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Endoplasmic Reticulum Protein Hsp47 Binds Specifically to the N-Terminal Globular Domain of the Amino-Propeptide of the Procollagen I α1(I)-Chain

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Abstract Hsp47, an endoplasmic reticulum-resident heat shock protein in fibroblasts, has gelatin-binding properties. It had been hypothesized that it functions as a chaperone regulating procollagen chain folding and/or assembly, but the mechanism of the hsp47-procollagen I interaction was not clear. Hsp47 could bind to both denatured and native procollagen I. A series of competition studies were carried out in which various collagens and collagen domain peptides were incubated with ^{35}[S]-methionine-labeled murine 3T6 cell lysates prior to mixing with gelatin-*Sepharose 4B beads*. The gelatin-bound proteins were collected and analyzed by gel electrophoresis and autoradiography. Collagenase digested procollagen I had the same effect as denatured intact procollagen, indicating that the propeptides were the major interaction sites. The addition of intact pro α1(I)-N-propeptide at 25 μg/ml completely inhibited hsp47 binding to the gelatin-Sepharose. Even the pentapeptide VPTDE, residues 86–90 of the pro α1(I)-N-propeptide, inhibits hsp47-gelatin binding. These data implicating the pro α1(I)-N-propeptide domain were confirmed by examination of polysome-associated pro α chains. The nascent pro α1(I)-chains with intact N-propeptide regions could be precipitated by monoclonal hsp47 antibody 11D10, but could not be precipitated by monoclonal anti-pro α1(I)-N-propeptide antibody SP1.D8 unless dissociated from the hsp47. GST-fusion protein constructs of residues 23-108 (NP1), 23-151 (NP2), and 23-178 (NP3) within the pro α1(I)-N-propeptide were coupled to Sepharose 4B and used as affinity beads for collection of hsp47 from 3T6 cell lysates. NP1 and NP2 both showed strong specific binding for lysate hsp47. Finally, the interaction was studied in membrane-free in vitro cotranslation systems in which the complete pro α1(I)- and pro α2(I)-chain RNAs were translated alone and in mixtures with each other and with hsp47 RNA. There was no interaction evident between pro α2(I)-chains and hsp47, whereas there was strong interaction between pro α1(I)-chains and nascent hsp47. SP1.D8 could not precipitate pro α1(I)-chains from the translation mix if nascent hsp47 was present. These data all suggest that if hsp47 has a "chaperone" role during procollagen chain processing and folding it performs this specific role via its preferential interaction with the pro α1(I) chain, and the pro α1(I) amino-propeptide region in particular.

Key words: Hsp47, endoplasmic reticulum protein, procollagen I, pro α1(I)-N-propeptide, heat shock protein

The individual pro α1(I) and pro α2(I) chains of the type I procollagen heterotrimer are translated from endoplasmic reticulum (ER) membrane-bound polyribosomes and inserted cotranslationally into the lumen of the ER. Each chain contains three conformationally distinct sequence domains: an amino-terminal N-propeptide, the central G-X-Y repeat domain, and the carboxyl-terminal C-propeptide. It has been demonstrated convincingly [Bachinger et al., 1980, 1981; Bruckner et al., 1981] that triple helix formation is initiated by interactions between pro α chain C-propeptide domains and proceeds in the carboxyl-terminal to amino-terminal direction. The heterotrimer [pro α 1(I)]$_2$[pro α2(I)] is the predominant product but stable, extracellular homotrimeric molecules, [pro α1(I)]$_3$, can also form. On the other hand, stable secreted [pro α2(I)]$_3$ homotrimers have never been detected.

In addition to the basic problem of how the pro α1(I) and pro α2(I) ribosomal-mRNA translation assemblies are directed to the same regions of the ER [Hu et al., 1995], the process of heterotrimer assembly raises several mechanistic questions: How are the chains selected for

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assembly? At what point do the independently synthesized chains register? How is the formation of [pro $\alpha 1(I)]_3$ homotrimer suppressed? What prevents formation of mismatched or otherwise improperly folded helices? The objective of the present study was to examine factors relating to chain selection and intracellular formation of the heterotrimer.

Veis and Kirk [1989] proposed that the procollagen I pro α-chains were coordinately synthesized but associated or registered cotranslationally only in the late stages of chain elongation, via the formation of interchain disulfide bonds between the C-terminal propeptide domains [Veis and Kirk, 1989; Kirk et al., 1987] prior to helix propagation. Both the selection of the appropriate set of chains and their folding to stable triple-helical molecules must be closely regulated processes so that the heterotrimer, rather than the homotrimer, is favored. These selection and assembly problems are not unique to collagen. The folding and assembly of a number of secretory heteromultimeric proteins within the ER are closely regulated.

It has been postulated that ER-resident proteins, such as BiP (grp78) [Bole et al., 1986], might actively participate in regulating the folding process so that malfolding and premature triple-helix formation are prevented [Pelham, 1989]. These ER-resident proteins constitute a subset of the larger group of proteins known as chaperones. They only transiently associate with the target molecule, and do not bind to the assembled/folded final product. Their release is accompanied by a target molecule conformational change, assisted by ATP/GTP hydrolysis or a pH change within the local compartment [Landry and Gierasch, 1994].

A putative collagen-specific chaperone protein, heat shock inducible glycoprotein hsp47, has been identified [Nagata et al., 1986], and found to colocalize with type I procollagen within the ER in BALB/3T3 cells [Nakai et al., 1990]. It was abundant within the ER, and immunostaining showed it to be concentrated on the cisternal side of the ER membrane in chick embryo fibroblasts [Saga et al., 1987]. The ability of hsp47 to reversibly bind to denatured collagen and gelatin-Sepharose suggested to Nakai et al. [1990] that it had a role in the processing and ER transport of procollagen, that is, a chaperone-like function. Nakai et al. [1992] demonstrated that anti-hsp47 coprecipitated procollagen and hsp47 from intact cells, and pulse-label experiments (±α,α′-dipyridyl to inhibit triple-helix formation) showed that the coprecipitated procollagen was detectable as long as the procollagen was present in the ER compartment. They postulated a role for hsp47 in intracellular processing of procollagen but the locus of procollagen association with hsp47 was not elucidated. Nandan et al. [1988] also demonstrated that hsp47, or its analogs, colligin and gp46, were localized to the ER. Sauk et al. [1994] and Ferreira et al. [1994] have also implicated hsp47 in the ER related processing of type I collagen.

The gelatin-binding properties of hsp47—the basis for its discovery [Nagata et al., 1986]—make it likely that the Gly-X-Y repeats within the potential helical domain of the individual pro α chains are binding sites. This would be concordant with a chaperone function but inhibition of pro α chain folding may not be it's main role. Paradoxically, hsp47 also has the capacity to bind to fully processed, triple-helical, native type I collagen [Nagata and Yamada, 1986]. This observation has led to the proposal that hsp47 might be a molecular chaperone, active during the movement of the procollagen through the ER compartment [Sauk et al., 1994]. Nakai et al. [1992] have also shown that other ER-resident proteins may join in the complexation with hsp47.

We reasoned that if hsp47 serves as a collagen chaperone, it should be directly associated with nascent polysome-associated pro α chains in the process of elongation. As shown below, this is the case. However, we have also found that in addition to its generalized binding to the G-X-Y triple helical domain (native or denatured), hsp47 interacts specifically with the pro α1(I) N-propeptide. Three approaches have been taken in exploration of this interaction. First, the interaction between hsp47 and polysome associated pro α chains from murine 3T6 cells has been examined. Second, constructs of specific domains of the collagen pro α chains have been used in collagen-binding competition experiments with hsp47. Finally, the interaction has been studied in membrane-free in vitro cotranslation systems. These data all suggest that while several ER-resident proteins might function as "accessory proteins" or chaperones during procollagen chain processing and folding, hsp47 may have a more specific role via its preferential interaction with the pro α1(I) chain, and the pro α(I) amino-propeptide region in particular.

EXPERIMENTAL PROCEDURES

Polysomes From 3T6 Cells

Cell culture. American Type Tissue Collection Swiss mouse 3T6 cells were grown to confluence in DMEM, containing 10 mM glutamine, 10% FBS, and 10 μg/ml ascorbate, plus 1,000 u/ml penicillin, 1 mg/ml streptomycin, and 0.25 μM fungizone at 37°C in 150 cm^2 flasks. Where labeled collagen was required, the media was removed and fresh DMEM containing 2 μCi/ml [^{14}C]-proline (Amersham, Arlington Heights, IL, sp. act. > 250 mCi/mmol) was added to the cells for 30 min at 37°C. One group of cells was heat shocked at 42°C for 4 h, and then labeled for 30 min at 42°C to verify that heat shock increased levels of hsp47, but all other experiments were carried out with nonstressed cells at presumed normal, constituitive hsp47 levels.

Polysome and nascent procollagen chain isolation. Protein synthesis was blocked by the addition of 100 μg/ml cycloheximide for 10 min. The media was removed and lysis buffer (LB; 4 ml/flask, 0.2% Triton-X, 0.05% Na^+ deoxycholate, 10 mM HEPES pH 7.5, 3 mM $MgCl_2$, 10 mM KCl, 0.25 M sucrose, 2 mM dithiothreitol, 0.2 mg/ml heparin, 100 μg/ml cycloheximide, 10 mM PMSF) was added at 4°C. The lysed cells were centrifuged at 12,000 rpm to remove nuclei and cell debris, then the polysomes were pelleted from the first supernatant by centrifugation at 27,000 rpm for 80 min in a *Beckman SW* 27 rotor. In some cases the polysomes were suspended in Q-Sepharose buffer and applied to a 2 ml Q-Sepharose Fast Flow column (Pharmacia, Piscataway, NJ) as described by Bergman and Kuehl [1977] and modified by Kirk et al. [1987] to separate tRNA bound (RET) and tRNA-free (FT) fractions containing nascent or polysome-associated fully elongated pro α chains, respectively.

Immunoprecipitations. Polysome pellets were resuspended in 100 μl NP40 buffer (1% Nonidet P40, 0.15 M NaCl, 50 mM Tris.HCl, pH 8.0, 2 mM N-ethylmaleimide, 2 mM PMSF, 5 mM EDTA, 2 μg/ml pepstatin). Immunoprecipitation of hsp47-associated proteins with monoclonal anti-hsp47 11D10 [Saga et al., 1987] was by the method of Nakai et al. [1990]. Immunoprecipitation with polyclonal anti-hsp47 was performed by the method of Saga et al. [1987] in PBS with 2% Triton X-100 (Pierce). Immunoprecipitation of collagen used the anti-pro α1(I) N-propeptide antibody SP1.D8 [Foellmer et al., 1983]. SP1.D8 was added at 0.1 μg/ml and incubated 24 h at 4°C. Protein G beads (Pharmacia) were added (100 μl) and incubation continued for 2 h at 4°C with end-over-end rotation. Beads were washed twice in PBS.

The term *immunoprecipitate,* used frequently in the presentation of results and discussions, may be misleading. In every case the final step in collection of the immune complex was interaction with protein G-beads followed by low speed centrifugation. The bead pellet is denoted as the "immunoprecipitate," but depending upon the circumstances, the beads contained varying amounts of the primary antigen. Thus, the question posed in most experiments was how much of a particular antigen the "immunoprecipitate" might contain. It was entirely possible that an "immunoprecipitate" of the protein G beads might not contain any antigen.

Collagenase digestion. Digestions were carried out in collagenase buffer (0.1 M Tris, pH 7.5, 0.4 M NaCl, 5 mM $CaCl_2$, 30 mM KCl, 10 mM PMSF, 0.5 mM N-ethylmaleimide, 2.5 mM benzamidine HCl) plus 27 units of Advanced Biofactures, Lynbrook, NY, Form III bacterial collagenase. Mixtures were incubated at 37°C for 1 h. Blank digestions in buffer alone were always run in parallel. The amount of bacterial collagenase used in these experiments was selected by titration of the digestion of the secreted procollagen I standards. It is possible that nascent collagen/hsp47 complexes could have been partially resistant to digestion, so that the digestion data represent minimal rather than maximal collagen content.

Gel electrophoresis. Samples for electrophoresis were suspended in Laemmli SDS-PAGE sample buffer and boiled for 5 min before loading onto gradient or uniform concentration gels. After electrophoresis the gels were either directly stained, or prepared for fluorography or autoradiography, or the contents of the gels were transferred to nitrocellulose. Blots were immunostained, as indicated in the figure legends, and/or exposed to Kodak (Rochester, NY) XAR film for up to 6 weeks.

Gelatin Binding Competition Experiments

Preparation of cell lysates. Human gingival fibroblasts (primary cultures) or the ATTC Swiss mouse 3T6 cells were cultured to confluence and then labeled for 24 h at 37°C with 0.5 μCi/ml [^{35}S]-methionine (Amersham, Sp. Act. > 500 mCi/mmol) in methionine-free DMEM

(Gibco, Grand Island, NY). The cells were used fresh or were aliquotted and stored at −70°C. The cells, fresh or thawed, were lysed in lysate buffer (LB; 1% Nonidet P40, 0.15 M NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 mM N-ethylmaleimide, and 2 mM PMSF), for 30 min on ice. The cell lysate was centrifuged at 15,000*g* for 20 min and the supernatant of the lysate was collected. The lysates were divided into aliquots and stored at −70°C.

Gelatin-Sepharose binding determinations. The [^{35}S]-methionine labeled cell lysate supernatant (250 μl) was mixed with 250 μl of a 50% (v/v) suspension of gelatin-Sepharose 4B (Pharmacia) in PBS and vortexed. These beads contain 1 to 2 mg gelatin/250 μl. After incubation at 4°C for 1 h the beads were collected by centrifugation and washed 3 times with an equal volume of LB. The washed gelatin-Sepharose beads were extracted with 250 μl Laemmli electrophoresis sample buffer by boiling the sample for 5 min at 100°C.

Direct competitors. Competition experiments were carried out using a variety of proteins and peptides: native rat type I procollagen (pNC), collected from 3T3 cell media by $(NH_4)_2SO_4$ precipitation; denatured pNC; bacterial collagenase digested pNC; synthetic peptides $(Pro\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}OHPro\text{-}Gly)_{10}$ (Peninsula Labs, Belmont, CA); 24 kDa bone peptide [phosphorylated bone α1(I) N-propeptide] [Fisher et al., 1987]; the SP1 peptide, Val-Pro-Thr-Asp-Glu, residues 85–89 of the pro α1(I) N-propeptide [Horlein et al., 1981]; NP1, the recombinant human pro α1(I) N-propeptide from residues 23–108 (from the signal peptide cleavage point to the junction between the most N-terminal portion and the propeptide G-X-Y sequence, including SP1); NP2, the recombinant human pro α1(I) N-propeptide from residues 23–151 (NP1 plus the propeptide G-X-Y domain); and, NP3, the recombinant human pro α1(I) N-propeptide from residues 23–170, including the N-telopeptide domain.

Each of the competitors was added to the [^{35}S]-Met labeled cell lysate to a final concentration of 1 μg/μl (or as otherwise specified) and incubated for 1 h at 4°C. The lysate (250 μl) was then added to 250 μl gelatin-Sepharose beads. Incubation at 4°C continued for an additional hour. The gelatin-Sepharose beads were collected by low speed centrifugation, washed twice with lysis buffer and then with 10 mM Tris-HCl, pH 6.8, buffer. The proteins bound to the beads were eluted by heating the beads to boiling in Laemmli-SDS-PAGE sample buffer for 5 min. Analysis was on 4–20% gradient SDS-PAGE. After electrophoresis the fixed gels were soaked in dimethyl sulfoxide overnight and dimethyl sulfoxide/2,5 diphenylpyrrazole for 3 h, rinsed with water, and dried. Fluorography followed the procedure of Bonner and Laskey [1974].

Preparation of GST-fusion proteins. cDNA sequences encoding the pro α1(I) N-propeptide globular domain (NP1) (residues 23–108), globular domain + propeptide G-X-Y domain (NP2) (residues 23–151), and the globular domain + G-X-Y domain + telopeptide (NP3) (residues 23–170) were prepared as GST-fusion proteins. Four oligonucleotides were designed for PCR:

1. Oligo F: TATCAAGGATCCCAAGAG-GAAGGCCAAGTC
2. Oligo R1: AATCTAGTCGACGGTTG-GTTTCTTGGTCGTT
3. Oligo R2: TATCATAGTCGACGGGAG-CAAAGTTTCCTC
4. Oligo R3: ACTCACAGTCGACAGGCACG-GAAATTCCTC.

The underlined sequence in oligo F denotes a BamH I site. Those in oligos R1–3 are Sal I sites. The pairs F-R1, F-R2, and F-R3 yielded NP1, NP2, and NP3, respectively. PCR was carried out for 30 cycles using pHHα1 [Hu et al., in press, 1995] as a template: 94°C, 1 min; 60°C, 1 min 20 s; 72°C, 1 min 40 s. The purified PCR products were digested by BamH I and Sal I and ligated in frame into the BamH I and Sal I sites of pGEX 4T-3 prokaryotic expression vector (Pharmacia). Recombinant plasmids were transformed into competent JM109 cells. The sequences at the GST-polypeptides insert junctions were verified by DNA sequencing (Sequenase Version 2.0, United States Biochemicals, Cleveland, OH).

Fusion protein expression was induced by 0.1 mM IPTG after the bacteria reached mid-log phase. The fusion proteins were purified from the bacterial sonicates by glutathione-Sepharose 4B beads (Pharmacia) according to the manufacturer's instructions. The purified fusion proteins were analyzed by SDS-PAGE. The NP2 and NP3 fusion proteins could be detected by SP1.D8 in immunoblots (data not shown). The NP1, NP2, and NP3 could also be obtained as the free polypeptides by cleavage of the gluta-

thione-Sepharose-bound fusion proteins with thrombin.

Preparation of NP1, NP2, and NP3 affinity beads and direct lysate component binding. The GST-fusion proteins containing from 3.5 to 4.8 mg of the desired peptides were dissolved in 0.05 M Tris.HCl at pH 8.0 and reacted with 500 U of thrombin at room temperature for 24 h. The reaction products were dialyzed vs. 0.1 M acetic acid overnight, and then dialyzed for 4 h against 1 × PBS. The GST protein was removed from each reaction mixture by passing it over a column of glutathione-Sepharose. The eluates were collected and lyophilized and coupled to CNBr activated Sepharose according to manufacturers protocols. The final beads contained 1–2 mg peptide per 250 μl, approximately the same as the gelatin-Sepharose. The lysate binding experiments were carried out as described above.

Polysome-associated procollagen binding competition experiments. Polysomes were collected from 5 groups of 3T6 cells (4 × 150 cm^2 flasks per group), each of which was labeled with 0.5 μCi/ml of ^{35}S-methionine as above. The polysomes were then incubated with the appropriate competitor and then with 5 μl of anti-hsp47 monoclonal antibody 11D10 [Saga et al., 1987] and immunoprecipitated with protein G beads, as above. Both immunoprecipitates and supernatants were collected. Group 1 served as a control. Both precipitates and supernatants in group 2 were digested with 24 U of bacterial collagenase at 37°C for 90 min after adjusting $CaCl_2$ concentration to 5 mM. Groups 3 and 4 were immunoprecipitated in the presence of 50 μg and 100 μg of synthetic SP-1, respectively. The immunoprecipitates of group 5 were incubated with NP40 buffer, pH 5.0, after immunoprecipitation at pH 8.0. All groups of immunoprecipitates and supernatants were loaded onto 5–15% gradient gels and fluorographed as described above.

In Vitro Translation and Immunoprecipitations

Preparation of RNA transcripts for in vitro cell free translation. Pro α1 and pro α2 RNAs were prepared from recombinant plasmids pHHα1 and pHHα2 as described by Hu et al. [in press, 1995]. The plasmids were linearized by Nde I digestion before transcription. The J6 cDNA clone [Wang and Gudas, 1990], corresponding to chick hsp47, was a kind gift from Dr. Sho-Ya Wang at SUNY-Buffalo. The full-length J6 cDNA was inserted into the EcoRI site in Bluescript SK(−), (Stratogene, La Jolla, CA) where the sense strand is under the control of the phage T3 promoter. The recombinant pBS-J6 was linearized by Hind III digestion. J6 in vitro RNA was then transcribed from the T3 promoter by phage T3 RNA polymerase. In each case, the in vitro transcribed RNA was purified from the transcription mixture by DNase digestion, phenol/chloroform extraction, and ethanol precipitation. The purity and integrity of the RNAs was analyzed by UV absorbance and denaturing agarose gel electrophoresis.

Cell-free translation. Rabbit reticulocyte lysate was used for all translations. The optimal K^+ concentrations for the three RNAs were different. Concentrations between 100 and 120 mM K^+ were best for both pro α1(I) and pro α2(I) RNAs but for J6 the optimal concentration was 80 mM K^+. Mixtures of (J6 + pro α1(I)) or (J6 + pro α2(I)) were prepared by co-translations. When the co-translations of J6 and the pro α chain RNAs were carried out, the best results were achieved at 100–120 mM K^+, with J6 RNA translated only slightly less efficiently. Optimal concentrations were 1–1.5 μg/50 μl J6 RNA and 2–5 μg/50 μl procollagen RNAs. Translations were carried out in 50 or 100 μl volumes at 30°C. The translation products were labeled with ^{35}[S]-Met at 0.8–1.0 μCi/μl, and analyzed by 4–15% SDS-PAGE and fluorography. The translations could be carried out efficiently in the absence of microsomal membranes, without any cotranslational processing of the procollagen chains or the hsp47. Cotranslational processing was evident in all three proteins when canine pancreatic microsomes (Promega, Madison, WI) were added to the translation mixture. Experiments in the presence of the canine pancreatic microsomes were complicated by the apparent presence of endogenous hsp47 in the microsomes.

Immunoprecipitation of pro α1(I) and hsp47 from translation mixtures. The SP1.D8 was used as described above to immunoprecipitate the pro α1(I) chains bearing the intact amino-propeptide region. The anti-gp46 monoclonal antibody was a gift from Dr. B. Sanwal. This antibody was directed specifically against the N-terminal region of rat gp46 (B. Sanwal, personal communication). Immunoprecipitations with anti-gp46 were carried out in RIPA buffer as described [Sefton et al., 1978]. All cell-free translation mixtures to be immunoprecipitated were equilibrated, on ice, in 5 vol of

IP buffer for 30 min in the presence of protein G′ beads. The supernatants were then incubated with 1–2 μl antibody on ice for 1 h. Protein G′ beads (Sigma, St. Louis, MO) were added and the mixtures incubated at 4°C with stirring on a rotary wheel for 1 h. Finally, the protein G′ beads were collected and washed 4× with IP buffer. Proteins bound to the beads were finally released in SDS-PAGE sample buffer and analyzed by 4–15% or 10% SDS-PAGE and fluorography.

RESULTS

Immunoprecipitation of Collagen-Containing 3T6 Cell Polysomes With SP1.D8 and 11D10

The initial observations which guided the course of these experiments were essentially two negative results. Swiss mouse 3T6 fibroblasts were grown to confluence and then labeled with [^{14}C]-proline at either 37°C following normal culture, or after they had been heat-shocked by incubation at 42°C for 4 h prior to labeling. Protein synthesis was terminated by the addition of cycloheximide and the cells were lysed. The polysomes were collected following the procedures described above. Gel electrophoresis under reducing conditions, followed by autoradiography (Fig. 1A, lane 3) showed the presence of many prominently labeled, polysome-associated newly synthesized or nascent protein components. As seen in Figure 1A, lane 4, some prominent bands (arrows) were susceptible to bacterial collagenase digestion, but a surprising number of proline labeled components were not. Since our objective was to analyze the composition of the collagen-containing polysomes, and since earlier studies had shown that collagen-related polysomes could be immunoprecipitated from polysome mixtures [Kirk et al., 1987], we selected the anti-pro α1(I)-N-propeptide antibody, SP1.D8 [Foellmer et al., 1983; Horlein et al. 1981], to specifically collect those polysomes bearing nascent pro α1(I) chains. Although the antibody could readily detect pro α1(I) and pN α1(I) chains in Western blots of standard media procollagen (Fig. 1B, lane 2) the SP1.D8 did not immunoprecipitate the polysome associated collagen (Fig. 1B, lane 3). Virtually no radioactivity was associated with the SP1.D8 polysome immunoprecipitate fraction.

The monoclonal antibody 11D10, and a polyclonal antibody to hsp47, have been shown [Nakai et al., 1992] to immunoprecipitate pro α1(I) chains from chick fibroblasts. We therefore used 11D10 to immunoprecipitate the 3T6 cell polysomes. In this case a radioactively labeled precipitate was obtained (Fig. 2, lane 4) and the majority of the counts were collagenase digestible (Fig. 2, lane 5). Although Nakai et al. [1992] had indicated that only pro α1(I)-related chains were in 11D10 immunoprecipitates, it was not possible to determine if all the collagenase sensitive bands were of pro α1(I) origin in the present experiment. Nevertheless, these data led to the conclusion that the hsp47 association with the collagen blocked the interaction of the SP1.D8 with pro α1(I) chains. The controls in this experiment were revealing. Media procollagen, used as the molecular weight marker, was also immunoprecipitated to a small extent with 11D10. Approximately 6 to 10% of the counts were collected by the protein G′ beads in the presence of 11D10 (Figure 2, lane 2).

Kirk et al. [1987] had demonstrated that the polysome-associated collagen of chick tendon fibroblasts could be divided into two pools which could be separated chromatographically on Q-Sepharose following the procedure of Bergman and Kuehl [1977]. Fully elongated, but not completely posttranslationally modified pro α chains appeared in the column flowthrough (FT) fraction. Nascent tRNA-bound chains were retained until eluted with a high salt buffer (RET). Accordingly, polysomes from the 3T6 cells were isolated and separated into FT and RET fractions to determine the distribution of collagen-associated hsp47 between these pools. The proteins in each fraction were divided into two portions. One pair of FT/RET fractions was boiled in SDS-gel running buffer to release any bound hsp47 [Nakai et al., 1989] and then immunoprecipitated with SP1.D8. The immunoprecipitate was dissociated, electrophoresed, and Western blotted with SP1.D8. The other pair of FT/RET fractions was directly immunoprecipitated with SP1.D8 without any effort to release bound hsp47 before electrophoresis and Western blotting with SP1.D8. These data are shown in Figure 3. Lane 1 presents the immunoblot of standard secreted media procollagen. As expected only the pro α1(I) chain was heavily stained. No stain was seen in the region of the pro α2(I) chains. When the FT and RET were boiled in Laemmli buffer for 5 min to dissociate any collagen-associated hsp47 prior to immunoprecipitation with SP1.D8, the fully elongated

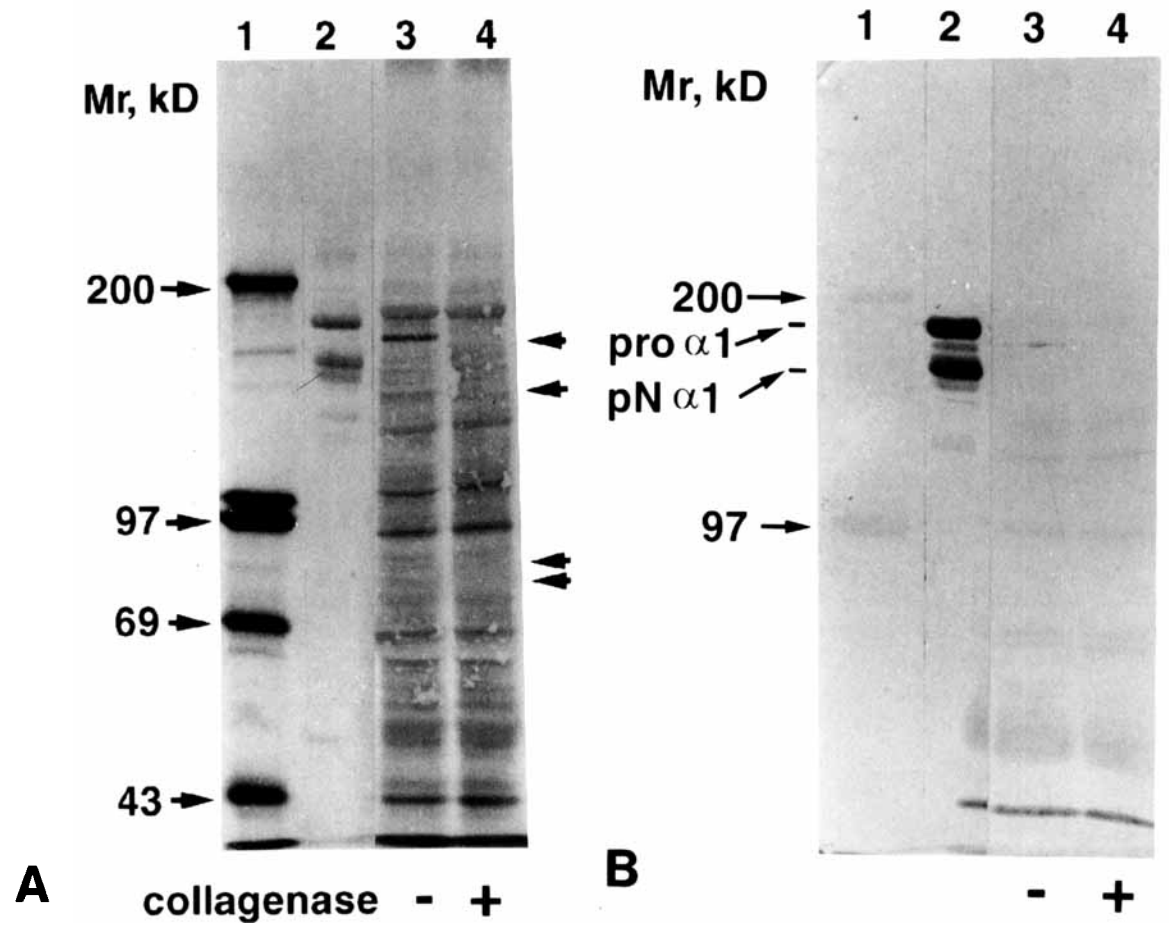

Fig. 1. The immunoprecipitation of [^{14}C]-proline labeled polysome-associated proteins from 3T6 cells by monoclonal antibody SP1.D8 against the pro α1(I)-N propeptide. **A:** An autoradiogram of the total [^{14}C]-Pro labeled polysomes from 3T6 fibroblasts. Exposed 4 weeks. *Lane 1,* [^{14}C]-Pro labeled protein standards; *lane 2,* [^{14}C]-labeled media procollagen standard; *lane 3,* [^{14}C]-Pro labeled polysomes; *lane 4,* the [^{14}C]-Pro labeled polysomes after collagenase digestion. **B:** A Western blot of the [^{14}C]-Pro labeled total polysomes immunoprecipitated with anti-pro α1(I)-N-propeptide antibody, SP1.D8. *Lane 1,* molecular weight standards; *lane 2,* media procollagen standard. The major bands correspond to pro α1(I) and pN α1(I). *Lane 3,* SP1.D8 immunoprecipitate of total polysomes; *lane 4,* total polysome precipitate after collagenase digestion. There was virtually no radioactivity in the material in lanes 3 and 4c confirming the lack of SP1.D8 interaction.

pro α1(I)-related chains were seen in the Western blots (lanes 2 and 3 of Fig. 3) of both fractions. These data showed that the FT and RET fractions did contain completed or nascent collagen chains which have the epitope for reaction with anti pro α1(I)-N-propeptide antibody SP1.D8. On the other hand, direct immunoprecipitation of FT and RET with SP1.D8 prior to boiling in Laemmli buffer and presumed separation from hsp47 (lanes 4 and 5 of Fig. 3) showed that no SP1.D8 reactive protein was available. These data confirmed that prior to boiling in SDS buffer, both the tRNA-free polysome-associated collagen pro α1(I) chains (FT) and the tRNA-bound nascent chains with completed intact N-propeptide regions (RET) were prevented from reacting with the SP1.D8 procollagen antibody.

Competition Studies: The hsp47-Procollagen Interaction

Procollagen. In their original studies, Nagata et al. [1986] determined the presence of hsp47 by its specific uptake from cell lysates on gelatin-Sepharose 4B beads. In the present work, 3T6 cells were labeled with [^{35}S]-methionine. After thorough washing the cells were lysed and the lysate supernatant was collected and aliquotted as described above. The lysates were then incubated with the gelatin-Sepharose beads containing 1–2 mg bound gelatin per 250 μl. The beads were washed in a standard protocol until

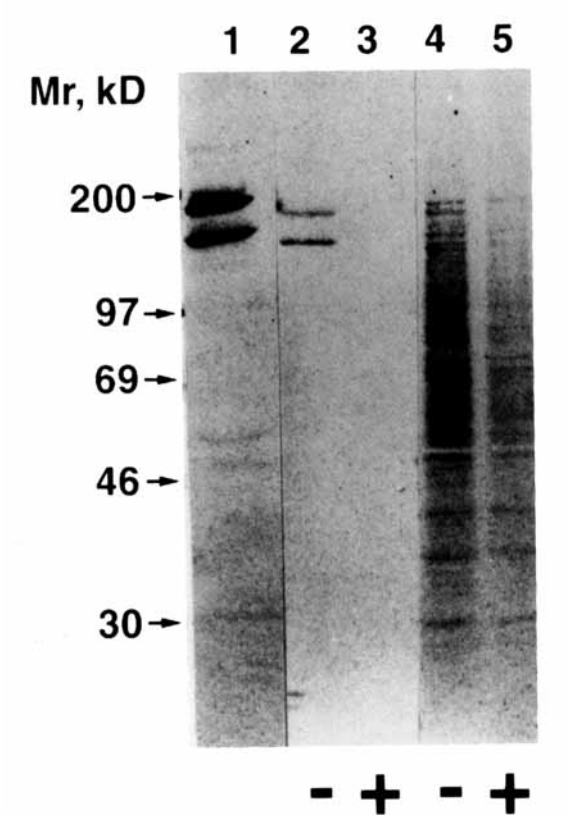

Fig. 2. The immunoprecipitation of [^{14}C]-proline labeled polysome-associated proteins from 3T6 cells by monoclonal antibody 11D10 against hsp47. An autoradiogram of the [^{14}C]-Pro labeled polysomes obtained by immuno-precipitation of the total polysomes with monoclonal antibody to hsp47, 11D10. *Lane 1,* media procollagen standard. *Lane 2,* the equivalent amount of media procollagen immunoprecipitated with 11D10 (≈6% by densitometry). The antibody evidently has a small, nonspecific interaction with procollagen. *Lane 3,* the immunoprecipitate of lane 2 plus collagenase. *Lane 4,* 11D10 immunoprecipitate of the polysomes (≈30% of the total polysome counts). *Lane 5,* the polysomes of lane 4 plus collagenase. The prominent lower weight, collagenase resistant bands may represent hsp47 nascent chains.

the radioactivity of the wash was essentially reduced to background, then boiled to release any bound labeled proteins. Equal volumes or counts of the bead supernatant were subject to electrophoresis and autoradiography.

Unlabeled procollagen was collected by ammonium sulfate precipitation from 3T6 cell culture media. This procollagen, at 1 μg/μl, was used directly, or after denaturation, or after denaturation and digestion by bacterial collagenase. Aliquots were added to the lysates for 1 h preincubation, then the gelatin-Sepharose beads were added and incubation continued for another h. The beads were washed as before and the bound radioactive lysate components were analyzed. The data are shown in Figures 4 and 5. In all of these experiments, the gelatin linked to the Sepharose beads was in excess of the amount of competitor added.

In the absence of any competitors, four main groups of cell lysate proteins were bound to the gelatin-Sepharose, proteins with M_r ~90,000, 70,000, 60,000, and 47,000 (Fig. 4). As anticipated, the 47 K band was the most prominent gelatin-bound component. However, the band appeared as a doublet of 46 and 47 K proteins (Fig. 4, lane 2). The 70 K proteins were present as a triplet, while there was a single 90 K protein. When denatured procollagen was added to the lysate, competition was evident. The binding of both the 46–47 K doublet proteins was reduced in intensity, as shown in Figure 5 (+ signs), but there was a more prominent reduction in the binding of the 46 K protein. Consistent with the data shown in Figure 2, lane 2, native procollagen also competed with the gelatin-Sepharose binding of the hsp46–47 proteins (Fig. 5, solid line) but to a lesser extent. Moreover, a different level of competition between the 46 and 47 K proteins was evident. The native procollagen was less effective than the denatured procollagen in competing with the 46 K component but virtually identical with respect to the 47 K component. The collagenase-digested procollagen (Fig. 5, diamonds) was almost identical to the intact native procollagen in its ability to compete with the gelatin-Sepharose. These data demonstrate that the interactions of hsp46–47 with procollagen are dependent on both the helix region and the intact propeptides, and that these interactions are independent and additive. The observation that the native and collagenase-digested procollagen had essentially equivalent effects suggested that the intact, native-folded triple-helical region has very weak binding to hsp46–47.

Helix-region competition. Two helix region analogs, synthetic peptides $(Pro\text{-}Pro\text{-}Gly)_{10}$ and $(Pro\text{-}Hyp\text{-}Gly)_{10}$ were added, at 1 μg/μl, to the cell lysate supernatant and incubated for 1 h before interaction with the gelatin-Sepharose beads. The bound proteins were eluted and the amounts quantitated by densitometry after gel electrophoresis. Figure 6 shows the differences between the amounts of protein bound by the gelatin sepharose and that bound in the presence of the peptides. The hydroxylated peptide

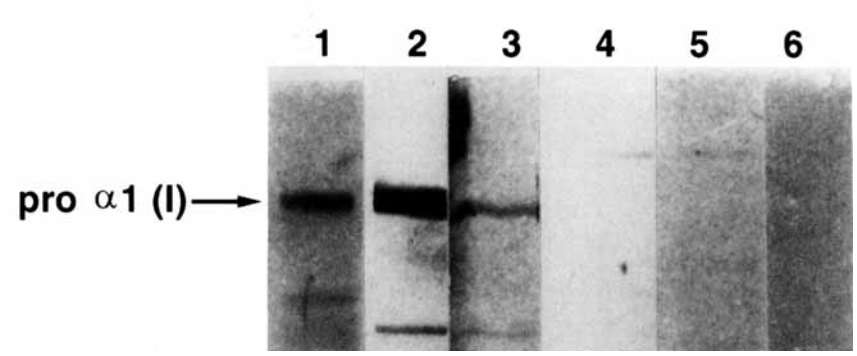

Fig. 3. The distribution of SP1.D8 precipitable components in the polysome fractions obtained after Q Sepharose fractionation. Detection by Western blotting and staining with SP1.D8. *Lane 1:* Standard media procollagen. *Lane 2:* Flowthrough fraction (FT), polysome-associated but not t-RNA bound. Fully elongated but not completely processed pro α1(I)-chains. FT was boiled in gel sample buffer to release any bound hsp47 prior to precipitation with SP1.D8. *Lane 3:* The retained fraction (RET) after boiling in gel sample buffer, before SP1.D8 reaction. There were many lightly brown stained bands in the gel migrating faster than pNα1(I). *Lane 4:* FT immunoprecipitated with SP1.D8 without prior boiling. The precipitate fraction was then electrophoresed and stained with SP1.D8. Essentially no SP1.D8 reactive material was in the immunoprecipitate fraction. *Lane 5:* The RET immunoprecipitate treated in the same way as the FT in lane 4. *Lane 6:* Immuno-precipitate of buffer blank.

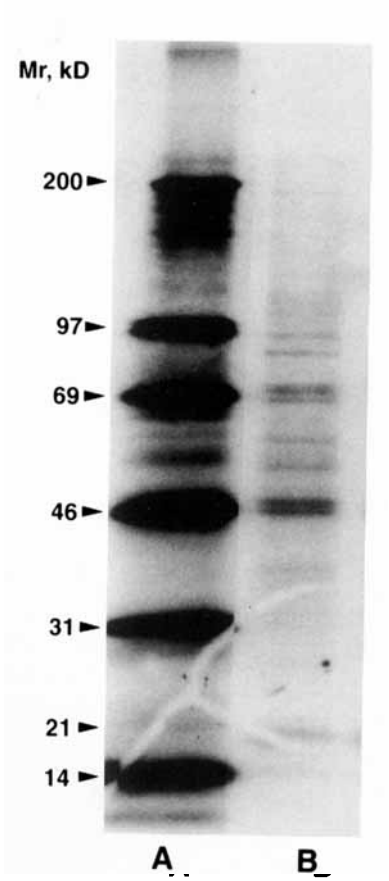

Fig. 4. Gelatin-Sepharose affinity bead collection of gelatin-binding components of the ^{35}[S]-Methionine labeled 3T6 cell lysate. *Lane A:* Molecular size markers. *Lane B:* The portion of the 3T6 cell lysate retained by the gelatin-Sepharose beads, eluted by boiling in SDS gel sample buffer. Note the clear doublet at 46–47 kD M_r.

provided virtually no competition for the binding of any lysate component to the gelatin-Sepharose. The $(Pro\text{-}Pro\text{-}Gly)_{10}$ did compete to a limited extent with the gelatin, and the principle competition was with proteins in the 46–47 K range.

Propeptide competition. The initial experiments were carried out using lysates of primary cultures of human gingival fibroblasts and the authentic phosphorylated N-propeptide of pro α1(I), isolated from bone, a small amount of which was available as a generous gift from Dr. Larry Fisher, National Institute of Dental Research. A small amount of synthetic SP-1 (Val-Pro-Thr-Asp-Glu), residues 85 to 89 of the pro α1(I)-N-propeptide, was also available. The SP-1 is just aminoterminal to the epitope for SP1.D8 [Wiestner et al., 1979, Horlein et al., 1981; Foellmer et al., 1983].

When the intact bone α1(I)-N-propeptide was added at 50 μg/ml to the gingival cell lysate-gelatin-Sepharose bead reaction mixture, binding of the M_r 70,000 component was reduced but binding of the 47,000 M_r band, attributed to hsp47, was completely inhibited (Fig. 7, lane 2). At 25 μg/ml propeptide, binding of the 70,000 M_r and minor component were as in the control, whereas binding of the hsp47 was still drastically inhibited (lane 3). The SP-1, at 100 μg/ml (Fig. 7, lane 4), had the same effect on hsp47 binding as the 25 μg/ml level of the intact pro α1(I)-N-propeptide. This effect was reduced in a dose dependent fashion, but was still evident at the 50 μg/ml level (Fig. 7, lane 5). Thus, both

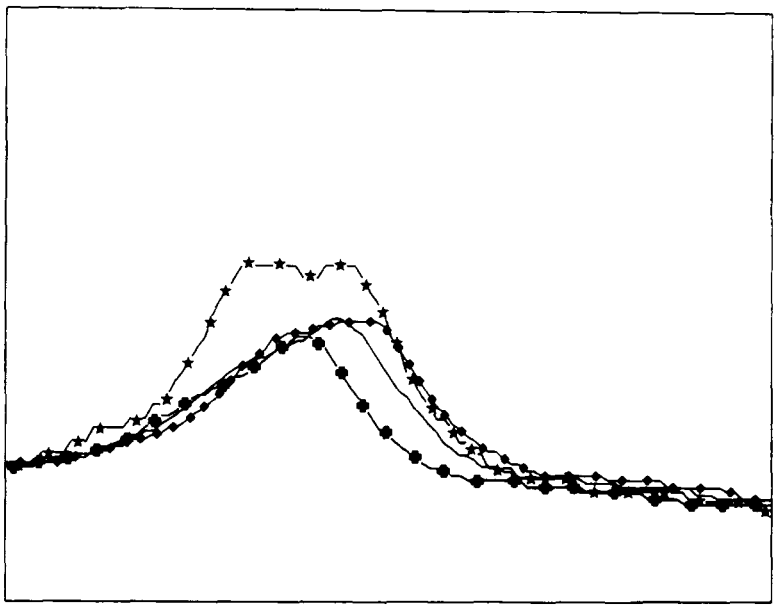

Fig. 5. Densitometric analysis of the competition between gelatin, native procollagen, denatured procollagen, and collagenase-digested procollagen for ^{35}[S]-Met labeled 3T6 cell lysate hsp47. Aliquots of the labeled cell lysate were preincubated with native procollagen, denatured procollagen or collagenase-digested procollagen under identical conditions, and then incubated with the gelatin-Sepharose beads. The affinity bound proteins were eluted, and analyzed electrophoretically as in the control experiment shown in Figure 4. Changes in the optical density of the 46–47 kDa doublet band were determined from the autoradiograms. All eluates were run on the same gel and all densitometric analyses were carried out with the same densitometer settings. Scans were run between base-line minima which were identical in every case. ☆-☆-☆, hsp46–47 kDa peak, no competitor; +-+-+, denatured procollagen; —, native procollagen; ◆-◆-◆, collagenase digested procollagen.

the pro α1(I)-N-propeptide and the small SP-1 pentapeptide domain of the pro α1(I)-N-propeptide had a strong and specific ability to compete with the interaction of hsp47 for the much larger quantity of gelatin (~1 mg) in the 250 μl of gelatin-Sepharose beads used in each assay. These data, though limited in scope because of the limited supply of peptides, clearly established that the α1(I)-N-propeptide, and the region near the SP1.D8 binding domain in particular, were major sites for interaction with hsp47.

Direct Binding Assays

To test this interaction more directly, the GST-NP1, NP2, and NP3 fusion human N-propeptide proteins were prepared. The recombinant propeptide domains were released by treatment of the GST with thrombin. The GST was removed by absorption on glutathione-Sepharose 4B beads. The free NP1, NP2, and NP3 were then coupled to CNBr-activated Sepharose to prepare affinity beads. [^{35}S]-Met labeled 3T6 cell lysates were then incubated as in the gelatin-binding competition experiments. The bound proteins were released and analyzed by gel electrophoresis, autoradiography, and quantitative densitometry (Fig. 8). NP1 and NP2 showed specific affinity for the hsp47. Surprisingly, in view of the strong competitive activity of the bone α1(I) N-propeptide, NP3, the complete α1(I) N-propeptide did not show any hsp47 binding. It is likely that this indicates that the recombinant NP3 was malfolded such that the C-terminal telopeptide section interacted to obstruct the hsp47 binding region or was coupled differently to the CNBr activated Sepharose. However, these data are clear in showing directly that the most N-terminal portion of the α1(I)-N-propeptide (NP1) has information sufficient to dictate significant hsp47 binding. The addition of the propeptide internal G-X-Y helix region (NP2) added substantially to the intensity of the hsp47 interaction.

Interactions During In Vitro Cotranslation of hsp47 and pro α(I)

Although informative, the cell lysate experiments are quite complex. Clearly, there are a number of gelatin-binding components in the cell lysates (Fig. 4). Furthermore, as shown in Figure 1, there are a number of nascent non-

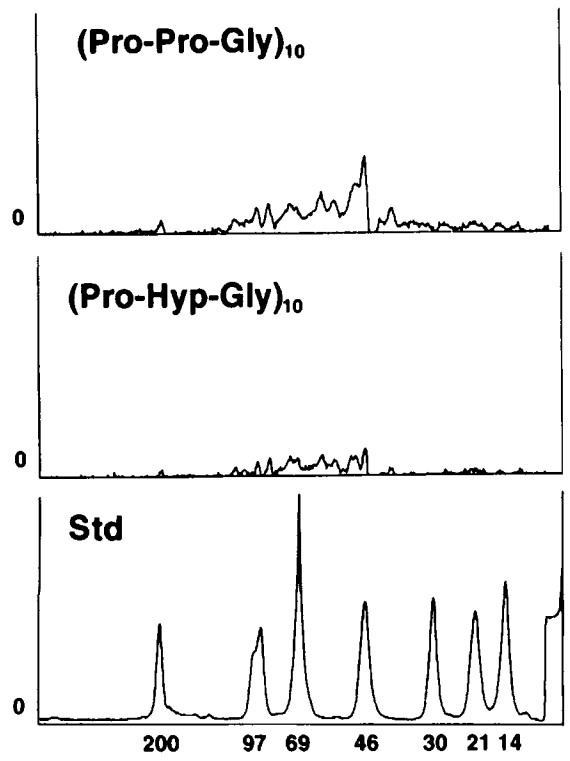

Fig. 6. The competition for binding of cell lysate constituents by helix region polypeptides. Aliquots of the labeled cell lysate were preincubated with 30-mer peptide analogs of the collagen helix region under identical conditions and then incubated with the gelatin-Sepharose beads. The affinity bound proteins were eluted, and analyzed electrophoretically as in the control experiment shown in Figure 4. The electrophoretograms were scanned densitometrically. The effect of the added peptide was determined as the difference between the density of the eluted bound proteins in the absence of the competitor and the presence of the competitor. A positive value for the difference indicates specific competition for gelatin binding of lysate proteins. **Upper panel:** Analog for the unhydroxylated helix region or nascent, unprocessed chain. **Middle panel:** Analog for the hydroxylated or processed helix region. **Lower panel:** Molecular weight markers. The $(Pro\text{-}Pro\text{-}Gly)_{10}$ demonstrated only a weak competition with the gelatin, but had strongest competition in the 46–47 kDa range. $(Pro\text{-}Hyp\text{-}Gly)_{10}$ was not competitive in the lysate binding assay at the concentration used.

collagenous proteins collected in the polysome preparations. To clarify the possible interactions between the pro α(I) chains and hsp47, cell-free translations of the appropriate in vitro transcribed RNAs (human pro α1(I), human pro α2(I), mouse J6 [Wang and Gudas, 1990]) were carried out separately and in mixtures. The translated proteins were then subject to immunoprecipitation with SP1.D8 and with anti-46K (antimouse gp46) antibody. Figure 9A shows the results of translations ± canine pancreatic microsomes. The pro α1(I), pro α2(I), and hsp47 (J6) RNAs all yielded the expected products, and translations in the presence of microsomes produced posttranslationally modified forms of the proteins. When subjected to immunoprecipitation with SP1.D8, the pro α1(I) RNA translation showed the presence of full and near-complete length pro α1(I) chains plus 3 bands in the 30 to 40 K M_r range, ascribable to the N-propeptide plus small lengths of helix domain (Fig. 9B, lanes 2 and 3). As a control, the pro α2(I) RNA translation products showed no reactivity with SP1.D8 (Fig. 9B, lanes 4 and 5). The immunoprecipitate of the cotranslation of hsp47 and pro α1(I) RNAs contained only traces of the pro α1(I) and hsp47 translation products (Fig. 9B, lanes 6 and 7). Both the full-length pro α1(I)-chains and the short, incompletely elongated 30–40 K chains were strongly inhibited from interaction with SP1.D8 by the presence of co-translated hsp47. These data are clear-cut in showing that the presence of hsp47 protein blocks the interaction between the pro α1(I)-N-propeptide and SP1.D8. Control immunoprecipitations of nascent hsp47 with SP1.D8 (not shown) showed no cross reactivity. Nevertheless, small but detectable amounts of hsp47, more prominently in the microsome-free preparation than in the presence of microsomes and consequent posttranslational modification, were present in the immunoprecipitate fraction. The same immunoprecipitates contained barely detectable amounts of near full length pro α1(I) or the nascent peptides (compare Fig. 9B, lanes 2, 3 and 6, 7). These data may reflect weak binding of hsp47 to multiple sites along the major collagen G-X-Y domain. In lane 7 of Figure 9B a faint band of near-full-length cotranslationally processed pro α1(I) can be seen, but the processed hsp47 band is weaker than in lane 6 where no cotranslational processing of either pro α1(I) or the hsp47 was possible. These data confirm the much weaker competition of the hydroxylated $(Pro\text{-}OHPro\text{-}Gly)_{10}$ for the hsp47 than the unhydroxylated $(Pro\text{-}Pro\text{-}Gly)_{10}$ shown in Figure 6.

In agreement with the literature [Saga et al., 1987; Nakai et al., 1990], the monoclonal hsp47 antibody 11D10 immunoprecipitated procollagen-related nascent chains from 3T6 cells, as shown in Figure 2, indicating that the antibody binds to the hsp47 at a site other than the procollagen chain binding site. The anti-46K antibody behaved in a different fashion. Cotranslations of pro α1(I), pro α2(I), and J6 RNAs were carried out. One aliquot of the mixture was

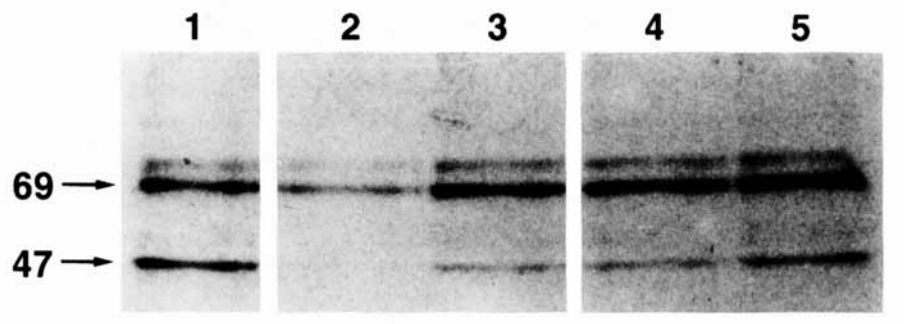

Fig. 7. The competition between gelatin, the pro α1(I) N-propeptide and SP-1 for binding to hsp47. Aliquots of a [^{35}S]-Met labeled human gingival cell lysate were preincubated with the bone pro α1(I)-N-propeptide, or the synthetic SP-1 peptide, under identical conditions at the concentrations noted below, and then incubated with the gelatin-Sepharose beads. The affinity bound proteins were eluted, and analyzed electrophoretically as in the control experiment shown in Figure 4. **Lane 1:** Control cell lysate bound to the gelatin-Sepharose beads. **Lanes 2, 3:** Bound lysate components after lysate incubation in 50 and 25 μg/ml bone α1(I)-N-propeptide, respectively. **Lanes 4, 5:** Bound lysate components after lysate incubation in 100 and 50 μg/ml SP-1, respectively. Note that in the gingival cell lysates the amounts of gelatin-binding 70 kDa and 47 kDa proteins are comparable whereas the amount of 46–47 kDa protein is relatively greater in the 3T6 cell lysates (Fig. 4). The α1(I)-N-propeptide at the 50 μg/ml concentration very effectively competes for the hsp47. At higher concentration, even the small 5 amino acid residue SP-1 peptide can compete with the gelatin affinity for hsp47.

immunoprecipitated with anti-46K, another aliquot with SP1.D8. The immunoprecipitate with anti-46K contained the hsp47 with only a very small amount of pro α1(I) (Figure 10, lane 2, arrowhead). On the other hand, immunoprecipitation with SP1.D8 yielded pro α1(I) chains but only a very small amount of hsp47 (Fig. 10, lane 4, arrowhead). However, the amount of pro α1(I) precipitated from the pro α1(I) + pro α2(I) + J6 RNA cotranslation mixture by SP1.D8 was substantially less than that harvested from a quantitatively identical translation of a mixture of pro α1(I) and pro α2(I) RNAs (Fig. 10, lane 3), indicating that the hsp47 was blocking the SP1.D8 binding site. Thus the anti-46K-hsp47 interaction domain utilizes the region of the hsp47 which is also involved in binding to the pro α1(I) chains so that anti-46K and pro α1(I) are competitors for hsp47 binding.

In vitro translation of J6 RNA alone yields a number of bands in the 44–47 kDa range whether the translations are in the presence or absence of microsomal membranes, (Fig. 11). Although the increased values of M_r in the presence of microsomes indicates that posttranslational modifications, such as glycosylation, were likely to have taken place, the multiple hsp47-related bands showed that translation of hsp47 might have been incomplete or interrupted and may not be entirely related to differences in posttranslational modifications. It should be noted that the optimum conditions for J6 RNA translation were not used, in favor of optimization of the collagen translations. How this observation fits with the clear-cut appearance of the differentially reactive bands of the 46–47 kDa doublet in the 3T6 cells (Figure 5) remains to be determined but, it is most likely that the 46 and 47 kDa bands represent the same protein core with different levels of post translational modification.

DISCUSSION

All of the data described above lead to the principal conclusion that the major site of strong interaction of hsp47 with procollagen I chains is within the domain of the pro α1(I)-N-propeptide. Although the gelatin ligand in gelatin-Sepharose can bind hsp47 from cell lysates, the gelatin is in large excess and the binding is weak. Shifting the pH to ≤ 6.3 [Saga et al., 1987; Nagata et al., 1988] is sufficient to release the basic hsp47 (pI ~ 9.0) from the affinity columns. On the other hand, as shown in Figure 7, 50 μg of bone α1(I)-N-propeptide was sufficient to completely inhibit binding of cell lysate hsp47 to 1–2 mg of gelatin per reaction in the competition experiments. The importance of the propeptide regions for interaction with hsp47 was further emphasized by the competition experiments with procollagen, Figure 5. Denatured, intact procollagen was the most effective competitor for gelatin binding, but intact procollagen and collagenase-digested procollagen were equal in their activity, showing the dominance of the intact propeptide domains in the interaction.

The $(Pro\text{-}OHPro\text{-}Gly)_{10}$ peptide was not an effective competitor of hydroxylated gelatin-

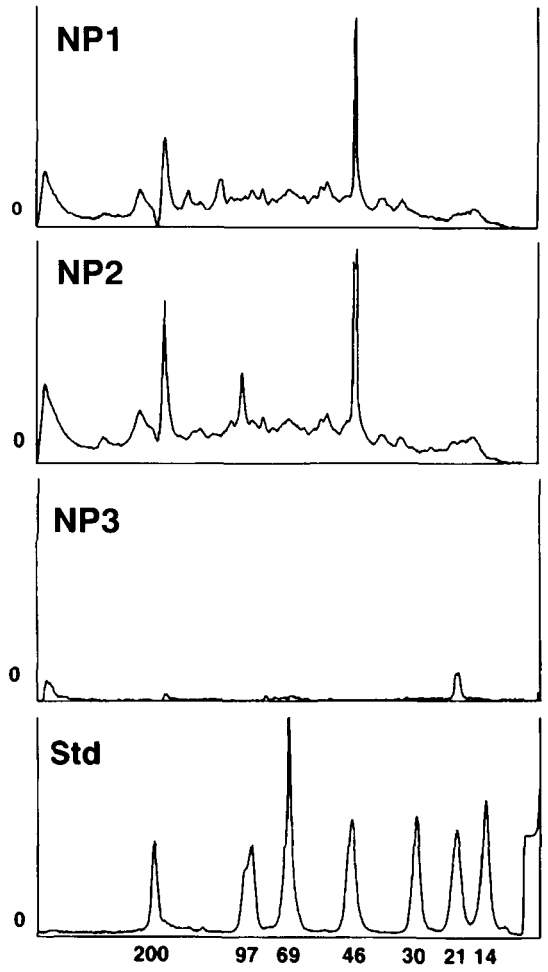

Fig. 8. Direct binding of 3T6 cell lysate constituents to α1(I)-N-propeptide constructs. The α1(I)-N-propeptide constructs were linked covalently to Sepharose 4B and used to determine the specific uptake of lysate proteins. The 3T6 cell lysate was incubated with the affinity beads in the standard protocol under the same conditions as the prior gelatin-binding studies. Under the washing protocols used there was always a small nonspecific binding of lysate proteins to the Sepharose 4B beads even in the absence of the propeptide adduct. The data presented are the densitometric scans of the electrophoretograms of the bound proteins, from which the background binding has been subtracted. The 46–47 kDa components are the major proteins taken up specifically by the NP1 and NP2.

hsp47 interaction at the concentration used. The gelatin in the gelatin-Sepharose affinity beads is hydroxylated. On the other hand, the unhydroxylated $(Pro\text{-}Pro\text{-}Gly)_{10}$ was able to compete with the gelatin binding at identical concentration, suggesting that the general interaction of the potential helix region in type I collagen unhydroxylated nascent chains might be stronger, and become important during the ER processing of the chains. However, this is unlikely since there is no evidence in either the present or prior work that hsp47 interacts with the pro α2(I)-chain [Ferreira et al., 1994; Sauk et al., 1994].

The direct binding studies with the recombinant pro α1(I), pro α1(I)-N-propeptide constructs verified this conclusion. NP1, the most amino-terminal domain of the N-propeptide has sufficient information to specifically take up hsp47 from the total cell lysate. However, the addition of the N-propeptide helical domain strengthened the hsp47-propeptide interaction as indicated by the enhanced binding of hsp47 to NP2 as compared with NP1 (Fig. 8). The anti-α1(I)-N-propeptide antibody, SP1.D8, was designed to bind to collagen specifically near the N-propeptidase cleavage site. It thus covers the region bridging from the SP-1 sequence in NP1 across the G-X-Y domain of NP2 and, on the basis of the data in Figure 8, it must compete directly with hsp47 in propeptide binding. The anti-46K antibody, which does not bind to collagen, must, however, bind strongly to hsp47 at the hsp47 propeptide binding domain so that it can extract hsp47 from its collagen complex. That is, the pro α1(I)-N-propeptide is a weak competitor to anti-46K. These conclusions are strengthened by the in vitro translation data. The co-translation of J6 and pro α1(I) RNAs ± membrane, leads to strong interactions between the nascent proteins which block the SP1.D8 reaction site. Nevertheless, in contrast to the use of the recently described polyclonal antibody to hsp47 (Sauk et al., 1994) or the monoclonal 11D10 to immunoprecipitate the hsp47-pro α1(I) complex, the use of the anti-46K antibody does not co-immunoprecipitate the pro α1(I) chains in the in vitro translation system.

It appears that the "hsp47" bound to the NP2 affinity column consists of both hsp46–hsp47 components whereas NP1 binds the "hsp47" component more strongly. The comparison of the denatured and collagenase digested procollagen-competition with gelatin (Fig. 5) provides similar information. That is, the Gly-X-Y domain has a stronger interaction with the 46 K component of the hsp46–47 proteins. It was entirely unexpected to find the complete N-propeptide construct, NP3, to be inactive. Two possible explanations might be that the N-telopeptide domain misfolds back over the hsp47 binding domain, preventing binding, or that the lysine in the NP3 telopeptide domain reacts with the CNBr-Sepharose more avidly than the

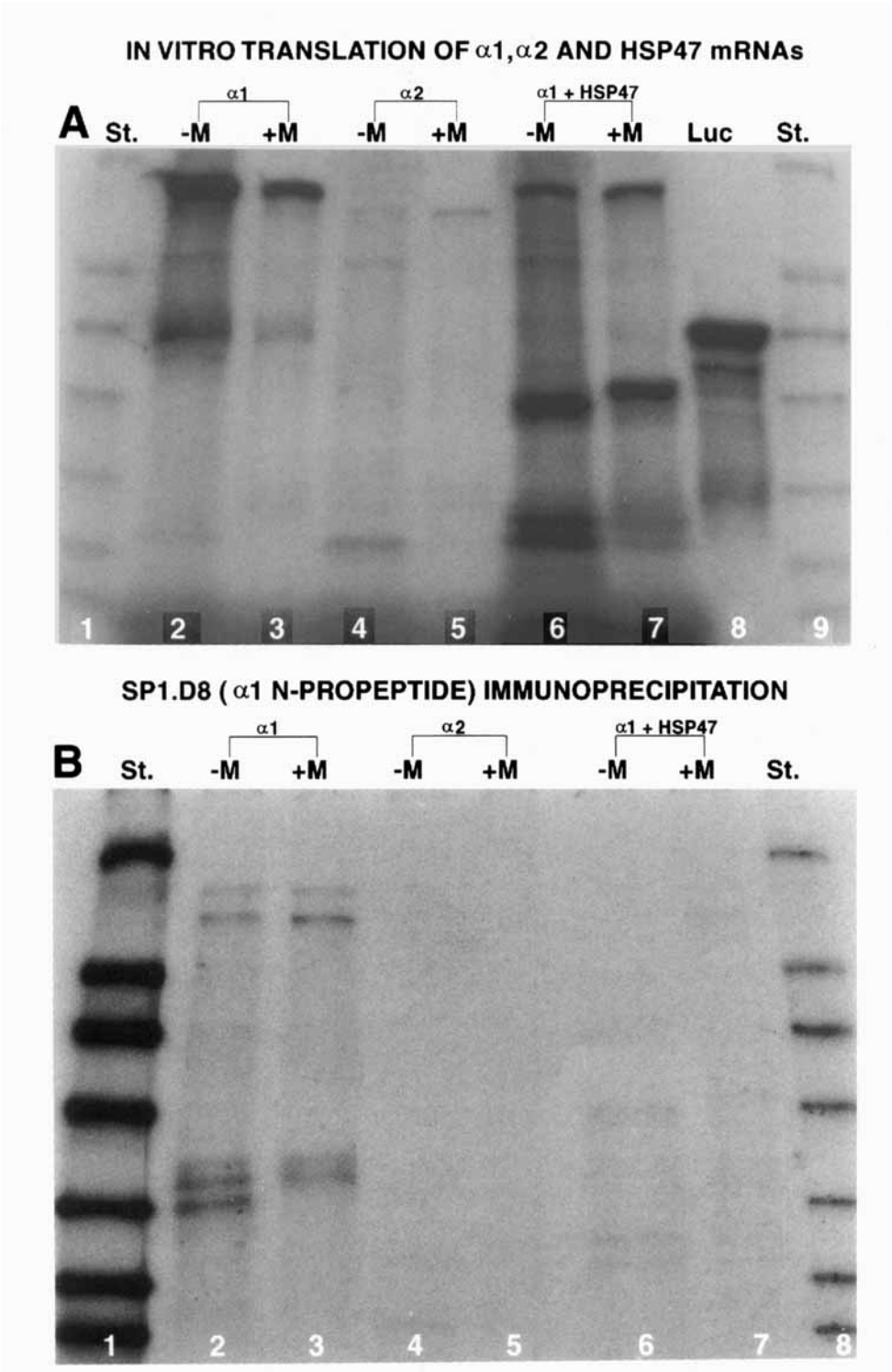

Fig. 9. The in vitro translation of pro α1(I), pro α2(I), and hsp47 RNAs in rabbit reticulocyte lysate in the absence or presence of canine pancreatic microsomes, and the precipitation of the reaction products with monoclonal antibody SP1.D8. **A:** The products from the translations pro α1(I), pro α2(I), and hsp47 RNAs in the rabbit reticulocyte lysate system. Pro α1(I) RNA and hsp47 RNA were co-translated. In each case, as indicated, the translations were ± pancreatic microsomes (M). *Lanes 1, 9:* Radioactive marker proteins, M_r from top to bottom, 200, 97.5, 69, 46, 30, 21.5, and 14 K. *Lanes 2, 3:* pro α1(I) RNA ± M. *Lanes 4, 5:* pro α2(I) RNA ± M. *Lanes 6, 7:* The co-translation of pro α1(I) and hsp47 RNAs ± M. Note that the amounts of full length pro α1(I) chain in lanes 2 and 3 closely approximate the amounts in lanes 6 and 7, and that there was abundant co-production of hsp47. *Lane 8:* The translation of standard luciferase RNA as a control to confirm the efficiency of the reticulocyte lysate. **B:** Equivalent aliquots of the translation mixtures in each case were taken for immunoprecipitation with SP1.D8, the collagen pro α1(I)-N-propeptide specific antibody. The immunoprecipitates were electrophoresed. *Lanes 1, 8:* Marker proteins as in A. *Lanes 2, 3:* pro α1(I) ± M. *Lanes 4, 5:* pro α2(I) ± M. *Lanes 6, 7:* [pro α1(I) + hsp47 cotranslation] ± M.

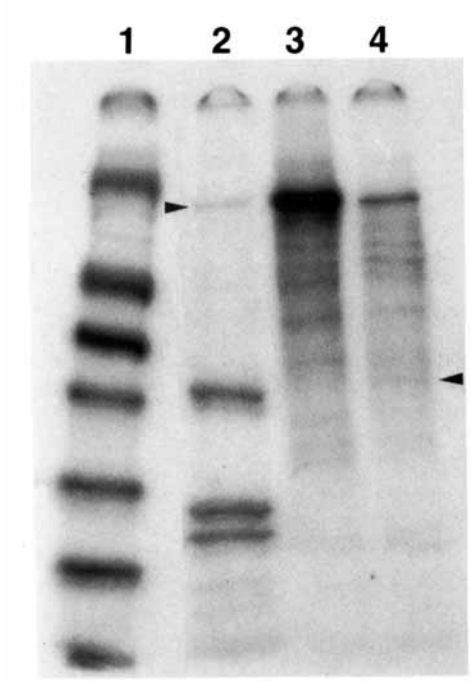

Fig. 10. A comparison of the immunoprecipitations of a co-translational mix of pro α1(I) + pro α2(I) and hsp47 RNAs (−M) with anti-46K and SP1.D8. *Lane 1:* Molecular size markers as in Figure 9A. *Lane 2:* The products of the translation immunoprecipitated with anti-46K. The arrowhead points to the small amount of pro α1(I) in immunoprecipitate. The lower bands indicate the hsp47 and two smaller incompletely elongated but anti-46K reactive hsp47 peptides. *Lane 4:* The products of the same translation mixture immunoprecipitated with SP1.D8. In this case pro α1(I) chains are evident, along with a weak band (arrowhead) showing the presence of a small amount of hsp47 in the immunoprecipitate. *Lane 3:* The SP1.D8 immunoprecipitate of a translation mixture containing only pro α1(I) and pro α2(I) RNAs in the same amounts as in the + J6 RNA cotranslation. As can be seen in Figure 9, the density of the pro α1(I) band in this lane is the amount of pro α1(I) available for immunoprecipitation. These data again show that the presence of hsp47 inhibits the immunoprecipitation of pro α1(I) chains by SP1.D8.

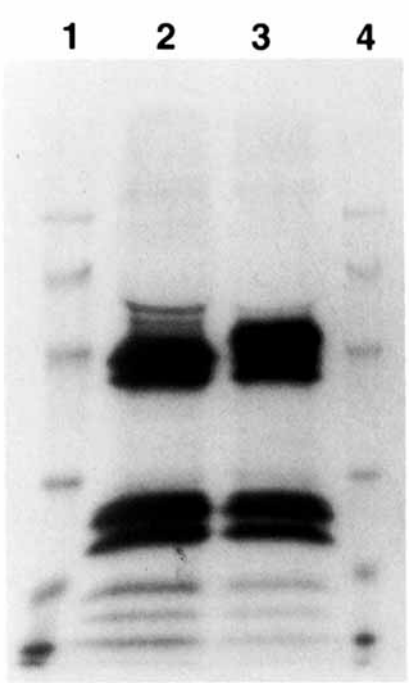

Fig. 11. The in vitro translation of J6 RNA ± M. *Lanes 1, 4:* Molecular size markers. *Lane 2:* J6 RNA −M. *Lane 3:* J6 RNA + M. In the presence of microsomes the translational processing of the full length hsp47 is evident. It is also clear that two major forms of hsp47 are produced in vitro. In the in vitro system-M several additional unexplained bands, typical of translational products resulting from premature chain termination or pausing, can be seen.

N-terminal amino group, drawing the potential reactive region too close to the Sepharose bead surface. This lysine, only 9 residues from the C-terminus of the telopeptide, may exist at the corner of a β-turn in the telopeptide [Helseth et al., 1979; Ananthanarayanan et al., 1985] in a reactive state. It is a substrate for hydroxylation and could be the preferred site for linkage to the CNBr-Sepharose in the construct. Further analysis of this question is being considered.

All of these data point to the conclusion that it is highly unlikely that hsp47 is a "chaperone" protein involved in regulating collagen triple helix folding. Nevertheless, in view of the facts that the triple helix formation proceeds from the carboxyl-terminal direction following chain registration consequent to interaction within the C-propeptides [Bachinger et al., 1980, 1981], and that pro α1(I) chains can readily form homotrimers [Olsen and Prockop, 1989] it does appear that some mechanism is required to prevent misfolding and to ensure proper chain selection. It has been argued that the procollagen chains themselves have all of the information required to make this selection and fold properly. Why then should there be a specific pro α1(I)-chain "chaperone" which has no apparent reactivity with the pro α2(I) chain? Further, why should this specific chaperone-like interaction be with the amino-terminal region of the pro α1(I)-chain, far spatially and temporally from the C-terminal domain where chain selection and folding initiate? It is instructive to consider and compare the structures of the pro α(I)-chain N-propeptide regions (Fig. 12). The N-terminal portion of the human pro α1(I)-N-propeptide (NP1) consists of 86 residues with 10 Cys forming 5 intrachain disulfide bonds stabilizing several loops into a globular domain of as yet un-

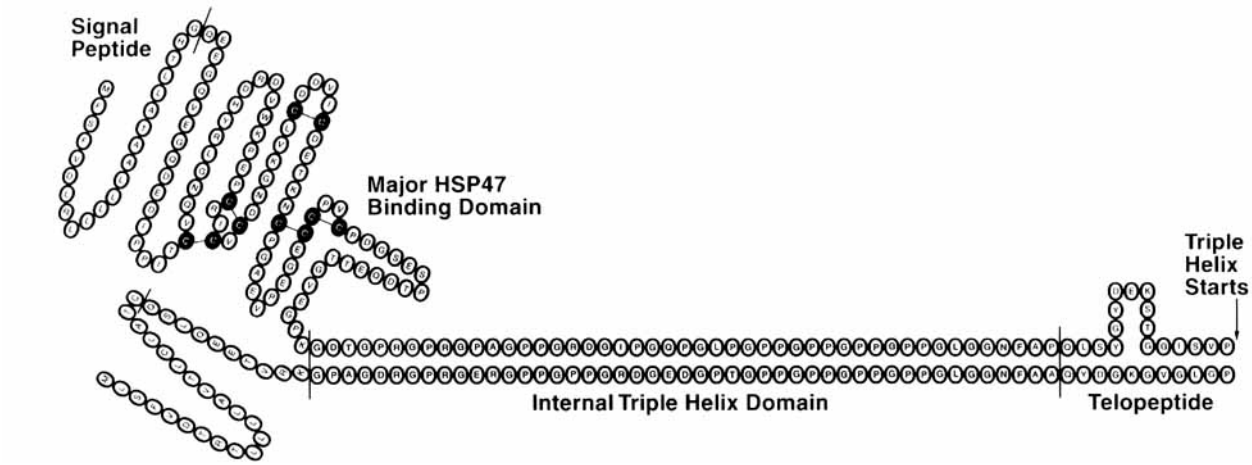

Fig. 12. Schematic plans of the N-propeptide regions of the pro α1(I)- and pro α2(I)-chains. The sequences of the chains for human procollagen I are shown, and numbered, from the signal peptide through the N-telopeptide. The sequences and overall organization of the pro α1(I) and pro α2(I)-N-propeptides are aligned so that their most C-terminal junctions with the major collagen triple helix are in register. They are further aligned so that the N-proteinase cleavage points and Gly-Xaa-Yaa domains are also in register for folding from the C-terminal end. Note that all three of the pro α2(I)-N-propeptide domains (telopeptide, Gly-Xaa-Yaa domains, and the N-terminal portions) chain are smaller than their corresponding pro α1(I) domains. When aligned in this way, the homologies between the regions which must fold in the compound triple helix are evident. The 4 (Gly-Pro-Pro) triplets align exactly and can hence form a particularly stable structure. The intrachain disulfide bonds of the N-terminal domain of the pro α1(I) constrain the C-terminal portion of the N-terminal propeptide to form a complex structure. All of the data presented point to this region as the site for strong binding of hsp47. However, the data also show the binding to be enhanced by some interaction with the Gly-Xaa-Yaa domain. The pro α2(I)-N-propeptide contains no disulfide bonds and, hence, does not have any specific binding to the hsp47. When the heterotrimer is formed, completion of the triple helix within the propeptide may weaken the hsp47 binding to the pro α1. If all three pro α1(I) chains in a potential homotrimer have bound hsp47, it may be more difficult to drive completion of the triple helix formation and, hence, may reduce the relative rate of formation of the homotrimer vs. heterotrimer. The **upper chain** is pro α1(I), the **lower** is pro α2(I). The Cys residues are shown in black, the triple helix domain residues are between the vertical bars.

known structure and conformation. This is followed by a stretch of 48 Gly-Xaa-Yaa residues which, in the completed molecule, are hydroxylated and participate in forming a very stable, collagenase-resistant triple helix. This is followed by a short connecting sequence which contains the N-proteinase cleavage site connecting to the 16 residue N-telopeptide. The NP2 construct covers the sequence to this point. In the remaining pro α1(I)-N-telopeptide, it is likely that there is a specific structure, with a β-turn involving the Asp^{160}-Glu^{161}-Lys^{162} sequence [Veis and George, 1994]. The NP3 construct includes the entire N-telopeptide. The counterpart pro α2(I)-N-propeptide-telopeptide also has three domains, but the N-terminal domain consists of only 10 residues, the Gly-Xaa-Yaa region includes 39 residues and the telopeptide 11 residues. It contains no internal disulfide bonds. The pro α2(I)-N-propeptide Gly-X-Y region participates in forming the very stable propeptide molecular triple helix. These are the first sequences synthesized and available for posttranslational hydroxylation, hence they must have a strong tendency to fold. Because the chains are coordinately synthesized and present in the same ER compartments it can be hypothesized that early interaction must be inhibited. Hence, by binding strongly in the region of the NP1-NP2 junction and covering the most amino-terminal portion of the helix domain, hsp47 would specifically inhibit premature stable molecular N-propeptide formation. As shown above, it is possible for a molecule such as anti-46K antibody to displace the propeptide bound to hsp47. The isolated SP-1 peptide, only 5 residues in length, but near a multiply disulfide linked region that brings the N-terminal portion of the Gly-Xaa-Yaa domain close to the globular domain, also can compete with hsp47 binding. This defines the region of NP2 involved, clearly overlapping the SP1.D8 binding domain. One can suggest that, in vivo, once C-terminal interaction registers the nearly completed molecules and triple helix formation proceeds along the major helix, the forced proximity of the pro α2(I)-chain N-propeptide helix domain can begin stable C ⇒ N propeptide helix formation and drive off the

hsp47 allowing final folding of the remaining N-propeptide region. It is also possible that difficulties in removing hsp47 from the pro α1(I)-N-propeptide prevents or reduces the rate of completion of folding of the pro α1(I) homotrimer. From this perspective hsp47 would be a very specialized "chaperone."

These considerations form the base of our present studies on the involvement of hsp47 in assembly of other homotrimeric and heteromeric fibrillar and basement membrane collagens.

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