A heparin binding, cell adhesion promoting domain, termed peptide F-9, from the B1 chain of human laminin, residues 641 to 660, i.e. RYVVLPRPV-CFEKGMNYTVR, has been investigated by $^1$H NMR (500 MHz) spectroscopy and CD spectropolarimetry. While small linear peptides in water solution normally exist in a number of fluctuating conformational states, CD data analysis of peptide F9 indicates the existence of some preferred average structural populations consisting of about 30% $\beta$-sheet, 22% $\beta$-turn, and 6% $\alpha$-helix. NMR structural analysis supports this observation and indicates specific sequences of preferred structural populations. Evidence for this is indicated by the presence of $d_{NN}$ nuclear Overhauser effect (NOE) populations and attenuated or absent $d_{NN}$ NOEs at short mixing times (0.1 s). $\gamma_{NN}$ coupling constants of 5 and 10 Hz, and chemical shifts significantly removed from random coil positions. The NH-terminal VVL sequence primarily exists in an extended chain conformation by virtue of large $d_{NN}$ NOEs and 9–10 Hz $\gamma_{NN}$ coupling constants. Residues C10–N16 have turn-like or helix character with a run of $d_{NN}$ and $d_{NN}$ NOEs and attenuated $d_{NN}$ NOEs. These midchain reversals include the lysine and asparagine residues proposed to be involved in heparin binding and N-glycosylation, respectively, to laminin peptide F-9.

Laminin is the major glycoprotein (850 kDa) of basement membranes where it serves as both a structural and biologically active component (Martin and Timpl, 1987). Laminin appears responsible for such interactions as cell adhesion and proliferation (Timpl et al., 1983), maintenance of the basal epithelial cell surface (Sugrue and Hay, 1981), and has been shown to play an important role in tumor cell metastasis (Terranova et al., 1980). Furthermore, there are reports that laminin and its receptors are present in greater amounts on highly metastatic cells compared to cells with low metastatic potential (Malinoff and Wicha, 1983; Malinoff et al., 1984; McCoy et al., 1984; Ito et al., 1985).

The three subunits of laminin (A, 400 kDa; B1, 215 kDa; and B2, 205 kDa) are disulfide-linked (Yurchenko et al., 1985).

$^*$This research was supported by National Institutes of Health Grants CA 29995, CA 21463, and DK 32660 and by Shared Instrumentation Grant RR-04040 from the National Institutes of Health.

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From rotary shadowing techniques, laminin appears cruciform in shape and consists of one long and three short arms, each of which possesses globular domains at their ends (von der Mark and Kuhl, 1985). Due to its size and shape, laminin can span the basement membrane and can bind various substances on cell surfaces (Martin and Timpl, 1987). For example, laminin binds heparin (Sakashita et al., 1980) at three distinct structural domains (Skubitz et al., 1988). Several biologically active peptides have been synthesized by solid phase techniques (Charonis et al., 1988) from one of these domains located on the B1 chain. In particular, one of these peptides, termed F-9, residues 641–660, i.e. RYVVLPRPV-CFEKGMNYTVR (Sasaki et al., 1987), shows specific binding to heparin and competes with laminin in promoting cell adhesion. Charonis et al. (1988) have also shown that acetylation of the lysyl 653 €-amino group greatly attenuates laminin F-9 heparin binding. Polyclonal antibodies raised against peptide F-9 bind to intact laminin (Skubitz et al., 1990), suggesting that the peptide F-9 sequence in laminin is solvent-exposed and that the peptide F-9 structure may be conserved relative to that in intact laminin.

Considering the biological significance of laminin-derived peptide F-9 in cell matrix molecular binding, the question of a possible preferred solution conformation arises. This present $^1$H NMR (500 MHz) and CD study is focused on secondary structure analysis of peptide F-9. While small linear peptides generally exist in solution as an ensemble of highly fluctuating, conformational states yielding average structural parameters, peptide F-9 seems to exist in preferred conformation(s).

**MATERIALS AND METHODS**

Pep tide Synthesis—The F-9 peptide of laminin, RYVVLPRPV-CFEKGMNYTVR (residues 641–660 in the intact laminin B1 chain), was synthesized by solid phase methods as previously described (Charonis et al., 1988) and HPLC-purified on a C-18 reverse phase column by using a linear acetonitrile-0.1% trifluoroacetic acid gradient. Pure F-9 was eluted at 30% acetonitrile; an oxidized, interchain disulfide-linked F-9 species was also found to elute at 35% acetonitrile. The oxidized species could be easily reduced by reaction with a 2 M excess of 2-mercaptoethanol prior to HPLC purification. Identity of the peptide was confirmed by amino acid analysis and amino-terminal sequencing. For simplicity, residues are assigned sequence numbers 1 through 20 rather than the residue positions in the parent laminin B1 chain given above.

$^1$H NMR Spectroscopy—Pep tide samples for NMR measurements were dissolved in either D$_2$O or H$_2$O/D$_2$O (9:1) at a concentration of 1 The abbreviations used are: HPLC, high pressure liquid chromatography; TSP, sodium 3-(trimethylsilyl)-1-propanesulfonate; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; COSY, two-dimensional scalar-correlated spectroscopy; HOHAHA, two-dimensional homonuclear magnetization transfer spectroscopy (HOMONUCLEAR HANNAH).
Circular Dichroism—CD spectra were measured on a JASCO J-720 automatic recording spectropolarimeter that had been calibrated with camphorsulfonic acid. Spectra were recorded over 185-280 nm using a 2-mm path length quartz cuvette. The scan speed was 10 nm/min. Curves were recorded digitally and fed through a data processor for signal averaging (16 times) and baseline subtraction. The ellipticity is reported as the mean residue ellipticity, $\theta$, in units of degrees cm$^2$/dmol. The program VARSELEC, provided by Dr. W. Curtis Johnson (Merck, Sharp and Dohme Research Labs), was used for secondary structure analysis.

Peptide concentration was determined by measuring the absorbance at 280 nm and by using a calculated extinction coefficient of 2980 M$^{-1}$ cm$^{-1}$. The peptide concentration used here was 40 mM in 20 mM sodium phosphate buffer. To prevent cysteine oxidation, a molar ratio of 2-mercaptoethanol was added to all NMR samples, and the pH was maintained at pH 3.5. At higher pH values, peptide F-9 solubility decreased, and cysteine oxidation rates were too rapid to maintain a homogeneous population during NMR experiments. Possible intermolecular disulfide bond formation was frequently monitored by HPLC and NMR spectral comparison; no such oxidation was observed in any data sets discussed in this paper. All spectra were acquired at 500 MHz on a General Electric GN-0-500 NMR spectrometer. The temperature was controlled at 288 K.

Sequential resonance assignments were obtained from COSY (Aue et al., 1976; Wider et al., 1984) and NOESY (Jeener et al., 1979) experiments. The mixing times in the NOESY spectra were varied at 100, 300, and 600 ms to check for possible spin diffusion and to allow normally weak NOEs to become more apparent. To further identify complete spin systems, HOHAHA spectra were collected by spin-locking with the MLEV-17 sequence (2ax and Davis, 1985) for 65 ms. The intense water resonance was suppressed by direct irradiation for 1.5 s. All spectra were acquired and processed in the phase-sensitive mode (States et al., 1989). The majority of two-dimensional NMR spectra were collected with 1K x 1K (t1 x t2) data points. 64 scans were accumulated per t1 experiment. Prior to Fourier transformation, the data sets were multiplied in both dimensions by a sine or squared sine function shifted by 30 to 60 degrees. 3J,HH coupling constants were estimated from double quantum filtered COSY spectra. All chemical shifts are reported downfield from TSP.

RESULTS

Circular Dichroism—The far-ultraviolet CD spectrum of laminin peptide F-9 is shown in Fig. 1. The spectrum gives a strong negative ellipticity maximum at 196 nm with a small shoulder near 218 nm. Weak positive and negative ellipticities are also observed at 230 and 235 nm, respectively. This spectrum differs appreciably from CD spectra of peptides known to assume random-coil conformations in solution. Although a negative signal near 200 nm could be associated with disordered structure, the broad nature of the band suggests some structural contributions. Analysis of this CD spectrum by using the variable selection method of Manavalan and Johnson (1987) indicates that laminin peptide F-9 is 6% (±3%) helix, 22% (±8%) $\beta$-turn, and 30% (±8%) $\beta$-sheet with 42% being considered random coil. Using these estimates, a spectral fit from the program VARSELEC (Manavalan and Johnson, 1987) is given by the dashed line in Fig. 1.

NMR Resonance Assignments—Spin systems were assigned using a standard approach (Wüthrich, 1986) by first analyzing COSY and HOHAHA spectra. Inspection of the HOHAHA spectrum (Fig. 2) permits many spin systems to be assigned by total J-correlation from backbone NH through terminal side chain CH resonances.

Sequential assignments were made by correlating NOESY and COSY/HOHAHA spectra. NOESY spectra were accumulated with mixing times of 0.1 s, 0.3 s, and 0.6 s to assure reasonable amplitudes for all through space $\alpha$CH $\rightarrow$ NH connectivities and to check for possible spin diffusion. Since the time dependence of the growth of NOE magnitudes (cross-peak volume integrals) was monotonic and generally changed much less between 0.3 s and 0.6 s than between 0.1 s and 0.3 s, it was assumed that spin diffusion did not significantly play a role in observed NOE magnitudes. Fig. 3 gives a 0.1-s mixing time NOE map showing sequence specific assignments. The positions of COSY cross-peaks are boxed-in, and solid lines connect sequential NOEs. A typical CO$^\delta$-type spectrum of the $\alpha$N fingerprint region is given in Fig. 4 for comparison in order to justify the locations of empty boxes in the NOE map. Starting at the well resolved G14 NH $\leftrightarrow$ K13 $\alpha$CH NOE cross-peak, sequential NOEs can be unambiguously traced to the carboxyl-terminal Y17 NH and to the amino-terminal P8 $\alpha$CH. The G14-M15 d$_{\alpha N}$ NOE could not be observed above the noise at 0.1 s mixing time; at 0.3 s and 0.6 s, it appeared relatively weak. The absence of an $\alpha$CH $\rightarrow$ NH COSY-type cross-peak in proline residues prevents uninterrupted $\alpha$CH $\rightarrow$ NH fingerprint tracing from P8 and likewise from P6 to their respective amino-terminal residues. This sequential assignment is made by observation of through-space NOE cross-peaks between proline $\delta CH_2$ resonances and the $\alpha$CH resonance of the amino-terminal neighbor. Specific proline assignments, however, are made by correlating NOESY cross-peaks between the proline $\alpha$CH and carboxyl-terminal neighbor NH resonances. Carboxyl-terminal residues were assigned unambiguously by correlations from the unique T18 to the terminal R20 residue. Sequential assignments from R1 to V4 were easily followed from L5. Mostly complete proton resonance assignments are given in Table I.

NMR Structure Analysis—Dominant solution structures for short linear peptides like laminin F-9 are often not found; rather, such peptides exist in a multitude of fluctuating conformational states. Highly fluctuating peptide structures in NMR experiments generally give rise to averaged NMR parameters characterized, for example, by $J_{\alpha N}$ coupling constants of 6.5-8 Hz for most amino acid residues (Gly is 5.6 Hz, and Phe is 9.4 Hz) (Kessler and Berrill, 1986); by similar magnitude $d_{\alpha NOE}$ and absent $d_{\alpha NH}$ NOEs, and by random coil chemical shifts (Bundib and Wüthrich, 1979). Evidence for preferred solution structure(s) in laminin F-9 comes from observation of $J_{\alpha N}$ coupling constants of 5 Hz for K13 and of 9-10 Hz for V3, V4, and L5; non-random coil chemical shifts; highly varied $d_{\alpha N}$ NOE magnitudes; and $d_{\alpha N}$ NOE cross-peaks between F11 and E12, and among K13, G14, M15, and N16 as shown in Fig. 5.
NMR of Laminin Peptide F-9

**FIG. 2.** HOHAHA spectrum of peptide F-9. The contour plot of the amide ($f_2$) and upfield ($f_1$) region for F-9 is displayed. The 10 mM sample was prepared with 20 mM phosphate at pH 3.5 and 288 K in 90% H$_2$O and 10% D$_2$O. The water resonance was suppressed by direct irradiation for 1 s prior to the preparation pulse. The data were collected as 1K X 1K hypercomplex data points. Prior to Fourier transformation, the data were multiplied by a 50°-shifted squared sine function in both dimensions. Labeling of resonances is described in the text.

Structural information derived from NMR data is summarized in Fig. 6. The amino acid sequence for F-9 is given by the accepted one-letter codes. Above the sequence, chemical shift differences for NH, $\alpha$CH, and $\beta$CH resonances relative to those for random coil positions taken from Wüthrich (1986) are indicated by the thickness of blocks as explained below. For this comparison, F-9 chemical shifts at pH 3.5 and 35 °C were used. Wüthrich (1986) used pH 7 and 35 °C. The lower pH is expected to affect chemical shifts for E12 and R20 where carboxylate group protonation occurs via this pH

**FIG. 3.** Fingerprint region of the NOESY spectrum of F-9. The backbone NH $\rightarrow \alpha$CH region of a NOESY contour plot has been correlated with information from a COSY experiment to exemplify sequential resonance assignments. The NOESY mixing time was 100 ms. Solution conditions are as described in the Fig. 2 legend. Labeling of resonances is as described in the text and has been done for most of the NOESY cross-peaks in this composite. COSY cross-peak positions are boxed and indicated by residue number. The data set was collected as 1024 hypercomplex free induction decays containing 1K words and then zero-filled to 1K in the $f_1$ dimension and multiplied by a 50°-shifted squared sine function prior to Fourier transformation.

**FIG. 4.** COSY contour plot for laminin peptide F-9. The $\alpha$N fingerprint region from a double quantum filtered COSY spectrum of peptide F-9 is shown. Cross-peaks are labeled as discussed in the text. Data sets were collected as 512 hypercomplex free induction decays each containing 2K words and were processed by zero-filling to 1024 in the $t_1$ domain and by multiplying the raw data by a 30°-shifted squared sine function in $t_1$ and $t_2$ prior to Fourier transformation. Other conditions are as described in the text.
change. Due to cysteine oxidation as discussed previously, higher pH values could not be used. Chemical shift differences less than 0.1 ppm in either direction were considered insignificant, and, therefore, are considered zero in this summary. A 0.1-0.2-ppm chemical shift difference is shown as a small filled-in block, while a difference greater than 0.2 ppm and then greater than 0.3 ppm is indicated by increasing block thickness. An F-9 value more upfield shifted than its random coil position faces downward, and a more downfield chemical shift difference faces upward. Chemical shift comparisons with random coil positions can be qualitatively, structurally informative when analyzed in conjunction with other data as presented here. These values should vary somewhat, especially where inter-residue contact domains and regions of significant conformational variance are involved. Not all resonances respond equally to these factors; therefore, it is difficult to be definitive about the origins of these chemical shift changes. When compared to random coil positions, many chemical shifts for F-9 are significantly changed. Changes at the amino and carboxyl termini, i.e. residues 1 and 20, may simply be due to terminal effects, whereas two intrasequence regions of significant change are noted: P6-R7-P8 and C10 through N16. Resonances belonging to these residues show the largest chemical shift differences.

Fig. 6 also summarizes those $^3J_{NN}$ coupling constants which vary from typical random coil values which, for most amino acid residues, generally range from 6 to 8 Hz (Kessler and Berman, 1986). Most $^3J_{NN}$ values for F-9 are 6.5-7.5 Hz, which for such a small, linear peptide are structurally uninformative; for that reason, these values are not printed in the figure. At the bottom of the figure is shown a summary of observed NOEs as defined by Wüthrich (1986). Since NOE magnitudes varied considerably at 0.1-s mixing time (Fig. 3), relative magnitudes are indicated by the thickness of bars; a thicker bar equals a larger NOE.

Amino-terminal $d_{NN}$ NOEs are not observed under any conditions, and $d_{NN}$ NOEs are consistently large suggesting a weighting toward an extended chain conformation. Moreover, amino-terminal residues V3-V4-L5 all show 8.5-10 Hz $^3J_{NN}$ coupling constants supporting the idea that the major conformational population in this short segment is extended chain structure. The Chou and Fasman (1978) predictive secondary
structure algorithm also indicates that the sequence YYVL has a high \( \beta \)-sheet potential.

The midpeptide segment which runs from about C10 through N16 also exists in a preferred conformation(s). It is typified by several \( d_{\alpha\alpha} \) and \( d_{\alpha\beta} \) NOEs as indicated in Figs. 5 and 6. Moreover, those residues which show \( d_{\alpha\alpha} \) NOEs also generally have attenuated \( d_{\alpha\beta} \) NOEs. In particular, the F11-E12 and G14-M15 \( d_{\alpha\alpha} \) NOEs at 0.1-\( s \) mixing time are relatively highly attenuated. Normally in small linear peptides, \( d_{\alpha\alpha} \) NOEs are averaged, showing similar magnitudes, and \( d_{\alpha\beta} \) NOEs are not observed. Significant structural elements for a turn centered at K13-G14 are noted. K13 has a \( J_{\alpha\beta} \) coupling constant of 5 Hz with a relatively medium to large \( d_{\alpha\beta} \) NOE with the G14 NH, which in turn shows an NOE to only one of its nondegenerate \( \alpha\beta \) resonances. At 0.1-\( s \) mixing time, the G14-M15 \( d_{\alpha\alpha} \) NOE cross-peak is nearly unobserved, while at 0.3 s, it appears weak. The expected G14-M15 \( d_{\alpha\beta} \) NOE, however, is observed even at 0.1 s. Moreover, at longer mixing times, a weak \( d_{\alpha\beta} \) NOE between K13 and M15 is observed. The \( d_{\alpha\alpha} \) NOE between M15 and N16 is small, while a \( d_{\alpha\beta} \) NOE is observed. Chemical shifts within this domain are also removed from random coil positions. In all, a multiple turn or helix-like conformation seems most probable within this midpeptide sequence domain.

The 4 carboxyl-terminal residues do not seem to exist in any preferred solution conformation. No \( d_{\alpha\alpha} \) NOEs are present; \( J_{\alpha\beta} \) coupling constants are mostly 7.5 Hz, and many chemical shifts are not significantly removed from random coil positions.

**DISCUSSION**

Short, linear peptides in water solution generally exist in an ensemble of highly fluctuating conformational states. Recent evidence, however, for example, from isolated ribonucleic acid S-peptide (20 residues) (Kim and Baldwin, 1984), carboxyl-terminal residues 69-96 from *Themisthe zostericola* myohemerythrin (Dyson et al., 1988a), and carboxyl-terminal residues 385-411 from human fibrinogen \( \gamma \)-chain (Mayo et al., 1990) indicate significant populations of helical structure. Highly populated \( \beta \)-turn conformations are evidenced by an immunogenic peptide from influenza virus hemagglutinin (Dyson et al., 1985, 1986, 1988b), fibronectin GRGDSP peptide (Reed et al., 1988), fibrinogen pro-\( \alpha \)-chain (Ni et al., 1989) and \( \gamma \)-chain (Mayo et al., 1990), and type IV collagen triple helix peptide IV-H1 (Mayo et al., 1991). In all these cases, preferred solution conformations are found.

Polycyclonal antibodies raised against laminin peptide F-9 bind to intact laminin (Skobits et al., 1990) suggesting that, in parent laminin, the peptide F-9 segment is solvent-exposed and that the conformation of peptide F-9 in solution is maintained sufficiently to show immunological cross-reactivity. The present NMR and CD structural study does indeed suggest that laminin-derived peptide F-9 exists in water solution with preferred conformation(s). CD data suggest approximately 30\% \( \pm 8\% \) \( \beta \)-sheet, 22\% \( \pm 8\% \) \( \beta \)-turn, and 6\% \( \pm 3\% \) helix potential. NMR data indicate that amino-terminal residues YVL prefer an extended chain structure, while mid-F-9 heptapeptide segment, i.e. CFEGKMN, exists in apparent multiple turn or helix conformations. Both proline residues predominantly exist in the normally observed trans conformation; moreover, proline \( d_{\alpha\beta} \) NOEs are large, suggesting backbone kinks in the structure. The remainder of the carboxyl-terminal segment, i.e. Y17 to R20, is structurally indescript. From NMR structural analysis, it is apparent that at least half of the 20 residues show some type of \( \beta \)-structure or multiple turn-like characteristics. Similar trends from CD and NMR, therefore, are generally noted. In fact, the C10-N16 segment, which by NM2 data is shown to be conformationally weighted toward multiple turn or helix-like structure, accounts for 35\% of the residues in F9. This is close to that value estimated by CD analysis, i.e. 22\% \( \pm 8\% \) \( \beta \)-turn plus 6\% \( \pm 3\% \) \( \alpha \)-helix. Although only the VVL sequence gives a clear indication of \( \beta \)-sheet structure, Y2 and the PRPVP sequence may also exist in \( \beta \)-sheet-like structure. This value from NMR data is then also in line with the CD conformational estimate.

The interaction of laminin with proteoglycans is important to the formation and, perhaps, degradation of basement membranes (Kleinman et al., 1985). Laminin peptide F-9 binds heparin (Charonis et al., 1988). Acetylation of K13 greatly attenuates the binding of heparin to peptide F-9 (Charonis et al., 1988), suggesting that this lysine residue plays a critical role in the heparin binding process. Interestingly, K13 in the midregion of laminin peptide F-9 is part of a preferred chain reversal domain. In fact, due to its position in the middle of this conformation, K13 is probably structurally constrained. Assuming that the conformation of the free peptide at pH 3.5 resembles that of the receptor-bound peptide at pH 7.4, it may be that this structure is important for heparin binding and, perhaps, binding of laminin to various proteoglycans.

Sasaki and collaborators (1987) have proposed 13 potential sites for \( N \)-linked glycosylation in the laminin B1 chain, as well as 14 sites in the homologous B2 chain. Each of these potential carbohydrate acceptor sites has the common amino acid sequence, N-X-S/T. Such a sequence is present at the carboxyl terminus of laminin peptide F-9 with N16, Y17, and T18. The important asparagine residue is on the carboxyl-terminal end of a chain reversal that runs from K13. Furthermore, this chain reversal appears helix-like with a continuous run of relatively short \( d_{\alpha\alpha} \) distances and relatively long \( d_{\alpha\beta} \) distances. This being the case, K13 would be proximal to N16 with one turn of the helix. Perhaps heparin interaction at K13 in peptide F-9 is structurally related to \( N \)-glycosylation in laminin. Once again, this assumes structural conservation between peptide F-9 as investigated here at pH 3.5 and the receptor-bound peptide at pH 7.4.

Since the amino-terminal fragment of peptide F-9 which contains the cell adhesion promoting activity\(^\ast\) seems to adopt a relatively well defined, extended chain structure, a computer search for homologous amino-terminal sequences was performed of all known protein and peptide sequences. Of the first 7 amino-terminal residues, a sequence of five amino acids, i.e. VVLPR, was found in five proteins: the hexon-associated protein (VIII) from mastadenovirus h2 (Galibert et al., 1979; Herisse et al., 1980), phosphoribosylamine-glycine ligase from *Bacillus subtilis* (Ebbole and Zalkin, 1987), and human angiotensinogen precursor (Kageyama et al., 1980), phosphoribosylamine-glycine ligase from *Saccharomyces cerevisiae* (Carr et al., 1985). It remains to be seen whether these amino-terminal amino acid residues of laminin peptide F-9 contain the functional activity attributed to any of these various bacterial, yeast, and human proteins. Sequence homology is also noted between the highly conserved amino-terminal regions of the \( \alpha \)- and \( \beta \)-chains of fibrinogen (Laudano et al., 1983) and the laminin peptide F-9 amino-terminal segment. The tripeptide PRP repeat, usually amino-terminally preceded by a Gly residue, is present in fibrinogen homologues. These fibrinogen terminal sequences likely function as binding sites for fibrinogen polymerization. The laminin F-9 peptide residues are not amino-terminal in
parent laminin, nor is an amino-terminal Gly residue present, but there may be sufficient structural homology to effect similar interactions. The (trans)P-R-(trans)P conformation observed in laminin peptide F-9 may be structurally and biologically significant.

CONCLUSIONS

In summary, these types of studies should provide information regarding the structural characteristics of cell adhesion promoting and glycosaminoglycan binding peptides derived from sequences of basement membrane and extracellular matrix molecules. Future NMR-peptide binding studies with low molecular weight heparin analogues and receptor protein binding domains will more accurately address the question of biologically active peptide F-9 structure.

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