

## Defining the domains of type I collagen involved in heparin-binding and endothelial tube formation

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**ABSTRACT** Cell surface heparan sulfate proteoglycan (HSPG) interactions with type I collagen may be a ubiquitous cell adhesion mechanism. However, the HSPG binding sites on type I collagen are unknown. Previously we mapped heparin binding to the vicinity of the type I collagen N terminus by electron microscopy. The present study has identified type I collagen sequences used for heparin binding and endothelial cell–collagen interactions. Using affinity coelectrophoresis, we found heparin to bind as follows: to type I collagen with high affinity ( $K_d \approx 150$  nM); triple-helical peptides (THPs) including the basic N-terminal sequence  $\alpha 1(I)87-92$ , KGH-RGF, with intermediate affinities ( $K_d \approx 2$   $\mu$ M); and THPs including other collagenous sequences, or single-stranded sequences, negligibly ( $K_d \gg 10$   $\mu$ M). Thus, heparin–type I collagen binding likely relies on an N-terminal basic triple-helical domain represented once within each monomer, and at multiple sites within fibrils. We next defined the features of type I collagen necessary for angiogenesis in a system in which type I collagen and heparin rapidly induce endothelial tube formation *in vitro*. When peptides, denatured or monomeric type I collagen, or type V collagen was substituted for type I collagen, no tubes formed. However, when peptides and type I collagen were tested together, only the most heparin-avid THPs inhibited tube formation, likely by influencing cell interactions with collagen–heparin complexes. Thus, induction of endothelial tube morphogenesis by type I collagen may depend upon its triple-helical and fibrillar conformations and on the N-terminal heparin-binding site identified here.

Cell surface proteoglycan interactions with type I collagen are likely a ubiquitous mechanism of cell adhesion, yet the interactive sites on these molecules are undefined. Consideration of the complexity of type I collagen structure is relevant to understanding its interactions with heparan sulfate proteoglycans (HSPGs) (see refs. 1 and 2 for review). Type I collagen is secreted as procollagen, which, after proteolytic cleavages, yields the triple-helical monomer composed of two  $\alpha 1$  and one  $\alpha 2$  chains. These monomers assemble in a regular staggered fashion into fibrils, which display the repeating D-period pattern, consisting of fine crossfibril bands (called the a, b, c, d, and e bands) in positively stained electron microscopy preparations.

Triple-helical type I collagen conformation is necessary for its high-affinity binding to HSPGs, or to heparin, a chemical analog of its heparan sulfate chains (3–5). Furthermore, the C-terminal triple-helical fragment of type I collagen, generated by vertebrate collagenase treatment, showed a higher affinity for heparin than the did the N-terminal fragment (4).

To localize more precisely the heparin-binding regions on type I collagen, we studied complexes between collagen monomers and heparin–albumin–gold particles by electron microscopy (6), and we observed heparin binding primarily to a region on the triple helix near the procollagen N terminus. In collagen fibrils, heparin–gold bound to the a bands region within each D-period, which is consistent with an N-terminal heparin-binding site on its monomers. The resolution of the mapping technique was insufficient to assign heparin-binding function to any particular protein sequence. Thus, amino acid sequences were inspected in relation to the heparin-binding locations observed by electron microscopy, searching for basic domains that might be suitable as heparin-binding sites. A highly basic sequence was found near the procollagen N terminus, and within the a bands fibril region, corresponding to amino acid residues 87–92 of the rat  $\alpha 1$  chain. To test the heparin-binding function of this or other sites of type I collagen, here we have studied how mimetic triple-helical peptides (THPs) including various collagen sequences interact with heparin. We have also explored the function of these sequences in endothelial cell interactions with type I collagen during endothelial tube formation.

### MATERIALS AND METHODS

**Cell Culture.** Human umbilical vein endothelial cells (HUVECs) were isolated as detailed in ref. 7. Cells were cultured in “complete medium” containing medium 199 (GIBCO/BRL) with 10% fetal bovine serum (FBS), 80  $\mu$ g/ml endothelial cell growth supplement (ECGS), 50–60  $\mu$ g/ml heparin from pig intestinal mucosa (Sigma; grade I-A), penicillin, streptomycin, and Fungizone, on gelatin-coated (7) tissue culture flasks (Falcon) and incubated at 37°C in 5% CO<sub>2</sub>/95% air. ECGS was isolated from bovine hypothalami (8).

**Collagen Preparation.** Type I collagen was isolated from rat tail tendons (3). Gelatin was prepared by denaturing a 2.5 mg/ml solution of acid-soluble rat tail collagen at 50°C for 45 min, or as an aqueous solution of 2 mg/ml gelatin (Sigma). Chicken type I procollagen was isolated as detailed in ref. 6, and human type V collagen was the gift of Steffen Gay of the University Hospital, Zurich.

**Preparation of Radiolabeled Heparin.** Whole heparin from pig intestinal mucosa (Sigma; grade I-A) was tyramine end-labeled and radiolabeled with Na<sup>125</sup>I (Amersham) (9) to a specific activity of  $\approx 1.40 \times 10^7$  cpm/ $\mu$ g. Radiolabeled heparin

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: ACE, affinity coelectrophoresis; Ahx, 6-aminohexanoic acid; GAG, glycosaminoglycan; GPP\*, Gly-Pro-Hyp (Hyp, 4-hydroxyproline); HSPG, heparan sulfate proteoglycan; HUVEC, human umbilical vein endothelial cell; PG, proteoglycan; SSP, single-stranded peptide; THP, triple-helical peptide.

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was then fractionated on Sephadex G-100, and the final  $\approx 12\%$  to elute was retained as the low  $M_r$  material of  $M_r \leq 6,000$ ; the remaining heparin, excluding the highest  $\approx 12\%$  in  $M_r$ , was retained as the medium  $M_r$  material (10, 11).

**Synthesis, Purification, and Characterization of Single-Stranded Peptides (SSPs) and THPs.** The synthesis and characterization of a generic THP containing 8 repeats of Gly-Pro-Hyp [(GPP\*)<sub>8</sub>, in which Hyp or P\* is 4-hydroxyproline] have been described previously (12). THPs containing sequences derived from the  $\alpha 1(I)$  or  $\alpha 2(I)$  chain of human type I collagen were synthesized (12–14) by using 9-fluorenylmethoxycarbonyl (Fmoc) methodology on an Applied Biosystems 431A peptide synthesizer. In the cases of difficult syntheses, some minor sequence adjustments were made to allow for efficient peptide assembly. All THPs were purified by a two-step reversed-phase HPLC procedure (15). The branch portion of the THPs was characterized by mass spectrometry, whereas the collagen-like sequence was characterized by Edman degradation sequence analysis (13, 16). THPs were homogeneous by analytical reversed-phase HPLC. SSPs containing sequences derived from the  $\alpha 1(I)$  or  $\alpha 2(I)$  chain of human type I collagen were synthesized by using Fmoc methodology on a Gilson automated multiple peptide synthesizer AMS 422 (17). Peptides were assembled on Fmoc-DMPAMP resin (Nova Biochem). All SSPs were purified by reversed-phase HPLC and characterized by Edman degradation sequence analysis, mass spectrometry, and analytical reversed-phase HPLC (18, 19). Circular dichroism (CD) spectroscopy was performed on a Jasco 710 spectropolarimeter (Easton, MD) in a 0.01-cm cell. Samples were dissolved in 0.5% acetic acid at concentrations of 0.5–1.0 mM. The triple-helicity of peptides was evaluated from CD spectra as detailed (20).

**Endothelial Tube Formation Assay.** Assays were performed as described (21). Briefly, 24-well tissue culture plates (Corning) were coated with 75  $\mu\text{g}$  of acid-soluble type I collagen. Endothelial cells, passages 4–6, were plated in confluent monolayers of  $3.0\text{--}3.4 \times 10^5$  cells per well in complete medium and were placed in the incubator. The next day, the medium was replaced with 500  $\mu\text{l}$  of fresh medium and either 75  $\mu\text{g}$  of acid-soluble type I collagen in 17.5 mM acetic acid or an equal volume of carrier was added. Some cultures received THPs in medium 199 either in place of the type I collagen or before its addition. The cultures were returned to the incubator for 4 hr and then were fixed in 0.4% formaldehyde in PBS and stained for study of morphology by using the Leukostat kit (Fisher).

**Collagen Fibrillogenesis Assay.** Type I collagen fibrillogenesis was studied as described in ref. 22, with slight modifications. To wells of 96-well Costar microplate dishes were added 100  $\mu\text{l}$  of 2 $\times$  buffer (0.28 M NaCl/60 mM sodium phosphate, pH 7.3) and either 50  $\mu\text{l}$  of 2–4 mg/ml aqueous solutions of THPs or water (controls). The solutions were mixed by pipetting, and 50  $\mu\text{l}$  of 0.4 mg/ml rat tail tendon type I collagen in 10 mM acetic acid was added, and the samples were mixed. Plates were covered with lids coated with fog-X (Unelko; Scottsdale, AZ) to reduce condensation, and absorbance at  $\lambda$

= 405 nm was monitored for 3 hr at 1.5-min intervals at 37°C in a microplate spectrophotometer (Molecular Devices).

**Electrophoretic Analysis of Binding of Heparin to Type I Collagen.** Binding of radioiodinated heparin to type I collagen and peptides was studied by affinity coelectrophoresis (ACE) (23), because we have shown that heparin–collagen binding affinities revealed by ACE match reasonably well with those obtained by other well established quantitative techniques for measuring binding interactions—e.g., see refs. 6 and 24–26. Collagen was dissolved in 0.5 M acetic acid at 2.4 mg/ml, and was serially diluted in 0.5 M acetic acid. Samples were neutralized with 0.5 M NaOH, mixed with warm 2 $\times$  ACE running buffer [1 $\times$  buffer is 50 mM sodium 3-(*N*-morpholino)-2-hydroxypropanesulfonate (MOPSO)/125 mM sodium acetate, pH 7.0], mixed with 2% agarose, and poured into agarose wells. Peptides were serially diluted in 1 $\times$  running buffer, then mixed 1:1 with 2% agarose and loaded into wells. Electrophoresis of radioiodinated heparins through the collagen- or peptide-containing wells was then conducted (23). Gels were dried and heparin mobility was measured with a PhosphorImager (Molecular Dynamics) by scanning each protein lane and determining relative radioactivity content per 88- $\mu\text{m}$  pixel along the length of the lane. Calculation of retardation coefficients (*R*), curve fitting of binding isotherms, and determination of apparent  $K_d$  values were as detailed in refs. 9 and 23.

## RESULTS AND DISCUSSION

Angiogenesis involves endothelial cell proliferation, migration, and capillary tube formation (27, 28). Endothelial cell surface–type I collagen interactions are believed important in angiogenesis. For example, angiogenesis in the chicken embryo was disrupted by inhibiting collagen triple helix formation with  $\alpha, \alpha$ -dipyridyl or by inhibiting collagen fibrillogenesis with  $\beta$ -aminopropionitrile (29). Furthermore, type I collagen expression precedes angiogenesis *in vitro* (30). The endothelium is proposed to exert pulling forces on matrices, forming “matrical tracks” for endothelial cord development (31). Endothelial cells grown between collagen gels form branching networks of tubes (32, 33), and in HUVEC monolayers, angiogenesis rapidly proceeds in the presence of type I collagen and sulfated glycosaminoglycans (GAGs) (21, 34). The signals mediated by type I collagen may be transduced by integrins (35), and it seems probable that HSPGs may also contribute to cell–collagen interactions of angiogenesis, because the endothelium produces HSPGs (36), which may bind collagens (24), and evidence suggests a role for GAGs in angiogenesis (34, 37, 38). Therefore, we have examined the structural basis of type I collagen–heparin interactions and their function in endothelial tube formation.

**THP Synthesis.** As functional probes for these studies, THPs and linear peptides containing collagen I sequences were synthesized by using a solid-phase covalent branching protocol (12, 13). The branch is incorporated at the C terminus of the triple helix, which is consistent with the natural nucleation of collagen triple helices from the C to the N termini. Branching

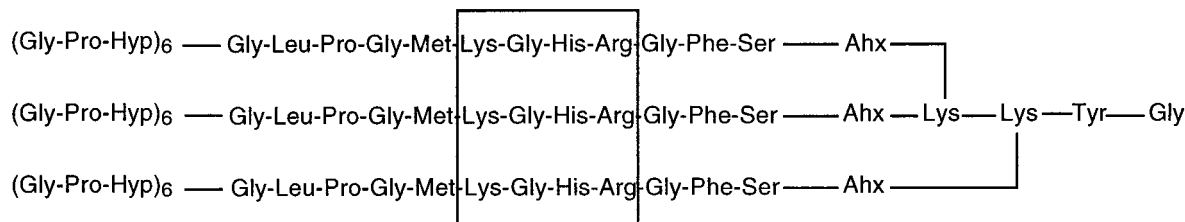


FIG. 1. Collagenous THP structure. Linkages between lysine and aminohexanoic acid groups carry 10–12 stretches of test amino acid sequences followed by 6 triplets of collagenous repeats that induce and stabilize the triple helix. This THP carries the heparin-binding site of the  $\alpha 1$  chain of type I collagen (boxed).

Table 1. Collagenous peptides used in this study

Peptide	Amino acid sequence
$\alpha 1(I)82-93$ THP	[(GPP*) <sub>6</sub> -GLPGMKGHRGFS-Ahx] <sub>3</sub> -KKYG
$\alpha 1(I)82-93X$ THP	[(GPP*) <sub>6</sub> -GFLGRSGMKGDH-Ahx] <sub>3</sub> -KKYG
$\alpha 2(I)85-94$ THP	[(GPP*) <sub>6</sub> -GFKGIRGHSG-Ahx] <sub>3</sub> -KKYG
$\alpha 2(I)85-94X$ THP	[(GPP*) <sub>6</sub> -GRSGFKGIH-Ahx] <sub>3</sub> -KKYG
$\alpha 1(I)787-796$ THP	[(GPP*) <sub>6</sub> -GQRGERGFPG-Ahx] <sub>3</sub> -KKYG
$\alpha 1(I)925-937$ THP	[(GPP*) <sub>6</sub> -GDRGIKGRGFSG-Ahx] <sub>3</sub> -KKYG
(GPP*) <sub>8</sub>	[(GPP*) <sub>8</sub> -Ahx] <sub>3</sub> -KKYG
$\alpha 1(I)82-94X$ SSP	GFLGRSGMKGPHG
$\alpha 2(I)85-94$ SSP	GFKGIRGHSG
$\alpha 2(I)85-94X$ SSP	GRSGFKGIHG
$\alpha 1(I)787-796$ SSP	GQRGERGFPG
$\alpha 1(I)925-937$ SSP	GDRGIKGRGFSG

THPS carry sequences corresponding to amino acid positions from the human sequences (49, 50) as numbered from the beginning of the type I collagen triple helix:  $\alpha 1(I)82-93$  THP and  $\alpha 2(I)85-94$  THP, which include the proposed N-terminal heparin-binding domain of type I collagen (6) or the same sequences in scrambled order as denoted by an X following the amino acid position designation;  $\alpha 1(I)925-937$  THP comprises a C-terminal site homologous to the proposed heparin-binding site. Scrambled sequences of these sites are also shown. Other THPs include one containing the slightly basic sequence at positions  $\alpha 1(I)787-796$  THP and another carrying eight repeats of the collagenous triplet Gly-Pro-Hyp, (GPP\*)<sub>8</sub>. SSPs carrying native or scrambled sequences selected from those carried by the THPs are denoted by SSP; those carrying scrambled sequences are denoted by X SSP.

was achieved by synthesizing (Lys)<sub>2</sub>-Tyr-Gly-Sasrin resin and incorporating Fmoc-6-aminohexanoic acid (Ahx) onto three amino termini to provide a flexible spacer. The collagen sequence of interest was then incorporated, followed by repeats of Gly-Pro-Hyp to induce triple helicity (Fig. 1). The THPs synthesized are shown in Table 1.

**Collagen-Heparin Binding.** The heparin-binding characteristics of native type I collagen and of the peptides were examined by ACE. For native type I collagen, significant heparin-binding affinities ( $K_d \approx 150$  nM) were observed as reported (6, 24) (Fig. 2 and Table 2). Interestingly, THPs carrying sequences that we proposed as a candidate heparin-binding site (6) [ $\alpha 1(I)82-93$  THP or  $\alpha 2(I)85-94$ ], a homologous sequence near the C terminus  $\alpha 1(I)925-937$  THP, and THPs containing scrambled sequences of these sites showed significant binding to low  $M_r$  heparin ( $K_d \approx 2-3 \mu M$ ); these affinities were only about one order of magnitude weaker than

Table 2. Apparent affinities of heparins for collagenous peptides and type I collagen

Collagen	Peptide	$K_d$ , nM	
		Low $M_r$ heparin	Medium $M_r$ heparin
Type I		165 ± 21	14 ± 0
	$\alpha 1(I)82-93$ THP	1,700 ± 80	
	$\alpha 1(I)82-93X$ THP	3,080 ± 680	
	$\alpha 2(I)85-94$ THP	3,500 ± 2,080	
	$\alpha 2(I)85-94X$ THP	2,000 ± 100	
	$\alpha 1(I)787-796$ THP	190,000 ± 184,000	
	$\alpha 1(I)925-937$ THP	1,630 ± 790	245 ± 21
	(GPP*) <sub>8</sub>	Not detectable	
	SSPs	Not detectable	

$k_d$  values are derived from binding plots such as those shown in Fig. 2. Peptide sequences are shown in Table 1. Each sample was tested for binding to low  $M_r$  heparin three to nine times, with an average of five times, and type I collagen and  $\alpha 1(I)925-937$  were each tested for binding to medium  $M_r$  heparin two times.

the affinity displayed by native type I collagen. The stronger affinities exhibited by collagen may be due to the fact that heparin-binding sites should be distributed at roughly 7.5-nm intervals across the fibril, and at 67-nm intervals along the length of the fibril; such multiple binding sites may contribute to cooperativity in heparin binding (6). Additionally,  $\alpha 1(I)925-937$  THP or type I collagen bound even more tightly to medium  $M_r$  heparin ( $K_d \approx 250$  nM and 14 nM, respectively). The heparin-binding  $K_d$  values of these peptides were  $\approx 250-3000$  nM (depending on heparin  $M_r$ ), values that are reasonably strong since the  $K_d$  values for the interactions between heparins and various native polyvalent forms of type I collagen were  $\approx 1.0$  to 200 nM (refs. 6 and 24 and unpublished data). In contrast, the basic control  $\alpha 1(I)787-796$  THP, the collagenous control peptide, and all of the single-stranded sequences representing native or scrambled sequences showed negligible affinities for heparin ( $K_d \gg 10 \mu M$ ) (Fig. 2 and Table 2).

That THPs containing the native-type sequences of the N- or C-terminal basic domains bind heparin similarly to those carrying scrambled sequences implies that the types and numbers of residues within a triple-helical conformation, and not their arrangement, confers high-affinity heparin binding to collagen. This binding likely requires the triple-helical conformation because the side chains of the basic amino acid residues are constrained to be clustered along a short linear distance and to project outwards, potentially promoting ligand interactions. Although the  $\alpha 1(I)82-93$  THP and  $\alpha 1(I)925-937$  THP differ in total number of charged residues, with the former containing up to 9 basic and the latter up to 12 basic and 3 acidic (depending upon histidine ionizations), their net charges are similar, and they bind heparin similarly. The  $\alpha 1(I)787-796$  THP, which carries a net charge of +3, and the uncharged (GPP\*)<sub>8</sub> THP bound heparin negligibly. Potentially, the many other type I collagen domains carrying net basic charges up to +6 might be expected to bind heparin at most with only weak affinities, especially because virtually all of these are close to clusters of acidic amino acids in the native protein (6). The acidic residues may influence proteoglycan (PG) binding by the basic domains through charge repulsion, or by forming salt bridges with them (39).

In the native protein, heparin-gold bound significantly to the N-terminal region, and although some binding was seen near the C-terminal region (6), there were not enough binding events to conclude that the latter region serves as a heparin-binding site. However, in the present study the basic C-terminal sequence  $\alpha 1(I)925-937$  THP bound heparin with high affinity. One way to explain these findings is to speculate that post-translational modifications near the C-terminal site

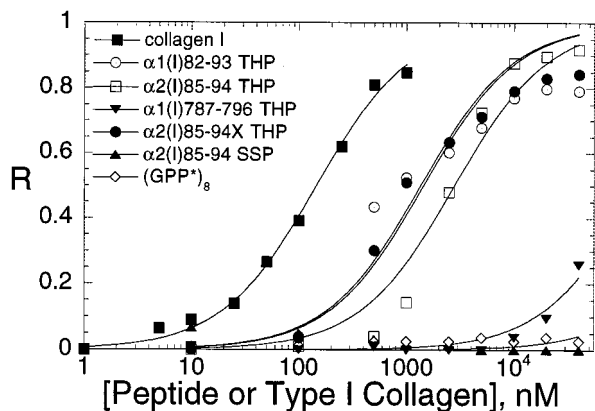


FIG. 2. Calculation of affinities of heparin for collagenous peptides and type I collagen. From ACE gel electrophoretograms, retardation coefficients ( $R$ ) for heparin migration were determined and plotted against protein concentration. Smooth curves represent nonlinear least-squares fits to the equation  $R = R_{\infty} / (1 + (K_d/[protein])^2)$ . THP sequences and apparent  $K_d$  values for heparin binding derived from ACE gels are shown in Tables 1 and 2.



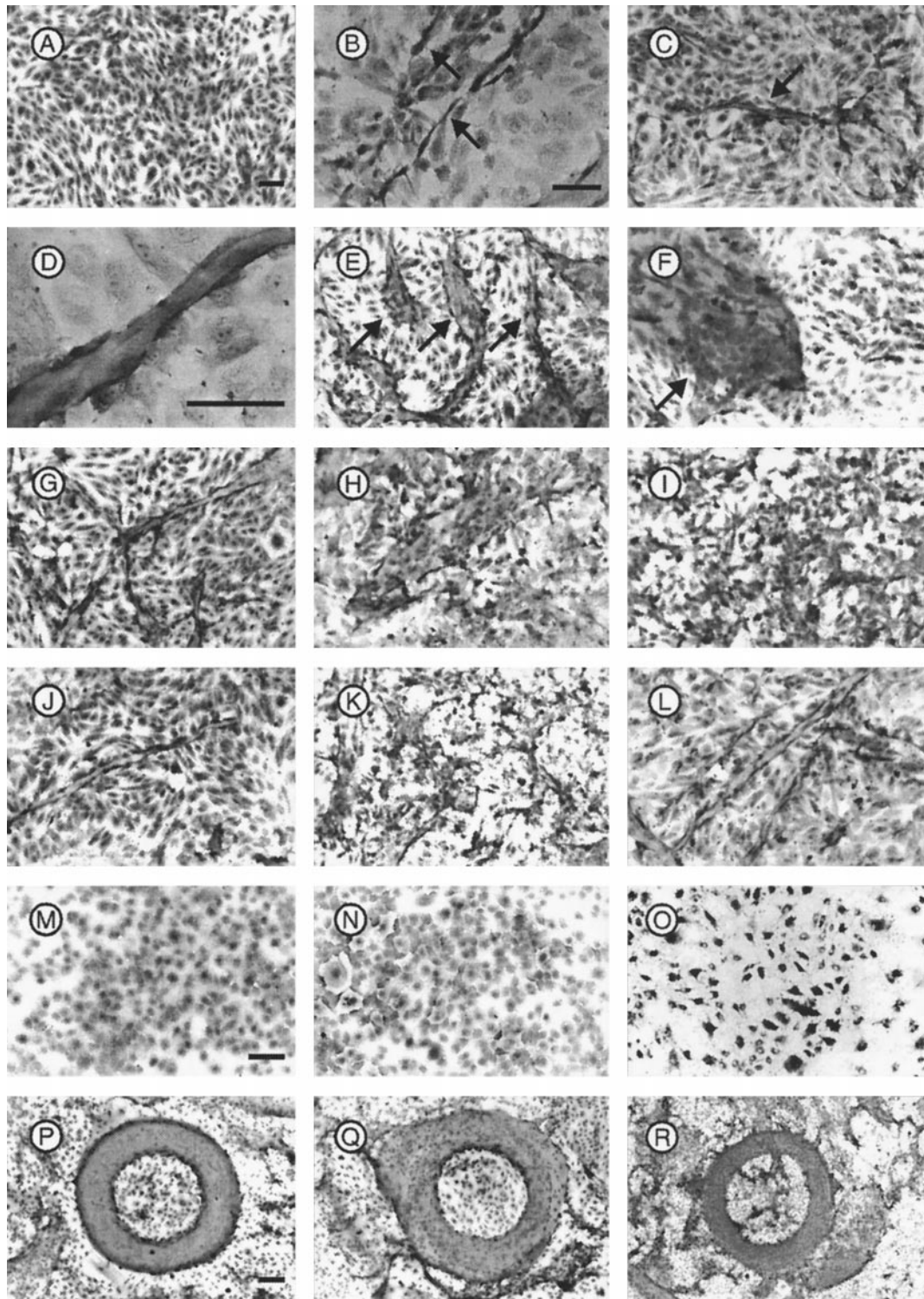


FIG. 3. Structural characteristics of type I collagen required for induction of endothelial tube formation. Effects of collagenous peptides on collagen-induced angiogenesis, or of various forms of collagen in place of native type I collagen were studied as described in the text. (A and M) Control HUVEC monolayers. (B-F and P-R) Cultures receiving 75  $\mu\text{g}$  of native type I collagen show cellular strands (B, arrows), capillary-like tubes (C, arrows; D), cellular cords (E, arrows), collagen clumps (F, arrow), and infrequently, large collagenous structures  $\approx 2$  mm in diameter (P-R). (N and O) Cultures receiving 125  $\mu\text{g}$  of denatured type I collagen (N), procollagen (data not shown), or type V collagen (O) in place of native type I collagen showed no tube formation. (G-L) Cultures exposed to THPs plus 75  $\mu\text{g}$  of native type I collagen. (G, H, and I) Effect of  $\alpha 2(\text{I})85-94$  THP at 100, 320, and 530  $\mu\text{g}/\text{ml}$  respectively. (J and K) Effect of  $\alpha 1(\text{I})925-937$  THP at 112 and 300  $\mu\text{g}/\text{ml}$  respectively. (L) Effect of  $\alpha 1(\text{I})787-796$  THP at 525  $\mu\text{g}/\text{ml}$ . Inhibition of tube formation was observed only in wells receiving the heparin-avid THPs. (Bar = 10  $\mu\text{m}$  for A-C, and E-L; 38  $\mu\text{m}$  for M-O; and 110  $\mu\text{m}$  for P-R.) On average each peptide was tested at each concentration in two wells per experiment, and in two to four experiments.

in the native protein, or other aspects of the local environment, such as the nature of the flanking sequences, may inhibit heparin binding to that site. Thus, these and past studies (6)

suggest that heparin-type I collagen interactions are sequence independent but site dependent, and likely rely on the basic triple-helical domain present at amino acid positions 87-94

near the N terminus of type I collagen monomer, and at multiple sites within native fibrils.

**Endothelial Cell–Type I Collagen Interactions.** To examine the structural features of collagen necessary for endothelial tube induction, we used a system in which endothelial tubes rapidly form in HUVEC monolayers in the presence of type I collagen and heparin (21, 34). Because tubes form within 4 hr, cell proliferation and migration are not involved and the assay is most representative of the endothelial tube formation phase of angiogenesis. Endothelial tubes formed in this way were found to have typical endothelial cell junctional complexes and to contain type I collagen (21, 32, 33). Type I collagen and sulfated GAGs were required for the effect, but laminin or type IV collagen and heparin were inactive (21, 34), and anti-VLA-2 antibodies, which block  $\alpha_2\beta_1$  integrin function, inhibited collagen-induced tube formation (35). We first tested the effects of various forms of collagen or the peptides (Table 1) as the inducer.

In response to the addition of 75  $\mu\text{g}$  of type I collagen to endothelial monolayers, we observed the appearance of thin lines of cells, endothelial tubes on top of the monolayers, thick cellular cords, and widespread deposition of collagen clumps (Fig. 3*B–F*), as detailed previously (21). Cell monolayers receiving 17.5 mM acetic acid (carrier) instead of collagen I retained the typical endothelial cobblestone morphology (Fig. 3*A* and *M*). On average, about 30 capillary-like tubes such as those shown in Fig. 3*C* and *D* were formed per culture, although each cell layer contained many large collagen strands and clumps, which appeared to be HUVEC-associated. In fact, widespread collagen deposition on the cell layers was obvious even by visual inspection of the cultures (data not shown). In a few experiments we observed the formation of large ( $\approx 2$  mm in diameter) circular structures associated with the HUVEC layers (Fig. 3*P–R*). These objects were grossly reminiscent of histological cross sections of blood vessels, but appeared to be composed mostly of collagen on the basis of their overall light pink staining, although many cells were associated with them. To test the role of type I collagen conformation in the induction of these endothelial behaviors, we tested whether denatured type I collagen (gelatin), which lacks triple-helical domains, or monomeric type I collagen (procollagen), which is triple helical but does not form fibrils, could be used in place of native type I as the inducer, but we found no effects (Fig. 3*N*, procollagen data not shown). Because type I collagen–heparin interactions were previously shown necessary for endothelial tube induction, we tested the strongest heparin-binding collagen, type V collagen, but no tube formation was observed, although a disruptive effect on the cell layer was evident (Fig. 3*O*).

Next, the sequence dependency of the type I collagen effect was examined by testing the activities of the various peptides from  $\approx 100$ – $600$   $\mu\text{g}/\text{ml}$  alone, in the absence of the type I collagen but in the presence of heparin. None of the peptides had any effect on the HUVEC layers up to 600  $\mu\text{g}/\text{ml}$  (data not shown). We next tested the peptides as competitive disrupters of type I collagen-mediated HUVEC tube induction, by their addition just before that of the collagen. Interestingly, the heparin-binding THPs inhibited type I collagen-induced effects in a concentration-dependent manner. Thus, the  $\alpha 2(1)85$ – $94$  THP and the  $\alpha 1(1)82$ – $93$  THP showed no effect on tube formation at 100  $\mu\text{g}/\text{ml}$  and caused a mild cell layer disorganization and inhibited most of the tube formation and collagen deposition at  $\approx 300$   $\mu\text{g}/\text{ml}$ ; at  $\approx 500$   $\mu\text{g}/\text{ml}$ , cell layers were highly disorganized and no tubes or collagen deposition were evident [Fig. 3*G–I*;  $\alpha 1(1)82$ – $93$  THP data not shown]. The  $\alpha 1(1)925$ – $937$  THP was even more active; although it had no effect at 100  $\mu\text{g}/\text{ml}$ , it abolished endothelial cell–collagen interactions at  $\approx 300$   $\mu\text{g}/\text{ml}$  (Fig. 3*J* and *K*). In contrast, the control  $\alpha 1(1)787$ – $796$  THP or (GPP\*)<sub>8</sub> (100–600  $\mu\text{g}/\text{ml}$ ), and all of the SSPs ( $\approx 200$ – $600$   $\mu\text{g}/\text{ml}$ ) showed no

effect on type I collagen-induced cellular behaviors [Fig. 3*L*; (GPP\*)<sub>8</sub> and SSP data not shown].

The disruptive effect of the heparin-binding THPs on endothelial cell–collagen interactions could be mediated by interfering with collagen fibrillogenesis, resulting in the formation of complexes that are inactive on the endothelial cells. Alternatively, the THPs could directly bind to the heparin in the medium or the cell surface HSPGs, thereby disrupting the interactions between collagen fibrils and heparin or the fibril–heparin complex and cells. In this regard, it is relevant to consider the effects of the THPs on collagen deposition. Thus, after type I collagen had been added to HUVEC cultures, many collagen clumps were distributed over the culture surfaces; in contrast, in cultures also treated with the most heparin-avid THPs, collagen deposition was inhibited in a concentration-dependent manner, whereas control THPs showed no effect (data not shown). Thus, the THPs may be acting at even the earliest step in HUVEC tube formation—i.e., by inhibiting collagen fibrillogenesis. Therefore we studied the effects of the THPs in a collagen fibrillogenesis assay in the absence of HUVECs. It was found that several of the THPs exert minor reproducible effects on both the time course and extent of collagen fibrillogenesis; however, their effects do not correlate with their activities on endothelial tube formation (data not shown). The finding that the THPs alone had no effect on the HUVECs, but when present with collagen caused cell layer disorganization, suggests that they may act by disrupting HUVEC interactions with collagen–heparin complexes. Finally, further work must establish whether the induction of endothelial tube formation by type I collagen and heparin, as well as the effects of certain THPs on this process, may also require the activity of additional culture medium or HUVEC-derived factors, such as fibronectin, a collagen- and heparin-binding protein shown to regulate angiogenesis (see, e.g., ref. 40). In summary, our studies indicate that induction of endothelial tube morphogenesis by type I collagen depends at least in part upon its triple-helical and fibrillar conformations, as well as the presence of the N-terminal heparin-binding site identified here.

*In vivo*, the requirement for sulfated GAGs in angiogenesis could be provided by HSPGs, or by type I collagen-associated PGs such as decorin. Interestingly, up-regulation of decorin expression correlates with endothelial tube formation (41). Furthermore, that native decorin influences collagen fibrillogenesis, whereas fragments of the core protein do not (42, 43), and that transgenic knock-out mice lacking this PG show altered collagen fibril structure (44) suggest that decorin or a similar PG could regulate fibril architecture or contribute to forming a collagen–GAG complex required as a template for vascular tube formation.

Other reports also implicate various other extracellular matrix domains as important in angiogenesis. Endostatin, a potent endothelial growth inhibitor, is a heparin-binding C-terminal fragment of type XVIII collagen (45). An RGD-containing sequence on the A subunit and the YISGR sequence on the B1 subunit of laminin promote endothelial cell attachment or tube formation, respectively (46, 47). Angiogenesis within fibrin requires the N-terminal sequence of polymerized fibrin II (48). It will be of interest to establish whether these extracellular matrix molecules and type I collagen act in concert to regulate angiogenesis *in vivo*.

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