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## The NC16A Domain of Collagen XVII Plays a Role in Triple Helix Assembly and Stability\*

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### Abstract

Collagen XVII/BP180 is a transmembrane constituent of the epidermal anchoring complex. To study the role of its non-collagenous linker domain, NC16A, in protein assembly and stability, we analyzed the following recombinant proteins: the collagen XVII extracellular domain with or without NC16A, and a pair of truncated proteins comprising the COL15-NC15 stretch expressed with or without NC16A. All four proteins were found to exist as stable collagen triple helices; however, the two missing NC16A exhibited melting temperatures significantly lower than their NC16A-containing counterparts. Protein refolding experiments revealed that the rate of triple helix assembly of the collagen model peptide GPP<sub>10</sub> is greatly increased by the addition of an upstream NC16A domain. In summary, the NC16A linker domain of collagen XVII exhibits a positive effect on both the rate of assembly and the stability of the adjoining collagen structure.

### Keywords

BP180; transmembrane collagen; protein stability; protein folding; nucleation site

## 1. Introduction

Collagen XVII (also known as BP180 or BPAG2) is a major transmembrane component of the epithelial anchoring complex [1,2], a structure that functions in maintaining the adhesion of cells in the basal layer of stratified epithelia to the underlying basement membrane. The importance of collagen XVII in maintaining tissue architecture is underscored by genetic and acquired skin diseases in which its loss or lack of function leads to blister formation at the level of the dermal-epidermal junction (reviewed in [3]).

Biochemical studies from several groups have established that the collagen XVII protein exists as a transmembrane homo-trimer, with an N-terminal globular head region comprising the intracellular domain, and a long, highly extended, semi-rigid extracellular domain corresponding to a series of collagen triple helices (Gly-Xaa-Yaa tripeptide repeats) separated by non-collagenous interruptions (see Fig. 1) [4].

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It has been hypothesized that collagen XVII and other members of the membrane-associated collagen sub-family undergo triple helix assembly in the N-terminal (membrane-proximal) to C-terminal direction [5], rather than in the C-terminal to N-terminal direction as is typical for the “classical” collagens. Therefore, the membrane-proximal non-collagenous linker region of collagen XVII (designated NC16A; see Fig. 1), has been identified as a likely candidate for the site of association and initiation of triple helix assembly.

Supporting this idea is the fact that NC16A contains a predicted  $\alpha$ -helical coiled-coil structure [4], as do the linker regions of the other members of the membrane-associated collagen sub-family [6]. Also it has been demonstrated that the carboxy-terminal region of collagen XVII is not required for triple helix formation [7]. More recently, Franzke and co-workers [8] reported that deletion of NC16A from full-length collagen XVII decreases its thermal stability. A disadvantage of using a transmembrane form of the protein for such an analysis is that a detergent is required for solubilization. In the present study we further probed the effects of NC16A on the stability and refolding of collagen XVII using soluble recombinant forms of the ectodomain of this protein.

## 2. Materials and Methods

### 2.1. Generation of recombinant forms of the collagen XVII ectodomain

The DNA vector pCEP4-sec180, which is based on the pCEP4 episomal expression vector (Invitrogen, Carlsbad, CA) and which encodes a secreted form of the collagen XVII ectodomain, was described previously [4,9]. pCEP4-sec180 was used as the template for PCR amplification of the segments of collagen XVII represented in Figure 1.

For use in the refolding experiments, a collagen model protein, consisting of 10 GPP triplets (GPP<sub>10</sub>), with an N-terminal NC16A moiety, was produced as a GST fusion protein using the pGEX-2T bacterial expression system (Pharmacia Biotech, Piscataway, NJ).

### 2.2. Peptide synthesis

A GPP<sub>10</sub> synthetic peptide (sGPP<sub>10</sub>) was synthesized using Fmoc chemistry on an Applied Biosystems 432A Synergy peptide synthesizer (Applied Biosystems, Foster City, CA). Peptide purity was confirmed by MALDI-TOF (matrix-assisted laser desorption time-of-flight) mass spectrometry.

### 2.3. Immunodetection

Three rabbit antisera reacting with distinct epitopes on the extracellular region of collagen XVII were used in this study. Rabbit sera R594 and R136 react with sites in NC16A and near the C-terminus of collagen XVII, as previously described [9]. Rabbit antiserum, R5151, a generous gift from Dr. Kim Yancey, recognizes sites within the C-terminal two-thirds of the collagen XVII ectodomain.

Immunoblotting was carried out as previously described [9]. When the His-tag was needed as a means of detection, a mouse anti-penta-His antibody (Qiagen, Valencia, CA) was used.

### 2.4. Concentration by immunoprecipitation or by nickel-NTA affinity-purification

Immunoprecipitation was performed as described previously [9] to detect small amounts of the metabolically radiolabeled recombinant proteins in column fractions. Alternatively, when the His-tag was needed as a means of concentration, the samples were incubated for 2 h with Ni-NTA agarose beads (Qiagen, Valencia, CA).

## 2.5. Gel filtration chromatography

The recombinant forms of collagen XVII were analyzed by gel filtration chromatography using a Superose-6 column (Amersham, Piscataway, NJ), as previously reported [9].

## 2.6. Calculations

The frictional ratio ( $f/f_0$ ) was obtained using the following equations [4]:

$$M_r = 6\pi N \eta r_s^2 s_{20,\omega} / (1 - \bar{v}\rho) \quad (1)$$

$$f / f_0 = r_s (4\pi N / 3M_r \bar{v})^{1/3} \quad (2)$$

where  $N$  is Avogadro's number ( $6.022 \times 10^{23} \text{ mol}^{-1}$ ),  $\eta$  is the viscosity of water at  $20^\circ\text{C}$  ( $1 \times 10^{-3} \text{ kg m}^{-1} \text{ s}^{-1}$ ),  $\bar{v}$  is the partial specific volume,  $\rho$  is the density of water at  $20^\circ\text{C}$  ( $0.998 \text{ g/mL}$ ),  $r_s$  is the Stoke's radius, and  $s_{20,\omega}$  is the sedimentation coefficient. The axial ratio,  $P$ , was calculated from the frictional ratio by fitting the protein as a cylinder using the equation:

$$f / f_0 = (2/3)^{1/3} [P^{2/3} / (-0.3 + \ln(2P))] \quad (3)$$

## 2.7. Glycerol gradient sedimentation

The sedimentation properties of the collagen XVII recombinant proteins were analyzed by glycerol gradient sedimentation (linear gradient, 10% to 30% v/v), as previously described [4].

## 2.8. Chemical cross-linking

The various truncated forms of collagen XVII were subjected to chemical cross-link analysis using DSS (disuccinimidyl suberate; Pierce, Rockford, IL), as previously described [9].

## 2.9. Sensitivity to proteolysis

The thermal stability of the recombinant proteins was determined as previously described [9]. When the affinity purification was done by Ni-NTA purification, the reaction was performed on the 0.5 M imidazole eluate. The results were quantified by densitometry and the data points were fitted to sigmoidal curves using Sigma Plot (Jandel Scientific). The melting temperature, i.e., the temperature at which 50% of the proteins are unfolded, was determined based on the  $x_0$  value from the resulting sigmoidal equation.

The rate of triple helix assembly of NC16A-GPP<sub>10</sub> was calculated by monitoring the recovery of resistance to trypsin digestion. After denaturation at  $70^\circ\text{C}$  for 20 min, 10  $\mu\text{l}$  aliquots were incubated at  $4^\circ\text{C}$  for varying lengths of time (from 5 min to 8 h). At the end of each incubation, duplicate samples received 10  $\mu\text{l}$  of trypsin (100  $\mu\text{g/mL}$ ; Sigma, St. Louis, MO) and were incubated at  $4^\circ\text{C}$  for 5 min. The reaction was stopped by adding 10  $\mu\text{l}$  of soybean trypsin inhibitor (500  $\mu\text{g/mL}$ ; Sigma, St. Louis, MO). After incubation, the samples were analyzed by SDS-PAGE and immunoblotting. The kinetic data were fitted to exponential curves using Sigma Plot (Jandel Scientific) software package. The initial rate of assembly is the calculated slope of the tangent to the curve at time zero.

## 2.10. Circular dichroism

To monitor the rate of triple helix assembly, the synthetic GPP<sub>10</sub> (sGPP<sub>10</sub>) was denatured by heating for 20 min at  $70^\circ\text{C}$ , and then quickly loaded into a thermostatted 1 mm path-length quartz cell at  $4^\circ\text{C}$  [10,11]. CD spectra of sGPP<sub>10</sub> were acquired on a Jasco J715 spectropolarimeter. Kinetics of refolding were determined by monitoring the temporal change

in ellipticity at a fixed wavelength of 228 nm. The ellipticity measurement was converted into the fraction of protein in the folded state following the equation:

$$FF = (\theta_{\text{obs}} - \theta_{\text{min}}) / (\theta_{\text{max}} - \theta_{\text{min}}),$$

where  $\theta_{\text{obs}}$  is the observed ellipticity and  $\theta_{\text{max}}$  and  $\theta_{\text{min}}$  are the ellipticities of the folded and unfolded proteins, respectively, acquired from melting curves. The initial rate of assembly was calculated as described above.

**Note about the refolding experiments**—Due to technical considerations, different methods were employed to monitor the refolding of sGPP<sub>10</sub> and NC16A-GPP<sub>10</sub>. There are two well-characterized methods for monitoring the assembly of collagens – CD spectropolarimetry and measuring resistance to proteolysis. While CD was ideal for following the folding of sGPP<sub>10</sub>, it was unsuitable for analyzing NC16A-GPP<sub>10</sub> because the collagen-specific portion of this peptide’s CD spectrum was obliterated by its non-collagenous moiety. The refolding of NC16A-GPP<sub>10</sub> was readily monitored using the trypsinization approach; however, this method could not be used for analyzing sGPP<sub>10</sub>, since this peptide is not trypsin sensitive (no Arg or Lys) and no antibodies are available for its detection in a Western blot.

### 3. Results

#### 3.1. Analysis of the conformation of the recombinant collagen XVII fragments

A series of recombinant forms of collagen XVII (shown schematically in Fig. 1) were expressed in the human kidney cell line, 293-EBNA (see Figure S1). To facilitate detection and purification, one protein, trunc- $\Delta$ N16-cHis, was engineered with a C-terminal His-tag. The protein partner for trunc- $\Delta$ N16-cHis, sec180-trunc, was prepared both with and without a C-terminal His-tag.

Chemical cross-link analysis (Figure 2) revealed homo-trimeric conformations for sec180 (panel A, lanes 1–3), sec180- $\Delta$ N16 (panel A, lanes 4–6) and sec180-trunc (panel B, lanes 1–3), as well as for sec180-trunc-cHis (data not shown), indicating that the presence of the His-tag did not affect the ability of the protein to trimerize. Significantly, the truncated protein lacking NC16A, trunc- $\Delta$ N16-cHis, assayed at the same subunit concentration as those used for the other four proteins, exhibited no cross-linked products (Fig. 2B, lanes 4–6). However, at a 35-fold higher subunit concentration, the homo-dimeric and homo-trimeric forms of trunc- $\Delta$ N16-cHis were observed (Fig. 2B, lanes 7–9), albeit with a low trimer-to-monomer ratio.

Gel filtration analysis of the collagen XVII recombinant proteins provided additional insight into the multimeric nature of these proteins. As examples, the chromatographic profiles of sec180 and sec180-trunc are shown in Figure S2. For each of these two recombinant proteins, two distinct peaks were identified -- the first one, with the higher Stoke’s radius, corresponds to the trimeric form and the second one to the monomeric form of the protein. The gel filtration results for all of the recombinant proteins are shown in Table I. Interestingly, trunc- $\Delta$ N16-cHis exhibited only a monomeric peak, presumably due to its very low trimer-to-monomer ratio, as shown by cross-linking.

The axial ratio  $P$ , the ratio of a protein’s length to width, can be deduced from the observed Stoke’s radius (Fig. S2 and Table I), sedimentation coefficient (data summarized in Table I) and molecular mass (see equations in Materials and Methods). The molecular mass of each trimer was calculated from the known amino acid sequence: sec180, 302.4 kDa; sec180- N16, 275.4 kDa; and sec180-trunc, 102.1 kDa. Using equation 1 and 2 to obtain the frictional ratio for each protein, and modeling them as cylinders (equation 3), the axial ratios of sec180,

sec180-N16 and sec180-trunc are 63, 81 and 79, respectively (Table I). For comparison, collagen I has an axial ratio of 200 with a subunit length of about 1000 amino acids [12].

### 3.2. Thermal stability of the recombinant collagen XVII fragments

The denaturation of the triple helical regions of the various collagen XVII recombinant proteins was measured at various temperatures by monitoring the degradation of the protein by trypsin. Representative results are shown in Figure 3A, and a summary of the thermal denaturation results is presented in Figure 3B. Sec180-trunc and sec180-trunc-cHis had very similar  $T_m$ 's of 42 and 43 °C respectively; however, their counterpart lacking NC16A, trunc- $\Delta$ N16-cHis, had a significantly lower  $T_m$  (36 °C;  $p = 0.022$ ), and its melting curve showed a more gradual transitional phase that extended from 25 °C to about 45 °C. The  $T_m$ 's of sec180 (46 °C) and sec180- $\Delta$ N16 (41 °C) were also shown to be significantly different ( $p = 0.007$ ). When the two proteins lacking NC16A (sec180- $\Delta$ N16 and trunc- $\Delta$ N16-cHis) were compared, the longer of the two, sec180- $\Delta$ N16, was shown to have a significantly higher  $T_m$  ( $p = 0.037$ ).

For each recombinant protein, we measured the percent of protein that is resistant to trypsin digestion at 4 °C. This provides information about the relative amounts of properly folded protein at steady state. As shown in Figure 3C, sec180-trunc-cHis exhibited a higher fractional level of trypsin-resistant material when compared with its counterpart that lacks the NC16A domain, trunc- $\Delta$ N16-cHis ( $p = 0.021$ ).

### 3.3. Refolding of model proteins

NC16A-GPP<sub>10</sub> and the sGPP<sub>10</sub> were used in a series of experiments to compare their initial rates of *in vitro* triple helix assembly either by quantifying the level of trypsin-resistant material for NC16A-GPP<sub>10</sub> or by following the ellipticity by circular dichroism at 228 nm for sGPP<sub>10</sub>. Figure 4A shows the refolding results obtained for NC16A-GPP<sub>10</sub> at 40  $\mu$ M and sGPP<sub>10</sub> at 200  $\mu$ M. Technical problems prevented us from measuring refolding of the two proteins at the same concentration. Forty  $\mu$ M was the maximum concentration that we were able to achieve for NC16A-GPP<sub>10</sub>, and refolding of sGPP<sub>10</sub> at 40  $\mu$ M was too slow to obtain an accurate initial rate. To circumvent this problem, we determined the initial rate of refolding of sGPP<sub>10</sub> at 40  $\mu$ M by extrapolation using a linear regression of the logarithm of the initial refolding rate of sGPP<sub>10</sub> versus the logarithm of the peptide concentration (Figure 4B). Based on this analysis, the rates of refolding of sGPP<sub>10</sub> and NC16A-GPP<sub>10</sub> (both at 40  $\mu$ M) were 2.3  $\mu$ M/hour and 61.7  $\mu$ M/hour, respectively, a 27-fold difference. The initial rate of sGPP<sub>10</sub> at 40  $\mu$ M obtained by extrapolation is based on the assumption that the reaction order does not change over the concentration range of 40–200  $\mu$ M. This is a reasonable assumption since Boudko *et al.* showed that this was the case for a GPP<sub>10</sub> peptide [10]. In addition, any change in reaction order that were to occur over this concentration range would result in a reaction rate lower than that obtained by the linear extrapolation and would increase the difference in initial rates exhibited by NC16A-GPP<sub>10</sub> and sGPP<sub>10</sub>.

## 4. Discussion

This study was designed to test the hypothesis that the NC16A domain of collagen XVII contains elements that influence the stability and rate of assembly of the adjacent triple helical domain. The approach involved structurally comparing, in a pair-wise fashion, recombinant forms of collagen XVII, with or without NC16A.

Our results demonstrated that the presence or absence of the NC16A domain had no major impact on the global conformation of the protein. The larger protein pair exhibited highly similar Stoke's radii, axial ratios and cross-linking results, consistent with a semi-rigid rod-like structure. A complete structural comparison of the shorter collagen XVII protein pair was

not possible, since the truncated protein without NC16A was expressed largely as a monomer. While the trimeric form of this protein was detectable by cross-linking only at high subunit concentrations, and was undetectable by gel filtration, it did yield a typical collagen spectrum by circular dichroism (data not shown). The dramatic differences in the trimer-to-monomer ratios for the two truncated proteins, revealed in both the protein structure and thermal denaturation analyses, indicated that the presence of NC16A leads to an increase in the steady state level of the correctly assembled protein form. This phenomenon is likely due to a difference in protein stability, although a difference in the rate of protein folding could also be a contributing factor.

The thermal denaturation experiments revealed that all four proteins were expressed in two distinguishable forms – one that is highly trypsin resistant (presumed to be folded correctly) and the other that is sensitive to trypsinization at 4 °C (presumed to be misfolded). For both pairs of collagen XVII proteins, the presence of NC16A resulted in a statistically significant increase in collagen triple helix stability (quantified by their melting temperatures), though this difference was more pronounced in the shorter protein pair. It is noteworthy that the denaturation of the truncated protein lacking NC16A occurred over a broad temperature range, suggestive of structural heterogeneity. This means that some of the trimers that do assemble might well be misaligned. These findings are consistent with previous data [8] and support the hypothesis that the NC16A domain facilitates chain association, forming a nucleus for triple helix assembly [10].

One key point from these studies is that two recombinant forms of collagen XVII – one missing the N-terminal NC16A and the other missing the C-terminal portion – are capable of folding into a stable conformation that resembles the native structure. Therefore, neither end of the ectodomain contains a site that is essential for proper folding. It must be stressed, however, that this does not exclude the terminal regions as potential sites of nucleation. Rather, the proper folding of these recombinant proteins is likely due to self-nucleation occurring at sites with sufficient numbers of GXP repeats, as shown for synthetic collagen peptides [11].

To address the question of whether NC16A contains a nucleation site, defined as a site that accelerates the folding of a protein, we measured the rate of triple helix assembly for the two collagen model peptides, sGPP<sub>10</sub> and NC16A-GPP<sub>10</sub>. Our analysis revealed that the NC16A-containing peptide refolded at a rate 27 times higher than that of its counterpart, sGPP<sub>10</sub>. Based on these data, we can conclude that NC16A has a positive effect on one, or both, of the processes associated with triple helix assembly, i.e., subunit association and helix propagation [10]. At the low subunit concentrations used in this study it is very likely that the rate limiting step was subunit association and not helix propagation [10]. To distinguish between these possibilities an in-depth kinetic analysis of the effects of NC16A on triple helix formation will be carried out.

In addition, our structural studies revealed important clues about the overall shape of collagen XVII. For example, the N-terminal one-third of the collagen XVII ectodomain (consisting of NC16A, col-15 and NC15) accounted for 76% of the Stoke's radius of the entire ectodomain (sec180) and exhibited an axial ratio very similar to that of a collagen triple helix of comparable size. In contrast, while sec180 and collagen I are very similar in terms of amino acid count, the axial ratio of sec180 is significantly lower. These findings strongly support a model in which the N-terminal one-third of the collagen XVII ectodomain is highly extended and rigid, while the remainder is highly flexible and might have a stable conformation in which this tail is folded back on itself (see Fig. S3). Such a model has previously been proposed based on ultrastructural data [3,13].



In conclusion, we have presented a systematic analysis of the structure of the membrane-proximal portion of the collagen XVII ectodomain. We have shown that the presence of the NC16A domain of collagen XVII confers a statistically significant increase in both the stability and the rate of assembly of the adjacent collagen triple helix.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## References

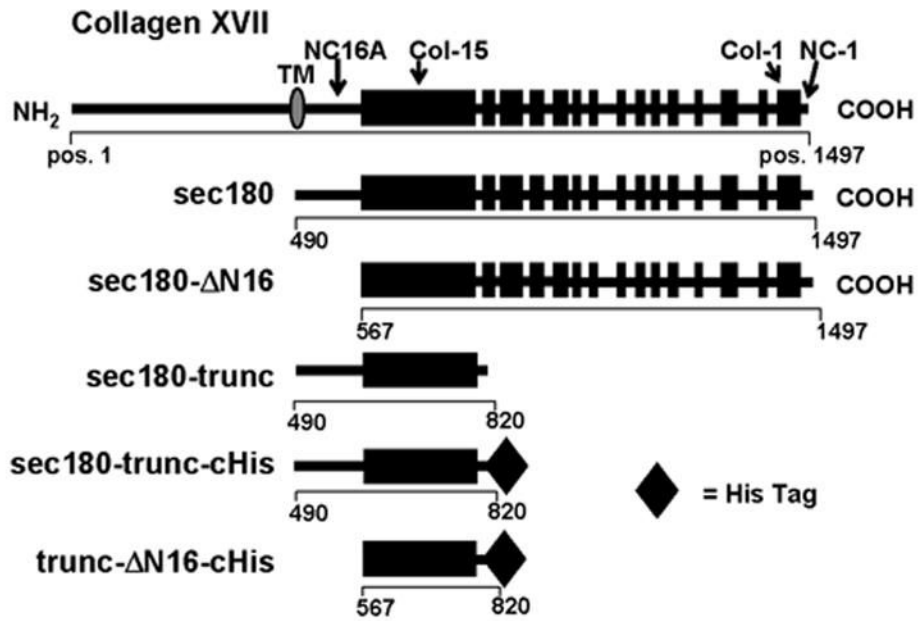
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## The abbreviations used are

- AA**  
amino acid
- CD**

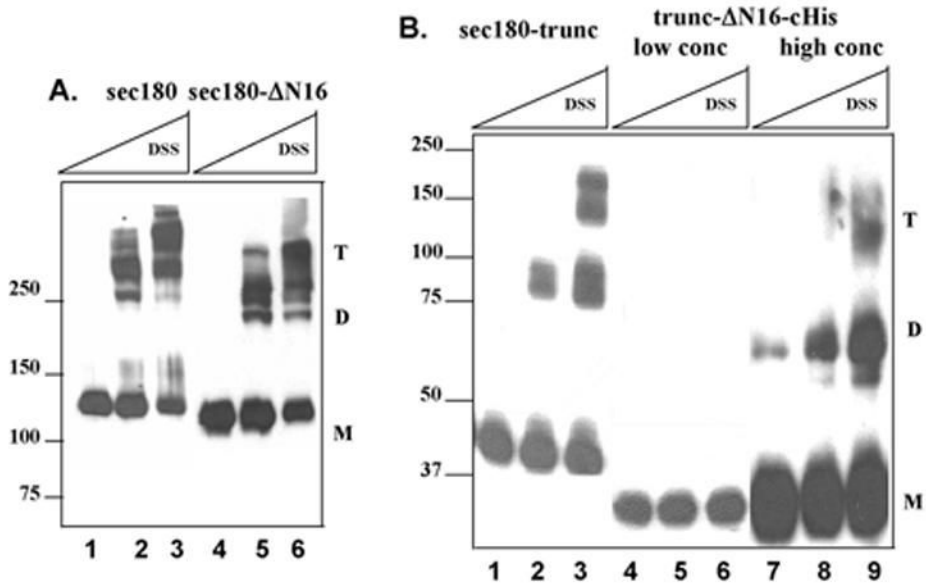
	circular dichroism
<b>Col</b>	collagen
<b>NC</b>	non-collagen
<b>DMSO</b>	dimethyl sulfoxide
<b>DSS</b>	disuccinimidyl suberate
<b>ECL</b>	enhanced chemiluminescence
<b>HRP</b>	horseradish peroxidase
<b>TM</b>	melting temperature





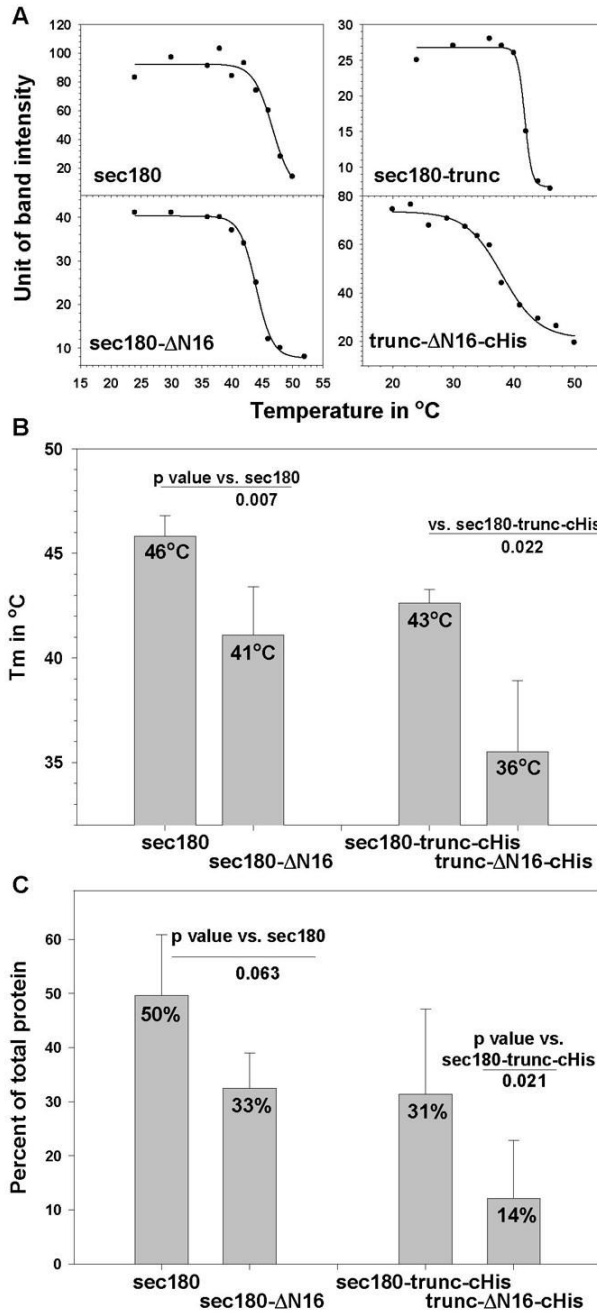
**Fig. 1. Schematic representation of recombinant proteins of collagen XVII**

The extracellular domain of type XVII collagen (top diagram) consists of 15 collagenous domains (solid boxes) and 16 non-collagenous domains (horizontal lines). Also shown are schematic representations of the recombinant proteins used in this study. The corresponding AA positions are indicated for the termini of each protein (from GenBank accession NM\_130778). The lengths (in AA) of sec180, sec180-ΔN16, sec180-trunc and trunc-ΔN16 are 1008, 931, 331 and 254, respectively. The His-tag, when present, adds 13 AA to the length of the protein. TM, transmembrane domain; pos, position.



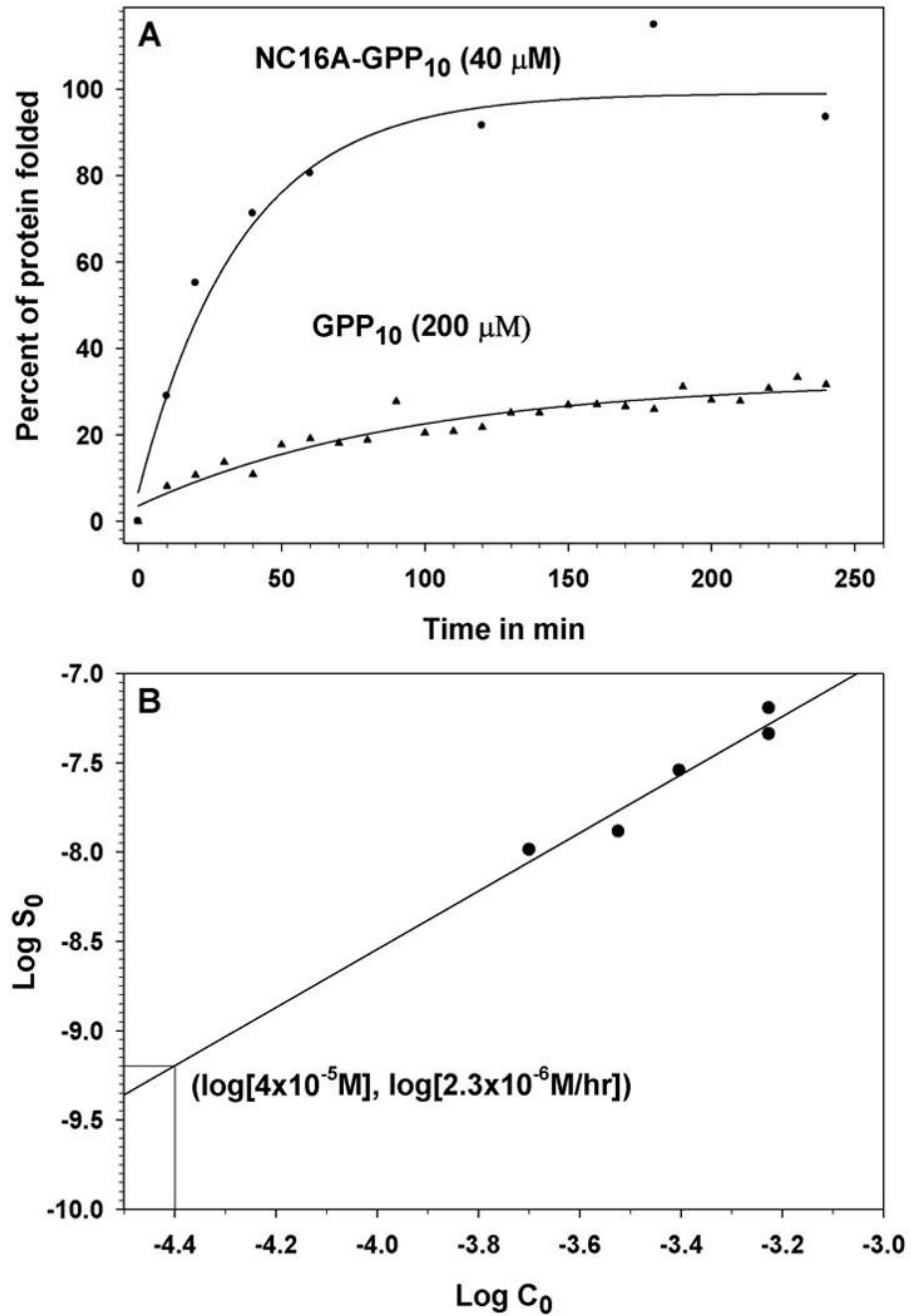
**Fig. 2. Cross-link analysis of collagen XVII recombinant proteins**

The collagen XVII truncation mutants were subjected to chemical cross-linking using disuccinimidyl suberate (DSS). *Panel A*, This figure is a Western blot of sec180 and sec180-ΔN16 treated with no DSS (lanes 1 and 4) or with DSS at 0.1 mM (lanes 2 and 5) or 0.5 mM (lanes 3 and 6). *Panel B*, This is a Western blot of sec180-trunc and trunc-ΔN16-cHis at low and high concentrations treated with no DSS (lanes 1, 4 and 7) or with DSS at 0.1 mM (lanes 2, 5 and 8) or 0.5 mM (lanes 3, 6 and 9). In both panels A & B, the positions of the monomer, dimer and trimer forms are indicated by "M", "D", and "T", respectively. The positions of the molecular weight markers are shown to the left of the blots.



**Fig. 3. Thermal denaturation analysis of collagen XVII recombinant proteins**

*Panel A*, This panel shows representative thermal denaturation curves that were generated by monitoring trypsin resistance. *Panel B*, The bar graph displays the mean melting temperature ( $T_m$ ; 4–6 experiments) and standard deviation obtained for each protein. *Panel C*, Each recombinant protein was incubated with or without trypsin at 4 °C. Shown in the bar graph are the mean percentages of protein resistant to trypsin (4–8 digestions) and the corresponding standard deviations.



**Fig. 4. Refolding of model proteins**

*Panel A*, This plot shows refolding curves for the model protein NC16A-GPP<sub>10</sub> (closed circles) and the synthetic collagen peptide sGPP<sub>10</sub> (closed triangles). *Panel B*, This plot shows the logarithm of initial rate (in M/sec) of triple helix formation of sGPP<sub>10</sub> as a function of the logarithm of total chain concentration (in M).

**Table 1**  
Structural parameters of collagen XVII recombinant proteins

Recombinant proteins	Average Stoke's radius (nm)		sedimentation coefficient	Axial ratio (P)
	Trimer	Monomer		
sec180	13.6 +/- 0.4	9.1 +/- 0.6	5.3 +/- 0.2	63
sec180- N16	14.3	9.0	5.5 +/- 0.4	81
sec180-trunc	10.3 +/- 0.07	5.1 +/- 0.4	2.6	79
sec180-trunc-cHis	11.2	5.3		
trunc- N16-cHis	undetectable	4.5 +/- 0.3		

The Stoke's radius of each protein was determined from gel filtration chromatographic data (see Figure 4), and the sedimentation coefficient was obtained from glycerol gradient sedimentation profiles. The axial ratio was calculated as described in the text. Each result represents the average of 2 to 5 runs. The standard deviation is given only when at least 3 runs were performed.