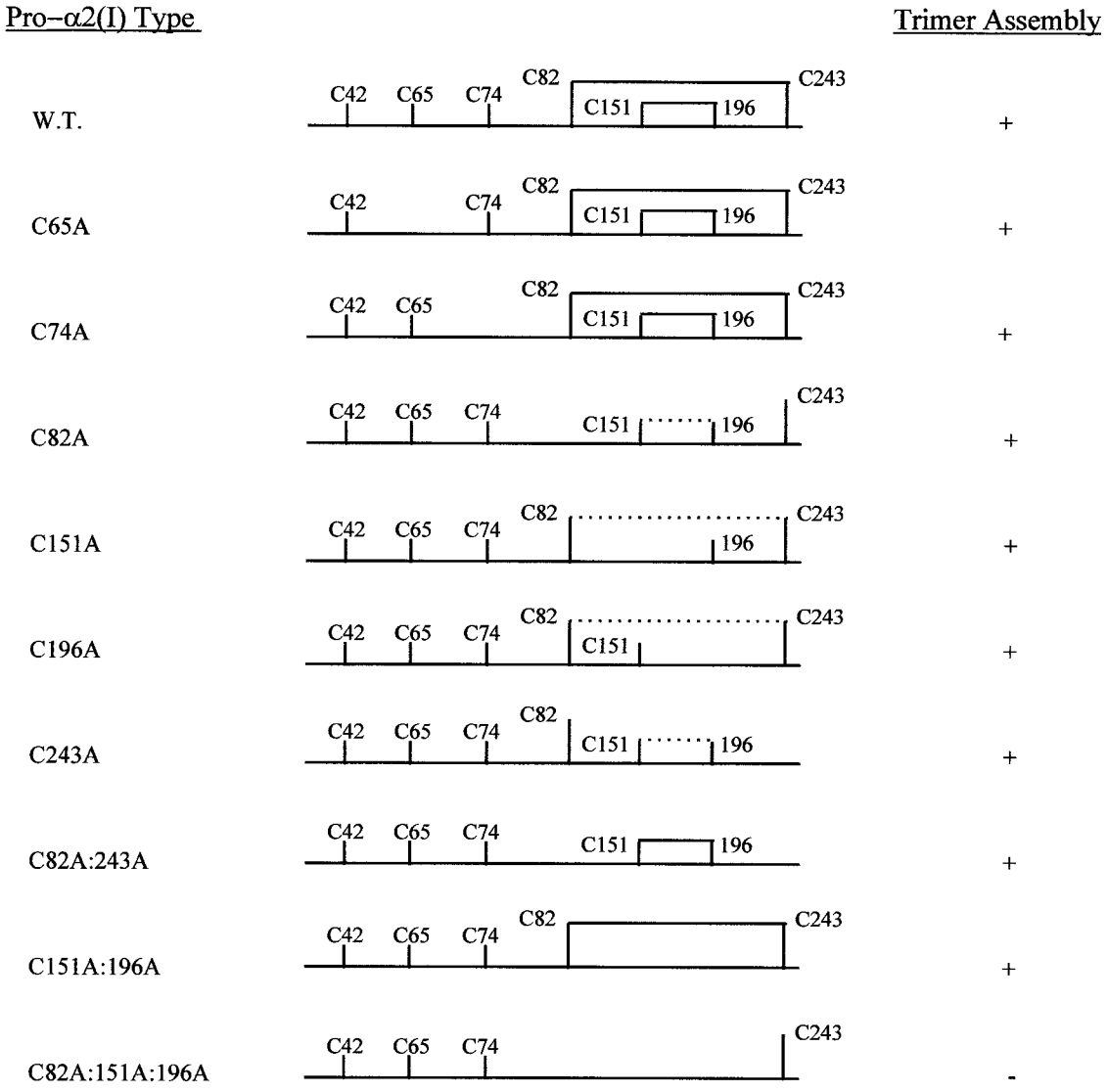

Fig. 4. A schematic summary of cysteine mutations and their effect on assembly of a stable type I collagen heterotrimer. The dotted lines denote disulfide bonds that cannot form due to the mutations in the protein.

collagen chains of the trimer together to promote helix assembly. Therefore, our mutagenesis data were in agreement with the findings of Beck et al. [1996] that the interchain disulfide bonds may stabilize the alignment and association of procollagen chains during assembly; however, they appear not to be essential. Since these cysteine residues are so highly conserved in the fibrillar collagens, it seems likely that some other reason for their presence remains to be discovered.

We faced several problems in these studies including low expression of procollagen chains from transfected cells, posttranslational modifications, and the intracellular degradation of unassembled procollagen chains, making it difficult to confirm that translation of the protein occurred. Since only one of the nine mutant cell lines did not produce assembled pepsin-resistant collagen, it is unlikely that the triple-mutated construct was not translated. We [Lim et al., 1998] and others [Deak et al., 1983] have previously used immunoprecipitation with human pro-α2(I) antisera to detect low levels of unassembled chains. However, this study used mouse pro-α2(I), which did not react specifically with available antisera tested in our laboratory.

Although in vitro studies provide important data on collagen assembly [Bulleid et al., 1992; Lees and Bulleid 1994], all the cellular components necessary may not be present in the

proper context to affect assembly exactly as in a living cell. Recent evidence suggests that assembly of collagen involves higher order structures in the lumen of the endoplasmic reticulum with the enzyme protein disulfide isomerase playing an important role in reorganizing aberrant disulfide bonds [Kellokkumpu et al., 1997]. Therefore, cell culture studies are important as well as in vitro experiments. The best approach to study collagen assembly, therefore, may necessitate a combination of in vitro and cell culture studies.

In conclusion, specific disulfide bonds of the pro-α2(I) C-propeptide are not necessary for collagen assembly, disproving previous hypotheses. Most important, the presence of at least one intrachain disulfide bond is required for assembly to proceed. Based on our results, we propose that the role of the intrachain disulfide bonds may be to provide entropic stabilization of a structure in the procollagen chains that protect the protein chains from intracellular degradation and allow assembly with pro-α1(I).

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