Identification of a Multifunctional, Cell-binding Peptide Sequence from the a1(NC1) of Type IV Collagen

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Abstract. We have previously identified three distinctive amino acid sequences from type IV collagen which specifically bound to heparin and also inhibited the binding of heparin to intact type IV collagen. One of these chemically synthesized domains, peptide Hep-I, has the sequence TAGSCLRKFSTM and originates from the a1(noncollagenous [NC1]) chain of type IV collagen (Koliakos, G. G., K. K. Koliakos, L. T. Furcht, L. A. Reger, and E. C. Tsilibary. 1989. *J. Biol. Chem.* 264:2313–2323). We describe in this report that this same peptide also bound to intact type IV collagen in solid-phase assays, in a dose-dependent and specific manner. Interactions between peptide Hep-I and type IV collagen in solution resulted in inhibition of the assembly process of this basement membrane glycoprotein. Therefore, peptide Hep-I should represent a major recognition site in type IV collagen when this protein polymerizes to form a network. In addition, solid phase-immobilized peptide Hep-I was able to promote the adhesion and spreading of bovine aortic endothelial cells. When present in solution, peptide Hep-I competed for the binding of these cells to type IV collagen- and NC1 domain-coated substrata in a dose-dependent manner. Furthermore, radiolabeled peptide Hep-I in solution also bound to endothelial cells in a dose-dependent and specific manner. The binding of radiolabeled Hep-I to endothelial cells could be inhibited by an excess of unlabeled peptide. Finally, in the presence of heparin or chondroitin/dermatan sulfate glycosaminoglycan side chains, the binding of endothelial cells to peptide Hep-I and NC1 domain-coated substrates was also inhibited. We conclude that peptide Hep-I should have a number of functions. The role of this type IV collagen-derived sequence in such diverse phenomena as self-association, heparin binding and cell binding and adhesion makes Hep-I a crucial domain involved in the determination of basement membrane ultrastructure and cellular interactions with type IV collagen-containing matrices.

TYPE IV collagen, a glycoprotein of basement membranes, has been described to have several important biological functions. A major function is the ability to self-assemble to a polymer with the form of a lattice (30, 36–39). Type IV collagen not only forms the main structural framework of most basement membranes, but also serves for the binding of other basement membrane components. For example, laminin (8, 20), heparan sulfate proteoglycan (13, 20), and entactin/nidogen (12) have been reported to bind to type IV collagen. An additional important function of type IV collagen is the ability to promote cell adhesion of various cell types, therefore serving as an adhesive protein, like laminin (1, 10, 16, 23).

To better understand several functions of this basement membrane collagen, we have examined the intact molecule and the major proteolytic noncollagenous (NC1)[1] and triple helix–rich domains (both from the a1[IV] and the a2[IV] chains) insofar as binding to cell surfaces (10, 16) is concerned. We have also examined the binding of heparin to intact type IV collagen, the above-mentioned proteolytic domains and peptide sequences from these domains (both from the a1[IV] and the a2[IV] chains). Previously three discrete amino acid sequences of type IV collagen were described that specifically bind to heparin and also inhibit the binding of heparin to type IV collagen (19). In the present report, we extend our earlier observations and describe additional functions of one heparin-binding peptide, peptide Hep-I. This peptide, which originates from the a1(NC1) chain (position 49–60 from the carboxyterminal area), also binds to type IV collagen and therefore should mediate, in part, the assembly process of this glycoprotein. In addition, we report here that peptide Hep-I also promotes the adhesion and spreading of bovine aortic endothelial cells and specifically binds to the surface of these cells.

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1. *Abbreviations used in this paper*: EHS, Engel-Holm-Swarm; NC1, noncollagenous.

Materials and Methods

Isolation of Type IV Collagen and Isolated NC1 Domain

Type IV collagen was isolated from the Engelbreth-Holm-Swarm (EHS) tu-

mor, a noninvasive murine tumor (26) as described previously (19, 30, 36). The concentration of type IV collagen was determined by the method of Waddell (33, 34).

The main (NC1) domain of type IV collagen was isolated by collagenase digestion of EHS-derived type IV collagen as described elsewhere (19, 30, 31). The concentration of isolated NC1 domain of type IV collagen was determined by the method of Lowry (22).

The major triple helical fragment of type IV collagen was isolated by a light digestion of EHS-derived type IV collagen with pepsin as reported elsewhere (16, 19). The concentration of the triple helical, pepsin-derived fragment (which lacks the NC1 domain) was determined by the method of Waddell (33, 34).

Peptide Synthesis and Purification

Peptides were synthesized on a solid phase support resin according to the technique of Barany and Merrifield (2, 19). The synthesized peptides were deprotected and released as reported previously (19). Peptides were checked for purity by HPLC, amino acid analysis and amino acid sequencing. In this study, we used interchangeably HPLC-purified and nonpurified peptides; however, in all cases, the biological activity of each peptide purified by HPLC was tested and found to be similar to that of the nonpurified peptides.

Iodination of Peptides

Several peptides were synthesized with an added tyrosine residue at their carboxy end and were then labeled with ^{125}I-Na (New England Nuclear, Boston, MA) by using the chloramine T method (18). Radiolabeled peptides were purified on SEP-PAK C-18 columns (Waters Associates, Milford, MA) and eluted with 50% acetonitrile in water containing 0.1% (vol/vol) TCA. Eluted peptides were lyophilized and stored at −70°C in a nitrogen atmosphere until further use.

Coupling of Peptides to Ovalbumin

Peptides Hep-I and 1 were coupled to ovalbumin via their cystein residues after activation of the ovalbumin by *m*-maleimido benzoyl-*N*-hydroxysuccinimide ester, according to the method of Liu et al. (21).

Assays for the Binding of Peptide Hep-I to Type IV Collagen

Solid Phase Binding Assays. 50 μl/well of type IV collagen (at 60 μg/ml), the pepsin-derived major triple helix-rich domain of type IV collagen (at 52 μg/ml), the major dimeric NC1 fragment of type IV collagen (at 20 μg/ml) and BSA (at 8 μg/ml) in PBS containing 0.02% azide were coated onto 96-well Immulon plates by drying at 29°C. We reported previously that, under these conditions, 40–50% of each added protein or fragment adhered to Immulon 1 plates (19). The plates were then blocked with BSA (19, 32), and increasing concentrations of 50 μl of ^{125}I-Hep-I or ^{125}I-peptide 1 (a control peptide) were added in a solution containing PBS and 2 mg/ml BSA, pH 7.4, and allowed to interact at 37°C for 2 h. The plates were then washed and the remaining bound radioactivity was solubilized and quantitated as described previously (19, 32).

In competition assays, a constant amount of ^{125}I-peptide (specific or control) was mixed with increasing concentrations of the corresponding unlabeled (specific or control) peptide and the experiment was otherwise performed as described above. The maximal excess of unlabeled peptide used was 400-fold.

These solid phase-binding assays were repeated a minimum of five times triplicate.

Rotary Shadowing Assays. The following solutions were then prepared in PBS: (*a*) type IV collagen only (250 μg/ml); or (*b*) type IV collagen (250 μg/ml) containing either peptide Hep-I or any one of the control peptides, peptide 1 and peptide 4, at 25, 50, 100, 200, 400, and 800 μg/ml. 200 μl of each of the incubated samples were subsequently incubated at 37°C for 1 h. Aliquots of the samples (containing 10–15 μg type IV collagen each) were then processed for rotary shadowing and examined by electron microscopy as described in earlier reports (19, 30–32).

Turbidity Assays. The following solutions were prepared: (*a*) type IV collagen only (at 250 μg/ml); (*b*) type IV collagen (250 μg/ml) containing isolated NC1 domain (20 μg/ml); (*c*) type IV collagen (250 μg/ml) containing isolated NC1 domain (20 μg/ml) and peptide Hep-I or any of the control peptides (100 μg/ml). The development of turbidity by these mixtures at 37°C was followed as described in earlier reports (30–32).

Assays for Interactions between Peptide Hep-I and Endothelial Cells

Bovine aortic endothelial cells were obtained from calf aortas and cultured and prepared for cell adhesion experiments as described elsewhere (16). Endothelial cells used in binding experiments were consistently derived from early passages (≤15 passages were used under our experimental conditions).

Solid Phase–binding Assays

Direct Cell-binding Assays. For these experiments, 96-well Immulon-1 plates were coated with the protein, fragment, or peptide of interest at a range of concentrations. The wells were then treated with a solution containing 2 mg/ml BSA in PBS (pH 7.4) for 2 h, at 37°C. [^{35}S]Methionine-labeled bovine aortic endothelial cells (0.5 mCi/10 ml, overnight) were washed three times with DME and were resuspended in DME containing 2 mg/ml BSA and 25 mM Hepes. The cells (~5,000 cells/well) were then added in the same buffer and were incubated at 37°C for 30–60 min. The plates were then washed with 200 μl of the same buffer three times and the bound radioactivity was released with lysis buffer and quantitated with a scintillation counter (LS 3801; Beckman Instruments, Inc., Palo Alto, CA).

Competition Solid Phase Cell-binding Assays

Competition of Cell Binding in the Presence of Peptides. 96-well, Immulon-1 plates were coated in this instance with type IV collagen at 10 μg/ml, isolated NC1 domain at 20 μg/ml, and BSA at 8 μg/ml in PBS. The assay was then performed as described above, with the following modifications. The cell-containing buffer was mixed with an equal volume of serial dilutions of peptide Hep-I or control peptide in the same buffer. The mixtures were incubated for 15 min at room temperature, were then added (100 μl/well) in the specific substrate-coated wells, and were further incubated for 60 min at 37°C. At the end of the incubation, the plates were washed, and bound radioactivity was solubilized and quantitated by scintillation counting.

Competition of Cell-binding in the Presence of Heparin and Chondroitin/Dermatan Sulfate Side Chains. For these experiments, 96-well Immulon-1 plates were coated with 50 μl/well of a solution of PBS containing (*a*) type IV collagen at 1, 10, or 60 μg/ml; (*b*) isolated NC1 domain (dimeric) at 20 or 60 μg/ml; and (*c*) peptide Hep-I at 50, 100, or 200 μg/ml, and further treated as described above. [^{35}S]methionine-labeled bovine aortic endothelial cells were suspended in DME containing 25 mM Hepes and 2 mg/ml BSA (pH 7.4) at a concentration of 10^5 cells/ml.

The resuspended endothelial cells were then mixed with an equal volume of serial dilutions of heparin or chondroitin/dermatan sulfate (Sigma Chemical Co., St. Louis, MO) in the same buffer. 100 μl from each mixture (containing ~5,000 cells) were added in the specific substrata-coated wells and were incubated for 1 h at 37°C. At the end of the incubation period, the plates were washed, and the remaining bound radioactivity was solubilized and quantitated by scintillation counting.

Most of the control peptides (Table I) were tested in two or more of the direct and competition solid-phase, cell binding assays. All the cell-binding assays (direct or competition) were repeated a minimum of five times in triplicate.

Assays for the Binding of ^{125}I-Hep-I to Endothelial Cells in Solution

Direct Binding. Unlabeled bovine aortic endothelial cells were suspended in DME containing 25 mM Hepes and 2 mg/ml BSA (pH 7.4) at ~10^5 cells/ml.

A constant amount of ^{125}I-Hep-I (~1 μg) was mixed with 250 μl of unlabeled peptide Hep-I (4 mg/ml in the same buffer), and the mixture was serially diluted in the same buffer. Subsequently, aliquots of 50 μl (~5,000 cells) of endothelial cells were added in Eppendorf tubes, followed by 50 μl of each peptide dilution at 0°C, and the samples were incubated at 4°C for 20 min. 900 μl of the same buffer were then added per Eppendorf tube, and the cells were pelleted by centrifugation in a microfuge (Beckman Instruments, Inc.), at 4°C (5 min, 500 *g*). The cells were washed three times and the remaining radioactivity was quantitated with a gamma counter (Gamma Trac-1193; TM Analytic Inc., Elk Grove Village, IL).

Competition of the Binding of ^{125}I-Hep-I to Endothelial Cells by Unlabeled Peptide Hep-I or Control Peptides in Solution. Unlabeled peptide Hep-I or control peptide ET-2 was serially diluted from a starting concen-

Table I. Control and Biologically Active Peptides Used

Peptide	Sequence	Origin	Reference
peptide Hep-I	TAGSCLRKFSTM	a1(IV)	19
peptide Hep-II	LAGSCLARFSTM	a2(IV)	19
peptide-1	DPLCPPGTKIL	a1(IV)	19
peptide-4	MFKKPTPSTLKAGELR	a1(IV)	19
peptide ET-2	GDSRTITTKGERGQP	a2(IV) (561-565 from NH_2: interruption)	this report
peptide J-61	GFPGSRGDTGPP	a1(IV)	10
	GRGDSP	Fibronectin; cell binding domain	26
	GRGDTP	a2(I)	3

tration of 2 mg/ml in DME containing 25 mM Hepes and 2 mg/ml BSA (pH 7.4). 5 μl (~5 μg) of ^{125}I-peptide Hep-I were then added to each serial dilution. Aliquots of ~5,000 endothelial cells in the same buffer were added in Eppendorf tubes followed by 50 μl of each peptide dilution at 0°C. The cell–peptide mixtures were then incubated for 20 min at 4°C, the cells were pelleted and washed, and the binding of ^{125}I-Hep-I in each case was quantitated. In these competition-binding assays, the excess of unlabeled control or Hep-I peptide was 400-fold at the highest peptide concentration. These assays (direct and competition) were repeated a minimum of three times in quadruplicate. Cell viability in the cell-binding assays was tested based on exclusion of trypan blue dye and was found in all cases to be >95%.

Assays for Cell Spreading in Response to Peptide Hep-I

These experiments were performed in 24-well tissue culture plates (Costar, Cambridge, MA) which had been previously coated with 350 μl type IV collagen (10 μg/ml), BSA (5 μg/ml), or peptide Hep-I (20 μg/ml) with the above-mentioned methodology (to achieve similar percent of protein and peptide bound to the tissue culture plastic when compared to the amount bound to Immulon-1, 96-well plates). The plates were blocked with BSA (19, 32), and unlabeled bovine aortic endothelial cells were then incubated with the coated substrates in DME containing 25 mM Hepes and 2 mg/ml BSA (pH 7.4) for 30–90 min at 37°C (~35,000 cells/well). At the end of the incubation time, the plates were washed three times and the cells were fixed by adding 1% glutaraldehyde in PBS, at room temperature. The remaining bound cells were examined by direct visualization with a Nikon Diaphot inverted phase light microscope for spreading. Spreading was quantitated based on the appearance of cells: a prerequisite for cells to be considered "spread" was a totally smooth, flattened appearance that indicated complete spreading. Rounded or partially flattened cells were considered to be in the category of nonspread cells. This type of experiment was performed in quadruplicate.

Results

The Binding of Peptide Hep-I to Type IV Collagen

When ^{125}I-peptide Hep-I was tested for binding to type IV collagen coated onto plastic, it bound in a dose-dependent and saturable manner. Approximately 70 ng of the radiolabeled peptide were bound to type IV collagen (~1.2 μg coated on plastic; reference 19) at the plateau level. This apparent saturation indicated that the binding should be specific (Fig. 1 *A*). Further evidence for the specific nature of the binding between type IV collagen and peptide Hep-I was obtained by experiments in which the binding of ^{125}I-Hep-I was efficiently competed in the presence of an excess of unlabeled peptide Hep-I (Fig. 1 *A*).

Peptide 1 (Table I), a control peptide of similar length and

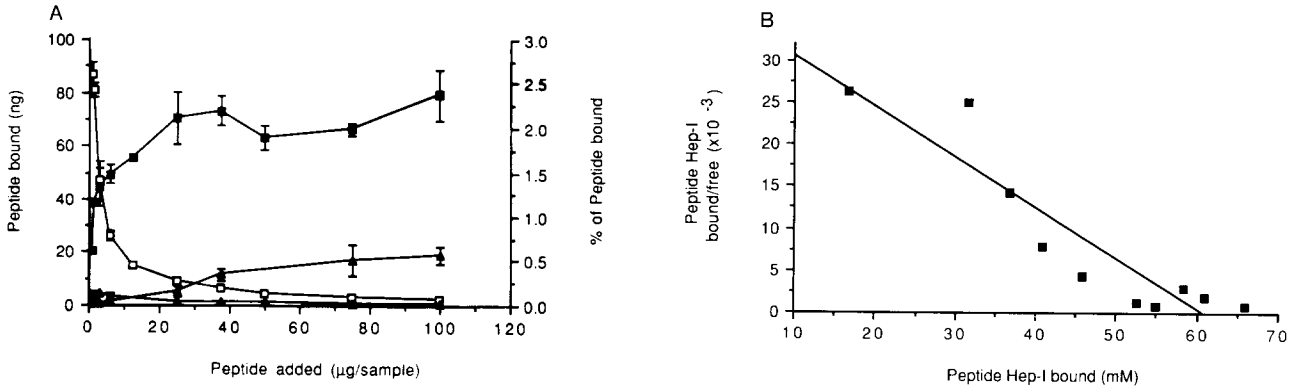


Figure 1. (*A*) Direct binding of peptide Hep-I to solid phase-type IV collagen and competition by unlabeled peptide. Type IV collagen at 60 μg/ml was coated onto 96-well plates (1.3 μg bound/well). For the direct binding, increasing concentrations of ^{125}I-labeled peptide Hep-I (■) or control peptide 1 (▲) were added (50 μl/well of serial dilutions from a starting concentration of 2 mg/ml) and were allowed to incubate for 2 h at 37°C. The radioactivity that remained bound after incubation and washing was plotted as nanograms of peptide bound per actual amount of peptide added per well. For competition, the binding of 5 μg ^{125}I-labeled peptide Hep-I (□) or control peptide (△) in the presence of increasing concentrations of unlabeled peptide Hep-I or peptide 1, respectively, was tested by incubation at 37°C for 2 h. The highest concentration of unlabeled peptide used was 2 mg/ml (400-fold excess). The remaining bound radioactivity after incubation and washes was plotted as percent radioactivity bound per actual amount of peptide added per well. (*B*) Scatchard analysis of a typical direct solid-phase binding experiment in which increasing concentrations of ^{125}I-peptide Hep-I were added to type IV collagen-coated wells (1.3 μg of type IV collagen coated/well). 10 different concentrations were used in triplicate wells to allow for quantitation of the binding.

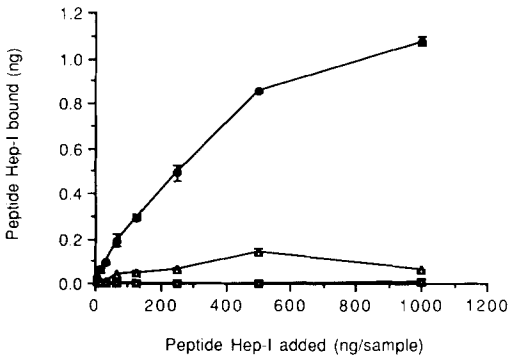


Figure 2. Binding of ^{125}I-Hep-I to solid phase: type IV collagen NC1 and triple helix-rich domains. Equimolar amounts of type IV collagen (60 μg/ml) (●), NC1 domain (20 μg/ml) (□), and triple helix-rich domain (52 μg/ml) (△) were coated onto 96-well plates and increasing concentrations of ^{125}I-Hep-I were added and allowed to incubate for 2 h at 37°C. The radioactivity that remained bound after incubation and washes was plotted per concentration of added peptide.

hydropathy value, bound to a much lesser extent to type IV collagen (maximal binding is ~20 ng) (Fig. 1 *A*). However, the obtained binding could not be competed even by a 400-fold excess of unlabeled control peptide 1, and, therefore, this binding should not be specific (Fig. 1 *A*). In this type of direct and competition-binding assay we used BSA coated onto plastic to test for nonspecific binding of the peptide. This binding was found to be <2% of the total binding of the peptide to type IV collagen-coated substrata at all the concentrations used (data not shown), and was subtracted accordingly before plotting the data (Fig. 1 *A*). Scatchard analysis of the solid-phase binding data (Fig. 1 *B*) suggested that a single class of affinity sites existed in type IV collagen for binding to peptide Hep-I, with an apparent K_d of 1.66 nM (R = 0.9209; $p<<0.001$).

In another series of experiments, the binding of increasing concentrations of ^{125}I-Hep-I to: type IV collagen, NC1 domain and triple helix-rich domain coated onto plastic wells was tested and compared. Under our experimental conditions, radiolabeled peptide Hep-I bound only to intact type IV collagen but failed to bind to either the NC1- or triple helix-rich domains (Fig. 2). Thus, the integrity of type IV collagen was apparently required for interaction(s) with peptide Hep-I.

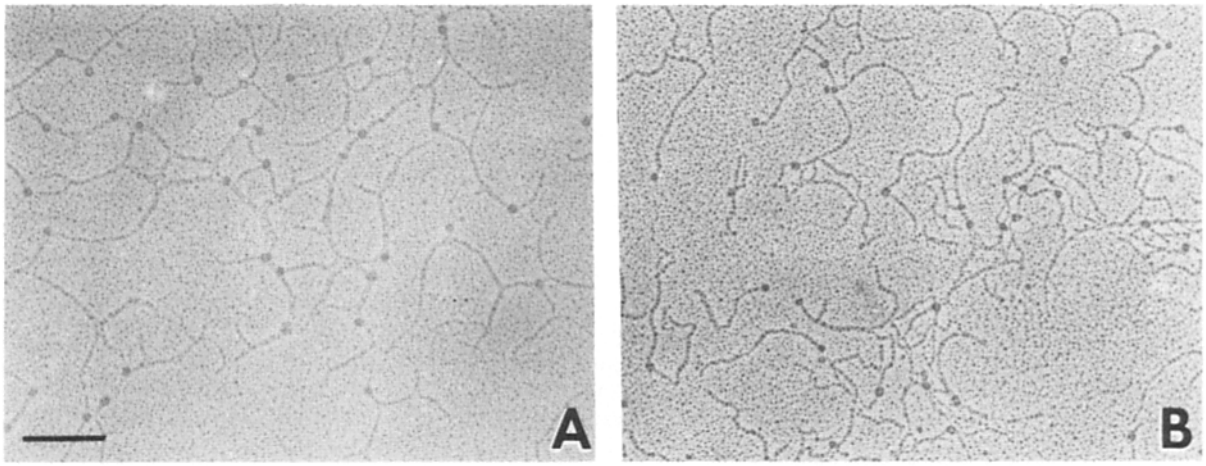
The effect of peptide Hep-I on the process of polymerization of type IV collagen was then tested by the technique of rotary shadowing in double-blind experiments. We and others have previously reported that upon incubation at 37°C, type IV collagen in neutral buffers forms a hexagonal quasi-regular structure (30, 38). For this type of assembly to occur, the NC1 domain is required to bind along the length of the rodlike part of type IV collagen so that subsequent lateral associations can form in a zipperlike manner (30, 37). This latticelike structure was quantitated and was observed to represent ~74% of the total type IV collagen present, the rest being either in dimeric/monomeric form or random aggregates. In the presence of NC1 domain, the lattice formation was decreased to ~30%, an indication that the NC1 domain bound to type IV collagen and competed for the binding of other adjacent type IV collagen molecules and lateral assembly as we reported previously (30, 31). When type IV collagen solutions were coincubated in PBS at 37°C with peptide Hep-I at a range of concentrations per milliliter, the lattice formation was also decreased, thus mimicking the effect of the intact NC1 domain (Fig. 3 *C*). Most of type IV collagen in the presence of peptide Hep-I occurred either in a dimeric/monomeric form or as random aggregates (Fig. 3 *B*). Two other NC1-derived, control peptides, peptides 4 (data not shown), and 1, did not have any effect on lattice formation when each was separately incubated with type IV collagen (Fig. 3, *A* and *C*).

Further evidence for a specific interaction between type IV collagen and peptide Hep-I and an effect on type IV collagen assembly was obtained by turbidimetric assays. In these assays, the turbidity of type IV collagen alone or in the presence of added NC1 domain with and without peptide Hep-I or other control peptides was assessed. When prewarmed at 37°C, type IV collagen (250 μg/ml) raised turbidity to a plateau value, with a typical curve (Fig. 4) (30, 38). Addition of isolated NC1 domain (20 μg/ml) resulted in ~30% decrease of the maximal turbidity of type IV collagen, as reported previously (30, 31). When peptide Hep-I was added in the incubation mixture, it further suppressed the development of turbidity by an additional ~20% (the total decrease of the maximal turbidity was therefore ~50%) (Fig. 4). Thus, peptide Hep-I had an effect similar to that of intact NC1 domain competing for lateral assembly of type IV collagen in turbidity experiments. Two other control peptides from the al(NC1) chain, peptide 1 and peptide 4 (Table I), did not have any additive effect on the maximal turbidity of prewarmed type IV collagen-NC1 domain mixtures (Fig. 4).

In summary, with the above-mentioned approaches: solid phase binding assays, turbidimetry and rotary shadowing, and peptide Hep-I appeared to specifically bind to intact type IV collagen and also inhibited the assembly process of this basement membrane collagen to a networklike polymer.

Role of Peptide Hep-I in Adhesion and Spreading of Bovine Aortic Endothelial Cells

In solid phase-binding experiments, the ability of peptide Hep-I to promote cell adhesion was tested in comparison to intact type IV collagen and the NC1 domain. As reported previously (16), both type IV collagen and also the isolated NC1 domain promoted the adhesion of bovine aortic endothelial cells in a dose-dependent manner. A concentration of type IV collagen as low as 0.05 μg/well resulted in ~20% adhesion whereas at 5 μg/well ~40% adhesion of added ^{35}S-labeled endothelial cells occurred. Intermediate concentrations of type IV collagen also resulted in a similar degree of adhesion. Similarly, NC1 domain coated at 0.5 μg/well resulted in ~25% adhesion of added endothelial cells. At a coating concentration of 2.5 μg/well and 5 μg/well, 30–35% endothelial cell adhesion was observed. Peptide Hep-I coated at the above mentioned concentrations of 0.5, 2.5, and 5 μg/well supported the adhesion of ~10% of added ^{35}S-labeled endothelial cells. Under our experimental conditions, 1–2% of the added peptide was bound to the plastic (19). When coated at higher concentrations (10–25 μg/well) a higher percentage of adhering cells (~25%) was obtained. Coupling of the peptide to ovalbumin did not enhance cell adhesion (data not shown). A control peptide with similar length and hydropathy value, peptide 1, either uncoupled



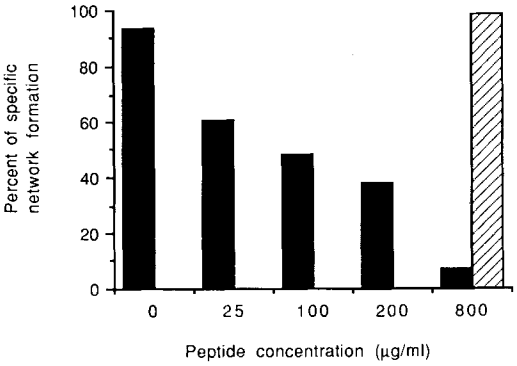


Figure 3. Rotary shadowing images of type IV collagen incubated for 7 h at 37°C in the presence of: (*A*) 800 μg/ml control peptide 1; (*B*) 200 μg/ml peptide Hep-I; (*C*) diagrammatic representation of the percentage of fields containing lateral associations in various samples of type IV collagen that was incubated at 37°C and then examined by rotary shadowing for network formation. Maximal network formation by type IV collagen only was considered to represent 100% of lateral associations under the experimental conditions used. (■) Type IV collagen containing 0, 25, 100, 200, and 800 μg/ml peptide Hep-1; and (▨) type IV collagen containing control peptide 1 (800 μg/ml). A minimum of 150 fields/specimen were scanned in double-blind experiments. Standard deviation was ≤5%.

or coupled to ovalbumin, did not promote any significant cell adhesion.

To further assess the specificity of the interaction(s) between peptide Hep-I and endothelial cell surfaces, the binding of bovine aortic endothelial cells to intact type IV collagen-coated substrata was competed by increasing concentrations of peptide Hep-I. In the presence of as low as 0.2 μg peptide (4 μg/ml) Hep-I, a ~25% decrease of endothelial cell adhesion occurred (Fig. 5). The maximal inhibition of adhesion to type IV collagen was ~50% and was reached when ~5 μg (100 μg/ml) of the specific peptide was coincubated with endothelial cells (Fig. 5). As a control, we used in the same range of concentrations peptide GRGDSP, which as we previously reported, did not have any effect on the pro-

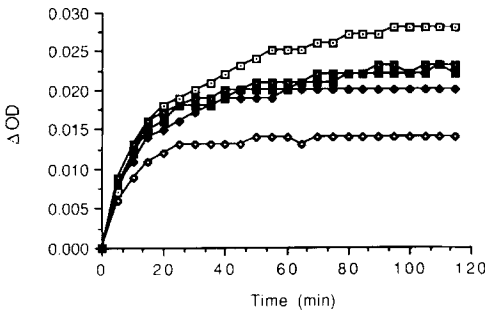


Figure 4. Turbidity of type IV collagen in the absence or presence of NC1 domain and various peptides. The following mixtures were examined and compared for the development of turbidity at 360 nm: type IV collagen (250 μg/ml) (⊡); type IV collagen (250 μg/ml) containing NC1 domain (20 μg/ml) (◆); and, in addition, either peptide Hep-I (100 μg/ml) (◇) or any of the control peptides, either peptide 1 (100 μg/ml) (■) or peptide 4 (100 μg/ml) (□). The temperature was maintained at 37°C throughout the course of the experiment.

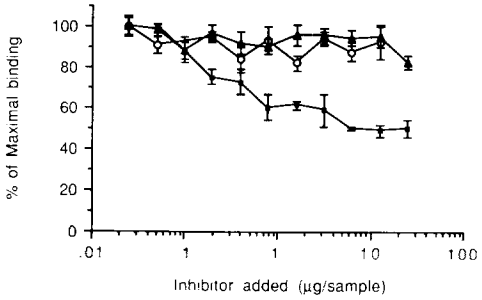


Figure 5. Inhibition of the binding of bovine aortic endothelial cells to solid-phase immobilized type IV collagen by various peptides in solution. [^{35}S]Methionine-labeled cells were incubated with increasing concentrations of peptide Hep-I (■), peptide GRGDSP (○), or peptide 1 (▲) for 15 min at room temperature. Approximately 5,000 cells/peptide concentration were then added per well in triplicate and were further incubated with type IV collagen (coated onto plastic at 10 μg/ml), for 60 min at 37°C. The remaining radioactivity after incubation and washes was expressed as percent cells bound per added concentration of inhibitor (highest concentration of inhibitor-peptide used: 500 μg/ml or 25 μg/well). The amount of cells bound in the absence of inhibitor was considered to represent maximal (100%) binding. Background binding to BSA was <2% and was subtracted.

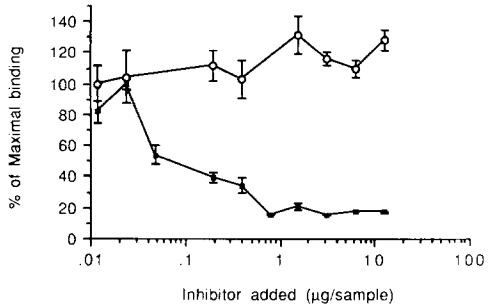


Figure 6. Inhibition of the binding of bovine aortic endothelial cells to solid-phase, immobilized NC1 domain by various peptides in solution. [^{35}S]Methionine-labeled cells were incubated with increasing concentrations of peptide Hep-I (■) or peptide GRGDSP (○) for 15 min at room temperature. Approximately 5,000 cells/peptide concentration were then added per well and were further incubated with NC1 domain-coated in plastic wells (20 μg/ml) for 60 min at 37°C. The remaining radioactivity after incubation and washes was expressed as percent of cells bound per added concentration of inhibitor, per well (highest concentration of inhibitor used was 500 μg/ml). The amount of cells bound in the absence of inhibitor was considered to represent maximal binding (100%). Background binding to BSA was <2% and was subtracted.

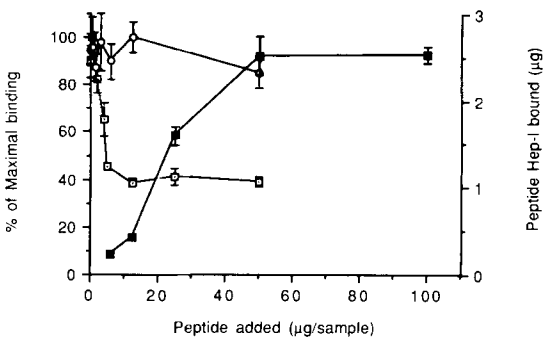


Figure 7. Binding of ^{125}I-peptide Hep-I to bovine aortic endothelial cells and competition of the binding of ^{125}I-peptide Hep-I to bovine aortic endothelial cells by unlabeled peptides in solution. Unlabeled cells were mixed with increasing concentrations of ^{125}I-Hep-I in solution and were incubated for 20 min at 4°C (highest concentration of peptide used was 2 mg/ml). The cells were then pelleted and washed and bound radioactivity was expressed as micrograms of peptide bound per microgram of added peptide (■). In addition, a constant amount of ^{125}I-Hep-I (5 μg) was mixed with increasing concentrations of either unlabeled peptide Hep-I (⊡) or unlabeled control peptide ET-2 (○) (maximal excess of unlabeled peptide used was 400-fold). Unlabeled cells were then incubated with each of the peptide mixtures for 20 min at 4°C. The cells were then pelleted and washed and the remaining radioactivity was plotted as percent of maximal binding per microgram of inhibitor added. Maximal binding was the binding obtained in the absence of inhibitor.

cess of adhesion of bovine aortic endothelial cells to type IV collagen (16) (Fig. 5) and peptide J-61, another RGD-containing peptide from the al(IV) (Table I) (data not shown). Additionally, peptide 1 (Table I), a peptide of similar length and hydropathy value to peptide Hep-I, also had no effect on the adhesion of endothelial cells to type IV collagen-coated substrata (Fig. 5).

The binding of endothelial cells to NC1 domain–coated substrata was dramatically decreased following preincubation of the cells with increasing concentrations of peptide Hep-I. Even in the presence of 0.1 μg (2 μg/ml) of peptide Hep-I, the final cell adhesion was ~50% (Fig. 6), and, with increasing peptide concentrations, the adhesion gradually decreased further; at a concentration of 1 μg (20 μg/ml) peptide Hep-I the obtained adhesion was as low as 20% or slightly less (Fig. 6). Control peptide GRGDSP did not have any effect on endothelial cell adhesion to NC1 domain-coated substrata following coincubation with the endothelial cells (Fig. 6).

Another approach that was used to confirm the specific nature of binding of peptide Hep-I to bovine aortic endothelial cells was the binding of ^{125}I-Hep-I in solution to the surface of these cells. This binding was shown to be dose-dependent and saturable (Fig. 7). Furthermore, the binding of radiolabeled peptide was competed by an excess of unlabeled peptide (maximal excess: 400-fold), further confirming the specificity of interaction(s) between endothelial cell surfaces and peptide Hep-I (Fig. 7). Peptide ET-2, another control peptide from the al(IV) (Table I), failed to compete for the binding of ^{125}I-Hep-I to endothelial cells (Fig. 7).

In cell spreading experiments, ~92.8% of attached endothelial cells were spread within 30 min when intact type IV collagen was the substrate (Fig. 8 *A*), and the percentage did not change substantially throughout the time course of the study. The spreading achieved on substrates of peptide Hep-I was ~60% of attached cells by 30 min (Fig. 8 *C*), 83% by 60 min and 84% by 90 min. Thus, a longer time was required for cells to attain maximal spreading in the case of peptide Hep-I–mediated adhesion when compared to intact type IV collagen-mediated adhesion. Adhesion and spreading to BSA at all time intervals were minimal (1.7–2.2% of added endothelial cells adhered) (Fig. 8 *B*).

The Effect of Heparin on Endothelial Cell Binding to Type IV Collagen, NC1 Domain and Peptide Hep-I

Because peptide Hep-I (from the al[NC1] chain) as well as the NC1 domain and intact type IV collagen specifically bound heparin (19), we tested the effect of heparin on the binding of endothelial cells to type IV collagen, NC1 domain, and peptide Hep-I–coated substrata. We observed that the binding of endothelial cells to intact type IV collagen at all concentrations used (1–60 μg/ml) was unaffected even at the highest concentration of glycosaminoglycans used (Fig. 10) (10 mg/ml, while the cells remained viable based on trypan blue exclusion). In contrast, the binding of endothelial cells to isolated, solid phase-immobilized NC1 domain was substantially decreased in the presence of increasing concentrations of heparin. A significant suppression of cell adhesion (~50%) was observed in the presence of ~0.300 μg/ml (15 μg/sample) heparin (Fig. 9). Chondroitin/dermatan sulfate glycosaminoglycan side chains also had a similar effect (data not shown).

Cell adhesion to peptide Hep-I was similarly suppressed by heparin (Fig. 9), whereas chondroitin/dermatan sulfate was effective in inhibiting cell adhesion only at the highest concentration used, a concentration of 10 mg/ml (500 μg/sample) (data not shown).

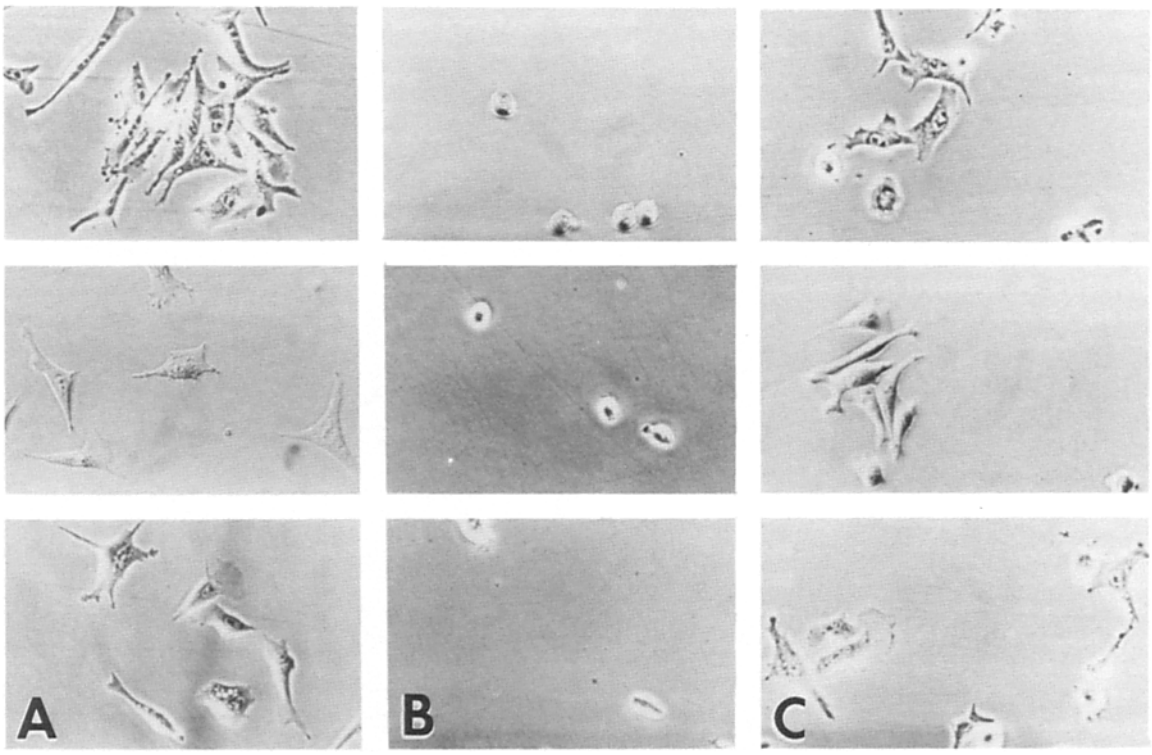


Figure 8. Light micrographs of bovine aortic endothelial cells following a 30-min adhesion to type IV collagen (*A*), BSA (*B*), and peptide Hep-I (*C*). Type IV collagen was coated at 10 μg/ml, BSA at 5 μg/ml and peptide Hep-I at 20 μg/ml. Magnification at 70.

Discussion

In this report, two distinctive functions of peptide Hep-I (Table I) from the al(NC1) chain of type IV collagen are described. A major function of this peptide is to specifically bind to type IV collagen. This binding results in inhibition of assembly of type IV collagen assessed both by turbidimetry and rotary shadowing.

We had reported earlier that the NC1 domain was necessary for the initiation of lateral associations by mediating the binding of adjacent collagen molecules (30). After diabetic modifications by in vitro nonenzymatic glucosylation of the NC1 domain, the ability of this domain to inhibit lateral associations by type IV collagen was perturbed (31). Such a functional impairment should lead to an altered assembly of glucosylated type IV collagen in newly synthesized basement membranes. Because lysine residues are the primary targets for nonenzymatic glucosylation (5, 6, 11), we selected three peptides from the al(NC1) chain that contained all the lysine residues (a total of five) present in this domain of the al(IV) chain (25, 28, 29). It was reasoned that if at least one of these lysine residues was part of a sequence binding to type IV collagen and if it were modified by glucosylation, this might account at least in part for the impairment of the function of glycated NC1 domain. The three al(NC1)-derived peptides that were synthesized were peptides 1, 4, and Hep-I (Table I). These peptides were then tested for binding to type IV collagen, and only peptide Hep-1 was found to specifically bind to this glycoprotein. The specificity of the interaction was demonstrated by the saturable nature of the binding and the efficient competition of the binding of ^{125}I-Hep-I to type IV collagen by an excess of unlabeled Hep-I. The affinity of the interaction(s) between type IV collagen and peptide Hep-I was substantial as indicated by the obtained K_d of 1.66 nM. This specific peptide, but not any of the other tested NC1-derived peptides, could also mimic the effect of intact NC1 domain when used at a range of concentrations (25–800 μg/ml) by competing for development of turbidity and lattice formation in rotary shadowing images. Thus, the sequence represented in peptide Hep-I should probably be a major molecular determinant in the noncollagenous domain that is used for recognition by type IV collagen. It is worth noting

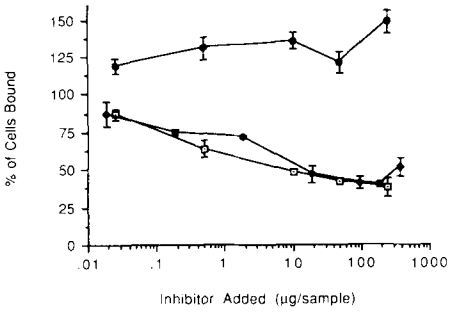


Figure 9. ^{35}S Methionine-labeled endothelial cells were mixed with increasing concentrations of heparin and were then added to type IV collagen-plated at 60 μg/ml (●); NC1 domain plated at 60 μg/ml (◆); and peptide Hep-I plated at 200 μg/ml (⊡). The mixtures were incubated for 60 min at 37°C. The radioactivity remaining after incubation and washes was expressed as percent cells bound per concentration after added competitor. The radioactivity obtained in the absence of competitor was considered to represent maximal binding (100%).

that only intact type IV collagen was able to bind to peptide Hep-I. Isolated dimeric NC1 domain failed to bind to this peptide, indicating that this sequence does not bind to itself or any other sequence of the NC1 domain. In addition, pepsin-derived type IV collagen also failed to bind to this specific peptide under our experimental conditions. Pepsin-derived type IV collagen lacks the NC1 domain but should also have several discontinuities of the triple helix-rich segment at least partially disrupted at the sites of cleavage by pepsin (4, 19). Therefore, the obtained data indicate that interactions between peptide Hep-I and the rodlike part of type IV collagen should probably occur primarily at sites where the triplet motif is interrupted by noncollagenous sequences.

Peptide Hep-I was also described in a previous report to specifically bind to heparin and to inhibit the binding of heparin to type IV collagen in a dose-dependent manner (19). Because heparin-related structures often occur in heparan sulfate proteoglycan and, in particular, because aortic endothelial cells have been reported to contain sequences identical to heparin in their heparan sulfate proteoglycan (24), we examined interactions between bovine aortic endothelial cells and peptide Hep-I. We had previously reported that aortic endothelial cells preferably adhered to type IV collagen and isolated NC1 domain as compared to fibronectin or laminin (16). In the present study, the adhesion of bovine aortic endothelial cells to substrates of type IV collagen, NC1 domain, and peptide Hep-I was compared. Peptide Hep-I was able to promote adhesion of this cell type in a dose-dependent manner (other cell types including microvascular endothelial cells and murine melanoma cells also adhered in preliminary observations). This cell adhesion was apparently specific for this sequence since it was not sustained in the presence of control peptides. Of specific interest is the lack of inhibition of adhesion of cells to type IV collagen and the NC1 domain by RGD-containing peptides that efficiently inhibit cell attachment to fibronectin (14–16, 27). Thus, these data confirm our previous report (16) in that they indicate an attachment to type IV collagen which is independent of RGD-containing sequences (including an RGD containing peptide from the al[IV], Table I). However, the adhesion of cells to both the intact type IV collagen and the isolated NC1 domain was dependent on peptide Hep-I; a partial inhibition (~50%) of endothelial cell adhesion to intact solid phase-type IV collagen was obtained under our experimental conditions. This finding indicates that multiple determinants in type IV collagen may interact with endothelial cell surfaces and that peptide Hep-I is one of them. A much higher inhibition of cell adhesion (~80%) was obtained on substrates coated with NC1 domain. Therefore peptide Hep-I should be a major determinant for cell recognition in the NC1 domain. Radiolabeled peptide Hep-I was also found to bind in solution to endothelial cells in a dose-dependent and saturable manner. An excess of unlabeled peptide efficiently competed for this binding, confirming the specificity of this interaction. The site(s) recognized by peptide Hep-I on cell surfaces remains to be elucidated. Because peptide Hep-I also bound heparin, the possibility exists that heparin-related side chains of one or more species of cell surface-heparan sulfate proteoglycan may partially mediate binding to the sequence represented in peptide Hep-I. This possibility is reinforced by the competition of adhesion of endothelial cells to NC1 domain and this specific peptide, in the presence of heparin. Because a substantial suppression of cell binding to NC1 coated onto plastic was obtained in the presence of heparin and chondroitin/dermatan sulfate glycosaminoglycan side chains, it seems likely that the NC1 domain may primarily mediate charge interactions between basement membrane collagen and endothelial cells. However, our experiments do not exclude the possibility that the protein core of one or more cell surface proteoglycans or additional glycoproteins or other components also serve for binding to peptide Hep-I, the NC1 domain or type IV collagen. The lack of competition by glycosaminoglycans for the binding of endothelial cells to intact type IV collagen implies that charge-mediated interactions (attributed to the side chains of cell surface proteoglycans) represent only one of many possible interactions and that other binding events should also occur which need to be explored.

Another point of interest was the ability of peptide Hep-I to sustain not only endothelial cell adhesion but a substantial cell spreading as well. Interactions between cells and extracellular matrix components including type IV collagen should comprise a series of events in a temporal scheme: (*a*) adhesion occurs; and then (*b*) cytoskeletal reorganization that leads to cell spreading, with the formation of focal adhesion and focal contacts (7, 35). Peptide Hep-I was able to promote the cytoskeletal organization required for spreading to occur, although a longer time interval was required when compared to intact type IV collagen.

The ability of a distinctive amino acid sequence of a large extracellular molecule, like basement membrane collagen, to perform more than one function could possibly allow for a multiplicity of regulatory events. For example, depending upon the local or temporal requirements, affinities of interactions, and availability of reacting candidates, this microdomain could have the flexibility to participate in a variety of possible interactions that in turn would result in a variety of structures. Thus, by the use of the same repertoire of reactants, structural and functional diversity could emerge depending upon different interactions (9). This then could account at least in part for the multiplicity of structure and function of different basement membranes.

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