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A Single Amino Acid Substitution within a Coiled-coil Motif Changes the Assembly of a 53-Amino Acid Protein from Two-dimensional Sheets to Filamentous Structures*

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The bacteriophage ϕ29 replication protein p1 self-interacts *in vitro*, generating highly ordered structures. Specifically, the 53-amino acid protein p1ΔN33, which retains the sequence of p1 spanning amino acids Met^{34} to Lys^{85}, assembles into two-dimensional protofilament sheets. The region of protein p1 located between residues Glu^{38} and Asn^{65} presumably forms an α-helical coiled-coil structure. Here we have examined the role of this coiled-coil sequence in the formation of protofilament sheets. Using sedimentation assays and negative-stain electron microscopy analysis, we demonstrate that residues Leu^{46}, Met^{53}, and Leu^{60}, but not Leu^{39}, are essential for p1ΔN33 assembly into sheets. Remarkably, replacement of Leu^{46} by Val shifts the pathway of molecular assembly, leading to the formation of filamentous polymers ~10 nm in diameter. These results show, for the first time, that a short coiled-coil motif can mediate protein assembly into protofilament sheet structures.

The α-helical coiled-coil is probably the most widespread subunit oligomerization motif found in proteins. Coiled-coils consist of two to five right-handed amphipathic α-helices that wind around each other to form a slightly left-handed supercoil (1, 2). The formation of a coiled-coil is dependent primarily on the presence of heptad repeat sequences of a form denoted [**abcdefg**]$_n$, where positions **a** and **d** are mostly occupied by hydrophobic residues, and positions **e** and **g** are frequently filled with charged residues.

The coiled-coil motif was first described as the main structural element of a large class of eukaryotic fibrous proteins such as tropomyosin (284 residues) and myosin (~1100 residues) (3). Structural analysis of *in vitro* assembled smooth muscle myosin filaments revealed that they are flat side-polar sheets in which adjacent molecules overlap by ~14 nm. A noteworthy feature of this assembly mechanism is that the packing is width-limiting but not length-limiting (4). Coiled-coils are also the main structural elements in the assembly of intermediate filament proteins (IFs),[1] a superfamily of 10-nm fibers ubiquitous in multicellular eukaryotes (5). Despite the rather low primary sequence identity, all the IFs have a long and central α-helical domain (~42 heptads), which is flanked by nonhelical end domains. The α-helical domain has the ability to form a parallel, unstaggered two-stranded coiled-coil. Formation of these dimeric structures constitutes the first step in the assembly of intermediate filaments, which proceeds further by different modes of dimer-dimer interactions (6). Cytoplasmic IFs readily assemble *in vitro* into ~10-nm wide filaments. These structures are similar in appearance to those seen in cells (7). Unlike cytoplasmic IFs, lamins, a class of nuclear IFs, are also able to assemble into paracrystal structures under certain *in vitro* conditions (8, 9). *In vivo*, different lamin proteins form the nuclear lamina, a thin fibrous structure immediately underlying the inner nuclear membrane of most eukaryotic cell nuclei (10).

Protein p1 from *Bacillus subtilis* phage ϕ29 has the capacity to polymerize *in vitro*. This small viral protein (85 residues) presumably assembles *in vivo* into a structure that associates with the bacterial membrane (11, 12). Recently, we have proposed that the membrane-associated p1 structure functions as a scaffold to which the ϕ29 replisome becomes attached to initiate viral DNA replication (13). Although the nature of p1 polymers *in vivo* is unknown, previous *in vitro* studies demonstrated that the 53-amino acid protein p1ΔN33, which retains the sequence of p1 spanning amino acids Met^{34} to Lys^{85}, assembles into large polymers that show a parallel array of longitudinal protofilaments. These structures are two-dimensional protofilament sheets whose length and width depends on the polymerization time (12). According to computer algorithms, protein p1 as well as p1ΔN33 contains a short α-helical coiled-coil sequence (~3 heptad repeats) (this work), suggesting that such a motif might be involved in the intermolecular interactions that lead to the formation of two-dimensional protofilament sheets. To our knowledge, coiled-coils as structural elements in the assembly of such structures have not been previously described. Negatively stained p1ΔN33 sheets examined in the electron microscope resemble polymers formed under particular *in vitro* conditions by FtsZ (14, 15), which forms the cytoskeletal framework for cell division in all bacteria (16), and by the α/β-tubulin heterodimer (17–19), which is the structural subunit of the eukaryotic microtubules (20). However, unlike protein p1ΔN33 (12), FtsZ (~40 kDa) and tubulin (~50 kDa each monomer) lack a coiled-coil motif (21, 22). Furthermore, polymerization of both proteins is regulated by GTP hydrolysis (23). Thus, the p1ΔN33 protofilament sheets must

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[1] The abbreviations used are: IF, intermediate filament protein; MalE, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

be structurally different from those formed by FtsZ and tubulin.

In this report and as an approach to understanding the assembly mechanism underlying the formation of p1ΔN33 protofilament sheets, we carried out kinetic experiments in which the p1ΔN33 assembly reaction was followed by sedimentation assays and negative-stain electron microscopy analysis. In addition, we performed a mutational analysis to examine whether the α-helical coiled-coil sequence present in protein p1ΔN33 is involved in the formation of sheet structures. Specifically, amino acid residues occupying the **d** position of the heptad repeats were replaced by other apolar amino acids. The results obtained indicate that a nucleation-elongation mechanism mediated by a coiled-coil sequence is involved in the formation of p1ΔN33 sheets. Furthermore, we show that a single conservative substitution within the hydrophobic core of such a coiled-coil motif is sufficient to shift the pathway of protein assembly from two-dimensional protofilament sheets to ~10-nm-wide filaments.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—*Escherichia coli* XL1-Blue (Stratagene) was used as host for the plasmids constructed in this work. Plasmid pMalE-p1ΔN33 has been previously described (12). It is based on the pMAL-c2 expression vector (New England Biolabs). To introduce single-amino acid substitutions in protein p1ΔN33, a two-step mutagenesis method based on the polymerase chain reaction was used. In the first step, two reactions (A and B) were carried out using plasmid pMalE-p1ΔN33 as template. Reaction A contained as primers the oligonucleotide *Hin*dIII (5′-CGACG GCCAGTGCCAAGCTTG-3′) and the oligonucleotide I, which carries the modified codon (L39A: 5′-ACCT TGAGGCAGAAAAGAAGATG-3′; L46A: 5′-GATGACTAAAGCAGAGCATGAA-3′; L46V: 5′GATGACTAAAGTAGAGCATGAA-3′; M53A: 5′-ATAAACTCGCGAAAAACGCATTG-3′; L60I: 5′-TTGTATGAGATTTCTAGGATG-3′). Reaction B contained as primers the oligonucleotide *Sac*I (5′-CGATGAAGCCCTGAAAGAC-3′) and the oligonucleotide II, whose sequence is complementary to oligonucleotide I. In the second step of the mutagenesis procedure, the polymerase chain reaction fragments obtained in the reactions A and B were mixed and used as template in a polymerase chain reaction that used oligonucleotides *Hin*dIII and *Sac*I (see above) as primers. The resulting polymerase chain reaction fragments, digested with *Hin*dIII and *Sac*I, were used to replace the *Hin*dIII-*Sac*I region of plasmid pMalE-p1ΔN33. In the mutant plasmids, as in the wild-type plasmid pMalE-p1ΔN33, there is a Gly codon between the Ile-Glu-Gly-Arg-encoding sequence and the Met^{34} codon of gene 1. Protease factor Xa cleaves after the sequence Ile-Glu-Gly-Arg. The nucleotide sequence of the inserts was confirmed by DNA sequencing using the dideoxy nucleotide chain termination method (24). Plasmid-containing cells were grown in LB broth (25) supplemented with ampicillin (100 μg/ml).

Protein Purification and Factor Xa Protease Cleavage—Purification of maltose-binding protein (MalE) fusion proteins has been described (12). Protein preparations were concentrated using a Microcon microconcentrator 10 (Amicon). The concentration of the MalE fusion proteins was measured by Lowry method using bovine serum albumin as a standard. For digestion with protease factor Xa (New England Biolabs), protein preparations were diluted to 16 μM in dialysis buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 10 mM β-mercaptoethanol). Factor Xa was added to the protein preparation at a ratio of 1.25% (w/w) the amount of fusion protein. The reaction was incubated at room temperature for the indicated time. The cleavage with factor Xa was monitored by SDS-Tricine-PAGE (26). The percentage of uncleaved fusion protein was calculated by densitometric scanning of the gel in a Molecular Dynamics 300A densitometer.

N-terminal Sequencing of the Wild-type and Mutated p1ΔN33 Proteins by Edman Degradation—MalE fusion proteins were treated with protease factor Xa. The digested protein preparations were loaded onto an SDS-Tricine polyacrylamide gel. After electrophoresis, the p1ΔN33 products were transferred electrophoretically to Immobilon-P membranes (Millipore) using a Mini Trans Blot (Bio-Rad) at 100 mA and 4 °C for 60 min. N-terminal sequencing was performed on an Applied Biosystems 473A protein sequencer.

Electrospray Mass Spectrometry—Analyses were performed with a quadrupole Hewlett-Packard 1100 MSD mass spectrometer by using an electrospray interface. MalE fusion proteins were treated with factor Xa. The digested preparations were fractionated in an HPLC apparatus (HP Series 1100) with an autosampler (injection volume 5 μl) equipped with a Zorbax 300 SB C_{18} column (150 × 2.1 mm, 5-μm particle size). The mobile phase was a mixture of solvent A (acetonitrile + 0.1% trifluoroacetic acid) and solvent B (0.1% trifluoroacetic acid in water) according to a step gradient over 40 min, changing from 85% B at 7 min to 5% B at 40 min at a flow rate of 0.2 ml/min. Detection was accomplished by using a diode array detection system Series 1100 (Hewlett-Packard), storing the signal at a wavelength of 220 nm and 280 nm. A personal computer system running Hewlett-Packard software was used for data acquisition and processing. In the atmospheric pressure electrospray ionization method, the eluted compounds were mixed with nitrogen in the heated nebulizer interface, and polarity was tuned to positive. Adequate calibration of electrospray ionization parameters (needle potential, gas temperature, nebulizer pressure) was required to optimize the response and to obtain a high sensitivity of the molecular ion. The selected values were: needle potential, 4000 V; gas temperature, 310 °C; drying gas, 10 ml/min; nebulizer pressure, 40 p.s.i.g. (1 p.s.i. = 6894.76 pascals). The fragmentor was jumped from 50 to 140 V for different values of *m/z*.

Sedimentation through Glycerol Gradients—Aliquots of the protein preparations were loaded on to 5-ml glycerol gradient (15–30% in dialysis buffer; see above) prepared in a Beckman polyallomer centrifuge tube (13 × 51 mm). Centrifugation was performed at 62,000 rpm and 4 °C in a Beckman SW.65 rotor for the indicated time. After centrifugation, ~170-μl fractions were collected by puncturing the bottom of the tubes. The material sedimented at the bottom of the tubes was resuspended in loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 30% glycerol). Aliquots from each fraction were analyzed by SDS-Tricine-PAGE (26).

Electron Microscopy—Protein preparations were diluted to 0.5–1.5 μM in dialysis buffer (see above) and immediately applied to carbon-coated copper grids for 2 min. Grids were then washed with a few drops of water and stained for 30 s with 1% uranyl acetate. Electron micrographs were taken in a Jeol 1010 electron microscope at 80 kV.

RESULTS AND DISCUSSION

Protein p1ΔN33 Has an α-Helical Coiled-coil Motif—Protein p1ΔN33 is an N-terminal-truncated p1 protein that retains the sequence spanning amino acids Met^{34} to Lys^{85}. This 53-amino acid protein has Gly as first residue (Fig. 1*A*). In solution, protein p1ΔN33 assembles into two-dimensional protofilament sheets (Fig. 1*B*) (12). Polymerization of p1ΔN33 into sheets takes place in the absence of auxiliary factors, suggesting that the information necessary to form such polymers relies upon its structure. According to the PHD secondary structure prediction program (27), the region of protein p1 located between residues Ile^{35} and Arg^{62} has a high probability (0.5–1) of adopting an α-helical conformation. Moreover, the COILS prediction program (1) revealed that the region of p1 spanning amino acids Glu^{38} to Asn^{65} has a high probability (0.98) of forming an α-helical coiled-coil structure (Fig. 1*A*). This structure is characterized by a repeating heptad motif (**a** to **g**), with the amino acid side chains being predominantly hydrophobic at the **a** and **d** positions of each heptad and hydrophilic elsewhere (1). Since each heptad repeat forms two α-helical turns, the **a** and **d** residues are located on the same face of the helix forming a hydrophobic core (Fig. 1*C*).

Site-directed Mutagenesis of the Putative Coiled-coil of p1ΔN33—It has been shown that interhelical hydrophobic interactions are a dominant factor in the stabilization of coiled-coils (28). Furthermore, a systematic mutational analysis in the yeast transcription factor GCN4 revealed that conservative substitutions targeting the hydrophobic residues at positions **a** and **d** alter the packing and stoichiometry of coiled-coils (29). To determine whether assembly of protein p1ΔN33 (referred to as wild-type protein) into protofilament sheets is mediated by a coiled-coil sequence, we introduced single substitutions at the four **d** positions: Leu^{39}, Leu^{46}, Met^{53}, and Leu^{60}. These residues were changed to Ala, Val, or Ile, as shown in Fig. 1*A*. The changes were designed taking into account coiled-coil predictions (1) and general suggestions for conservative substitutions (30).

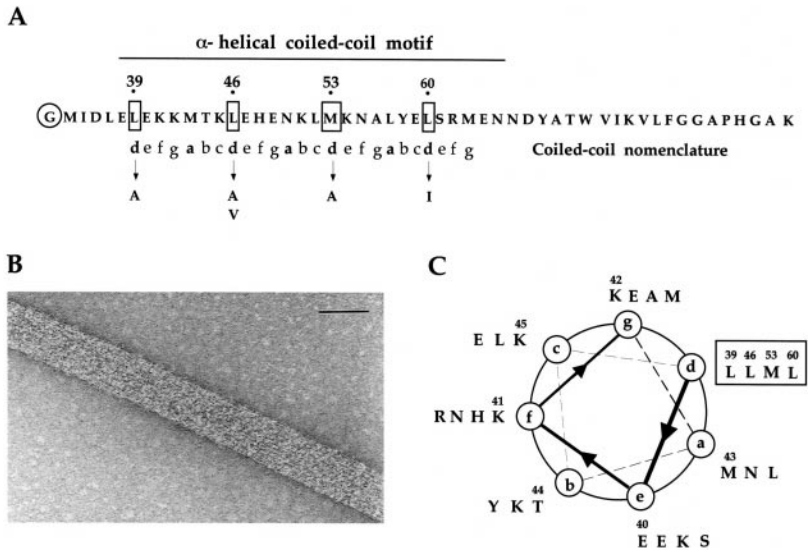

FIG. 1. **Relevant features of p1ΔN33, a truncated p1 protein that lacks the N-terminal 33 amino acids.** *A*, amino acid sequence of the 53-amino acid protein p1ΔN33. This protein has Gly as the first residue. It retains the region of p1 spanning amino acids Met^{34} to Lys^{85}. The amino acid position refers to the p1 sequence (12). The predicted α-helical coiled-coil sequence is indicated. Heptad repeats are denoted $[\mathbf{a}bc\mathbf{d}efg]_n$. Residues at the **d** positions were changed to Ala, Val, or Ile, as indicated. *B*, electron micrograph of a negatively stained p1ΔN33 protofilament sheet (12). The *scale bar* represents 50 nm. *C*, helical wheel projection of residues Leu^{39} to Met^{63} of the p1ΔN33 sequence. Residues at position **d** are *boxed*.

Characterization of the Wild-type and Mutated p1ΔN33 Proteins—Like the wild-type protein, the mutated p1ΔN33 proteins (L39A, L46A, L46V, M53A, and L60I) were fused to the maltose-binding protein (MalE) to facilitate their purification (see "Experimental Procedures"). The purified MalE fusion proteins, concentrated to 16 μM, were further treated with protease factor Xa. This protease cleaves after the sequence IEGR, which separates MalE from p1ΔN33. SDS-Tricine-PAGE analysis of digested MalE-p1ΔN33 preparations showed that only two products with the mobility expected for MalE (42.4 kDa) and p1ΔN33 (6.1 kDa) were generated (Fig. 2*A*). The same result was obtained when the mutated MalE fusion proteins where cleaved with factor Xa (not shown). Furthermore, to determine accurately the molecular mass of the wild-type and mutated p1ΔN33 products, digested MalE fusion proteins were fractionated by HPLC. Two fractions, which corresponded to the MalE and p1ΔN33 monomeric forms, were obtained. Each fraction was analyzed by electrospray-mass spectrometry. As shown in Table I, the molecular mass of the p1ΔN33 products differed from the expected value by 3.37–6.24 Da. In all the cases, the measured molecular mass of the MalE component was 42476.48 ± 6.93 Da. These results, together with N-terminal sequencing of the wild-type and mutated p1ΔN33 proteins (not shown), demonstrate that factor Xa cleavage occurs exclusively at the specific site.

Assembly of p1ΔN33 into Protofilament Sheets Can Be Envisaged as a Two-step Process—Sedimentation assays through glycerol gradients showed that MalE-p1ΔN33 (48.5 kDa) sedimented at the position of 88 kDa (Table II). Therefore, this fusion protein is likely a dimer throughout the concentration range analyzed (1.5–16 μM). Previous studies demonstrated that, after adding factor Xa to a MalE-p1ΔN33 preparation, protein p1ΔN33 self-associated into protofilament sheets (Fig. 1*B*), whereas MalE remained in solution as monomer (12). These findings reveal that MalE, when fused to p1ΔN33, provides a steric hindrance for p1ΔN33 assembly. This feature has been crucial in studying the assembly properties of protein p1ΔN33 in solution. To this end, factor Xa was added to a MalE-p1ΔN33 protein preparation (16 μM). At different times, samples of the reaction mixture were analyzed by SDS-Tricine-PAGE (Fig. 2*B*). This analysis allowed us to determine the percentage of uncleaved MalE-p1ΔN33 and, thus, to estimate the concentration of protein p1ΔN33 in the reaction. The partially digested samples were also analyzed by sedimentation through glycerol gradients (Fig. 3) and negative-stain electron microscopy. After 4 h of factor Xa addition, when the concentration of p1ΔN33 was 6 μM, p1ΔN33 complexes sedimenting as the native MalE protein were detected (Fig. 3*A*). The same sedimentation position was observed at 8 h, when the p1ΔN33 concentration was 9 μM. Such a position corresponded to 45 kDa, as determined by increasing the centrifugation time of the sample in the presence of different marker proteins (Fig. 3*B*). The MalE-p1ΔN33 molecules still present in the preparation sedimented as 88 kDa (Table II). At these times, negatively stained specimens were not visualized in the electron microscope. These results indicated that, after removal of MalE by cleavage with protease factor Xa, protein p1ΔN33 was able to self-associate into small oligomers, which were accumulated. Interestingly, at 19 h, when the concentration of p1ΔN33 was 14 μM, the amount of p1ΔN33 complexes sedimenting at the position of 45 kDa decreased, and p1ΔN33 complexes migrating faster appeared (Fig. *3A*). Moreover, the amount of protein p1ΔN33 recovered at the bottom of the glycerol gradient increased (Fig. 3*C*). Hence, assembly of p1ΔN33 into larger structures was taking place. Negative-stain electron microscopy analysis of this preparation showed that protofilament sheets (Fig. 1*B*) with different widths (14–62 nm) were formed. From these results we conclude that assembly of p1ΔN33 into protofilament sheets can be envisaged as a two-step process: (i) a nucleation step that leads to the accumulation of small oligomers, and (ii) an elongation step in which these oligomers are assembly intermediates able to generate protofilament sheets.

We also performed kinetic experiments in which factor Xa was added to a less concentrated MalE-p1ΔN33 protein preparation (4 μM). In this case, at 48 h of addition of factor Xa, when the concentration of p1ΔN33 was 3.5 μM, negatively stained sheets were visualized by electron microscopy (not shown). Therefore, p1ΔN33 polymerization is a function not only of protein concentration but also of time.

Residues Met^{53} and Leu^{60}, but Not Leu^{39}, Are Essential for p1ΔN33 Assembly into Protofilament Sheets—We next investigated whether residues Met^{53} and Leu^{60} of p1ΔN33 are necessary for assembly into protofilament sheets (Fig. 1). To this end, we constructed the MalE-p1ΔN33.M53A and MalE-p1ΔN33.L60I fusion proteins, in which residues Met^{53} and Leu^{60} of p1ΔN33 were replaced by Ala and Ile, respectively. In contrast to MalE-p1ΔN33, the MalE-p1ΔN33.M53A fusion protein sedimented in glycerol gradients at the position of 52 kDa, which may correspond to the monomeric form (Table II). At 8 h of addition of factor Xa to a MalE-p1ΔN33.M53A protein preparation (16 μM), the p1ΔN33.M53A concentration was 12 μM (Fig. 4*A*). It was measured as described for the wild-type pro-

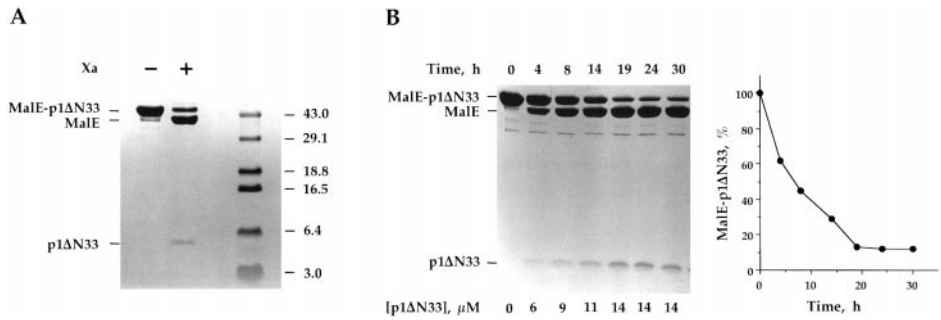

FIG. 2. **Treatment of MalE-p1ΔN33 with protease factor Xa.** *A*, SDS-Tricine-PAGE analysis of a MalE-p1ΔN33 preparation (16 μM) digested with factor Xa. The molecular weight of prestained proteins (Life Technologies, Inc.) used as markers is indicated in kilodaltons. *B*, factor Xa was added to a MalE-p1ΔN33 protein preparation (16 μM) at a ratio of 1.25% (w/w) of the amount of fusion protein. The reaction mixture was incubated at room temperature. At the indicated times, samples were analyzed by SDS-Tricine-PAGE. The gel was stained with Coomassie Blue. The concentration of p1ΔN33 at different times of addition of factor Xa was determined by calculating the percentage of uncleaved MalE-p1ΔN33 by densitometric scanning of the gel.

TABLE I
Comparison of expected and measured molecular masses (Da) of the wild-type and mutated p1ΔN33 proteins

Protein	Expected values[a]	Measured values[b]
p1ΔN33	6103	6106.91
p1ΔN33.L39A	6061	6064.37
p1ΔN33.L46A	6061	6064.79
p1ΔN33.L46V	6089	6093.45
p1ΔN33.M53A	6043	6049.24
p1ΔN33.L60I	6103	6106.92

[a] Molecular mass from the predicted amino acid sequence.
[b] Molecular mass from electrospray-mass spectrometry.

TABLE II
Sedimentation position of the MalE fusion proteins in glycerol gradients

Aliquots of the protein preparations (1.5–16 μM) were loaded on to a 15–30% glycerol gradient and subjected to centrifugation for 20 h as indicated under "Experimental Procedures."

Fusion protein	Position
	kDa
MalE-p1ΔN33	88
MalE-p1ΔN33.L39A	84
MalE-p1ΔN33.L46A	115
MalE-p1ΔN33.L46V	112
MalE-p1ΔN33.M53A	52
MalE-p1ΔN33.L60I	92

tein (Fig. 2*B*). At this time, p1ΔN33.M53A sedimented at the position of 12 kDa, whereas MalE sedimented as 45 kDa. Sedimentation of p1ΔN33.M53A at such a position was also observed at later times of factor Xa addition (not shown). Furthermore, protein complexes migrating faster were not detected, and negatively stained structures were not visualized. These results indicate that protein p1ΔN33.M53A cannot generate highly ordered structures.

The ability of protein p1ΔN33 to form sheets was also affected when residue Leu^{60} was changed to Ile. Although the MalE-p1ΔN33.L60I fusion protein behaved in solution as the wild-type protein (Table II), the assembly properties of p1ΔN33.L60I, after being cleaved from MalE, were different. As shown in Fig. 4*B*, at 8 h of addition of factor Xa to a MalE-p1ΔN33.L60I preparation (16 μM), the p1ΔN33.L60I concentration was 14 μM. At this time, protein p1ΔN33.L60I sedimented at the position of 25 kDa. This position did not change as a function of time (not shown). Moreover, negatively stained specimens were not visualized. Thus, small p1ΔN33.L60I oligomeric structures are formed, but they cannot assemble further into larger arrays.

We have also studied the assembly properties of protein p1ΔN33.L39A, in which residue Leu^{39} was changed to Ala (Fig. 1*A*). Like MalE-p1ΔN33, protein MalE-p1ΔN33.L39A behaved in solution as a dimer (Table II). Furthermore, after being cleaved from MalE, protein p1ΔN33.L39A was able to generate protofilament sheets (Fig. 6) following the same assembly pathway than the wild-type protein (Fig. 3).

Collectively, the above results demonstrate that residues Met^{53} and Leu^{60}, but not Leu^{39}, of protein p1ΔN33 are involved in the formation of sheets. Specifically, the change of Met^{53} to Ala as well as the change of Leu^{60} to Ile generates structures that are not able to function as assembly intermediates.

Replacement of Leu^{46} by Val in p1ΔN33 Leads to the Formation of 10-nm Filamentous Structures—To find out whether residue Leu^{46} of protein p1ΔN33 is essential for assembly into protofilament sheets, we changed this amino acid residue to Val (protein p1ΔN33.L46V) or Ala (protein p1ΔN33.L46A) (Fig. 1*A*). These mutated proteins were fused to MalE. Sedimentation analysis through glycerol gradients showed that both fusion proteins sedimented slightly faster than the wild-type MalE-p1ΔN33 protein (Table II). We next examined the assembly properties of p1ΔN33.L46V and p1ΔN33.L46A after being cleaved from MalE by sedimentation assays (Fig. 5) and negative-stain electron microscopy analysis (Fig. 6). At 4 h of addition of factor Xa to a MalE-p1ΔN33.L46V protein preparation (16 μM), the p1ΔN33.L46V concentration was 7 μM (Fig. 5*A*). It was measured as described for the wild-type protein (Fig. 2*B*). At this time, p1ΔN33.L46V complexes migrating faster than MalE were detected. At 8 h of addition of factor Xa, samples of the reaction mixture were subjected to centrifugation for 11 h (Fig. 5*A*) or for 20 h in the presence of protein markers (Fig. 5*B*). At this time, most of the p1ΔN33.L46V complexes sedimented at the position of 115 kDa, although complexes migrating more slowly were also observed (Fig. 5*A*). After 19 h of factor Xa addition, the concentration of p1ΔN33.L46V was 14 μM. At this time, the amount of complexes sedimenting at the position of 115 kDa was lower (Fig. 5*A*). This decrease correlated with an increase in the amount of protein p1ΔN33.L46V recovered at the bottom of the glycerol gradient (Fig. 5*C*). Therefore, assembly of larger structures was taking place. Analysis of this protein preparation by negative-stain electron microscopy showed that long filamentous polymers were formed (Fig. 6). These filaments had different lengths and were ~10 nm in diameter.

The behavior of protein p1ΔN33.L46A in solution was different (Fig. 5*A*). As in the case of p1ΔN33.L46V, p1ΔN33.L46A complexes sedimenting at the position of 115 kDa were observed at 4 and 8 h of factor Xa addition, when the concentration of p1ΔN33.L46A was 8 and 14 μM, respectively. However, in contrast to p1ΔN33.L46V, the amount of protein detected at such a position did not decrease at 19 h (Fig. 5*A*). Moreover,

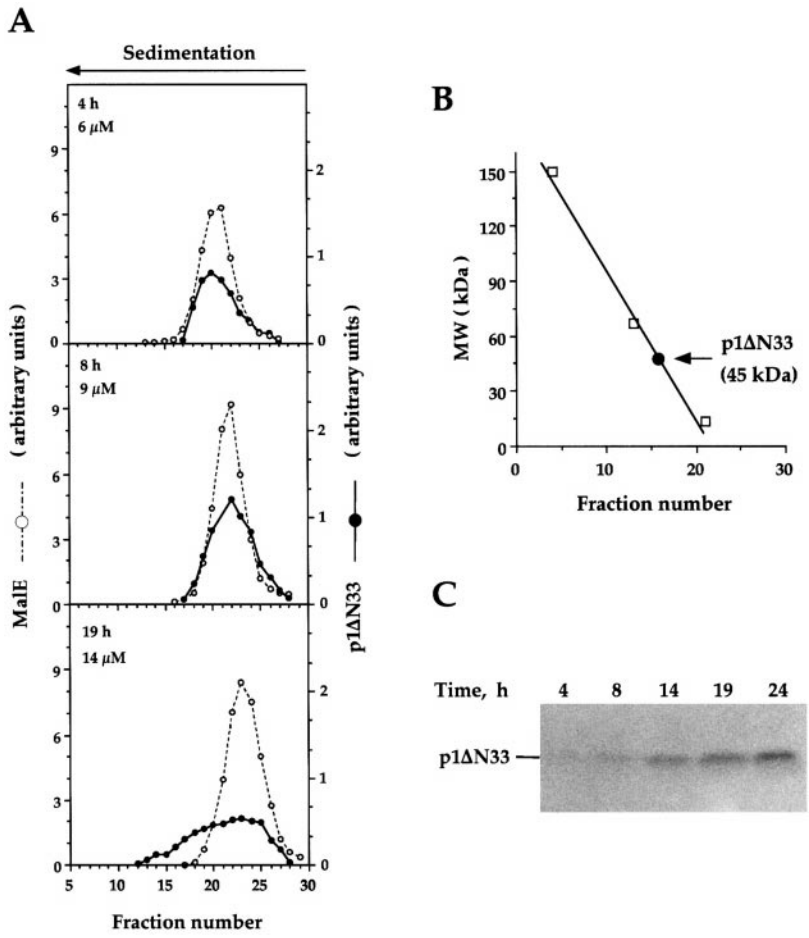

FIG. 3. **Assembly of p1ΔN33 into sheets.** Partially digested MalE-p1ΔN33 samples from the reaction mixture shown in Fig. 2*B* were analyzed by sedimentation through glycerol gradients (15–30%). *A*, samples taken at the indicated times were subjected to centrifugation for 11 h, as indicated in "Experimental Procedures." Sedimentation is from right to left. After fractionation of the glycerol gradients, aliquots from each fraction were analyzed by SDS-Tricine-PAGE. Densitometric scanning of the gel stained with Coomassie Blue was used to determine the amount of MalE and p1ΔN33 in each fraction (arbitrary units). *B*, a sample taken at 8 h, when the p1ΔN33 concentration was 9 μM, was also centrifuged for 20 h in the presence of different marker proteins (*open boxes*): alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and φ29 single-stranded DNA-binding protein (13.3 kDa). After fractionation of the glycerol gradient, aliquots from each fraction were analyzed by SDS-Tricine-PAGE. The fraction at which the maximal amount of each protein appeared was determined. The maximal amount of MalE was detected in the same fraction as the maximal amount of p1ΔN33. *C*, samples taken at the indicated times were subjected to centrifugation for 11 h, as shown in *panel A*. After fractionation of the glycerol gradients, the material sedimented at the bottom of the tubes was resuspended in loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 30% glycerol). Equivalent volumes were analyzed by SDS-Tricine-PAGE. The gel was stained with Coomassie Blue.

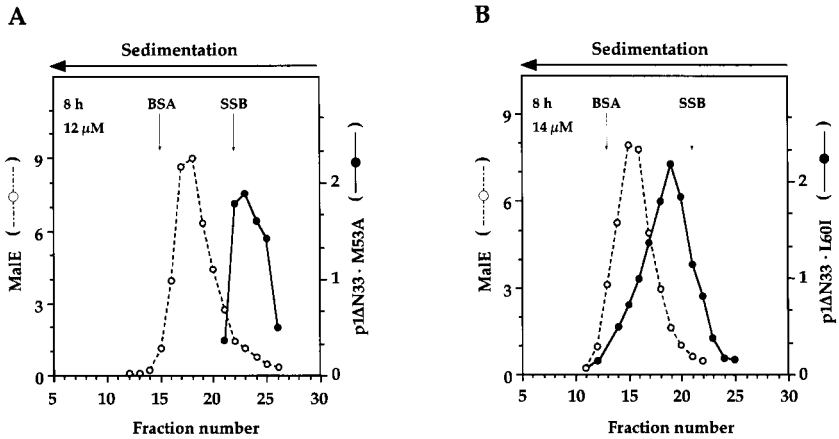

FIG. 4. **Sedimentation studies of protein p1ΔN33.M53A (*A*) and protein p1ΔN33.L60I (*B*).** The corresponding MalE fusion protein (16 μM) was treated with factor Xa for 8 h. At this time, when the concentration of p1ΔN33.M53A and p1ΔN33.L60I was 12 μM and 14 μM, respectively, the reaction mixtures were loaded on to a 15–30% glycerol gradient and subjected to centrifugation for 20 h. As markers, bovine serum albumin (*BSA*; 67 kDa) and φ29 single-stranded DNA-binding protein (*SSB*; 13.3 kDa) were loaded in the same gradient. After fractionation, aliquots from each fraction were analyzed by SDS-Tricine-PAGE. Densitometric scanning of the gels stained with Coomassie Blue was used to determine the amount of the different proteins in each fraction (arbitrary units). The native MalE protein sedimented at the position of 45 kDa. The *arrows* indicate fractions at which the maximal amount of each marker was detected.

neither protein p1ΔN33.L46A was found at the bottom of the glycerol gradient nor were negatively stained specimens visualized (not shown). Thus, protein p1ΔN33.L46A is deficient in the formation of higher-ordered structures.

The above results demonstrate that residue Leu^{46} of p1ΔN33 is essential for assembly into protofilament sheets. Specifically, replacement of Leu^{46} by the hydrophobic amino acid Val is sufficient to change the pathway of molecular assembly, generating 10-nm filamentous structures. These structures seem to be constituted by a discrete number of protofilaments adopting a helical array. Like formation of p1ΔN33 sheets, assembly of p1ΔN33.L46V into filaments can be envisaged as a two-step process. During the nucleation step, oligomeric structures are accumulated that show a sedimentation behavior different from the p1ΔN33 assembly intermediates. Furthermore, replacement of Leu^{46} by Ala results in a protein that can self-interact into oligomeric structures. These oligomers show a sedimentation behavior similar to the p1ΔN33.L46V assembly intermediates. However, despite this similarity, they must be structurally different because the p1ΔN33.L46A oligomers can-

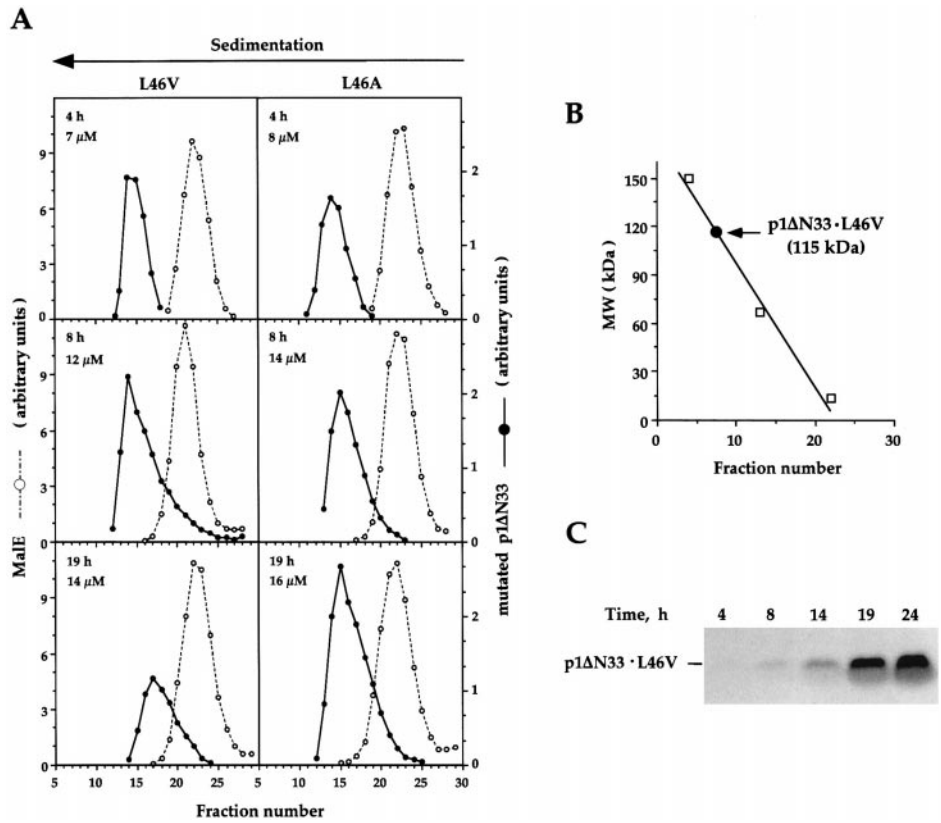

FIG. 5. **Sedimentation studies of p1ΔN33.L46V and p1ΔN33.L46A.** *A*, factor Xa was added to the corresponding MalE fusion protein (16 μM) at a ratio of 1.25% (w/w) the amount of fusion protein. At different times, samples of the reaction mixture were analyzed by SDS-Tricine-PAGE (not shown) to determine the concentration of the mutated p1ΔN33 protein, as indicated in the legend to Fig. 2*B*. Such samples were also analyzed by sedimentation through glycerol gradients (15–30%). Centrifugation time was 11 h. After fractionation of the gradients, the amount of the different proteins in each fraction (arbitrary units) was calculated by densitometric scanning of SDS-Tricine polyacrylamide gels stained with Coomassie Blue. *B*, a MalE-p1ΔN33.L46V preparation (16 μM) was digested with factor Xa for 8 h. Then the reaction mixture was subjected to centrifugation in a 15–30% glycerol gradient for 20 h. As markers (*open boxes*), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and φ29 single-stranded DNA-binding protein (13.3 kDa) were used. The fraction at which the maximal amount of each protein appeared was determined. Native MalE sedimented at the position of 45 kDa (not shown). *C*, a MalE-p1ΔN33.L46V preparation (16 μM) was digested with factor Xa. At the indicated times, samples were subjected to centrifugation in glycerol gradients for 11 h, as shown in *panel A*. After fractionation, the material sedimented at the bottom of the tubes was resuspended in loading buffer. Equivalent volumes were analyzed by SDS-Tricine-PAGE. The gel was stained with Coomassie Blue.

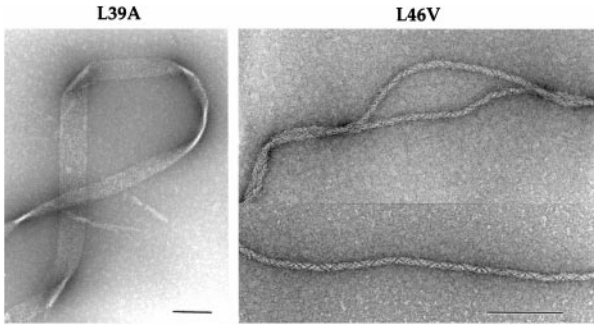

FIG. 6. **Electron micrographs of p1ΔN33.L39A and p1ΔN33.L46V structures.** Protease factor Xa was added to the corresponding MalE fusion protein (16 μM). At 19 h, samples were diluted and treated for negative staining. *Left*, protofilament sheet formed by protein p1ΔN33.L39A. When attached to the carbon, sheets tend to fold over, indicating that both sides are identical. Sheets with different widths were visualized. These structures were identical to the sheets formed by the wild-type protein. *Right*, filamentous structures formed by p1ΔN33.L46V. Although filaments of various lengths were visualized, they were always ~10 nm wide. The *upper panel* shows two filaments crossing each other. The *scale bars* represent 100 nm.

not assemble further into larger arrays.

Conclusion—Protein p1 has a short α-helical coiled-coil sequence located between Glu[38] and Asn[65] (~3 heptad repeats). This motif is also present in p1ΔN33, an N-terminal-truncated p1 protein that self-associates *in vitro* into two-dimensional protofilament sheets. We show in this study that assembly of the 53-amino acid protein p1ΔN33 into sheets can be envisaged as a two-step process. First, a nucleation step leads to the accumulation of small oligomeric structures. And second, these structures are able to function as assembly intermediates. In addition, we demonstrate that residues Leu[46], Met[53], and Leu[60] of p1ΔN33 are essential for assembly into sheets. These amino acid residues are located at the hydrophobic **d** position of the heptad repeats. Interestingly, substitution of residue Leu[46] with the smaller hydrophobic amino acid Val allows protein p1ΔN33 to assemble into long filaments, whose diameters are ~10 nm. These findings are of particular relevance because they show for the first time that (i) a short coiled-coil motif functions to assemble a small protein into two-dimensional protofilament sheets and (ii) a single conservative substitution targeting the hydrophobic core of the coiled-coil sequence is sufficient to change the pathway of molecular assembly from two-dimensional sheets to 10-nm filamentous structures.

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A Single Amino Acid Substitution within a Coiled-coil Motif Changes the Assembly of a 53-Amino Acid Protein from Two-dimensional Sheets to Filamentous Structures

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