Mechanism of Laminin Chain Assembly into a Triple-Stranded Coiled-Coil Structure†

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ABSTRACT: Laminin, a basement membrane glycoprotein, is a heterotrimer with α, β, and γ chains held together by a triple-stranded α-helical coiled-coil structure. Recently, a short peptide sequence at the C-terminus of the α-helical domain of each chain was identified as a critical site for the initiation of laminin chain assembly. Synthetic peptides, B1 and B2 (51-mers from the mouse laminin β1 and γ1 chains, respectively) and M (55-mer from the laminin α2 chain), containing these sites were able to assemble into a triple-stranded coiled-coil structure with chain-specific interactions [Nomizu, M., Otaka, A., Utani, A., Roller, P. P., & Yamada, Y. (1994) *J. Biol. Chem. 269*, 30386−30392]. Here we focus on the mechanism of laminin assembly and examine the conformation and stability of the peptides under various conditions using circular dichroism (CD) spectroscopy. Dependence on chain length for the conformation and stability of trimers suggests that 51-mers for laminin β1 and γ1 chains and a 55-mer for the laminin α2 chain are critical to attain high thermal stability (T_m = 62 °C), similar to the larger fragments (approximately 200-mers) and to intact laminins. Since the conformation and stability are dependent on pH and the B1 and B2 monomers and the B1−B2 dimer conformations are partially destroyed at neutral pH, it is likely that they contain intra- and/or interchain repulsions by acidic residues. Moreover, the B1−B2 dimer was significantly more stable under acidic conditions, while the B1−B2/M trimer appears to dissociate into separate B1−B2 and M peptides at pH 2. Urea-induced denaturation showed that the B1−B2/M was more stable than the B1−B2, while both complexes showed virtually identical guanidine hydrochloride denaturation curves. Our data indicate that ionic interactions between B1−B2 and M are critical for the specific trimer formation. We propose a mechanism for laminin assembly: (1) A heterodimer B1−B2 is preferentially formed and creates an acidic pocket which provides a less stable structure due to intra- and intermolecular repulsions between acidic amino acids. (2) A basic site in the M peptide interacts specifically with the acidic pocket of the B1−B2 dimer and results in assembly into a more stable triple-stranded coiled-coil structure.

Laminins are large heterotrimeric glycoproteins specifically located in the basement membranes. Several laminin isoforms have been identified with at least eight genetically distinct subunits (Paulsson, 1993; Burgeson et al., 1994). They serve diverse biological functions including the promotion of cell adhesion, growth, migration, and differentiation, and influencing the metastatic potential of tumor cells (Martin et al., 1988; Timpl, 1989; Beck et al., 1990; Engel, 1993). Laminin-1 consists of three chains designated α1, β1, and γ1, which form a cruciform-shaped molecule. Chain assembly within the laminin family is mediated through the α-helical long arm region of each chain via a parallel triple-stranded α-helical coiled-coil structure in the long arm region of laminin with each chain contributing approximately 570 amino acids (Engel et al., 1981; Sasaki et al., 1987, 1988; Sasaki & Yamada, 1987; Beck et al., 1990, 1993). Elucidation of the molecular mechanism of laminin assembly is critical for a better understanding of the biopathway (Morita et al., 1985) and biological functions of laminins.

It has been shown that proteolytic fragments E8 and C8−9, consisting of either the carboxy third or the entire long arm of laminin-1, respectively, can be reconstituted after denaturation and reduction to a structure indistinguishable from that of the native molecule (Paulsson et al., 1985; Hunter et al., 1990, 1992). Recently, we have demonstrated that a short sequence from the carboxy terminus of the long arm of each chain is required to initiate the assembly of the laminin chains (Utani et al., 1994, 1995). Site-directed mutagenesis studies suggested that charged amino acid residues within these short sequences were important for the specific formation of double- and triple-stranded coiled-coil structures (Utani et al., 1994, 1995). Based on these previous studies where various lengths of recombinant proteins were used, we focused on the C-terminal region (approximately 50 amino acids) of the long arm, which was deemed to be the critical region of dimer and trimer assembly. Three chemically synthesized peptide segments of the three subunits corresponding to this region were found to assemble into

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double- and triple-stranded coiled-coil structures with chain-specific interactions, and the heterotrimer of the synthetic peptides showed a high thermal stability ($T_m = 62$ °C) similar to those of the recombinant trimers (approximately 200 amino acids long) (Nomizu et al., 1994). Further, the hydrophobic residues, such as the isoleucine in the laminin α2 and γ1 chains, were found to be crucial for stabilizing the heterotrimeric coiled-coil structure (Nomizu et al., 1994).

Double-stranded coiled-coil structures such as tropomyosin (Zhu et al., 1992; Zhou et al., 1992; Monera et al., 1993, 1994) and the dimerization domain of the DNA binding proteins c-Fos and c-Jun (O'Shea et al., 1989, 1992) have previously been studied in detail using synthetic peptide segments. Ionic interchain interactions at the "*e*" and "*g*" positions of the heptad repeat motif $(abcdefg)_n$ were shown to be essential, and destabilization of the Fos homodimer by acidic residues was shown to provide a major thermodynamic driving force for the preferential Fos−Jun heterodimer formation (O'Shea et al., 1992). The critical factors contributing to the stability of heterotrimeric coiled-coil structures, however, are less well understood compared to the double-stranded coiled-coil structures. Here we focus on the mechanism of coiled-coil formation in the laminin-2 molecule which consists of the α2, β1, and γ1 chains. We determined conformations and stabilities of trimers consisting of the N-terminal truncated peptides based on our previous synthetic peptides (B1, 51-mer from β1; B2, 51-mer from γ1; M, 55-mer from α2). We also measured the effects of pH, denaturation agents, and salt on the conformation and stability of the monomers, dimer, and trimer to determine the influence of intra- and/or interchain interactions between the B1, B2, and M peptides. Based on the present and previous studies, we proposed a mechanism of the laminin trimer assembly: A basic region of the M peptide specifically interacts with an acidic pocket of the B1−B2 dimer, followed by assembly into a triple-stranded coiled-coil structure.

MATERIALS AND METHODS

Synthesis of Peptides. All peptides were synthesized using the *tert*-butyloxycarbonyl based strategy of solid-phase peptide synthesis as previously reported (Nomizu et al., 1991a, 1992, 1994). Using a 4-methylbenzhydrylamine resin (Stewart, 1976), the respective amino acids corresponding to the peptides were assembled in a stepwise manner using an Applied Biosystems Peptide Synthesizer, Model 431A (Foster City, CA), according to a single coupling protocol. The following side chain protecting groups were used: Asp, cyclohexyl; Arg, tosyl; Cys, 4-methylbenzyl; Glu, Ser, and Thr, benzyl; His, benzyloxymethyl; Lys, 2-chlorobenzyloxycarbonyl; Tyr, 2-bromobenzyloxycarbonyl. The protected peptide resins were deprotected using the two-step hard acid deprotection/cleavage method (Nomizu et al., 1991b), which consists of consecutive treatments with 1 M trimethylsilyl bromide−thioanisole in trifluoroacetic acid (TFA)[1] in the presence of *m*-cresol and ethanedithiol at 4 °C for 30 min and with anhydrous hydrogen fluoride in the presence of *m*-cresol and ethanedithiol at 4 °C for 1 h. Resulting crude peptides were purified by gel filtration (Sephadex G-10 eluted with 1 M acetic acid) and reverse-phase high-performance liquid chromatography (HPLC) on a Vydac 5C18 column using a gradient of H_2O/acetonitrile containing 0.1% TFA. Purity and identity of the synthetic peptides were confirmed by analytical reverse-phase HPLC and amino acid analysis. All peptide concentrations in this investigation were determined by amino acid analysis. Amino acid analyses were performed at the Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan. Ion spray mass spectra were obtained with a Sciex API*III*E triple quadrupole mass spectrometer: ion spray mass spectra (reconstructed): *m*/*z* 6212.60 (6212.72 calcd for M, $C_{267}H_{463}N_{81}O_{88}$), 6013.59 (6012.41 calcd for B1, $C_{263}H_{435}N_{71}O_{87}S$), 5780.09 (5779.28 calcd for B2, $C_{248}H_{416}N_{72}O_{84}S$), 11793.43 (11789.16 calcd for B1−B2, $C_{511}H_{849}N_{143}O_{171}S_2$).

Preparation of Disulfide-Bonded Dimer Peptides. Heterodimers were prepared by mixing of the B1 and B2 peptides (B1+B2, $B1_{41}$+$B2_{41}$, and $B1_{32}$+$B2_{31}$). B1 peptides (5 mg) and B2 peptides (5 mg) were mixed in 50 mM sodium phosphate buffer (pH 7.4, 5 mL), and the solution was kept at 20 °C for 72 h. The resulting disulfide-bonded B1−B2 heterodimers (B1−B2, $B1_{41}$−$B2_{41}$, and $B1_{32}$−$B2_{31}$) were purified by reverse-phase HPLC.

Nondenaturing Gel Analysis. Nondenaturing gel analysis was performed as described by Utani et al. (1994). Equimolar mixtures of the peptides were incubated for at least 2 h. The final concentrations of the total peptides were 10 μM in the mixtures. The products were analyzed by electrophoresis on a 4% nondenaturing gel with Coomassie Brilliant Blue staining.

Determination of Conformation and Thermal Stability of the N-Terminal Truncated Peptides. Monomers and dimers were dissolved in 50 mM sodium phosphate buffer (pH 7.4) at final concentrations of 10 μM and 5 μM, respectively. Using the solutions, various combinations of mixtures were prepared and reconstituted at 20 °C for 12 h. Total peptide concentrations of all samples were 10 μM (monomer, 10 μM; dimer, 5 μM; trimer, 3.3 μM). Using the peptide solutions, spectra were recorded from 250 to 190 nm on a Jasco Model J-500A/DP-501N CD spectropolarimeter in Hellma cells, with a 1 mm path length, at 20 °C. The fractional helical content for each peptide was calculated according to the assumption (Chen et al., 1974) that for 100% α-helix the mean residue ellipticity, $[\theta]$, at 222 nm is $[\theta]_{222} = -36300(1 - 2.57/X)$, where X stands for the number of amino acids in the peptide. To determine thermal melting curves of the dimers and trimers, the mean residue ellipticity signal in the CD spectra was measured at 222 nm at different temperatures. Temperature was gradually increased from 20 to 80 °C in 5 °C intervals.

pH Effect on Conformation and Stability of Peptides. Since B1 and B2 peptides contain free SH groups, the stock solutions of the peptides were prepared at a final concentration of 200 μM in H_2O containing 20 mM dithiothreitol (DTT). The other stock solutions of M and B1−B2 (200 and 100 μM in H_2O, respectively) were prepared, and a mixture of the solutions (B1−B2/M, 66.7 μM) was reconstituted at 20 °C for 2 h. The stock solutions were diluted with 50 mM sodium phosphate solutions (pH 2−10) to a final total peptide concentration of 10 μM (monomer, 10 μM; dimer, 5 μM/chain; trimer 3.3 μM/chain). CD spectra were recorded from 250 to 190 nm as described above.

Thermal stabilities of B1−B2 (5 μM) and B1−B2/M (10 μM) at various conditions (pH 2−10) were determined.

[1] Abbreviations: CD spectroscopy, circular dichroism spectroscopy; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

Table 1: Conformation and Thermal Stability of Synthetic Laminin Peptides[a]

	$B1_{32}-B2_{31}$			$B1_{41}-B2_{41}$			B1−B2		
peptide[b]	f^H (%)[c]	$[\theta]_{222/208}$[d]	T_m (°C)[e]	f^H (%)	$[\theta]_{222/208}$	T_m (°C)	f^H (%)	$[\theta]_{222/208}$	T_m (°C)
none	38	0.83	39	50	0.88	39	56	1.07	43
M_{30}		ND			ND		53	0.96	44
M_{40}		ND		48	0.89	42	59	0.97	48
M_{48}	33	0.78	39	55	0.90	47	62	1.00	51
M	34	0.80	40	59	0.93	46	68	1.04	62

[a] The dimers ($B1_{32}-B2_{31}$, $B1_{41}-B2_{41}$, and B1−B2) and the monomers (M, M_{48}, M40, and M30) were mixed in equimolar levels and analyzed using CD spectroscopy. ND: not determined. [b] The sequences of peptides are shown in Figure 1. The α-helical content of peptides M, M_{48}, M_{40}, and M_{30} was between 21 and 26%. [c] The mean residue ellipticities at 222 nm were measured at 20 °C in 50 mM sodium phosphate, pH 7.4. The fractional helical content, f^H, for each peptide mixture was calculated according to the assumption (Chen et al., 1974) that for a 100% α-helix and X number of amino acids in the peptide the mean residue ellipticity $[\theta]_{222} = -36300(1 - 2.57/X)$. [d] The ratios of mean residue ellipticities at 208 and 222 nm, $[\theta]_{222/208}$, are given as a relative measure for the coiled-coil interaction between helices (see text). [e] T_m value was obtained from the thermal transitional curves of the dimers and the trimers.

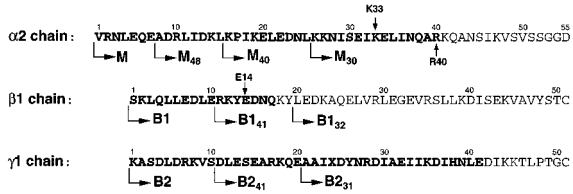

FIGURE 1: List of synthetic peptides. B1: mouse laminin β1 chain positions 1735−1785 (Sasaki et al., 1987). B2: mouse laminin γ1 chain positions 1548−1598 (Sasaki & Yamada, 1987). Met (position 1571) was replaced with norleucine (X = norleucine). M: mouse laminin α2 chain positions 2095−2149 (Bernier et al., 1995) [corresponding to amino acid residues 2079−2133 of the mouse laminin α1 chain (Sasaki et al., 1988)]. N-Terminal positions of truncated peptides of B1, B2, and M are shown by arrows. All peptides have C-terminal amides. Critical regions for trimer assembly are written in boldface type (Utani et al., 1994, 1995). E14 (mouse laminin β1 chain position 1746) and K33 and R40 (mouse laminin α2 chain positions 2127 and 2134, respectively), which were previously found to be critical residues for trimer assembly (Utani et al., 1994, 1995), are indicated by arrows.

Guanidine Hydrochloride and Urea Denaturation. Stock solutions of M, B1−B2, and B1−B2/M were prepared in 50 mM sodium phosphate buffer at a final total peptide concentration of 200 μM (M, 200 μM; B1−B2, 100 μM; B1−B2/M, 66.7 μM). The stock peptide solutions were diluted with 50 mM sodium phosphate buffer (pH 7.4) containing various concentrations of guanidine hydrochloride or urea, and the mean residue ellipticity in the CD spectra was measured at 222 nm.

Salt Effects on Conformation and Stability of Peptides. The peptides were dissolved in 50 mM sodium phosphate buffer (pH 7.4) at a final total peptide concentration of 20 μM. Since B1 and B2 peptides contain free SH groups, the monomers were dissolved in the same buffer with 3 mM DTT. The peptide solutions (200 μL) were diluted with 200 μL of the same buffer containing 1−4 M NaCl. Conformation and thermal stability were determined by CD spectroscopy as described above.

RESULTS

Conformation and Stability of N-Terminal Truncated Laminin-2 Peptides. The synthetic peptides from mouse laminin-2 are listed in Figure 1. The conformations of the monomers (M, M_{48}, M_{40}, M_{30}), dimers (B1−B2, $B1_{41}-B2_{41}$, $B1_{32}-B2_{31}$), and various equimolar mixtures thereof were examined by circular dichroism (CD) spectroscopy at 20 °C (Table 1). $B1_{41}-B2_{41}$ (50% helix) and $B1_{32}-B2_{31}$ (38% helix) exhibit α-helical spectra with lower α-helix contents compared to that of B1−B2 (56%). M_{48}, M_{40}, and M_{30} showed a low degree of α-helicity, similar to that found for the M peptide (Nomizu et al., 1994). M_{48} and M_{40} were found to interact with the B1−B2 dimer and significantly enhance α-helical contents of the equimolar mixtures, but M_{30} was found to be significantly less effective in binding to the dimer than M_{48} and M_{40}. On the other hand, $B1_{41}-B2_{41}$ significantly increased α-helicity on mixing with either M or M_{48}, but M_{40} did not enhance α-helical conformation with this N-terminally truncated dimer. None of the N-terminal truncated trimers showed high α-helical content comparable to that of B1−B2/M. These results suggest that the N-terminal regions of the peptides are critical for interactions to form a highly ordered coiled-coil structure.

The ratio between the intensities of the bands at 222 and 208 nm can be regarded as a measure for the degree of coiling of the α-helices around each other. The $n-\pi^*$ transition (222 nm CD band) is mainly responsive to the α-helical content. The $\pi-\pi^*$ excitation band at 208 nm polarizes parallel to the helix axis and is sensitive to whether the α-helix is monomeric or is involved in tertiary contacts with other α-helices (Cooper & Woody, 1990; Zhou et al., 1992; Greenfield & Hitchcock-Degregori, 1993). Monomeric α-helices show a higher intensity of the 208 nm band than interacting helices even through the α-helical content remains the same. Therefore, changes which enhance the ratio of the intensities of the two bands can be interpreted as an additional probe for the formation of stable coiled-coil structures than the absolute value of the molecular ellipticity at 222 nm alone.

Thermal stabilities of the N-terminal truncated dimer and trimer peptides were determined by monitoring changes in the CD spectra (222 nm) at various temperatures (Table 1). Using large recombinant chains consisting of one-third of the C-terminal long arm (α2−221, containing 221 amino acid residues from 1929 to 2149 of the α2 chain; β1−217, containing 217 residues from 1570 to 1786 of the β1 chain; γ1−217, containing 217 residues from 1391 to 1607 of the γ1 chain), we previously reported that the T_m value of the β1−217−γ1−217 dimer (63 °C) was comparable to that of the β1−217−γ1−217/α2−221 trimer (64 °C) (Utani et al., 1995). Furthermore, the T_m value of the B1−B2/M trimer (62 °C) was similar to those of the recombinant dimer and trimer, but the B1−B2 dimer was significantly less stable (T_m = 43 °C) (Nomizu et al., 1994). Thermal stabilities of $B1_{41}-B2_{41}$ and $B1_{32}-B2_{31}$ (T_m = 39 °C each) were found to be lower than that of B1−B2. $B1_{41}-B2_{41}$/M showed

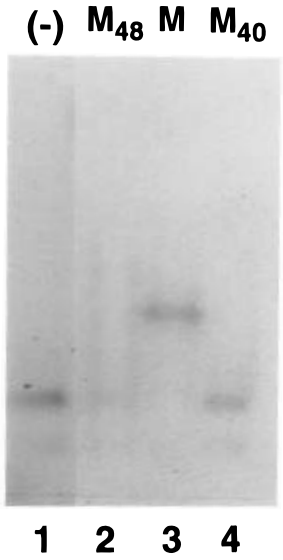

FIGURE 2: Nondenaturing gel analysis for trimer assembly. Various M chain peptides were mixed with equimolar amounts of B1−B2. The products were analyzed by 4% nondenaturing gel electrophoresis and stained with Coomassie Brilliant Blue. The α2 components of the mixture are listed on the top of the each lane.

higher thermal stability (T_m = 46 °C) than that of $B1_{41}$−$B2_{41}$, but the T_m value of the trimer was lower than that of B1−B2/M. When the N-terminal truncated M peptides were used for trimer assembly with B1−B2, thermal stabilities of the trimers were decreased as sizes of the M peptides were decreased (T_m values of B1−B2/M_{48}, B1−B2/M_{40}, and B1−B2/M_{30} are 51, 48, and 44 °C, respectively). These results suggest that the 51-mer for the laminin β1 and γ1 chains and the 55-mer for the laminin α2 chain are critical lengths for each chain to form a stable triple-stranded coiled-coil structure, with the stability equivalant to that of intact laminin.

Trimer formation of M peptide with B1−B2 was examined by nondenaturing gel electrophoresis using various sizes of M peptides (Figure 2). The B1−B2 dimer migrated as a single band when analyzed in the absence of the M peptides (Figure 2, lane 1). All M peptides including M, M_{48}, and M_{40} remained on top of the gel (data not shown) without B1−B2, similar to the recombinant M chains reported previously (Utani et al., 1994, 1995). The equimolar mixtures of B1−B2 and M showed a single band which migrated slower than the B1−B2 dimer (Figure 2, lane 3). The addition of M_{40} to B1−B2 did not significantly change the mobility of the B1−B2 dimer (Figure 2, lane 4). When M_{48} was incubated with B1−B2, most of the B1−B2 dimer formed a complex with M_{48} which migrated as diffused bands (Figure 2, lane 2). These results also suggest that M and B1−B2 can form a trimer and that the minimum size of the M peptide for stable trimer formation was at least 55 amino acids.

pH Dependence on Conformation and Stability of Laminin-2 Peptides. Using CD spectroscopy at 20 °C, we examined the pH effects on conformations of M, B1, B2, B1−B2, and B1−B2/M to elucidate the significance of ionic interactions (Figure 3). At a neutral pH, α-helix contents

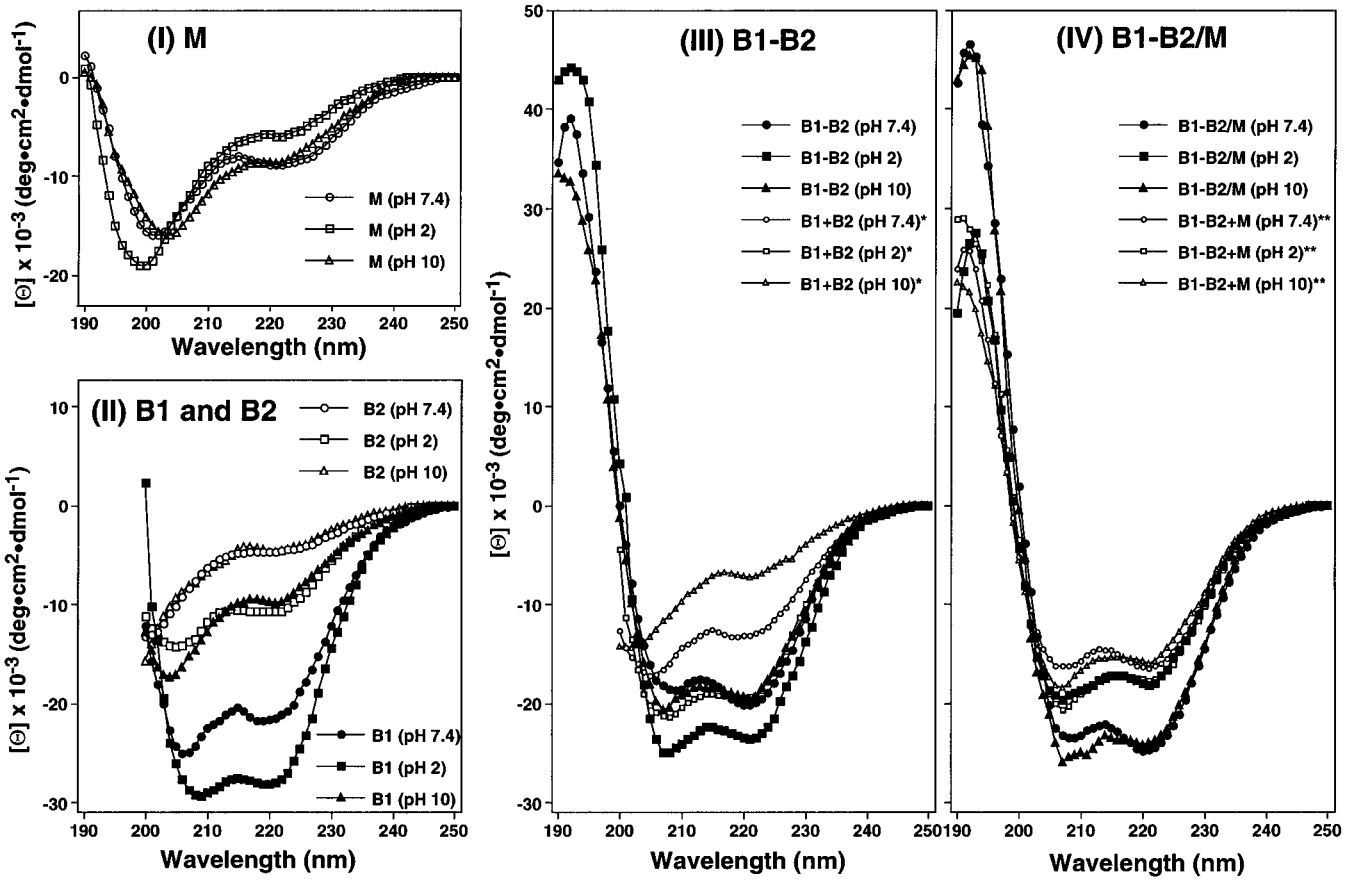

FIGURE 3: Circular dichroism (CD) spectra of the synthetic peptides at different pH solutions. (I) M peptide (10 μM); (II) B1 and B2 peptides (10 μM); (III) B1−B2 dimer (5 μM); (IV) B1−B2/M trimer (3.3 μM). CD spectra were recorded at 190−250 nm in 50 mM sodium phosphate solutions at different pH (2, 7.4, and 10) at 20 °C. *B1+B2, sum of the spectra of B1 and B2. **B1−B2+M, sum of the spectra of B1−B2 and M.

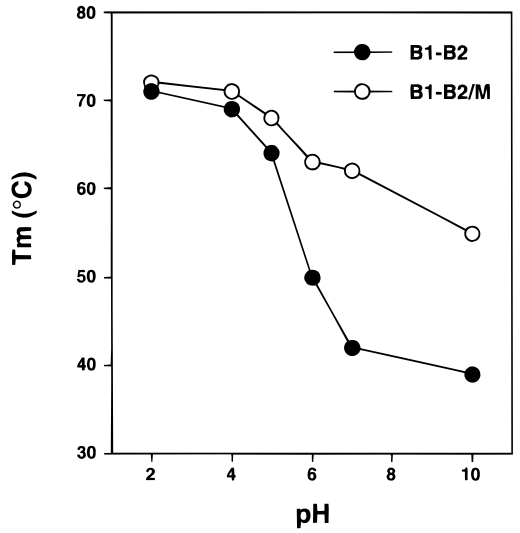

FIGURE 4: pH dependence of thermal stability for the dimer and the trimer. T_m values, melting temperature determined from the midpoint of the thermal unfolding transition, were determined from thermal transition curves. Thermal transition curves of B1−B2 and B1−B2/M in different pH solutions were determined by circular dichroism (CD) spectroscopy at 222 nm.

of B1, B2, and M are 61%, 14%, and 26%, respectively, while at a low pH (pH 2.0) the α-helicities of B1 (80% helix) and B2 (31% helix) were significantly increased and the α-helicity of M (17% helix) was decreased. At a high pH (pH 10), the CD spectra of B2 and M peptides did not change, but the α-helicity of B1 (28%) was significantly decreased compared to those of each peptide at a neutral pH. The results suggest that there are intrachain ionic repulsions by acidic amino acids in B1 and B2 peptides and that these peptides are favored to form α-helical structures under acidic conditions. The B1−B2 dimer at basic and neutral conditions (pH 7.4 and 10) showed similar CD spectra (55% and 56% α-helix, respectively), while at pH 2 the α-helicity of B1−B2 (66%) was significantly increased. These results indicate that there are intra- and/or interchain repulsions by acidic amino acids in the B1−B2 dimer, and these repulsions partially destroyed its α-helical conformation. B1 and B2 monomers possessed high α-helical structure at pH 2, whereas the CD spectrum of B1−B2 showed a higher α-helical content than the sum of those of B1 and B2 monomers under the same conditions. The hydrophobic interactions of B1 and B2 chains are expected to favor an increase in the α-helical contents in the disulfide-bonded B1−B2 coiled-coil structure at pH 2. At acidic conditions, the α-helicity of B1−B2/M (50%) was lower than those of the trimer at neutral and basic conditions (69% and 67% α-helix, respectively). The CD spectrum of B1−B2/M was found to be comparable to the sum of the CD spectra of B1−B2 and M at pH 2. These results suggest that B1−B2/M is dissociated into B1−B2 and M at these acidic conditions.

Next we determined the pH dependence of the thermal stabilities of B1−B2 and B1−B2/M by monitoring changes in the CD spectra (222 nm) at various temperatures (Figure 4). The thermal stability of B1−B2 is strongly pH-dependent. The thermal stabilities of the dimer and the trimer were significantly increased at low pH conditions, and B1−B2 and B1−B2/M showed the highest T_m values at pH 2 (71 and 72 °C, respectively). The result suggests that B1−B2/M is dissociated to the dimer and monomer, and the melting curve of B1−B2/M at pH 2 is primarily dependent on the conformational change of the B1−B2 dimer. While at higher pH the T_m values for the dimer and the trimer were decreased slightly, the T_m values for B1−B2 and B1−B2/M were 39 and 56 °C at pH 10, respectively. These results suggest that there are intra- and/or interchain repulsions by acidic amino acids in B1−B2 chains. These repulsions could create a less stable double-stranded coiled-coil structure of B1−B2 at neutral and basic conditions. At acidic conditions, these repulsions are minimized by protonation of carboxylic acid side chains of aspartic acid or glutamic acid, and the dimer is stabilized largely by hydrophobic interactions.

Urea and Guanidine Hydrochloride Denaturation. The conformational stability of B1−B2 and B1−B2/M was determined by monitoring changes in the CD spectra (222 nm) at various concentrations of urea and guanidine hydrochloride. As shown in Figure 5, the intensity of the CD signal for the B1−B2 dimer and the B1−B2/M trimer was decreased as the urea or guanidine hydrochloride concentrations were increased, reflecting the loss of α-helix structure. The urea concentration at which 50% of peptide is unfolded for B1−B2/M ($[urea]_{1/2}$ = 3.3 M) was higher than that of B1−B2 dimer ($[urea]_{1/2}$ = 2.4 M), while the [guanidine hydrochloride]$_{1/2}$ value of B1−B2/M (2.2 M) was similar to that of B1−B2 ([guanidine hydrochloride]$_{1/2}$ = 2.1 M). The denaturation profiles of B1−B2 in the presence or absence of M chain, respectively, show a striking difference dependent on whether urea or guanidine hydrochloride are used as a chaotropic agent (Figure 5A,B). Whereas the degree of unfolding caused by guanidine hydrochloride is indistinguishable from each other for both of the protein mixtures, B1−B2 becomes more stable in the presence of M chain ($\Delta[urea]_{1/2}$ = 0.9 M). Based on the observations of Monera et al. (1993, 1994a,b), we conclude that, due to its ionic nature, guanidine hydrochloride masks interchain ionic interactions and gives a measure of the coiled-coil stability based on the strength of the interchain hydrophobic interactions. Since urea is uncharged and it cannot mask electrostatic interactions, the difference in $\Delta[urea]_{1/2}$ values may be interpreted as the total net stability of the peptide structure which is the sum of electrostatic and hydrophobic contributions. Thus, stability values from urea denaturation can be consistent with temperature denaturations. Indeed, the addition of M chain to the B1−B2 dimer increases the T_m from 43 to 62 °C (Table 1). In our case, the positively charged guanidinium ions might bind to the acidic pocket of B1−B2 which neutralizes the side chains and allows them to interact more closely with the hydrophobic interface.

Salt Effects on Conformation and Stability of Peptides. CD spectra of peptides were determined in the presence of various concentrations of NaCl (0−2 M) in 50 mM sodium phosphate buffer (pH 7.4) (Figure 6A). B1 and M peptides significantly increased α-helical contents as concentrations of NaCl were increased from 0 to 2 M (B1, from 61% to 100%; M, from 26% to 66%). The α-helical contents of B2, B1−B2, and B1−B2/M did not change at the high concentrations of NaCl. Thermal transition curves of the dimer B1−B2 and trimer B1−B2/M in 50 mM sodium phosphate buffer (pH 7.4) in the presence or absence of 2 M NaCl were determined by monitoring changes in the CD spectra (222 nm) at various temperatures (Figure 6B). The thermal stabilities of the dimer and trimer were not significantly affected by 2 M NaCl. Salt-induced conformational

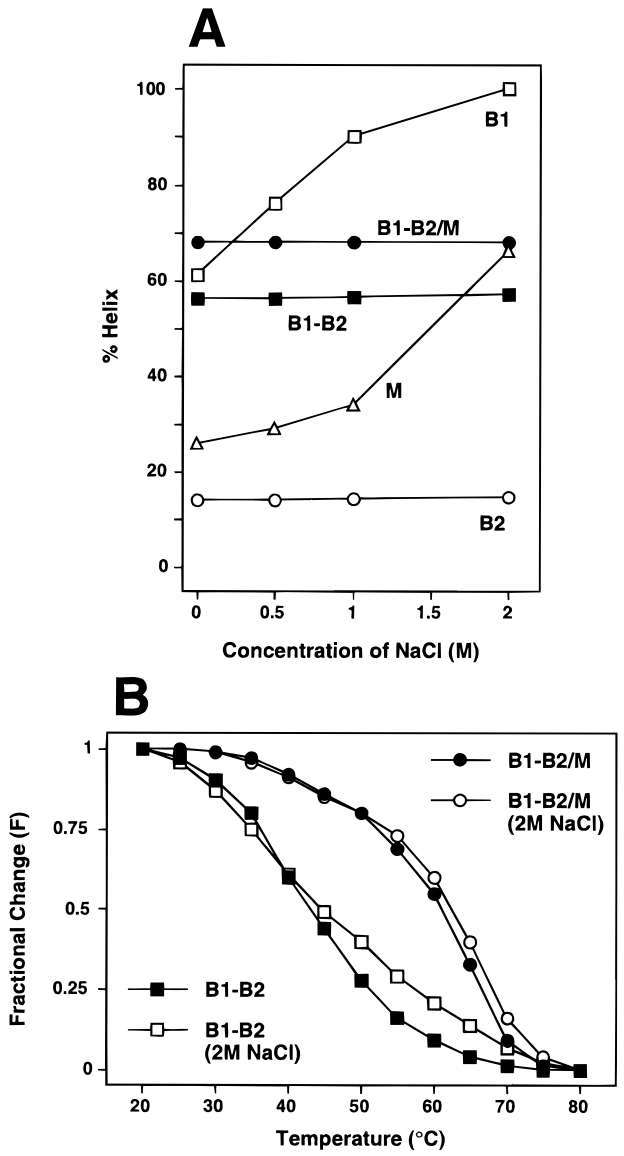

FIGURE 5: Urea (A) and guanidine hydrochloride (B) denaturation profiles of the B1−B2 dimer and B1−B2/M trimer in 50 mM sodium phosphate buffer (pH 7.4) at 20 °C. The plots represent the fractional change from the α-helical to random coil conformation, by circular dichroism spectroscopy at 222 nm. The fractional change (F) was determined from $F = ([\theta] - [\theta]_n)/([\theta]_n - [\theta]_r)$, where $[\theta]$ is the ellipticity measured at any particular denaturant concentration and $[\theta]_n$ and $[\theta]_r$ are the ellipticity of the native (folded) and denatured (unfolded) states, respectively.

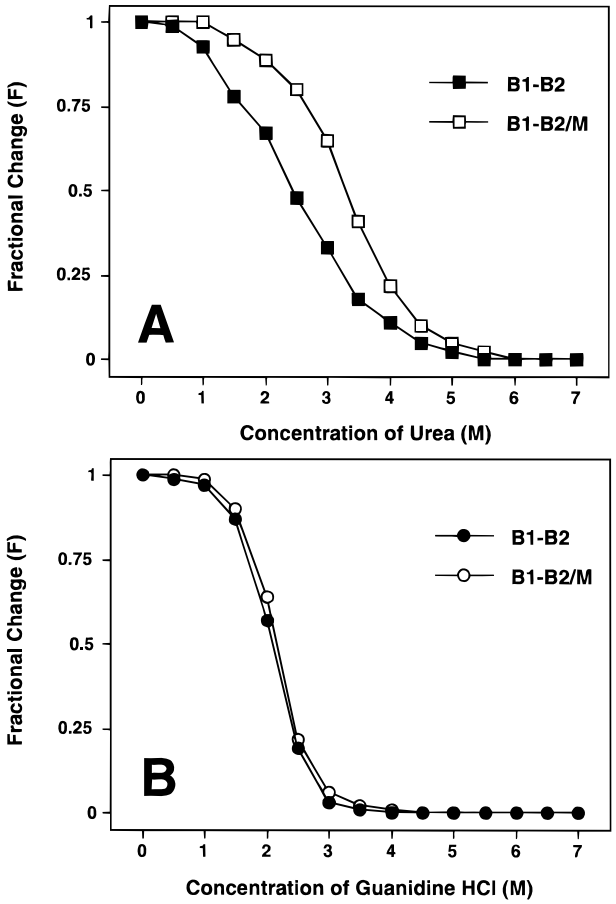

FIGURE 6: Salt effects on the conformation (A) and thermal stability (B) of the peptides in 50 mM sodium phosphate buffer (pH 7.4) containing different concentrations of NaCl (0−2 M) at 20 °C. (A) The α-helical contents of the peptides were calculated according to the method of Chen et al. (1974). (B) Thermal transition curves of the dimer and the trimer were determined in 50 mM sodium phosphate buffer (pH 7.4) with or without 2 M NaCl. The plots represent the fractional change from the α-helical to random coil conformation, as measured by circular dichroism spectroscopy at 222 nm. The fractional change (F) was determined from $F = ([\theta] - [\theta]_n)/([\theta]_n - [\theta]_r)$, where $[\theta]$ is the ellipticity measured at given temperature and $[\theta]_n$ is the ellipticity at 20 °C. $[\theta]_r$ is the ellipticity of the random coil conformation which was measured at 80 °C.

transitions have been reported and explained by several mechanism (Goto et al., 1990; Goto & Aimoto, 1991). Since salts can bind to charged amino acid residues and inhibit ionic repulsions of peptides, it is conceivable that the B1 and M peptides increase their α-helicities due to inhibiting ionic repulsions under high salt conditions.

DISCUSSION

Laminin assembly has been studied previously using proteolytic laminin-1 fragments (Paulsson 1985; Hunter et al., 1990, 1992) and recombinant laminin proteins covering different lengths of the C-terminal regions of laminin long arms (Utani et al., 1994, 1995; Kammerer et al., 1995; Antonsson et al., 1995). These studies suggest that the C-terminal sequence of the long arm of each chain is essential for the triple-stranded coiled-coil formation and that ionic interactions with their sequences were important for specific chain assembly. Recently we demonstrated specific assembly of short laminin-2 peptide segments (B1, B2, and M) into a triple-stranded coiled-coil structure (Nomizu et al., 1994). The T_m value (62 °C) of the B1−B2/M trimer was comparable to that of the recombinant trimers (approximately 200 amino acids long), and isoleucine residues in the laminin α2 and γ1 chains were critical for stability of the triple-stranded coiled-coil structure (Nomizu et al., 1994). Here we have focused on mechanisms of the laminin chain assembly, and the conformation and stability of the synthetic laminin-2 peptides.

The size-dependent conformation and stability of trimer using the N-terminal truncated synthetic peptides indicates that the 51-mer for laminin β1 and γ1 chains and the 55-mer for the laminin α2 chain are minimum sizes for each chain to assemble into the stable triple-stranded coiled-coil structure complex similar to that of the intact laminin-1 and laminin-2 molecules.

CD studies of the peptides at different pH and at various salt concentrations suggest that B1 and B2 monomer peptides

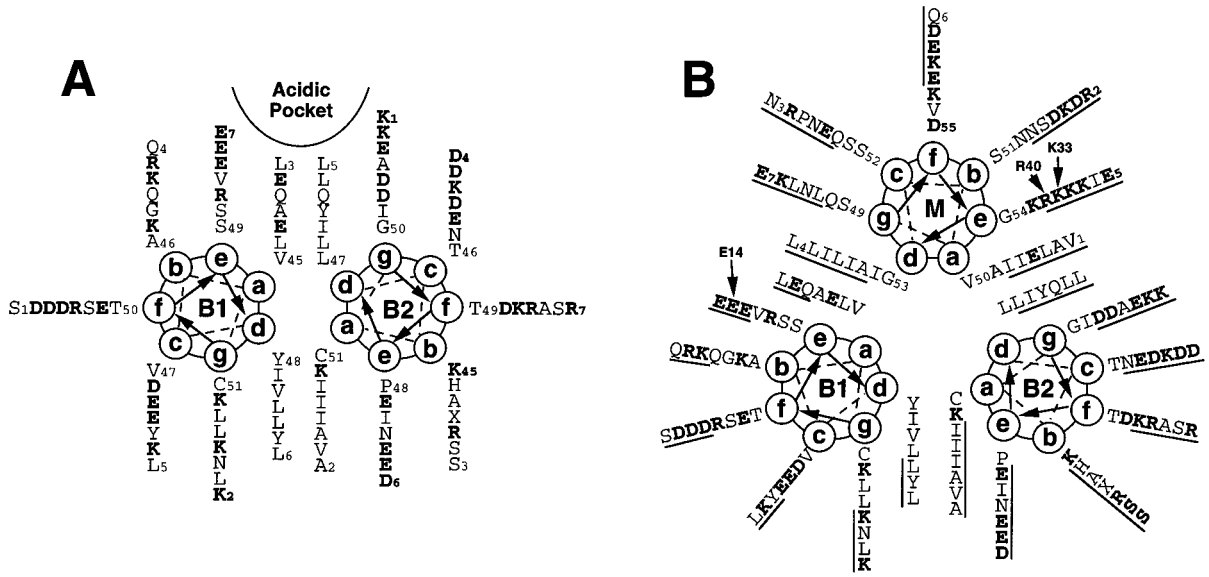

FIGURE 7: Coiled-coil helical wheel representation of the sequences of B1−B2 dimer (A) and B1−B2/M trimer (B). Charged residues are written in boldface type. Residues shown by underlines or side lines in the trimer model are critical regions for trimer assembly (B1, 1−17; B2, 1−41; M, 1−40). E14 (mouse laminin β1 chain position 1746) and K33 and R40 (mouse laminin α2 chain positions 2127 and 2134, respectively), which were previously found to be critical residues for trimer assembly (Utani et al., 1994, 1995), are indicated by arrows.

contain intrachain repulsions by acidic residues, the M peptide has ionic repulsions, and the B1−B2 dimer has intra- and/or interchain repulsions by its acidic amino acid side chains. At neutral and low salt conditions, these repulsions partially destroy their α-helical structure. Moreover, these intra- and/or interchain repulsions of the B1−B2 dimer significantly decrease the thermal stability of the double-stranded coiled-coil structure. At pH 2, the CD spectrum of B1−B2/M was comparable to the sum of the CD spectra of B1−B2 and M, and the thermal stability of the trimer (T_m = 72 °C) was comparable to that of the dimer (T_m = 71 °C), while B1−B2/M and B1−B2 showed similar guanidine hydrochloride denaturation curves. These results indicate that the B1−B2/M trimer dissociates to the B1−B2 dimer and M monomer either at the low pH condition or in the guanidine hydrochloride containing solutions. These findings suggest that ionic interactions between the B1−B2 dimer and M are critical for the formation of the triple-stranded coiled-coil structure. The intra- and/or interchain repulsions in the B1−B2 dimer by acidic residues seem to be compensated by the heterotrimer assembly with M peptide into the triple-stranded coiled-coil structure, in which interchain ionic interactions are highly contributory.

The B1 and B2 peptides were aligned into a heptad repeat model, $(abcdefg)_n$ (Figure 7A). In the helical wheel model, "*a*" and "*d*" are hydrophobic positions and stabilize the coiled-coil structure by hydrophobic interchain interactions. Interchain interactions of "*e*" and "*g*" positions by charged residues have been previously found to be critical for specific formation of the double-stranded coiled-coil structure (O'Shea et al., 1992). In the B1−B2 dimer model, there are 10 acidic amino acids and 6 basic amino acids in the "*e*" and "g" positions, respectively (Figure 7A). Ionic interactions between the amino acids in the "*g*" position of B1 and in the "*e*" position of B2 are suggested to be well organized. However, there are eight acidic amino acids in the "*a*" and "*e*" of B1 and three basic amino acids in the "*g*" of B2, and these acidic residues form an acidic pocket in the B1−B2 molecule. Thus, it is likely that there are intra- and/or interchain repulsions by the acidic residues in the acidic pocket. This interpretation is in agreement with the experimental observations from the pH dependence CD studies of B1−B2 dimer.

A helical wheel model of trimer is shown in Figure 7B. Hydrophobic amino acids (isoleucine residues) in the M and B2 peptides were shown to be critical for assembly and stability of the triple-stranded coiled-coil structure (Nomizu et al., 1994). In the trimer model, five of six isoleucines in the M peptide and four of six of the amino acids in B2 are located in the "*a*" and "*d*" positions. These isoleucine residues in the "*a*" and "*d*" positions play an important role in stabilizing the triple-stranded coiled-coil structure as hydrophobic amino acids. Previously we showed critical sites on each chain for trimer assembly using the C-terminal truncated recombinant proteins (Utani et al., 1994, 1995). In this paper, we show that the N-terminal regions of B1, B2, and M are important for trimer formation. Together, these data suggest that B1(1−17), B2(1−41), and M(1−40) are critical regions for trimer formation (in Figure 7B, these regions are indicated). We showed previously that the glutamic acid, E14 (position 1746 in the laminin β1 chain), is critical for trimer assembly but not important for the dimer formation in our reconstitution study substitution of the glutamic acid residue by alanine of the recombinant proteins (Utani et al., 1994). In the B1−B2 dimer, this glutamic acid (E14) could be repelling the other closely located acidic amino acids, such as the glutamic acids in the "*a*" and "*e*" positions of B1 and/or the glutamic acid in the "*g*" position of B2. In trimer formation, the glutamic acid E14 can interact with the lysine in the "*g*" position of M, and this ionic interaction could critically contribute to the stabilization of trimer assembly. Further, the recombinant laminin α2 chains (approximately 200-mer), in which a lysine (K33, position 2127 in the laminin α2 chain) and/or an arginine (R40, position 2134 in the laminin α2 chain) were substituted with alanine, significantly decreased their propensity for trimer formation (Utani et al., 1995). It is thus likely that these basic residues in the "*e*" position of M interact with aspartic acids in the "*g*" position of B2.

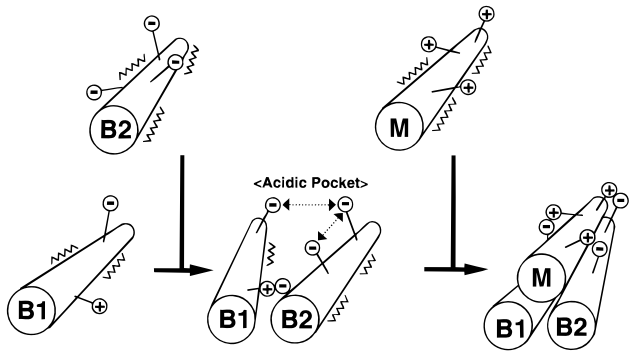

FIGURE 8: Proposed mechanism of laminin-2 peptide assembly into a double- and triple-stranded coiled-coil structure. Dashed arrows show ionic repulsions. Wavy lines show conformational unstability. The side chains of the basic amino acids (arginine and lysine) and acidic residues (aspartic acid and glutamic acid) are represented by + and −, respectively.

Based on the experimental data and the dimer and trimer helical wheel model, we propose two steps for laminin chain assembly (Figure 8). The first step is B1−B2 dimer formation. B2 has strong intrachain repulsions by acidic amino acids, and the α-helical structure of the peptide is significantly destabilized. B1 also has intrachain repulsions by its negatively charged amino acids, but its α-helical conformation is not affected as much as B2. The conformational instability of the B2 peptide is a driving force, in that B1 and B2 are preferentially assembled into a double-stranded coiled-coil structure. It was shown previously that the B1−B1 homodimer could be formed in the absence of B2 and that the thermal stability of the B1−B1 homodimer was higher than that of the B1−B2 heterodimer (Nomizu et al., 1994). However, the B1−B2 heterodimer is formed preferentially in the B1+B2 mixture. The preferential formation of the heterodimer is likely due to kinetic interactions between each chain (Nomizu et al., 1994). The second step is B1−B2/M trimer formation. The B1−B2 dimer is relatively unstable because it has intra- and/or interchain repulsions by acidic amino acids which create an acidic pocket. M is a basic peptide, and its α-helical conformation is partially destroyed by ionic repulsions. The basic residues of the M peptide can specifically interact with the acidic pocket of the B1−B2 dimer to form a stable triple-stranded coiled-coil structure.

Although the triple-stranded coiled-coil region of laminin consists of about 570 amino acids of each chain, only a short C-terminal sequence of each chain is active for efficient initiation of trimer formation (Utani et al., 1994, 1995). Our previous studies with in vitro reconstitution and DNA transfection analysis indicate that the N-terminal portions of the long arm of laminin are not capable of heterotrimer formation. The short sequence of each chain could function as a nucleation site to initiate chain interactions which lead to the completion of assembly in a C- to N-terminal direction. Although our proposed mechanism is based on the use of the short peptides, it is in good agreement with the processes of laminin assembly in vivo. Laminin-1 in mouse PYS tetracarcinoma cells is assembled intracellulary from α1, β1, and γ1 chains (Cooper et al., 1981). Pulse−chase labeling experiments also provide similar results in which $\beta-\gamma$ dimer is formed first followed by trimer formation with the α chain (Peters et al., 1985; Morita et al., 1985; Lissitzky et al., 1988; Aratani & Kitagawa, 1988). These studies also suggest that the trimer formation is a prerequisite for translocation in the Golgi apparatus and only complete trimers are secreted. There is also agreement in these studies that posttranslational modifications including glycosylation are not required for chain assembly. It is likely that the intact laminin chains assemble through the proposed two-step mechanism.

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