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Role of the Pro-α2(I) COOH-terminal Region in Assembly of Type I Collagen: Truncation of the Last 10 Amino Acid Residues of Pro-α2(I) Chain Prevents Assembly of Type I Collagen Heterotrimer

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Abstract Procollagen (Type I) contains a noncollagenous COOH-terminal propeptide (C-propeptide) hypothesized to be important in directing chain association and alignment during assembly. We previously expressed human pro-α2(I) cDNA in rat liver epithelial cells, W8, that produce only pro-α1(I) trimer collagen (Lim et al. [1994] Matrix Biol. 14: 21–30). In the resulting cell lines, α2(I) assembled with α1(I) forming heterotrimers. Using this cell system, we investigated the importance of the COOH-terminal propeptide sequence of the pro-α2(I) chain for normal assembly of type I collagen. Full-length human pro-α2(I) cDNA was cloned into expression vectors with a premature stop signal eliminating the final 10 amino acids. No triple-helical molecules containing α2(I) were detected in transfected W8 cells, although pro-α2(I) mRNA was detected. Additional protein analysis demonstrated that these cells synthesize small amounts of truncated pro-α2(I) chains detected by immunoprecipitation with a pro-α2(I) antibody. In addition, since the human-rat collagen was less thermostable than normal intraspecies collagen, wild-type and C-terminal truncated mouse cDNAs were expressed in mouse D2 cells, which produced only type I trimers. Results from both systems were consistent, suggesting that the last 10 amino acid residues of the pro-α2(I) chain are important for formation of stable type I collagen. J. Cell. Biochem. 71:216–232, 1998.

Key words: assembly of type I collagen; COOH-terminal propeptide; pepsin-resistant heterotrimers; interspecies collagen molecule; thermal stability

Collagen is a family of multidomain proteins composed of three α chains wound into a triple helix conformation [Linsenmayer, 1990]. The process of assembly is closely regulated since many cell types produce more than one collagen type [Gay et al., 1976]. For example, fibroblasts co-express as many as six homologous procollagen chains necessary for formation of collagen type I, III, and V. Type III collagen is a homotrimer composed of three pro-α1(III) chains, whereas the type I and V collagens are normally heterotrimers. The stoichiometry of type I collagen is usually two pro-α1(I) chains to one pro-α2(I) chain. However, type I collagen is found as a homotrimer of three α1(I) chains (referred to as α1(I) type I trimers) occasionally in tumors [Moro et al., 1977; Minafra et al., 1988], liver cirrhosis [Seyer et al., 1977, 1982; Rojkind et al., 1992], inflamed gingiva [Narayanan et al., 1978, 1985], and cell culture [Smith and Niles, 1980; Diduch et al., 1993]. There have been no reports of homotrimer formation of the normal α2(I) chains. In fact, pro-α2(I) chains are not assembled and are rapidly degraded in Mov-13 cells that have blocked synthesis of pro-α1(I) chains [Dziadek et al., 1987]. The stability of the pro-α2(I) chain seems to require association with pro-α1(I) chains.

The current view of the intracellular steps in collagen biosynthesis [see Olsen, 1992 for review] suggests that collagen is translated as a

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pre-procollagen chain, which becomes membrane bound after signal peptide formation in a complex with a signal recognition particle. During growth of the nascent chain in the endoplasmic reticulum, multiple posttranslational modifications occur that are characteristic of collagen, such as hydroxylation of proline and lysine followed by glycosylation of hydroxylysine. If assembly is delayed, the collagen molecule becomes overmodified, which alters thermal stability of the final triple helical molecule. Once translation is complete, the noncollagenous COOH-terminal propeptide (C-propeptide) of type I collagen seems to play a pivotal role in directing chain association and possibly alignment. The C-propeptides fold and intrachain disulfide bonds form prior to chain association and stabilization by interchain disulfide bridges [Doege and Fessler, 1986]. Type I procollagen is assembled by association of three chains at the COOH-terminal region after release from the ribosomes [Fessler et al., 1975; Harwood et al., 1976; Bruckner et al., 1981,1984]. Disulfide bonds between procollagen chains form within the COOH-terminus prior to triple helix formation [Schofield et al., 1974; Uitto et al., 1981] and the formation of the helix is propagated from the COOH- to NH_3- terminal direction [Engel, 1991]. Many of these steps are mediated by chaperone proteins, both general chaperones such as BiP [Gething and Sambrook, 1992; Chessler and Byers, 1993], and specific chaperones such as colligin/hsp47 [Nakai et al., 1992; Jain et al., 1994; Sauk et al., 1994], which specifically binds to collagenous chains. The mechanism that assures proper assembly and alignment is not well understood. It is probable that chain selection is determined at an early stage in the folding pathway by specific regions within the C-propeptide.

Our previous studies indicated that a N-acetoxy-acetyl-aminofluorene (AAF) transformed rat liver epithelial-like cell line, W8, produced α1(I) type I trimer, whereas the parent cell line, K16, produced collagen heterotrimers [Smith and Niles, 1980]. When a human pro-α2(I) cDNA was transfected and expressed in rat W8 cells [Lee et al., 1988; Lim et al., 1994], the expressed pro-α2(I) chain assembled with pro-α1(I) chains producing a triple helical pepsin-resistant collagen heterotrimer. The production of heterotrimer in W8 cells enhanced cell adhesion to substratum and inhibited anchorage independent growth in soft agar [Lim et al., 1994]. However, there was concern that the human-rat collagen formed by transfected human cDNA into rat W8 cells would have different thermal stability than rat-rat collagen produced by the parent cell line K16.

Therefore, a second transfection system using mouse cDNA [Phillips et al., 1991] and mouse cells that produce α1(I) type I trimer was developed. Two cell lines, D1 and D2, were previously cultured from mouse bone marrow cells and characterized in terms of osteogenic characteristics and collagen biosynthesis [Diduch et al., 1993]. D1 cells displayed various osteogenic characteristics such as high levels of alkaline phosphatase, increased cyclic adenosine monophosphate upon treatment with parathyroid hormone, expression of osteocalcin mRNA, and the ability to form a mineralized matrix in vitro. D2 cells, in contrast, do not exhibit these osteogenic characteristics. Most importantly, the D2 cells produce α1(I) type I trimer as judged by RNA analysis and metabolic radiolabeling. A full-length mouse α2(I) cDNA has been constructed for use in transfection studies [Phillips et al., 1991]. Thus the mouse D2 cells provide a unique system for transfection of wild-type and mutant α2(I) cDNA to create recombinant intraspecies type I heterotrimers in order to study the role of α2(I) in trimer assembly.

Our approach in this study is to test whether certain amino acids are critical for assembly by expressing normal and mutated pro-α2(I) cDNA in cells that produce type I trimers and to determine if the pro-α2(I) assembles to form heterotrimer molecules containing α2(I). Since frame-shift mutations in the last amino acids in the C-propeptide [Pihlajaniemi et al., 1984; Chipman et al., 1993] lead to type I trimer production and degradation of pro-α2(I), we hypothesized that the last amino acids could be part of a signal mechanism or nucleation site for assembly. In this work, we test this hypothesis by transfecting mouse and human α2(I) constructs with and without translation stop signals eliminating the last 10 amino acids. These constructs were expressed in two cell lines that produce type I trimer, W8 cells and D2 cells [Diduch et al., 1993]. This work demonstrates that the last 10 amino acids are critical for normal collagen heterotrimer production and secretion.

MATERIALS AND METHODS

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 Bio-Lab (Beverly, MA). A 14 bp palindromic (5'-TTAAGTTAACTTAA-3'), multiple reading frame stop linker (Smurfit Ochre stop linker, Pharmacia Biotechnology, Piscataway, NJ) was ligated into the plasmid to produce a stop codon. All sequencing was performed using a Sequenase 7-deaza-dGTP Sequencing kit (United States Biochemicals, Cleveland, OH). RNA extraction in certain experiments 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. PCR was performed on a thermo cycler (MJ Research, Watertown, MA).

Cells and Culture

All cells (K16, W8, D1, or D2) were grown in Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum, 1% penicillin G-streptomycin sulfate, 1% glutamine, and 1% sodium pyruvate at 37 °C in a 5% CO_2 atmosphere as described earlier [Smith and Niles, 1980; Lim et al., 1994]. Cells were subcultured by dissociating confluent cells with 5 mg/ml trypsin-EDTA solution. Usually, 200,000–500,000 cells were seeded in a T75 flask and the cells reached confluence in 5–7 days.

Plasmid Constructs

The pSTL29 plasmid (provided by Dr. Daniel Greenspan) contained the full-length pro-α2(I) cDNA coding sequence with stop codon and a SV40 small t-splice site and SV40 early polyadenylation signal driven by a Rous sarcoma virus LTR promoter. The pSTL29 plasmid was constructed from recombinant plasmid pSTL22 as previously described [Lee et al., 1988, 1990]. In order to increase transcription, we constructed a plasmid, pAL29, with α2(I) coding sequence driven by the stronger CMV promoter as previously described [Lim et al., 1994].

Two plasmids with full-length mouse α2(I) cDNA were used. The first plasmid, pHβAPr-1-neo-mα2(I), which contains the full-length mouse pro-α2(I) cDNA driven by the human β-actin promoter [Gunning et al., 1987] was kindly provided by Dr. R. Wenstrup. A smaller plasmid, pc3mα2(I), was created by digesting pHβAPr-1-neo-mα2(I) with HindIII/BamHI to remove the mouse α2(I) cDNA sequence. This 4.3 kb fragment was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA), which contained the CMV promoter and HindIII/BamHI sites in the multicloning region. Both mouse plasmids contained a neomycin resistance gene for selection.

To produce the human truncated α2(I) expression vector, the pSTL29 plasmid was digested with EcoRI/HindIII and subcloned into pBluescript. The new plasmid, pEH was digested with EcoRI and blunt-ended with klenow. A 14 bp palindromic (5'-TTAAGTTAACTTAA-3'), multiple reading frame stop linker (Smurfit Ochre stop linker, Pharmacia Biotechnology) was ligated into the plasmid. Confirmed clones with termination codons at the EcoR1 site were either digested with NotI/HindIII and recloned back into the pMS6 expression vector (pAL29t10) or digested with BamHI/HindIII and recloned into backbone from pSTL29 (pSTL29t10).

A similar mutation was also created in the mouse pro-α2(I) cDNA, using the Smurfit Ochre stop linker described above. The linker was ligated with an EcoR1/blunt-ended fragment of the vector pTZ18UCterm, which contained the last 710 bp of the mouse pro-α2(I) cDNA. The mouse pro-α2(I) sequence with the inserted stop codon was excised from this vector by digestion with Afl II and Afl III and ligated with a similarly digested vector pTZ18Umα2, which contained the remainder of the mouse pro-α2(I) sequence. The entire pro-α1(I) sequence with the premature stop (4.3 kb) was excised by digestion with Ase I, blunt-ended with Klenow, and digested with Hind III. This fragment was ligated with the vector pcDNA3, which was digested with Bsp120I, blunt-ended with Klenow, and digested with Hind III.

Transfection and Isolation of Stable Cell Lines

Supercoiled plasmids were transfected into W8 or D2 cells by either calcium phosphate method as previously described [Ritzenthaler et al., 1991; Lim et al., 1994], or by Lipofectin Transfection System (Gibco Life Technologies). Selection of cell lines transfected with constructs containing the neomycin resistant gene was performed as previously described [Lim et al., 1994] by replating cells into medium supple-

mented by G418 (0.6 mg/ml). The resistant colonies were all combined and maintained in medium containing 0.2 mg/ml G418, a neomycin derivative. Several cell lines were established and examined in this report as listed in Table 1.

The cell lines are named by the original cell type, the promoter in the construct and protein expressed. The truncated α2(I) protein is listed as t10 for truncation of 10 amino acids.

RNA Isolation and Analysis

Total cellular RNA was isolated from cell cultures using the guanidine-thiocyanate procedure [Chirgwin et al., 1979]. Total RNA (25 µg) was separated by electrophoresis on a 1% agarose (w/v) 0.66 M formaldehyde gel with ethidium bromide (20 µg/100ml) and transferred to nitrocellulose paper (BA-S 85 nitrocellulose, Schleicher & Schuell, Keene, NH). Nitrocellulose blots were hybridized with a 24 bp antisense oligonucleotide probe complementary to a sequence in human α2(I) exon 6 that was labeled by a 3' end labeling system (Promega Corp., Madison, WI) ($1x10^6$ CPM per lane) or with the C-propeptide rat cDNA probe, pα2R2 [34].

In some experiments, mRNA extraction was performed using TRIzol reagent (Gibco BRL Corp., Grand Island, NY) and mRNA was analyzed using reverse transcriptase followed by PCR (3′ RACE System Gibco Life Technologies) using the manufacturer's protocol. The forward primer (5'- CATCCAGGCCCAAC CTGTAAACACCCCAGC-3') annealing to the pro-α2(I) sequence and the reverse primer (5'- GCATTTAGGTGACACTATAGAATAGGGCA -3') annealing to the 3' untranslated region in the plasmid cDNA was used for the cDNA synthesis reaction and PCR annealing at 59°C. Products from the PCR reaction were separated on agarose gels and stained with ethidium bromide.

Radioactive Labeling

Confluent cells were preincubated in serum-free Dulbecco's modified Eagle's medium supplemented with 50 or 25 µg/ml ascorbic acid. After 2 hours, cells were labeled with 2 µCi/ml L-[^{14}C]proline for 24 hours [Smith and Niles, 1980], or with 10 µCi/ml L-[^{14}C]proline and 10 µCi/ml L-[^{14}C]glycine for 30 minutes or 2 hours. Both radiolabeled media and cell layers were harvested with addition of protease inhibitors to final concentrations of 20 mM EDTA, 10 µM phenylmethanesulfonyl fluoride (PMSF), and 1 mM p-chloromercuribenzoate (PCMB) in 50 mM Tris, pH 7.4 as previously described [Lim et al., 1994]. The cell layers in some experiments (see Figs. 2, 3, and 7) were extracted with 50 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl, 5 mM EDTA, 1 mM NEM, 1 µg/ml leupeptin, and 1 µg/ml pepstatin.

Collagen Precipitation and Polyacrylamide SDS Gel Electrophoresis

The labeled collagen in the media was precipitated with either 25% saturated ammonium

TABLE I. Cell Line and Transfected Plasmids[a]

Cell line	Cell species	Plasmid name	Promoter	Expressed protein
K16	Rat	none	none	none
W8	Rat	none	none	none
W8-CMVα2	Rat	pAL29	CMV	human α2(I)
W8-RSVα2	Rat	pSTL29	RSV	human α2(I)
W8-CMVα2t10	Rat	pSTL29t10	CMV	human α2(I) −10 amino acids
W8-RSVα2t10	Rat	pAL29t10	RSV	human α2(I) −10 amino acids
W8-neo	Rat	pSV2-neo	SV40	neomycin
D1	mouse	none	none	none
D2	mouse	none	none	none
D2-βApr-mα2	mouse	pHβApr-1-neo-mα2	β-Actin	mouse α2(I)
D2-CMV-mα2	mouse	pc3mα2	CMV	mouse α2(I)
D2-CMV-mα2t10	mouse	pc3mα2t10	CMV	mouse α2(I) −10 amino acids
D2-CMV	mouse	pcDNA3	CMV	neomycin

[a]The cell lines are named by the original cell type, the promoter in the construct and protein expressed. The truncated α2(I) protein is listed as t10 for truncation of 10 amino acids.

sulfate overnight at 4°C or 3:1 (v/v) ethanol overnight at 20°C. Precipitates and homogenized cell layers were then resuspended in 0.5N acetic acid and pepsin-treated (0.1 mg/ml) overnight at 4°C. After repeated lyophilization, pepsin-resistant proteins were resuspended in electrophoresis sample buffer and separated by 5%, 6%, or 7.5% polyacrylamide SDS gel electrophoresis at 10 volts per cm^2 for 4–5 hours [Smith and Niles, 1980]. Standard preparations of type I collagen, either extracted lathyritic rat skin collagen or radiolabeled chicken calvaria collagen [Smith and Niles, 1980] were used as molecular markers on all gels since collagen does not migrate as a globular protein. The gels were fixed in 50% methanol, 40% water, and 10% glacial acetic acid for 30 minutes, enhanced in Enlightning solution (Dupont) for 30 minutes, vacuum dried, and exposed to x-ray films.

Salt Fractionation of Radiolabeled Collagen

Radiolabeled cell layers and media were pepsin-treated (0.1 mg/ml) overnight at 4°C. Pepstatin was added to a final concentration of 1 µM to inactivate the pepsin. The pepsin-resistant collagen was salt fractionated to separate type I collagen from type I trimers. Samples with addition of lathyritic rat skin type I collagen carrier (5 g/ml) were dialyzed against 2.6 M sodium chloride, 0.05 M Tris (pH 7.6) overnight at 4°C in order to precipitate the heterotrimers. After centrifugation at 20,000 rpm for 30 minutes, the precipitate was recovered and washed twice with 80% cold ethanol. Sodium chloride was added to the supernatant to a final concentration of 4.4 M in order to precipitate the remaining homotrimers. The 4.4 M NaCl precipitates were also collected by centrifugation and washed twice with 80% cold ethanol. Both 2.6 M and 4.4 M NaCl precipitates were redissolved in 400 mM NaCl, 100 mM Tris, pH 7.4.

Thermal Stability Assay

Thermal denaturation temperatures were determined following an adaptation of the method described by Wenstrup et al. [1991]. Multiple aliquots (45,000 cpm/45 µl) were placed in a thermal cycler (MJ Research) at the initial temperature of 20°C and programmed to increase temperature 2–5 degrees every 12 min. At the end of each 10 min temperature plateau, an aliquot was removed, cooled to 20°C for 30 seconds, and the enzymes, trypsin (100 µg/ml) and chymotrypsin (250 µg/ml), were added for 2 minutes at 20°C. The reaction was terminated by adding of 10 µM phenylmethanesulfonyl fluoride and 10x SDS electrophoresis sample buffer. A control aliquot remained at 4°C and another was incubated at 20°C without enzymes. Digestion products and controls were analyzed by 7.5% polyacrylamide SDS gel electrophoresis to determine the melting temperatures of the different type I collagen molecules.

Reduction, Alkylation, and Immunoprecipitation

The labeled procollagen of both cell layer and media were reduced and alkylated using the following adaptation of a previously described method by Deak et al. [1983]. All samples were heat denatured by addition of urea to a final concentration of 6 M at 55°C for 20 min. Samples were brought up to 1.5 M EDTA and disulfide bonds were reduced by incubating with 50 mM DTT at 37°C for 3 hours. Alkylation was performed by addition of iodoacetic acid to a concentration of 0.1 M followed by incubation in the dark at room temperature for 1 hour. Samples were then dialyzed against 0.1 M Tris-HCl buffer pH 7.5.

Samples were immunoprecipitated with a human pro-α2(I) antibody, LF116, made to the amino-terminal telopeptide of α2(I) (kindly provided by Dr. L. Fisher). The α2(I) antibody was diluted to 1:1,000 in a binding buffer containing 0.15 M NaCl, 1 mg/ml BSA, and 0.01% sodium azide in 0.1 M HCl pH 7.5. After incubation on ice for 2 hours, GammaBind G Sepharose beads (50 µl) was added and incubated in the sample overnight at 4°C on a rotator. Immunoprecipitates were collected by centrifugation in microcentrifuge at 3,000 rpm for 15 minutes at 4°C. After pellets were washed gently three times with phosphate buffer saline containing 1% triton-X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (PBSTDS), they were resuspended in sample buffer with 8 M urea and 50 mM DTT. The samples were boiled for 10 minutes and analyzed by polyacrylamide SDS gel electrophoresis as described earlier.

RESULTS

As previously described [Smith and Niles, 1980; Lee et al., 1988], the chemically transformed cell line, W8, produces α1(I) homotrimers with no α2(I) chains, whereas the parent cell line, K16, produces normal collagen heterotrimers. When W8 cells were transfected with plasmid constructs containing the full-

length human α2(I) cDNA, pAL or pSTL29, the cells expressed α2(I) mRNA as judged by Northern blots and S1 nuclease protection assays [Lee et al., 1988; Lim et al., 1994]. The α2(I) protein formed heterotrimers as judged by the appearance of pepsin-resistant collagen containing α2(I).

In this study, we transfected constructs expressing truncated pro-α2(I) into W8 cells in order to determine whether the last amino acids were important for assembly of heterotrimers. Two constructs (pSTL29t10 and pAL29t10) were prepared that translated α2(I) truncated by 10 amino acids. After the mutations were confirmed by sequencing, each construct was transfected into W8 cells with pSV2neo and stable cell lines were selected by resistance to neomycin. The stable cell lines (W8-RSVα2t10 and W8-CMVα2t10) contained mRNA for α2(I) as judged by Northern blot analysis (Fig.1A, lanes 3 and 4). This RNA was translatable in cell free extracts (data not shown).

Collagen production by transfected cells was assayed first by pepsin-treating media and cell layer proteins to examine the triple-helical collagen accumulation during a 24-hour period. No heterotrimers could be demonstrated in cells transfected with truncated α2(I) when media and cell layer radiolabeled proteins were separated by 7.5% polyacrylamide SDS gel electrophoresis (Fig. 1B, lanes 5 and 6). Pepsin-resistant pro-α2(I) chains could be detected in the media and cell layer fractions from control cells, K16, or W8 cells transfected with wild-type pro-α2(I) expression construct (Fig.1B, lanes 2 and 4).

Next, experiments were performed by incubating cells with [^{14}C]proline and [^{14}C]glycine for shorter time periods. Cell layer proteins were extracted with NP-40 and protease inhibitors to detect newly synthesized pro-α2(I) chains. The K16 cells and W8 cells transfected with wild-type α2(I) construct (cell lines W8-RSVα2 and W8-CMVα2) produced a high amount of pro-α2(I) as compared to pro-α1(I) during a 30-minute pulse (Fig. 2B, lanes 1, 3 and 4). K16 and W8 cells transfected with wild-type pro-α2(I) secreted collagen into the media as a heterotrimer within 30 minutes (Fig. 2A, lane 1). The W8 cells produced an α1(I) procollagen whose secretion was delayed compared to K16 cells (Fig. 2A compare lane 2 to lane 1). W8 cells transfected with full- length pro-α2(I) cDNA synthesized procollagen with secretion kinetics similar to K16 procollagen (Fig. 2A, lanes 3 and 4 compared to lane 1), whereas the cells transfected with truncated constructs, cell lines W8-RSVα2t10 and W8-CMVα2t10, resembled W8 procollagen (Fig. 2A, lanes 5 and 6 compared to lane 2) .

Fibroblasts from an osteogenesis imperfecta (OI) patient with a frame shift mutation in the C-propeptide of pro-α2(I) produced small amounts of pro-α2(I) that were rapidly degraded (36). The production of pro-α2(I) could be detected by immediately denaturing cell extracts with 6 M urea, reducing the disulfide bonds with DTT and alkylating with iodoacetamide followed by immune precipitation. Following the same method, proteins were immunoprecipitated using an antibody to the human α2(I) N-telopeptide, LF116 (kindly provided by Dr. Larry Fisher). This antibody reacted with only human α2(I) collagen or precursor proteins on Western analysis (data not shown). As seen in Figure 3 (lanes 7 and 8), pro-α2(I) chains were precipitated from cells transfected with truncated constructs. The truncated pro-α2 was visible after 30 minutes and increased after 2 hours. Therefore, the pro-α2(I) protein was translated from the expressed mRNA. Some pro-α1(I) chains were visible, which suggested there was some cross reaction between pro-α chains, or the pro-α1(I) chains were co-precipitating due to low solubility in neutral salt buffers.

Since a human cDNA for pro-α2(I) was transfected into rat cells, there was concern that the resultant heterotrimer might not be as thermally stable as the normal molecule. The mutation in the polypeptide could have simply enhanced the instability of the molecule causing blocked assembly. Therefore, thermal stability of the heterotrimers was tested by determining the temperature at which the triple helical structure denatured and became susceptible to proteases using an adaptation of the method of Wenstrup et al. [1991]. For this experiment, the heterotrimer was separated from the α1(I) type I trimer by differential salt precipitation whereby heterotrimer precipitates at 2.6 M NaCl and α1(I) type I trimer precipitates at high salt, 4.4 NaCl [Moro and Smith, 1977; Lee et al., 1988; Lim et al., 1994]. As shown in Figure 4A, the human-rat hybrid heterotrimers from W8-CMVα2 cells had a lower melting temperature (Tm 41°C) than the endogenous rat heterotrimers from K16 cells or the rat α1(I)

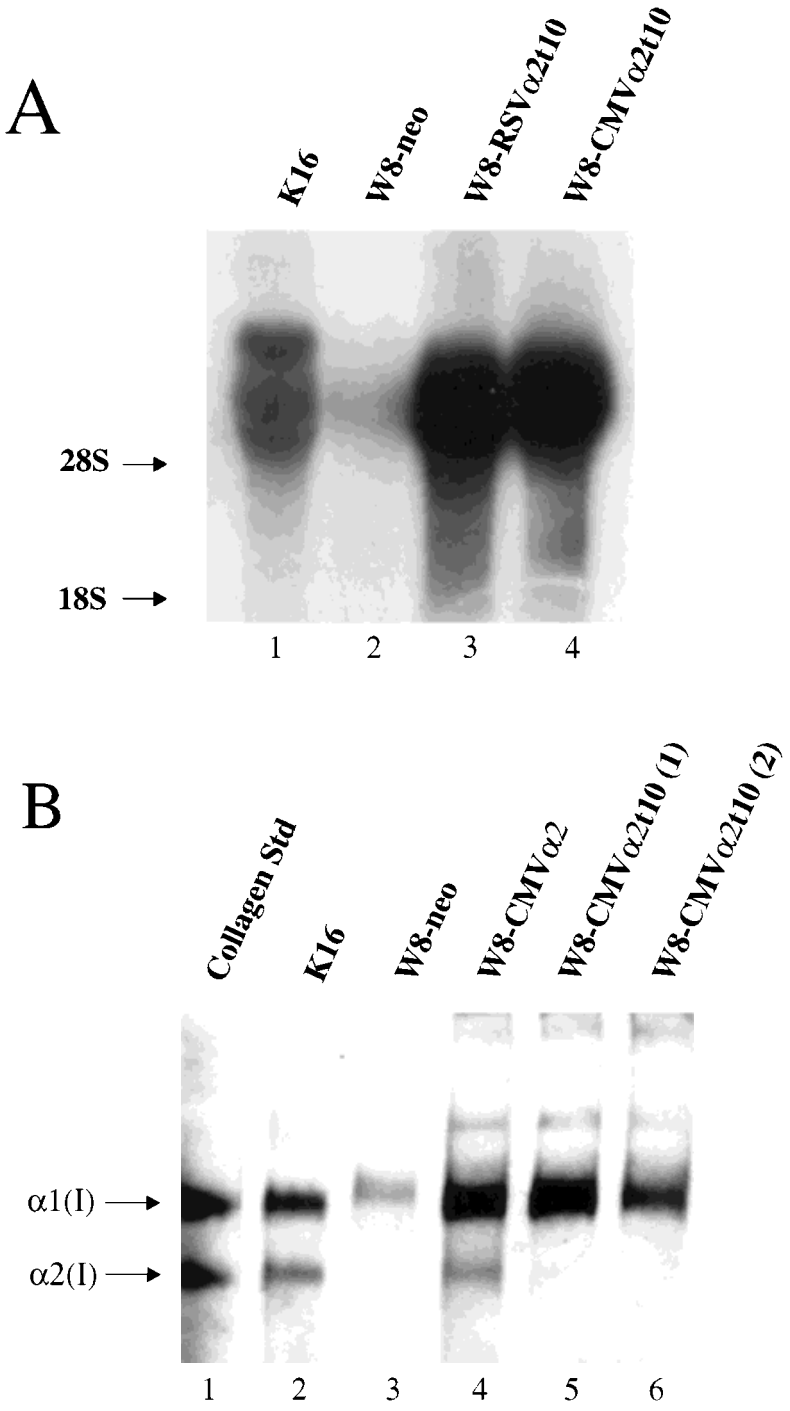

Fig. 1. **A:** Northern blot analysis detects the presence of pro-α2(I) mRNA in total RNA extracted from W8 cells transfected with expression constructs (pSTL29t10, pAL29t10) containing truncated pro-α2(I) cDNA. Total RNA (25 µg) was isolated from the different cell lines (K16, w8-neo, W8-RSVα2t10 and W8-CMVα2t10), separated by electrophoresis on a 1% agarose gel containing formaldehyde, and transferred to nitrocellulose as described in the experimental section. Specific pro-α2(I) mRNA was hybridized with a [^{32}P] labeled antisense oligonucleotide from the sixth exon of the pro-α2(I). Arrows indicate the position of the 18S and 28S forms of rRNA visualized by ethidium bromide. **B:** No pepsin-resistant α2(I) collagen chains could be detected in media from W8 cells transfected with expression constructs (pSTL29t10, pAL29t10) containing truncated α2(I) cDNA. Radiolabeled media proteins secteted by cells (K16, W8, W8-CMVα2, W8-CMVα2t10, W8-CMVα2t10) during a 24 hour incubation with [^{14}C]proline were ethanol-precipitated, resuspended in 0.5 M acetic acid, pepsin-treated and separated on a 7.5% polyacrylamide gel by SDS polyacrylamide gel electrophoresis. The products were visualized by autoradiography as described in the experimental section. A [^{14}C]proline-labeled chicken calvaria collagen standard (Std) of 30,000 CPM was separated in the **first lane.** CMVα2t10(1) and CMVα2t10(2) refer to two stable cell lines established by transfecting plasmid (pAL29t10) into W8 cells in two separate experiments. Arrows indicate the position of the α1(I) and α2(I) standards.

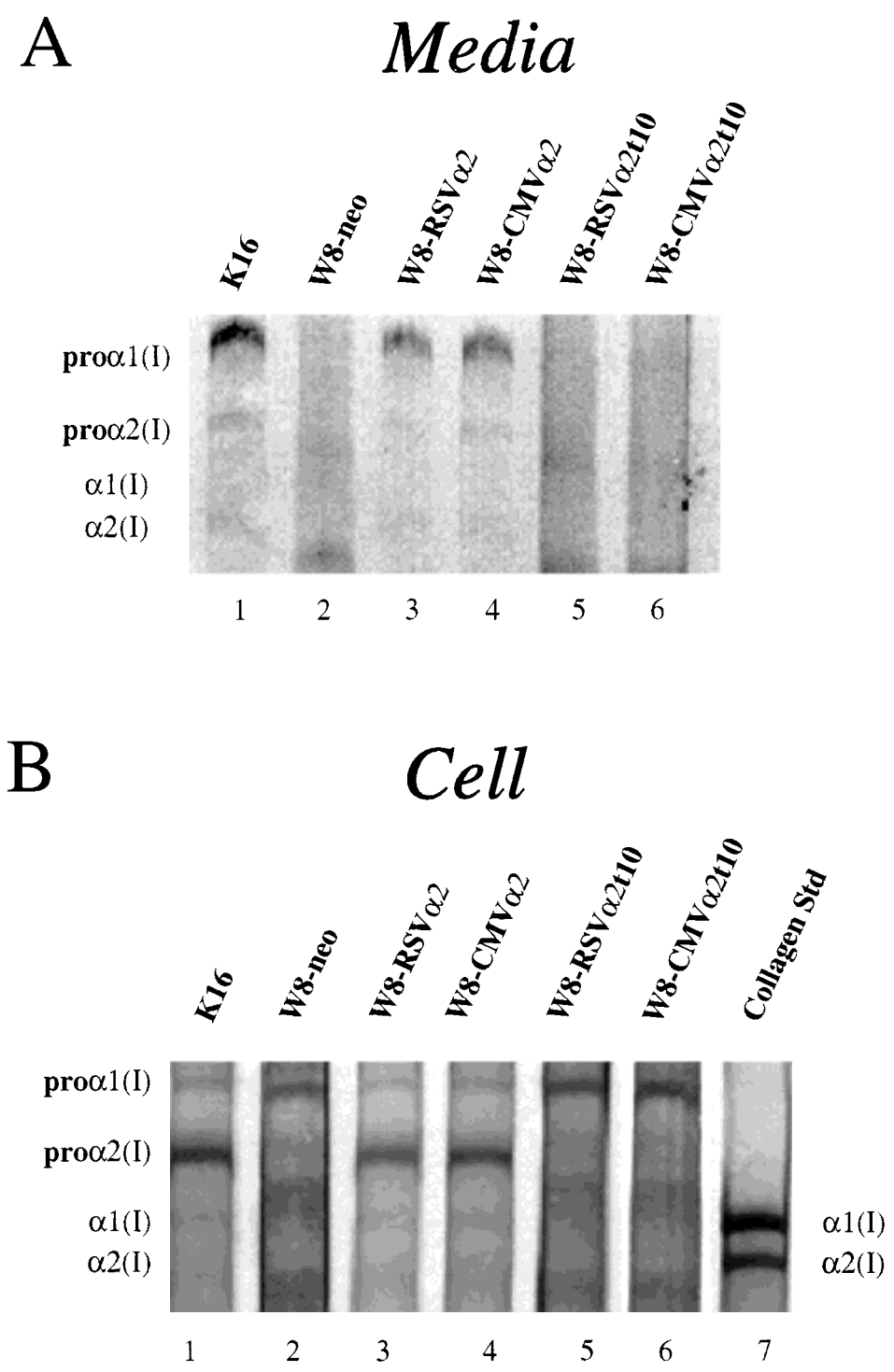

Fig. 2. Autoradiograph of media **(A)** and cell layer **(B)** proteins from cells radiolabeled for 30 min. Cells (K16, W8-neo, W8-RSVα2, W8-CMVα2, W8-RSVα2t10, and W8-CMVα2t10) were grown to confluence in 6-well cluster dishes. The confluent cells were incubated with [^{14}C]proline for 30 min. Medium was removed and protease inhibitors were added. Three equal aliquots (300 µl) were ethanol-precipitated, separated by electrophoresis on a 7.5% SDS polyacrylamide gel and exposed to x-ray film for three days. A [^{14}C]proline-labeled chicken calvaria collagen standard (Collagen Std.) of 30,000 CPM was used as a molecular weight marker.

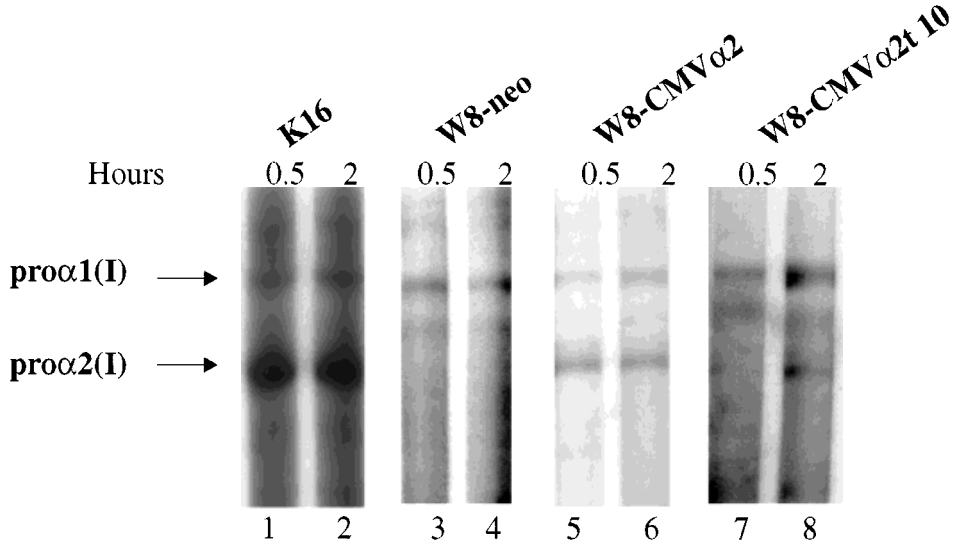

Fig. 3. Pro–α2(I) could be detected in media after reduction, alkylation of radiolabeled cell extracts. Each cell line (K16, W8, W8-CMVα2 and W8-CMVα2t10) was plated at a density of 500,000 cells per T25 flask and grown to confluence. Confluent cells were incubated with [^{14}C]proline for 0.5 or 2 hours. Cell layers were harvested with protease inhibitors, heat denatured, reduced and alkylated with iodoacetic acid as prescribed in experimental methods section. Equal aliquots of each sample (300 µl) were immunoprecipitated with a specific human α2(I) antibody, LF116, made to the amino-terminal telopeptide of α2(I) at a dilution of 1:1,000. Immunoprecipitates bound to GammaBind G Sepharose beads were collected by centrifugation and gently washed three times with PBS buffer containing detergents. Heat denatured samples were separated by electrophoresis on a 5% SDS polyacrylamide gel, dried and visualized by autoradiography. Arrows indicate position of pro–α1(I) and pro–α2(I) collagen chains.

type I trimers from W8 cells (Tm 43 °C). These results suggested that interspecies collagen heterotrimers were less thermal stable.

In order to eliminate the possible problem of an unstable heterotrimer caused by species differences, the full-length mouse pro-α2(I) cDNA driven by a human β-actin promoter with a neomycin-resistance gene, pHβAPr-1-neo-mα2(I), was stably transfected into α1(I) type I trimer-producing D2 mouse cells. Northern blot analysis demonstrated the presence of pro-α2(I) mRNA (Fig. 5A) and pepsin-resistant heterotrimers were secreted into the media (Fig. 5B). Homotrimer was also secreted in the media as demonstrated by the greater than 2:1 ratio of α1(I) to α2(I) (Fig. 5B) and the salt precipitation (Fig. 4B). The mouse-mouse heterotrimers produced by D2-βApr-mα2 cells had a higher melting temperature (Tm 43°C) than the human-rat heterotrimers (Tm 41°C) (Fig. 4B). More importantly, the heterotrimers of the transfected mouse cells had the same melting temperature as normal heterotrimers from the D1 cells (Fig. 4B). Therefore, the D2 cells expressing the mouse cDNA for pro-α2(I) was potentially a better system for examining the effects of mutations on collagen type I assembly.

Since the human cDNA was driven by viral promoters, the mouse cDNA was cloned into pcDNA3 vector, which has a CMV promoter. The resultant mouse construct contained a 10 amino acid truncation mutation similar to the human α2(I) cDNA (see Materials and Methods for construction details). Both the full-length cDNA [pc3mα2(I)] and mutated cDNA [pc3mα2T10(I)] were stably transfected into D2 cells and the presence of the mRNA was confirmed by using RT-PCR (Fig. 6A). No pepsin-resistant heterotrimers could be detected in media secreted by the cells transfected with mutated pro-α2(I) cDNA. Pepsin-resistant pro-α2(I) chains could be detected in media secreted by the D2 cells transfected with the full-length pro-α2(I) cDNA as well as by the D1 control cells (Fig. 6B). This data corroborates that the last 10 amino acids are important for assembly of the heterotrimer.

Collagen synthesis by D1 cells was compared to collagen synthesis by D2 cell lines expressing wild-type mouse pro-α2(I) within shorter labeling periods (1, 2, 4, 8 hours). Unlike K16 cells, the control cell line, D1, had a close to 2:1 ratio of pro-α1(I) to pro-α2(I). The transfected D2 cells maintained a barely detectable level of

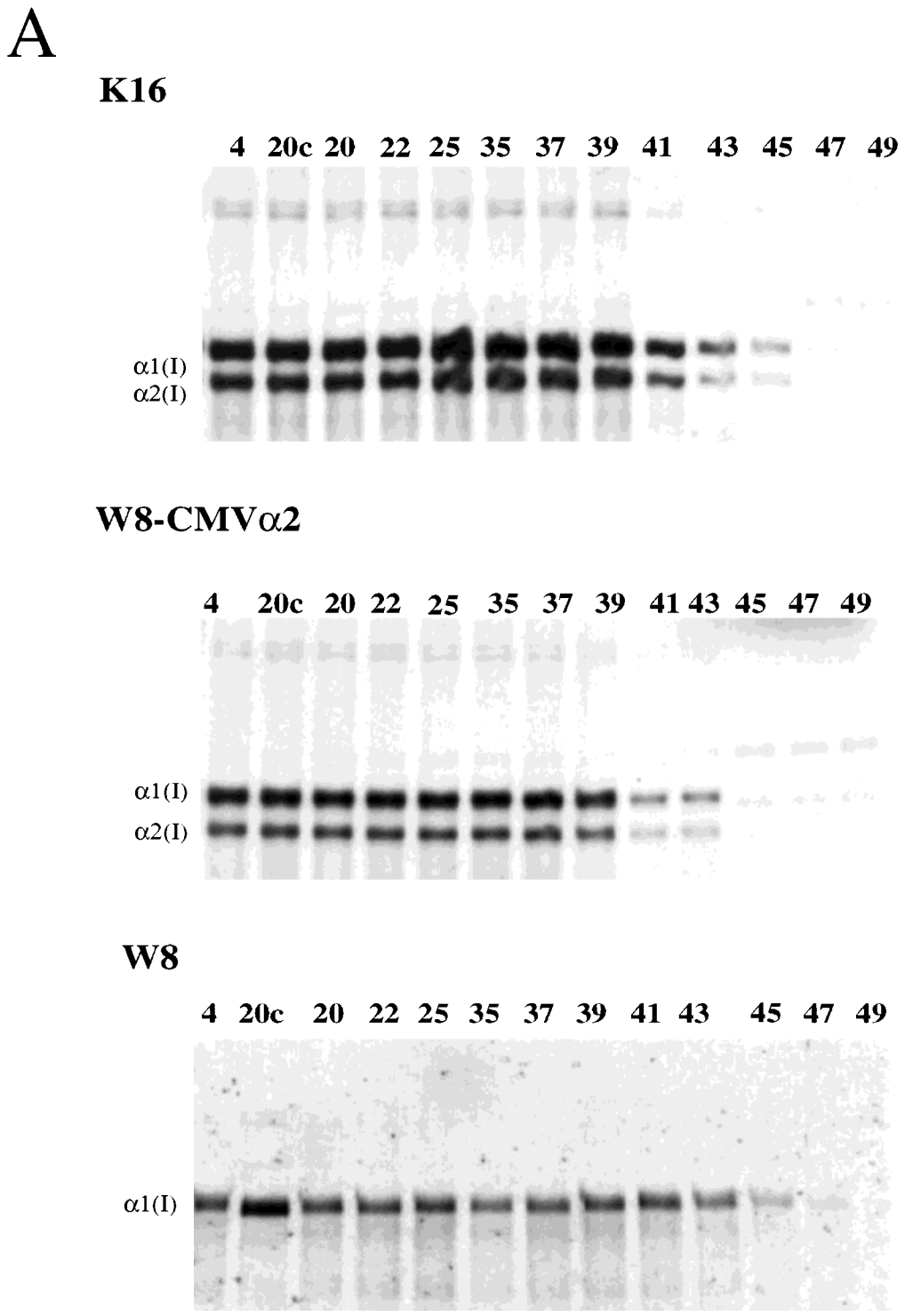

Fig. 4. Autoradiographs comparing thermal denaturation of type I collagen heterotrimers and homotrimers. Aliquots of media containing [^{14}C]proline-labeled procollagen were digested with pepsin to produce collagen molecules. Type I collagen heterotrimers were differentially separated by salt fractionation using 2.6 M NaCl, 0.05 M Tris (pH 7.6) overnight at 4°C. Aliquots of 45 µl samples were incubated for 12 min. at temperatures indicated above each lane. The samples were rapidly cooled and digested with trypsin and chymotrypsin as described in experimental section. Collagen chains were separated on a 5% polyacrylamide gel by SDS polyacrylamide gel electrophoresis. **A.** Collagen type I heterotrimer secreted by K16, W8-CMVα2, and W8 cells. **B.** Collagen type I heterotrimer secreted by D1, D2, and D2-βAPr-mα2 cells.

B

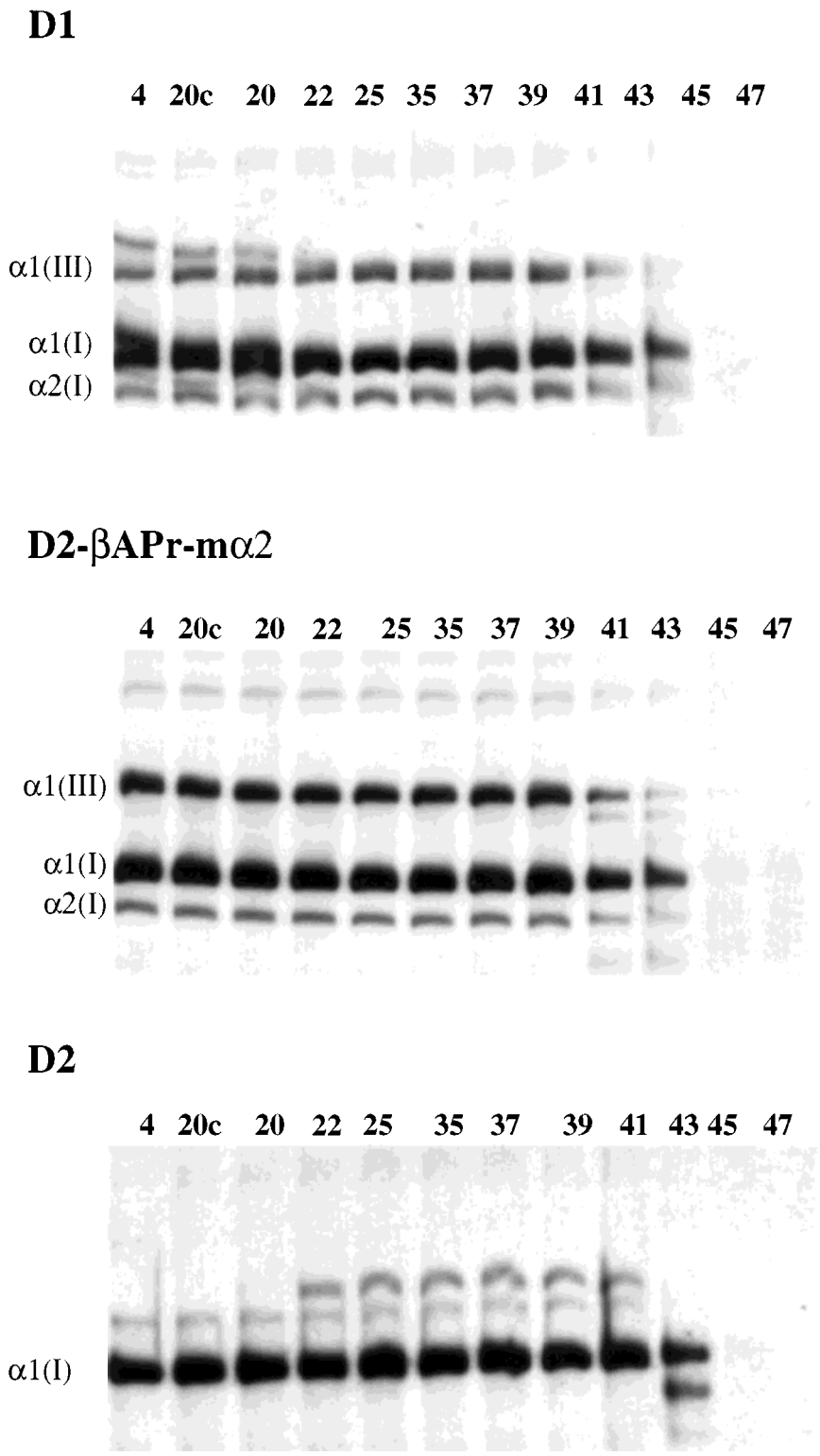

Figure 4. (Continued)

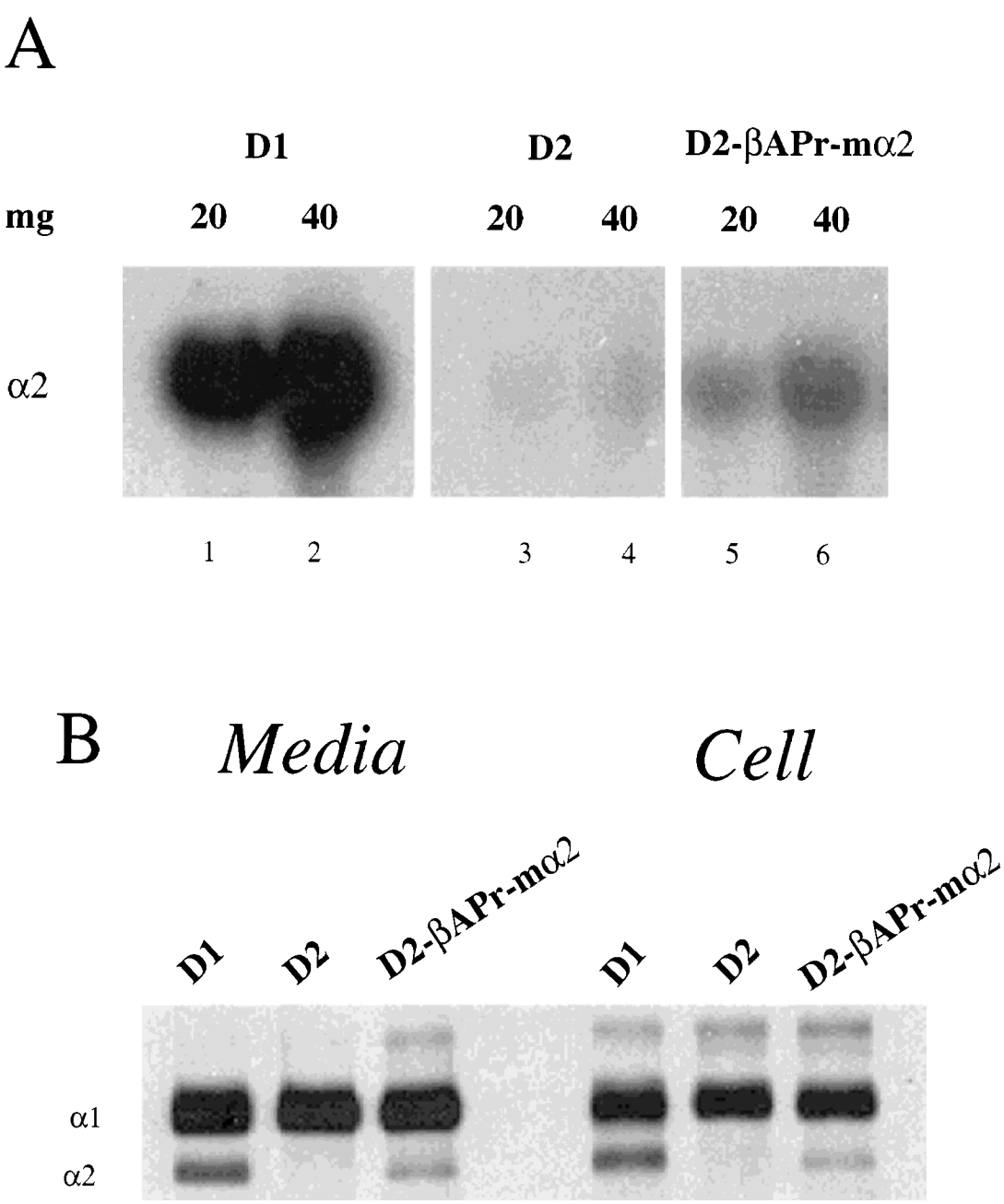

Fig. 5. **A:** Northern blot analysis demonstrates the presence of mouse α2(I) mRNA in total RNA extracted from D2 cells transfected with expression construct (pHβAPr-1-neo-mα2) containing mouse α2(I) Total RNA (20 or 40 μg) was isolated from the different cell lines (D1, D2, D2-βAPr-mα2) and separated by electrophoresis on a 1% agarose gel containing formaldehyde. The RNA in the gel was transferred to nitrocellulose and subjected to hybridization with a [^{32}P] labeled cDNA probe to rat pro-α2(I) (32). **B.** Pepsin-resistant α2(I) collagen chains could be detected in media from D2 cells transfected with expression construct (pHβAPr-1-neo-mα2) containing mouse α2(I) cDNA. Radiolabeled pepsin treated media proteins secreted by cells (D1, D2, D2-βAPr-mα2) during a 24 hour incubation with [^{14}C]proline were separated on a 7.5% polyacrylamide gel by SDS polyacrylamide gel electrophoresis.

pro-α2(I) within the cells even though there was secretion of heterotrimer. In addition, the D2 cells secreted 2–3 times more pepsin-resistant α1(I) type I collagen during 8 hours than the D1 cells (Fig. 7A). Expression of pro-α2(I) in D2 cells reduced the level of pro-α1(I) synthesized close to the levels synthesized by D1 cells (Fig. 7B). Therefore, although W8 and D2 cells

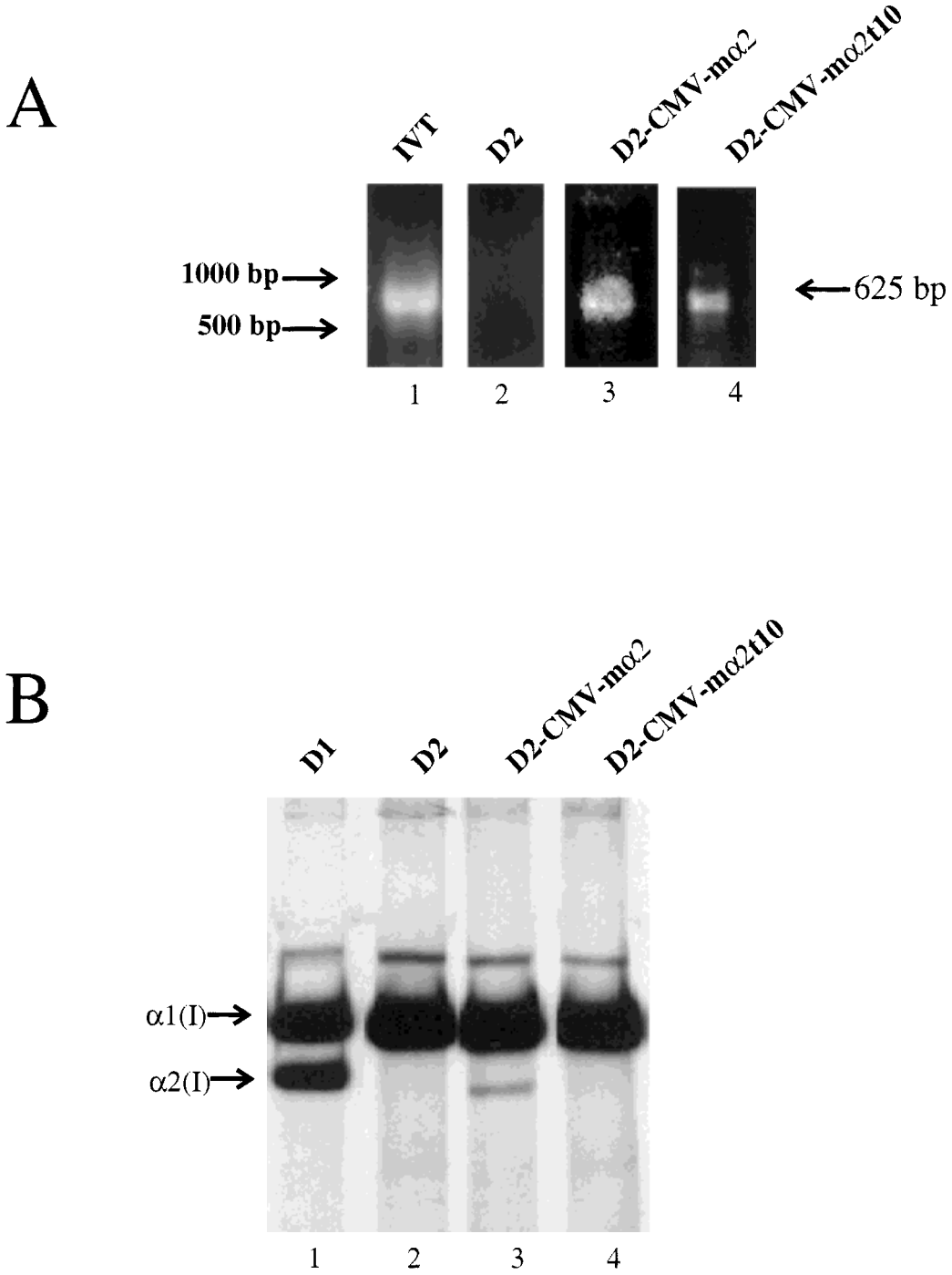

Fig. 6. **A:** RT-PCR detection of mouse pro-α2(I) mRNA in total RNA extracted from D2 cells transfected with expression constructs containing wild type (pc3mα2) or truncated mouse pro-α2(I) (pc3mα2T10). RT-PCR was performed on RNA from cells (D2, D2-CMV-mα2, and D2-CMV-mα2t10) and in vitro transcribed RNA from pc3mα2 (lane IVT) using the reverse primer for the cDNA synthesis reaction. A forward primer to the pro-α2(I) sequence and a reverse primer in the plasmids 3′ untranslated region were used for PCR amplification of the cDNA. The sequences of the primers are in the experimental section. **B:** No pepsin-resistant α2(I) collagen chains could be detected in media from D2 cells transfected with expression constructs (pc3mα2t10) containing truncated mouse pro-α2(I) cDNA. Radiolabeled media proteins secreted by cells (D1, D2, D2-CMV-mα2, and D2-CMV-mα2t10) during a 16 hour incubation with [^{14}C]proline were ethanol precipitated, resuspended in 0.1 M acetic acid, pepsin-treated and separated on a 6% polyacrylamide gel. The products were visualized by autoradiography as described in Materials and Methods. Arrows indicate that positions of α1(I) and α2(I).

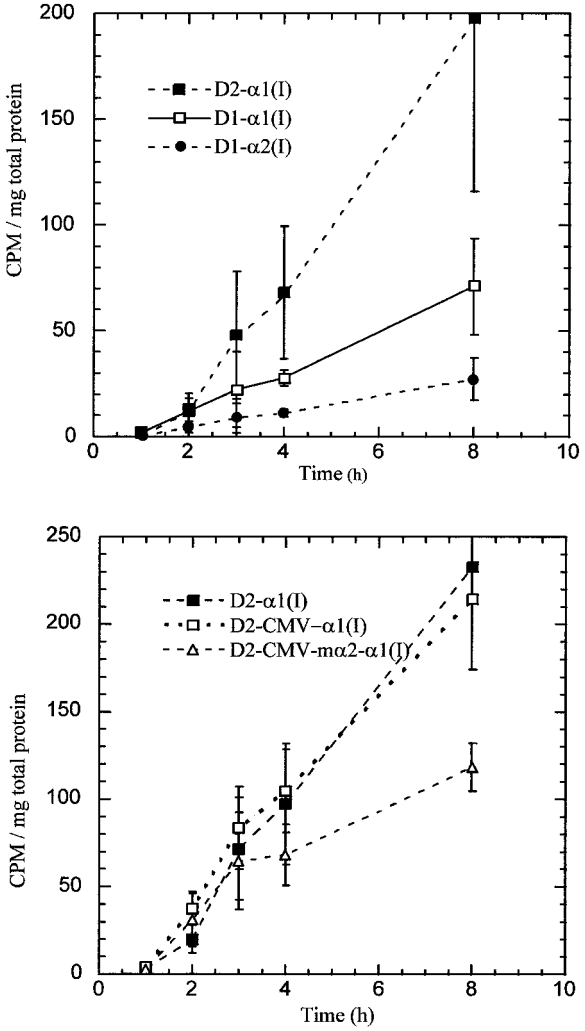

Fig. 7. **A:** Time course of collagen production in D1 and D2 cells. D1 and D2 cells were metabolically radiolabeled with [^{14}C]-proline for 1, 2, 3, 4, and 8 hours. Media and intracellular proteins were pepsin-digested and the proteins were separated on 6% polyacrylamide gels. The radioactivity in α1(I) and α2(I) bands on the dried polyacrylamide gel was counted using an InstantImager flat bed counter. Error bars represent the standard error for the values obtained for each time point in 3 experiments. Symbols represent α1(I) chain synthesis from D2 cells (--■--), α1(I) from D1 cells (–□–), and α2(I) from D1 cells (--●--). **B:** Expression of mouse Pro-α2(I) in D2 cells decreases the amount of detected α1(I) secreted from the cells. Cells (D2, D2pc3 and D2-CMV-mα2) were metabolically radiolabeled with [^{14}C] proline for 1, 2, 3, 4, and 8 hours. The media proteins were pepsin treated and separated on 6% polyacrylamide gels. The radioactivity in pro-α1(I) bands for 3 identical experiments were measured using an Instant Imager. Error bars represent the standard error for values obtained for each time point in 3 experiments. Symbols represent α1(I) chain synthesis from D2 cells (--■--), control plasmid pcDNA3 transfected D2 cells, D2-CMV cells, (--□--), D2-CMV-mα2 cells (--Δ--).

both make α1(I) type I trimer, they express, assemble, and secrete this collagen in a cell-specific manner.

DISCUSSION

In order to establish the function of the terminal amino acids of pro-α2(I) chain in the collagen assembly, a mutation causing a premature stop was inserted into two pro-α2(I) cDNAs (human and mouse) eliminating the last 10 amino acids (FVDIGPVCFK) of the pro-α2(I) chain. Although specific pro-α2(I) mRNA was detected in total cell RNA (Figs. 1A, 6A) confirming transcription of the pro-α2(I) plasmid, pepsin-resistant type 1 heterotrimer with truncated α2(I) collagen could not be detected in W8 cells (Fig. 1B) or D2 cells (Fig. 5B). The results from two cell systems were consistent. Therefore, it is likely that the truncation mutation at the COOH-terminus blocked assembly of the pro-α2(I) with pro-α1(I).

Naturally occurring mutations in the C-propeptide noted in osteogenesis imperfecta in humans [Byers and Steiner , 1992; Lachman et al., 1992] and mice [Chipman et al., 1993] suggest that assembly of pro-α2(I) chains with two pro-α1(I) molecules required the last portion of the C-propeptide. A 4-base pair frameshift deletion altered the last 33 amino acids in the pro-α2(I) chain [Pihlajaniemi et al., 1984]. Immunoprecipitable mutated pro-α2(I) chains were synthesized, but were rapidly degraded and not incorporated into a triple helix [Deak et al., 1983]. Using a similar technique, this study demonstrated that newly synthesized unassociated truncated pro-α2(I) chains could be detected (Fig. 3). The fact that the truncated precursor chain could be detected only after immunoprecipitation suggests that newly translated pro-α) chains did not assemble into a stable triple helix procollagen. Our studies, thereby, reduce the number of amino acids that are critical for assembly from 33 to the last 10 amino acids in the C-propeptide.

Included in the last 10 amino acids is a cysteine residue that is involved with intramolecular bonding within the C-propeptide. Disulfide-bonds form before formation of the triple helical structure [Fessler and Fessler, 1974, Uitto et al., 1981; Doege and Fessler, 1986] and this process has been hypothesized to aid in the initial stages of procollagen trimerization. A site-directed mutation converting the last cysteine to alanine has been tested in our laboratory

[Doyle and Smith, 1998]. This mutation alone was not sufficient to block assembly of pro-α2(I) with pro-α1(I). Since these last 10 amino acids are highly conserved between the fibrilar collagens (I, II, III, V) and between species this region may be essential for collagen assembly of other collagen types.

Recent evidence demonstrate that completed procollagen chains remain associated with the ER membrane after synthesis through a weak association with the C-propeptide [Beck et al., 1996]. This association could be dependent on the last amino acids in the protein. Alternatively a membrane-bound protein could be involved. A variety of molecular chaperones are known to interact with newly synthesized collagen pro-α chains including BiP/HSP78, which interacts with many native polypeptides [Nakai et al., 1992] and HSP 47/colligin, which is specific for collagen [Nakai et al., 1992; Jain et al., 1994; Sauk et al., 1994]. These molecular chaperones have diverse functions that may be influenced by the presence of the last amino acids.

Since a human pro-α2(I) cDNA was transfected into rat cells, there was concern that species differences between human and rat would alter posttranslational processing of the collagen chains. Stability and the rate of secretion of heterotrimers could be affected because of the interspecies differences in addition to the differences in the last 10 amino acids. The thermal stability of the heterotrimers salt-precipitated from the different clonal cell lines were tested. The human-rat hybrid heterotrimer was less thermostable when compared to its control rat-rat heterotrimer (Fig. 4), indicating that the chimeric molecule has an altered triple helical structure.

A complete mouse pro-α2(I) collagen cDNA was constructed by and obtained from Dr. Richard Wenstrup [Phillips et al., 1991]. Its sequence was 90% identical in amino acid composition and 87% identical in nucleotide composition to the human collagen chains [Phillips et al., 1992]. The mouse pro-α2(I) cDNA was transfected into mouse bone marrow cells, D2 cells, which produced only type I trimers [Diduch et al., 1993]. Most importantly, the heterotrimers of the transfected mouse cells had the same melting temperature as the normal mouse heterotrimers from the control D1 cells (Fig. 4), implying that interspecies collagen chains did cause a difference in the stability of the protein. As a consequence, the heterotrimer formed with the truncated human pro-α2(I) and rat pro-α1(I) might have been undetectable due to the instability of the interspecies hybrid heterotrimer and not the truncation of the last 10 amino acids. However, confirmation with the mouse system establishes that the last 10 amino acids are important for assembly.

Our results demonstrate that the secretion of the α1(I) type I trimer in W8 cells or in W8 cells transfected by truncated constructs is slower than heterotrimer secretion in K16 cells or W8 cells transfected by wild-type constructs (Fig. 2). Therefore, assembly of the homotrimer might be a slower process in these cells than normal heterotrimer assembly. If assembly of collagen is slow, the collagen posttranslational modifying enzymes have more time to act on the newly synthesized unfolded collagen resulting in overmodified collagen chains. Hydroxylation of proline is important for the stabilization of the triple helix because the hydroxyl groups contributes to the formation of hydrogen bonds between the collagen chains. However, overhydroxylation of the collagen chains increases the thermal stability as well as intracellular retention of the collagen molecules [Willing et al., 1988]. The homotrimers in both cell systems did have a higher melting temperature supporting the assumption that the chains were overmodified during assembly.

In contrast, the steady-state levels of collagen chains differed in the two cell systems. K16 cells and W8 cells transfected with wild-type pro-α2(I) maintained a steady-state level of pro-α2(I) greater than pro-α1(I). D1 cells, however, maintained a normal collagen chain ratio (2:1) of pro-α1(I) to pro-α2(I) within the cell. In addition, the kinetics of homotrimer secretion by D2 cells was the same as heterotrimer secretion in D1 cells, suggesting that the homotrimer assembly was not slower in D2 cells.

Our results strongly support the hypothesis that the last 10 amino acids in the C-propeptide region are important for the proper alignment and registration of the three pro-α chains prior to the folding of the triple helix structure. A reasonable model to propose for assembly of type I collagen is that there is an association of the newly formed complete unfolded nascent chains on the membranes in a region where there is co-localization of the nascent pro-α1(I) and pro-α2(I) chains. The association with the membrane could reside in the C-propeptide at

the last 10 amino acids. Chaperone proteins such as BiP or HSP47/colligin probably maintain the nascent chain in an unfolded, undegraded configuration until folding can begin. Other molecules residing in the ER membrane, such as hydroxylases and cis-trans isomerases, are involved in folding of the collagen chains. The resulting conformational folding of the C-propeptide may be important in proper assembly and alignment of the individual collagen chains. The conformational determinant for proper assembly may require the last 10 amino acids and intramolecular disulfide bond stabilization.

In conclusion, we have demonstrated that the last 10 amino acid residues in the pro-α2(I) chain is required for proper assembly of pepsin-resistant type I collagen. Additionally, the presence of the full-length pro-α2(I) chain enhances the secretion of the type I collagen. The mouse cell system secretes a more normal triple helical molecule and, therefore, has been used for investigations concerning the role of cysteine and disulfide bonds in the pro-α2(I) C-propeptide [Doyle and Smith, 1998]. This system should be useful in future mutational studies to determine exactly which amino acids are essential for fibril formation or in studies to examine the role of the pro-α2(I) chain in cell-matrix interactions.

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