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Structural and functional analysis of the baculovirus single-stranded DNA-binding protein LEF-3

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Abstract

The single-stranded DNA-binding protein LEF-3 of *Autographa californica* multinucleocapsid nucleopolyhedrovirus consists of 385 amino acid residues, forms oligomers, and promotes Mg^{2+}-independent unwinding of DNA duplexes and annealing of complementary DNA strands. Partial proteolysis revealed that the DNA-binding domain of LEF-3 is located within a central region (residues 28 to 326) that is relatively resistant to proteolysis. In contrast, the N-terminus (27 residues) and C-terminal portion (59 residues) are not involved in interaction with DNA and are readily accessible to proteolytic digestion. Circular dichroism analyses showed that LEF-3 is a folded protein with an estimated α-helix content of more than 40%, but it is structurally unstable and undergoes unfolding in aqueous solutions at temperatures near 50 °C. Unfolding eliminated the LEF-3 domains that are resistant to proteolysis and randomized the digestion pattern by trypsin. The structural transition was irreversible and was accompanied by the generation of high molecular weight (MW) complexes. The thermal treatment inhibited DNA-binding and unwinding activity of LEF-3 but markedly stimulated its annealing activity. We propose that the shift in LEF-3 activities resulted from the generation of the high MW protein complexes, that specifically stimulate the annealing of complementary DNA strands by providing multiple DNA-binding sites and bringing into close proximity the interacting strands. The unfolded LEF-3 was active in a strand exchange reaction suggesting that it could be involved in the production of recombination intermediates.

Keywords: Baculovirus; Single-stranded DNA-binding protein; LEF-3; Replication; Recombination

Introduction

Autographa californica multinucleocapsid nucleopolyhedrosis virus (Ac*M*NPV) belongs to the family Baculoviridae and contains a circular double-stranded DNA genome of 134 kb that encodes approximately 150 genes (Ayres et al., 1994). Ac*M*NPV is widely used for the generation of recombinant viruses for the expression of foreign genes and as a model for analysis of baculovirus molecular biology. Six viral factors, including a transactivator of early gene transcription IE-1, DNA polymerase, DNA helicase, a DNA primase called LEF-1 (*L*ate *E*xpression *F*actor 1), primase-associated factor LEF-2, and a single-stranded DNA-binding protein (SSB) LEF-3 are essential for replication of viral DNA in transient assays (Kool et al., 1994; Lu and Miller, 1995). AcMNPV LEF-3 has a molecular mass of 44.5 kDa and forms multimers in solution (Evans and Rohrmann, 1997). It binds specifically to ssDNA (Hang et al., 1995) and promotes $Mg^{2+}$and ATP-independent unwinding of partial DNA duplexes (Mikhailov, 2000) and annealing of complementary strands (Mikhailov et al., 2005). In nuclei of infected cells, LEF-3 localizes to virus replication factories and virogenic stroma (Okano et al., 1999). The abundance of this protein in infected cells, its preferential binding to ssDNA, and its unwinding and annealing activity, suggests that LEF-3 is a member of a diverse family of SSB proteins. Despite minimal sequence homology and a highly diverse structure, different SSBs likely play similar roles in DNA metabolism. By destabilizing DNA duplexes, SSBs facilitate various transitions of DNA that accompany replication, repair, and recombination. In addition, they serve as accessory factors for other proteins and enzymes. LEF-3 has been demonstrated to interact with baculovirus DNA helicase (Chen et al., 2004; Evans et al.,

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1999; Wu and Carstens, 1998) and alkaline nuclease (Mikhailov et al., 2003). It remains unclear how the multiple functions of LEF-3 are regulated in vivo. But in model experiments with oligonucleotides, the DNA-binding, unwinding, and annealing activities of LEF-3 are regulated by redox factors with the oxidized state favoring annealing and the reduced state favoring unwinding (Mikhailov et al., 2005).

In this report, we analyzed the LEF-3 structure by physical methods and by partial proteolysis. LEF-3 was found to be structurally unstable, and it underwent unfolding under heating to approximately 50 °C. The thermal treatment decreased the DNA binding and unwinding activities of LEF-3 but, surprisingly, stimulated its annealing activity. We propose that the functional switch in LEF-3 activities results from a change in the protein structure and the generation of high molecular weight (MW) protein species and may reflect an intrinsic biological function of this protein.

Results

The baculovirus single-stranded DNA-binding (SSB) protein LEF-3 promotes Mg^{2+}- and ATP-independent unwinding of DNA duplexes (Mikhailov, 2000) and annealing of complementary DNA strands (Mikhailov et al., 2005). Both LEF-3 activities function in a competitive manner, and the balance between unwinding and annealing is determined by the ratio of LEF-3 to DNA and by the redox state (Mikhailov et al., 2005). Mild thermal treatment dramatically and irreversibly changed the properties of LEF-3 and affected its unwinding and annealing activities. After heating for 10 min at 50 °C, LEF-3 lost the ability to unwind partial DNA duplexes but acquired an ability to anneal DNA strands (Fig. 1a). The change in activity was observed after LEF-3 incubation at 50 °C or higher temperatures but not after incubation at lower temperatures (Fig. 1b). These data suggested that LEF-3 is structurally unstable and undergoes structural transition at temperatures near 50 °C that shifts the balance between its activities in favor of annealing.

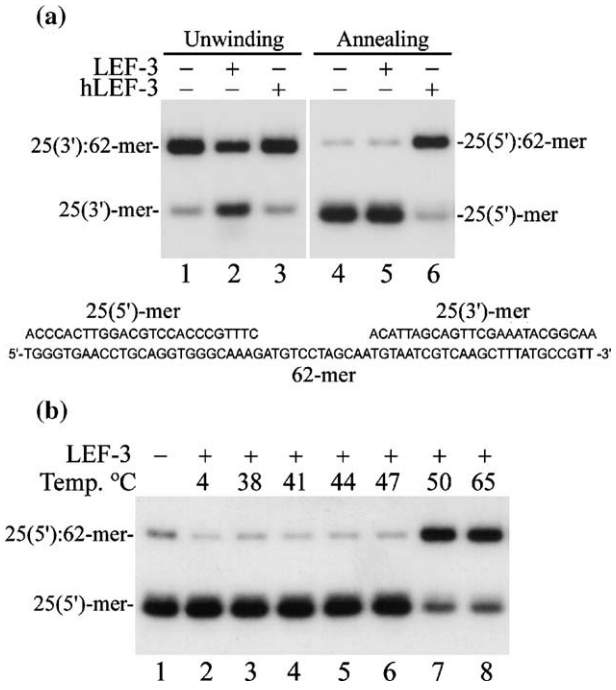

Fig. 1. (a) Inactivation of the unwinding activity and the induction of the annealing activity after heat treatment of LEF-3. The assay was carried out in standard 10-μl reactions containing 0.5 nM of ^{32}P-labeled 25(3′):62-mer partial duplex (lanes 1 to 3) or a mixture of 0.5 nM of ^{32}P-labeled 25(5′)-mer and 1 nM of 62-mer (lanes 4 to 6). The control LEF-3 sample ("LEF-3") and the LEF-3 sample heated for 10 min at 50 °C ("hLEF-3"), each in a concentration of 0.25 μM were added respectively to the reactions 2, 5 and 3, 6. The reactions were incubated for 60 min at 30 °C and then analyzed by PAGE. Lanes 1 and 4 represent control reactions lacking LEF-3. The sequences of 25(5′)-mer, 25(3′)-mer, and 62-mer are shown below the panel. (b) The heat-induced change in the annealing activity of LEF-3. The annealing assay was carried out as in panel a above. The LEF-3 samples were incubated for 10 min at the temperatures indicated above respective lanes and were added to the reactions 2 to 8 in concentration of 0.25 μM. The reactions were incubated for 60 min at 30 °C and then analyzed by PAGE. Lane 1 represents control reaction lacking LEF-3.

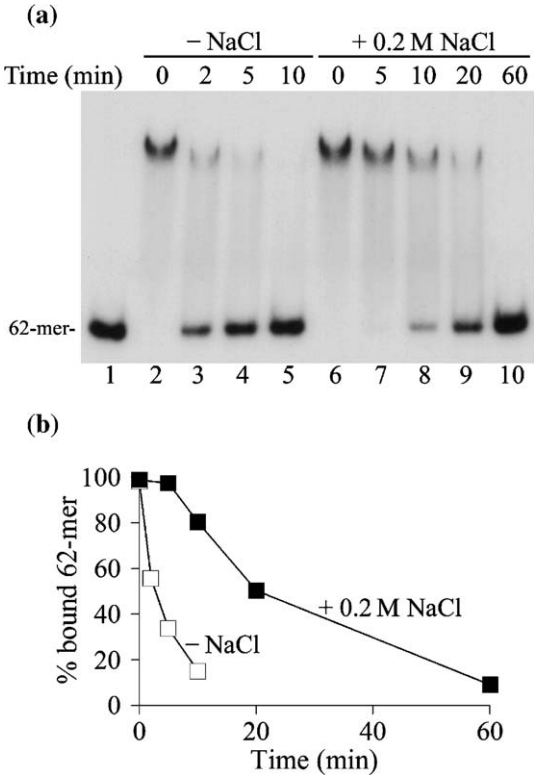

Fig. 2. Inactivation of the DNA-binding activity of LEF-3 in the course of thermal treatment. (a) The LEF-3 samples diluted to 90 nM in a buffer (10 mM Tris–HCl, pH 7.5, 50% glycerol, 1 mM DTT, and 0.2 mM EDTA) (lanes 2–5), or in the same buffer containing 0.2 M NaCl (lanes 6–10) were incubated for the indicated times at 50 °C, then cooled on ice and used for the DNA-binding assay. The standard 10-μl reactions contained 0.2 nM of ^{32}P-labeled 62-mer. After LEF-3 (18 nM) addition, the reactions were incubated for 15 min at 22 °C and then analyzed by EMSA. Lane 1 represents a control reaction lacking LEF-3. (b) The decrease in DNA-binding activity of LEF-3 in the course of thermal treatment in the presence of 0.2 M NaCl (filled symbols) and in the absence of salt (open symbols). The gel shown in panel a was used for quantification.

The analysis of LEF-3 binding to DNA by using electrophoretic mobility shift assay (EMSA) revealed a drop in LEF-3 affinity for ssDNA in the course of thermal treatment. Incubation of LEF-3 at 50 °C caused a rapid decline in its binding to a 62-mer oligonucleotide probe (Fig. 2a, lanes 2–5). The addition of 0.2 M NaCl to the LEF-3 samples before the treatment (lanes 6–10) markedly slowed the inactivation rate,

suggesting that the monovalent salt stabilizes the protein structure and a repulsion of charged groups in LEF-3 contributed to the structural transition caused by heating. The next experiments were designed to elucidate the changes in LEF-3 structure caused by thermal treatment.

To examine the folding and thermal stability of LEF-3, we recorded circular dichroism (CD) spectra and performed a thermal denaturation experiment (Fig. 3). Two structural characteristics, the α-helix content (parameter H) and the extended-β-strand content (parameter E), were calculated by using the CDsstr software (Johnson, 1999). The spectrum of LEF-3 at room temperature (22 °C) was characteristic of a folded, helical protein with an estimated α-helix content higher than 40% (Fig. 3a). In the interval from 22 to 40 °C, the CD spectrum of LEF-3 was steady showing that the α-helixes and β-strands comprised respectively 43.5 ± 2.5% and 13.2 ± 2.1% of the LEF-3 structure. These data confirmed that LEF-3 is stable in this temperature interval. Under further heating from 40 to 50 °C, the CD spectrum underwent a change to another pattern with approximately two-fold lower α-helix content and higher β-strand content. In the interval from 50 to 70 °C, the α-helixes and the β-strands comprised respectively 20.6 ± 2.2% and 32.1 ± 2.2% of the LEF-3 structural motifs. These data indicated that the helical structure of LEF-3 was disrupted by the thermal treatment. The CD melting curve at 222 nm (Fig. 3b) confirmed that LEF-3 was unfolded cooperatively under heating in the interval from 40 to 50 °C with an apparent T_m value of approximately 45 °C. The heat-induced structural transition of LEF-3 was irreversible because cooling of the denatured LEF-3 sample from 70° to 22 °C did not restore the original CD spectrum typical of the native protein (Fig. 3b).

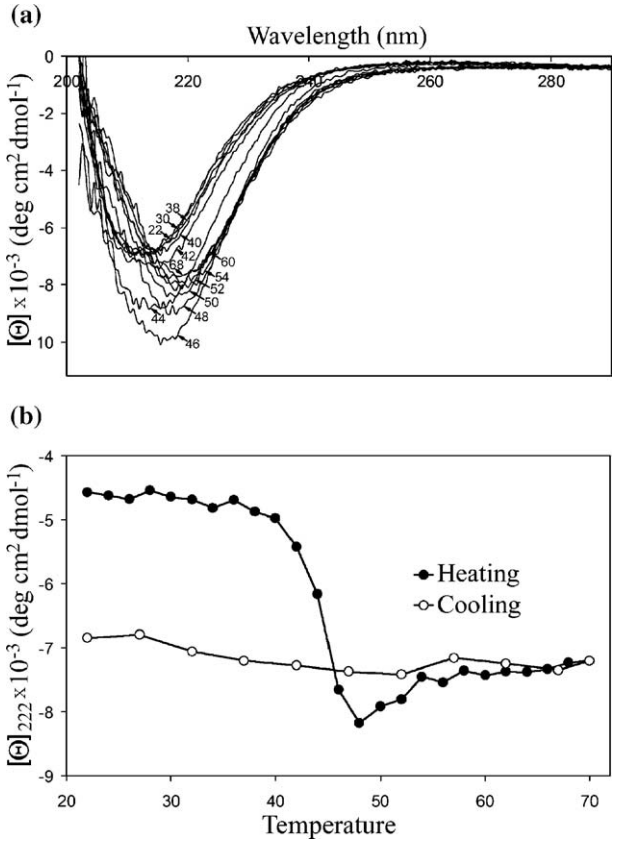

Fig. 3. Change in the circular dichroism (CD) spectrum of LEF-3 in the course of thermal denaturation. The CD spectra from 198 to 290 nm were recorded at 2 °C intervals under heating from 22 to 70 °C and then at 5 °C intervals under cooling from 67 to 22 °C. The LEF-3 sample (1 mg/ml) was in a buffer containing 2.5 mM Tris, pH 7.5, 0.2 M NaCl, 2.5% glycerol, 0.05 mM EDTA, and 1 mM DTT. (a) The representative CD spectra of LEF-3 obtained at indicated temperatures in the course of heating. (b) The ellipticity of LEF-3 recorded at 222 nm as a function of temperature during the heating (filled circles) and subsequent cooling (open circles).

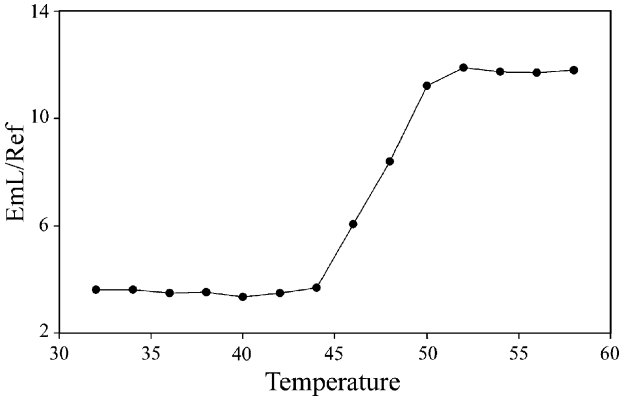

Fig. 4. Change in light scattering of LEF-3 in the course of thermal denaturation. Light scattering of LEF-3 sample was recorded at 400 nm at 2 °C intervals under heating from 32 to 58 °C and is expressed as a ratio to a signal from a control sample lacking LEF-3. LEF-3 (0.8 mg/ml) was in a buffer containing 2 mM Tris, pH 7.5, 0.2 M NaCl, 2% glycerol, 0.04 mM EDTA, and 1 mM DTT.

In parallel to the analysis of the CD spectra, we measured the light scattering of LEF-3 samples in the course of the thermal denaturation experiment. The light scattering was steady under heating to approximately 44 °C, and then it increased dramatically in the interval from 44 to 50 °C (Fig. 4). This result suggested that the structural transition of LEF-3 at the temperature near 50 °C was accompanied by protein oligomerization or aggregation. The protein oligomerization likely prevents proper renaturation of the heated LEF-3 at lower temperatures (Fig. 3b). To confirm the multimerization of LEF-3 after the thermal treatment, gel filtration experiments were performed (Fig. 5). In order to prevent nonspecific

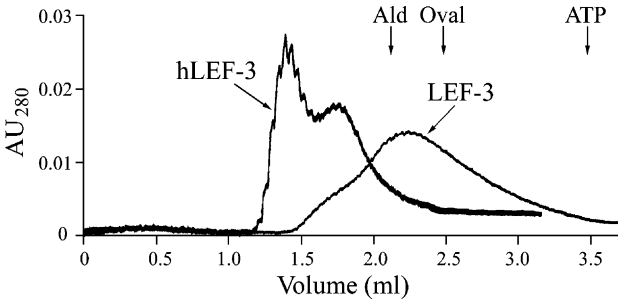

Fig. 5. Gel filtration of LEF-3. Native LEF-3 or heat-treated LEF-3 (hLEF-3) each at 13 μg in 50 μl of column buffer (0.4 M NaCl, 10 mM Tris–HCl, pH 7.5, 10% glycerol, 1 mM EDTA, and 1 mM DTT) were applied to a 3.4-ml column of Sephacryl S-300 HR. The column was processed at a rate of 0.1 ml/min, and absorbance was registered at 280 nm (AU_{280}). Aldolase (Ald, 158 kDa), ovalbumin (Oval, 45 kDa), and ATP were used for column calibration.

aggregation of protein during this experiment, high salt (0.4 M NaCl) was included in the running buffer. The native LEF-3 (line "LEF-3") runs through the Sephacryl S-300 HR gel as a protein with a mass estimated to be ~100 kDa based on its elution relative to aldolase (158 kDa) and ovalbumin (45 kDa). After thermal treatment, LEF-3 was comprised of heterogeneous multimeric species (line "hLEF-3") that eluted ahead of the native protein up to the position of the exclusion volume (~1.4 ml) of the column. Native LEF-3 forms multimers in solution (Evans and Rohrmann, 1997). The data shown in Figs. 4 and 5 indicate that the unfolding of LEF-3 under thermal treatment augments its capacity to produce multimeric and aggregated forms.

To examine the protein folding and the heat-induced structural transition in more detail, we performed limited proteolysis of LEF-3 using trypsin and analyzed the digestion products by SDS-PAGE (Fig. 6a). The digestion pattern of native LEF-3 revealed protein domains that are relatively resistant to trypsin. Major resistant domains corresponded to fragments with apparent molecular masses (in kDa) of 42.6 (I fragment), 36 (XL fragment), 35 (L fragment), and 26 (S fragment). Thermal treatment of LEF-3 before digestion by trypsin randomized the cleavage and eliminated the digestion pattern specific for the native protein (see alternate lanes in Fig. 6a). This result confirmed that heat treatment disrupts the regular helical structure of LEF-3 and causes protein unfolding. At the early stage of the cleavage reaction, trypsin removed a fragment of approximately 2 kDa from one terminus of native LEF-3 and generated the 42.6-kDa fragment I (lane 3). The respective cleavage site appeared to be readily accessible to trypsin thus suggesting that the terminal fragment is exposed externally in the intact protein. To identify the exposed terminus of LEF-3, we compared the cleavage patterns of wild-type (wt) LEF-3 and the truncated LEF-3 which was tagged with 25 additional amino acids including six histidines at the N terminus (Fig. 6b). Due to the presence of 3.1-kDa fragment at the N-terminus, the His-tagged LEF-3 migrated slower than wt LEF-3 under SDS-PAGE (compare lanes 1 and 2 in Fig. 6b). Within 5 min, trypsin produced the 42.6-kDa fragment I from both, the His-tagged LEF-3 and wt LEF-3 (lanes 3 and 4), and then the digestion patterns of both LEF-3 species were indistinguishable. This result suggests that the initial cleavage by trypsin occurred at the N-terminal region, and the N-terminal fragment of approximately 20 residues is exposed from the folded and more compact central and C-terminal portions of LEF-3.

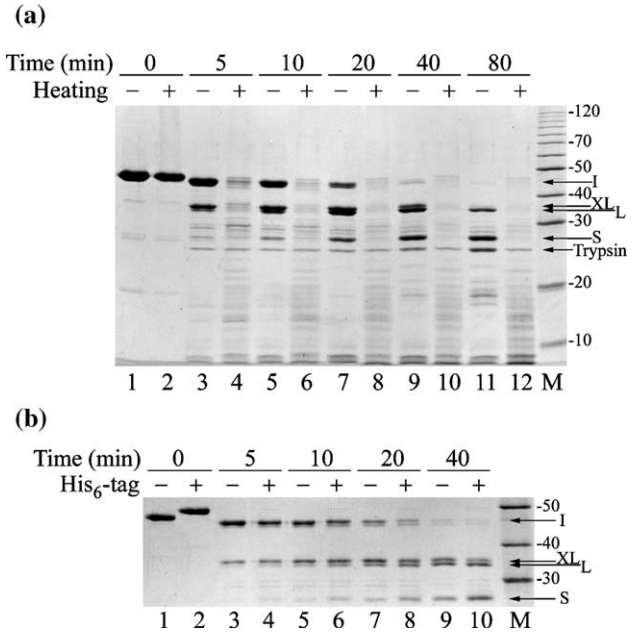

Fig. 6. Limited proteolysis of LEF-3 by trypsin. (a) Time course of trypsin digestion of native LEF-3 (marked by "−") and LEF-3 unfolded by heating for 10 min at 50 °C (marked by "+"). The 10-μl reactions containing 2 μg of LEF-3 and 0.1 μg of trypsin were incubated at 25 °C for a time indicated above respective lanes and terminated by the addition of 5.1 μl of loading buffer (3 mM PMSF, 190 mM Tris, pH 6.8, 4.3 M 2-mercaptoethanol, 6% SDS) and by boiling for 3 min. 12-μl portions from the reactions were analyzed by SDS-13% PAGE. Trypsin was not added to the time zero reactions (lanes 1 and 2). (b) Time course of trypsin digestion of wt LEF-3 (marked by "−") and LEF-3 tagged with His_6 at the N-terminus (marked by "+"). The 10-μl reactions containing 1 μg of LEF-3 and 0.05 μg of trypsin were incubated at 25 °C for the times indicated above respective lanes and then were analyzed by SDS-10% PAGE.

We used MALDI/TOF mass spectrometry to determine precisely the size of the major proteolytic fragments of LEF-3. The mass spectra were obtained for six time points during a 40-min cleavage reaction with trypsin. Three spectra, for time 0, 10, and 40 min are shown in Fig. 7. The following average molecular masses (±standard deviation) were obtained respectively for fragments I, XL, L, and S: 42594 (±9), 35760 (±3), 34864 (±4), and 25522 (±3). To map the cleavage sites that generated fragments XL, L, and S, the digestion products were transferred from SDS-polyacrylamide gel to an Immobilon-P membrane, and the fragments were sequenced by Edman degradation. The N-terminal residues of fragments XL, L, and S were respectively MAMAS, KIRE-Y, and FSQMIQ. The terminal sequences and the masses of fragments XL, L, and S allowed us to map the proteolytically stable domains in LEF-3 (Fig. 8). Two possible C-ends are shown for fragments XL, L, and S due to the presence of adjacent recognition sites for trypsin in the C-terminal regions of these fragments. The location of fragments XL, L, and S in LEF-3 structure suggests that trypsin starts cleavage of the native protein in the terminal regions, whereas the central portion of protein was more proteolytically stable. The rather random cleavage of LEF-3 after the heat treatment (Fig. 6a) indicates that LEF-3 unfolding exposed the sites located in the compact central region to proteolytic attack.

In order to clarify functional roles of the major proteolytically resistant domains of LEF-3 in DNA binding, we analyzed a trypsin digest of LEF-3 by column chromatography on ssDNA cellulose (Fig. 9). The largest peptide, fragment I, which lacks the N-terminal 19 amino acid residues was found to bind tightly to ssDNA and was eluted in a range of 0.8 M to 1.0 M NaCl. This is similar to the profile for native LEF-3 (data not shown). The fragments XL and L, which lack portions of both the N- and C-terminal regions, were mostly eluted at 0.8 M NaCl, one fraction ahead of fragment I. This result indicated that the affinity for ssDNA of both fragments, XL and L, was

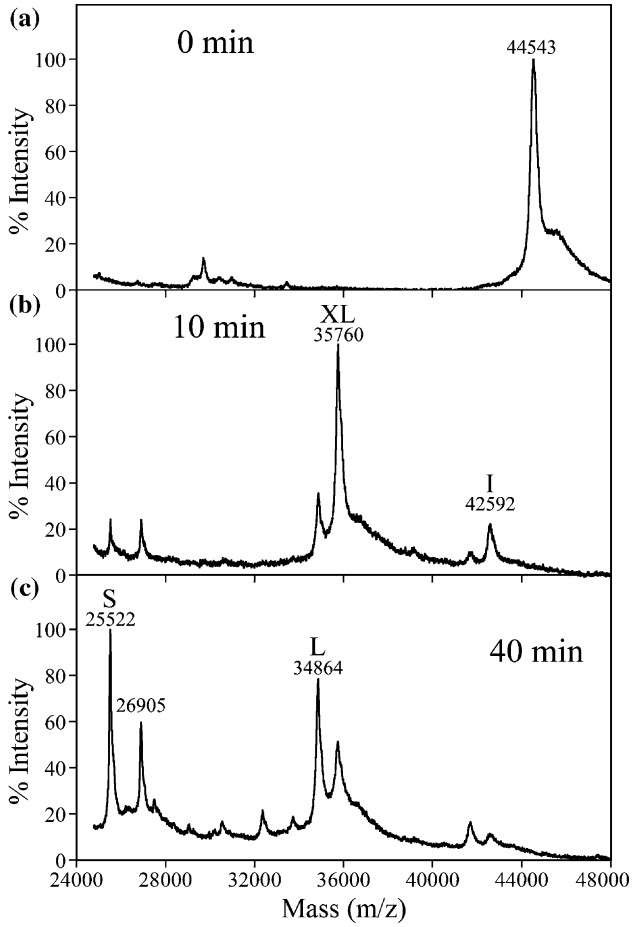

Fig. 7. MALDI-TOF mass spectrometry of the LEF-3 tryptic fragments. LEF-3 was digested with trypsin at a 1:20 trypsin/LEF-3 ratio for 40 min at 25 °C, and the portions were taken from the reaction at different time points. The figure shows the mass spectra obtained for the times indicated. The average masses of major tryptic fragments I, XL, L, and S are shown above their signals. The peak with the largest mass (44543) represents uncleaved LEF-3.

high but slightly lower than that of fragment I. In contrast to fragments I, XL, and L, the smallest fragment S (encompassing residues 28 to 313 or 316) showed a relatively low affinity for

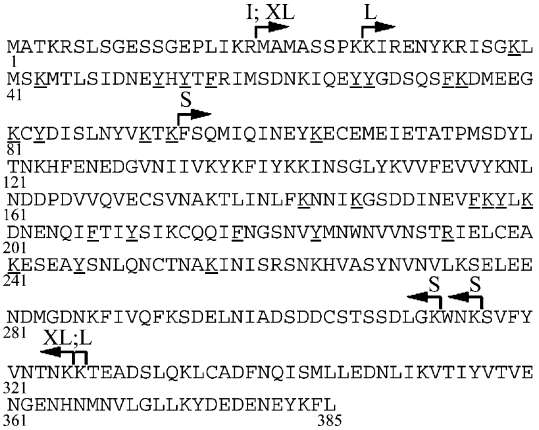

Fig. 8. Location of major tryptic fragments I, XL, L, and S in the LEF-3 sequence. Two possible carboxyl termini are shown for the fragments XL, L, and S due to the presence of adjacent recognition sites for trypsin in the C-terminal regions. The amino acid residues that form two putative DNA-binding motifs of LEF-3 are underlined.

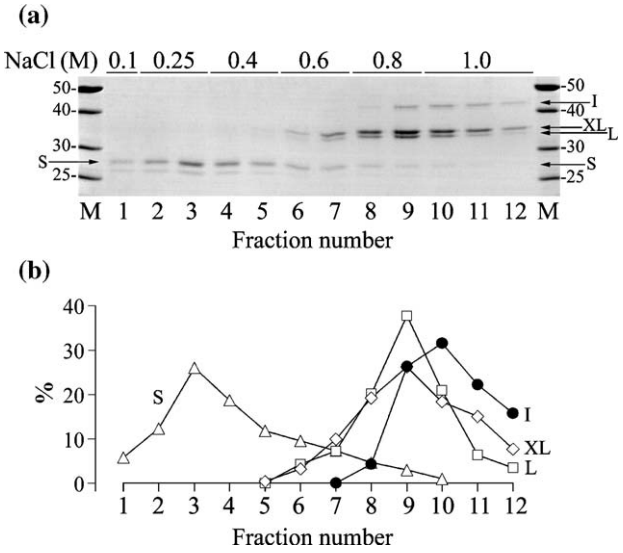

Fig. 9. Chromatography of a partial digest of LEF-3 by trypsin on ssDNA cellulose. LEF-3 (200 μg) was digested by trypsin as described under "Materials and methods". After loading of the sample onto a 0.5-ml column of ssDNA cellulose, the column was washed with 2 ml of a buffer containing 0.1 M NaCl, 10 mM Tris, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF and then processed with 0.5 ml of the same buffer (fraction 1) and with 1-ml portions of the buffer containing NaCl in concentration of 0.25 M (fractions 2 and 3), 0.4 M (fractions 4 and 5), 0.6 M (fractions 6 and 7), 0.8 M (fractions 8 and 9), and finally with the buffer containing 1 M NaCl (fractions 10–12). (a) Ten-microliter portions from the fractions were analyzed by SDS-13% PAGE followed by Coomassie staining. (b) The yield of major tryptic fragments I, XL, L, and S in fractions eluted from ssDNA cellulose at different salt concentrations. The gel shown in panel a was used for quantification. The total yield of each fragment in the fractions was taken as 100%.

ssDNA and was eluted from the column by the 0.1 M wash and then over a range spanning 0.25 to 0.4 M NaCl. The high affinity of fragment L for ssDNA suggests that the central portion of LEF-3 (residues 28 to 326) forms the DNA-binding domain, whereas the N-terminal 27 residues and C-terminal 59 residues do not interact with DNA.

The heat treatment also dramatically changed the fluorescence properties of LEF-3. After heating for 10 min at 60 °C, the emission of LEF-3 dropped approximately 6-fold, and the fluorescence peak was shifted by approximately 10 nm to longer wavelengths (data not shown). Under excitation at 280 nm, the native and heat-treated LEF-3 produced maximum fluorescence signals respectively at 320 nm and 330 nm. Under excitation at 292 nm, the native and heat-treated LEF-3 showed fluorescence maxima respectively at 326 nm and 337 nm. Ac*M*NPV LEF-3 contains twenty-two tyrosine and two tryptophan residues that contribute to the intrinsic fluorescence. The marked decrease in the protein fluorescence and the "red shift" in the emission spectra observed after heat treatment both suggested that the aromatic amino acids were more exposed to solvent in the heated LEF-3 than in the native protein. These data were in agreement with results of LEF-3 proteolysis, and they confirmed that the thermal treatment caused protein unfolding and made the buried amino acids more accessible.

The increase in the annealing activity of LEF-3 after thermal treatment (Fig. 1a) suggests that the unfolded form of LEF-3 is

capable of performing physiologically important reactions. To confirm this, we examined the ability of heated LEF-3 to initiate a strand exchange reaction that is an intermediate step in homologous recombination. This reaction requires the transfer of a DNA strand from a donor duplex molecule to an acceptor ssDNA molecule. Two linear DNA fragments of 5310 bp and 2289 bp were prepared from the replicative form (RF) of M13mp9 DNA, and they were used as donors of complementary (−) strands to the circular (+) strand of M13mp9 DNA. To generate single-stranded 5′-tails in the (−) strands, the linear DNA fragments were treated with Exonuclease III, which is a 3′→5′ exonuclease, and then they were labeled with ^{32}P at the 3′-ends. The production of chimeric DNA molecules containing both the circular (+) strand and the ds fragment annealed via its expanded 5′-tail was monitored by electrophoresis in an agarose gel. Native LEF-3 in a concentration range from 5 to 500 nM was inactive in the exchange reaction. Lane 1 in Figs. 10a, b demonstrate the absence of the chimeric DNA molecules after the reaction in the presence of 500 nM LEF-3. In contrast, the thermally activated LEF-3 in a similar concentration range promoted the dose-dependent generation of the chimeric DNA molecules (lanes 4 to 7). The enhanced activity of the unfolded LEF-3 in the strand exchange reaction was also demonstrated in experiments with partial duplexes pre-treated with Ac*M*NPV alkaline nuclease that possesses 5′→3′ exonuclease activity and forms a complex with LEF-3 that likely participates in homologous recombination (data not shown). These results confirmed that the unfolded LEF-3 promotes physiologically important reactions under conditions at which the folded protein is inactive.

Discussion

The baculovirus DNA-binding protein LEF-3 has a high affinity for ssDNA and presumably plays multiple roles in replication, repair, and recombination of the viral genome. Probing LEF-3 structure by trypsin digestion (Figs. 6–9) provided information about protein domains and their possible function. Accessibility of the N-terminal and C-terminal portions of LEF-3 to proteolytic attack suggests that the N-terminus (19 residues) and C-terminus (~60 residues) are exposed from the more resistant central portion of protein that forms a DNA-binding domain. The N-terminal region presumably does not bind to DNA because deletion of N-terminal 19 residues (fragments I and XL) or 27 residues (fragment L) did not dramatically affect DNA binding (Fig. 9). The LEF-3 nuclear localization signal is located within the N-terminal 56 amino acid residues (Chen and Carstens, 2005). Therefore, the N-terminal region likely interacts with factors involved in nuclear transport and may form a domain separate from the more compact central core. Deletion of C-terminal 59 or 60 residues (fragments XL and L) also did not cause a major change in LEF-3 affinity for ssDNA indicating that this region does not interact directly with DNA. This domain of LEF-3 may be involved in protein–protein interaction because deletion of the C-terminal 15 amino acids eliminates the ability of LEF-3 to interact with itself (Evans et al., 1999). If fragments XL and L lack a domain essential for oligomerization, they may bind DNA in a noncooperative manner. That might be responsible for the decrease in the apparent affinity of these fragments for ssDNA in comparison to fragment I that retains an intact C-terminus (Fig. 9). The relatively high affinity of fragment L for ssDNA suggests that a central portion of LEF-3 located within residues 28 to 326 forms the DNA-binding domain. Basic and aromatic amino acids of DNA-binding proteins are thought to interact with DNA. The L fragment contains two motifs matching the consensus sequence, K/R-X(2–5)-K/R-X(4–12)-F/Y-X(2–14)-F/Y-X(6–13)-F/Y-X(1–19)-K/R-X(3–26)-F/Y/W-X(6–11)-R/K, found in prokaryotic and eukaryotic SSBs (Wang and Hall, 1990). The first and second motifs are separated by a stretch of 78 residues, and they are located at positions 39 to 104 and 183 to 256, respectively. We have demonstrated previously that LEF-3

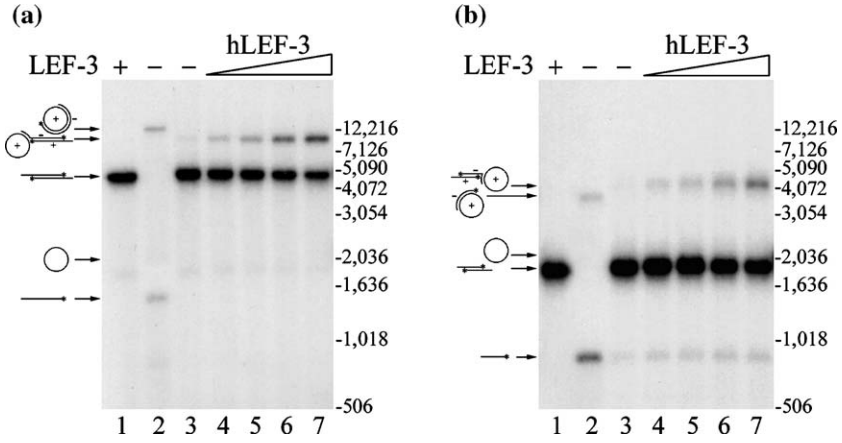

Fig. 10. The strand exchange reaction promoted by heat-activated LEF-3. The assay was carried out in 10-μl reaction mixtures containing 0.01 nM of ^{32}P-labeled partial duplexes of 5310 bp (a) or 2289 bp (b), 0.4 nM M13mp9 ssDNA, 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 15% glycerol, 100 μg/ml BSA, 2 mM DTT. After the addition of native LEF-3, 0.5 μM (lane 1), or unfolded LEF (hLEF-3), 0.12 μM (lane 4), 0.25 μM (lane 5), 0.5 μM (lane 6), and 0.75 μM (lane 7), the mixtures were incubated at 37 °C for 1 h. Lane 3 shows a control reaction lacking LEF-3. Reactions were terminated by the addition of SDS (0.5%), treated with proteinase K (150 μg/ml), and then analyzed by electrophoresis in a 1% agarose gel. Lane 2 shows the products obtained by boiling of respective ^{32}P-labeled duplexes followed by cooling in the presence of M13mp9 ssDNA.

has two available binding sites for oligonucleotide dT_{17}, but only one site for larger oligonucleotide dT_{31} (Mikhailov et al., 2005). We think that the two structurally separate LEF-3 domains with small DNA-binding sites may actually form one large DNA-binding site. Deletion of the first putative DNA-binding motif up to residue 94 (fragment S) markedly decreased the protein affinity for ssDNA but did not completely abolish DNA binding, presumably due to the presence of the second binding motif.

The characterization of LEF-3 by physical methods showed that this protein is structurally unstable, and its structure is altered by heating to 50 °C. The thermal treatment decreased approximately two-fold the α-helix content of LEF-3 (Fig. 3) and eliminated the protein domains resistant to proteolysis (Fig. 6). The structural transition was also accompanied by a marked decrease in the protein fluorescence and by a "red shift" of the fluorescence spectra that indicated the exposure of buried aromatic amino acids to a solvent. All these results proved that LEF-3 undergoes unfolding at temperatures near 50 °C. The structural instability of SSB proteins may be intrinsically connected to their function. The modes of interaction of SSBs with DNA are highly sensitive to ionic conditions (reviewed in Chase and Williams, 1986; Meyer and Laine, 1990), and structural transitions accompany their binding to DNA (Blackwell et al., 1996; Dekker et al., 1998; Dudas et al., 2001; Gomes et al., 1996; Uprichard and Knipe, 2003; Villemain and Giedroc, 1993). Multiple protein–protein interactions, post-translation modifications such as phosphorylation, and redox factors may affect both the structure and function of SSBs. In addition, multimerization may serve as a specific mechanism for regulation of SSB proteins. The bacteriophage T4 gene 32 protein (gp32) forms oligomers in solution (Carroll et al., 1975) and protein chains on ssDNA (Kowalczykowski et al., 1981). The herpes simplex virus protein ICP8 forms long filaments in solution (O'Donnell et al., 1987). Multimerization of the adenovirus DNA-binding protein (DBP) serves as the driving force for DNA unwinding (Dekker et al., 1997). The baculovirus SSB protein LEF-3 forms oligomers, predominantly trimers, in solution (Evans and Rohrmann, 1997). Oxidation of LEF-3 stimulates generation of high molecular weight (MW) oligomers, presumably via dimerization of LEF-3 trimers by inter-protein crosslinks (Mikhailov et al., 2005). In a previous report (Mikhailov et al., 2005), we showed that a balance between unwinding and annealing activities of LEF-3 is determined by its redox state with the oxidized state favoring annealing and the reduced state favoring unwinding. To explain the regulation of LEF-3 activities by redox factors, we proposed that the high MW oligomers generated by oxidation specifically stimulate the annealing of complementary DNA strands by providing multiple DNA-binding sites and bringing into close proximity the interacting strands (Mikhailov et al., 2005). This hypothesis is consistent with the data obtained in this report. The heat-induced LEF-3 unfolding resulted in protein oligomerization and aggregation as revealed by the dramatic increase in light scattering of LEF-3 samples after the thermal treatment and by increase in apparent mass of heated protein under gel filtration (Figs. 4 and 5). The high MW complexes were presumably stabilized by associations other than disulfide bonds because the complexes also appeared after heating LEF-3 samples in the presence of a reducing agent (20 mM DTT) (data not shown). Thermal unfolding decreased the affinity of LEF-3 for ssDNA (Fig. 2) and inhibited its unwinding activity (Fig. 1). However, paradoxically, unfolding stimulated the annealing activity of LEF-3. The increase in the annealing activity of LEF-3 after the thermal treatment was demonstrated in experiments with both, short oligonucleotides (Fig. 1) and long fragments of phage M13 DNA (Fig. 10). The elevated annealing activity of the high MW complexes suggests that they may mimic physiologically relevant activities of LEF-3 multimers in the nuclei of infected cells. Insects have to survive at a broad range of temperatures, and they have developed a highly sophisticated defense mechanism against heat shock (for references see Feder and Hofmann, 1999). Although to ensure productive infection and virus spread, baculoviruses may have to tolerate heat shock conditions, the specific viral defense mechanisms allowing them to contend with elevated temperatures are unknown. LEF-3 is an essential viral protein which accumulates to a high level in the nucleus (Okano et al., 1999). The unwinding and annealing activities of LEF-3 act in a competitive manner (Mikhailov et al., 2005). The shift in LEF-3 activity from DNA unwinding to annealing may help to preserve the integrity of the viral genome at high temperatures, thus reflecting a specific defense mechanism of baculoviruses against heat shock. It is possible that other factors that interact with LEF-3 in the replication and recombination complexes may decrease the energy (and temperature) required for its structural transition. This may allow the transition to occur at temperatures lower than 50 °C. In addition, the activity of the unfolded LEF-3 in the strand exchange reaction (Fig. 10) suggests a possible involvement of this LEF-3 form in homologous recombination that likely plays a vital role in the baculovirus DNA replication cycle (Kazuhiro Okano, unpublished). As shown previously (Mikhailov et al., 2005), oxidation induces oligomerization of LEF-3 and markedly stimulates its annealing activity. Therefore, the activated multimeric species of LEF-3 may be commonly produced in the infected cell by mechanisms other than heat shock.

Materials and methods

Cells and recombinant baculoviruses

Spodoptera frugiperda 9 (Sf9) cells were cultured in Sf900II serum-free media (Invitrogen), penicillin G (50 units/ml), streptomycin (50 μg/ml, Whittaker Bioproducts), and fungizone (amphotericin B, 375 ng/ml, Flow Laboratories) as previously described (Harwood et al., 1998). Recombinant baculovirus vAcLEF3 for overexpression of Ac*M*NPV LEF-3 under the control of the *polh* promoter was previously described (Mikhailov et al., 2004). For overexpression of His-tagged LEF-3, a recombinant baculovirus vAcHisLEF3 was obtained using the Bac-to-Bac baculovirus expression system (Invitrogen) following the manufacturer's instructions.

DNA substrates

The following oligonucleotides were used for assay of LEF-3 activities: 62-mer, TGGGTGAACCTGCAGGTGGGCAAA-GATGTCCTAGCAATGTA ATCGTCAAGCTTTATGCCGTT; 25(3′)-mer, AACGGCATAAAGCTTGACGATTACA; and 24-mer (5′-P)CTTTGCCCACCTGCAGGTTCACCC. The 62-mer and 25(3′)-mer were labeled at the 5′-end by using T4 polynucleotide kinase and [γ-^{32}P]ATP (Perkin-Elmer). To obtain the 25(5′)-mer, the 24-mer was 3′ end-labeled with [α-^{32}P] 3′-deoxyadenosine 5′-triphosphate (cordycepin 5′-triphosphate) (Perkin-Elmer). For the unwinding assay, the ^{32}P-labeled 25(3′)-mer was annealed with a 1.5 molar excess of the (5′-P)62-mer.

Purification of LEF-3

Ac*M*NPV LEF-3 was purified from Sf9 cells infected with wild-type Ac*M*NPV or with the recombinant baculovirus vAcLEF3 as described previously (Mikhailov et al., 2004) and was stored at −20 °C in 10 mM Tris–HCl, pH 7.5, 50% glycerol, 1 mM dithiothreitol (DTT), 0.2 mM EDTA, and 0.2 M NaCl, or in the same buffer without NaCl. The His-tagged wt LEF-3 was expressed in recombinant baculovirus vAcHi-sLEF3 under the control of the *polh* promoter and purified as described (Mikhailov et al., 2005). To obtain unfolded LEF-3, the samples were diluted in the storage buffer without NaCl to concentration less than 400 μg/ml and incubated for 10 min at 50 °C.

Assay for LEF-3 activities

The annealing and unwinding assay was carried out in 10-μl reaction mixtures containing 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2 mM DTT, 12.5% glycerol, and 100 μg/ml BSA. The unwinding assay contained 0.5 nM of ^{32}P-labeled 25(3′):62-mer partial duplex. The annealing assay contained a mixture of 0.5 nM of ^{32}P-labeled 25(5′)-mer and 1 nM of 62-mer. After addition of LEF-3, the reactions were incubated for 1 h at 30 °C, then treated with SDS (0.5%) and proteinase K (150 μg/ml) for 15 min at room temperature, and analyzed by 6% polyacrylamide gel electrophoresis (PAGE) in 20 mM HEPES, pH 8.0, 0.1 mM EDTA. For DNA-binding assay, the 10-μl reactions mixtures contained 0.2 nM of ^{32}P-labeled 62-mer, 20 mM Tris–HCl, pH 7.5, 2 mM DTT, 12.5% glycerol, 100 μg/ml BSA, and NaCl as indicated. After LEF-3 addition, the binding reactions were incubated for 15 min at 22 °C and then analyzed by electrophoretic mobility shift assay (EMSA) using a 6% polyacrylamide gel as described previously (Mikhailov and Bogenhagen, 1996). The gels were dried on DE-80 paper and exposed to X-ray film.

DNA strand exchange reaction

The replicative form of M13mp9 DNA of 7599 bp (10 μg) was digested for 3 h at 37° with 20 U of endonuclease *Xmn*I to produce dsDNA fragments of 5310 and 2289 bp and then treated for 4 min at 37° with 3 U of *Exo*III from *E. coli* to digest approximately 10% of the DNA and generate ss 5′-tails in the fragments. The fragments were labeled by using [α-^{32}P]dCTP and Klenow (−Exo) DNA polymerase in a reaction mixture lacking one cold dNTP (dATP), and then the fragments were purified by electrophoresis in agarose gel. The assay was carried out in 10-μl reaction mixtures containing 0.01 nM of the ^{32}P-labeled 5310-bp or 2289-bp DNA fragments, 0.4 nM ss M13mp9 DNA, 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 15% glycerol, 100 μg/ml BSA, and 2 mM DTT. After addition of LEF-3, the mixtures were incubated at 37 °C for 1 h. Reactions were terminated by the addition of SDS (0.5%), treated with proteinase K (150 μg/ml), and then analyzed by electrophoresis in a 1% agarose gel.

Analysis of LEF-3 structure by circular dichroism

The circular dichroism (CD) spectra were recorded using a J-720 Spectropolarimeter (JASCO Co) in a cell with a 0.5-mm path length at 0.5-nm intervals from 198 to 290 nm. LEF-3 samples (1 mg/ml) were in a buffer containing 2.5 mM Tris, pH 7.5, 0.2 M NaCl, 2.5% glycerol, 0.05 mM EDTA, and 1 mM DTT. Each spectrum was recorded twice, averaged and corrected for the buffer contribution. The structural parameters H (the α-helix content) and E (the extended-β-strand content) were calculated from the CD spectra by using the CDsstr software (Johnson, 1999). For thermal denaturation experiments, samples were equilibrated for 3 min at 2 °C intervals under heating at 0.3 °C/min from 22 to 70 °C and then at 5 °C intervals under cooling at 0.9 °C/min from 67 to 22 °C using a thermoelectric temperature control.

Gel filtration

Gel filtration was carried out using a 3.4-ml column (0.7 × 8.8 cm) of Sephacryl S-300 HR (Amersham Biosciences) in a buffer containing 0.4 M NaCl, 10 mM Tris–HCl, pH 7.5, 10% glycerol, 1 mM EDTA, and 1 mM DTT. Protein samples were loaded in 50 μl of the buffer, the column was processed at a rate of 0.1 ml/min under constant hydrostatic pressure, and absorbance was registered at 280 nm using a 2-mm path length flow cell.

Analysis of LEF-3 structure by limited proteolysis with trypsin

The proteolysis of LEF-3 (2 μg) was carried out with sequencing grade modified trypsin (Promega) (0.1 μg) in 10-μl mixtures containing 50 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, and 2 mM DTT. The digestion reactions were carried out at 25 °C for the indicated times and were terminated by the addition of 5.1 μl of loading buffer (190 mM Tris, pH 6.8, 4.3 M 2-mercaptoethanol, 6% SDS) containing 3 mM phenylmethylsulfonyl fluoride (PMSF) and by boiling for 3 min. The samples were analyzed by SDS-13% PAGE. To determine the N-terminal sequences of the proteolytic fragments, 100 μg of LEF-3 was digested with 5 μg of trypsin for 20 min at 25 °C

and fractionated by SDS-10% PAGE. The fragments were transferred onto Immobilon-P transfer membranes (Millipore) and subjected to Edman degradation (Molecular Structure Facility, University of California, Davis). For chromatography of LEF-3 fragments on ssDNA cellulose, LEF-3 (200 μg) was digested by trypsin (6 μg) for 30 min at 25 °C in 400 μl of a mixture containing 0.1 M NaCl, 50 mM Tris–HCl, pH 7.5, 10% glycerol, 1 mM EDTA, and 1 mM DTT. The digestion was terminated by chilling on ice and by the addition of PMSF to 2 mM, and the sample was immediately loaded onto a 0.5-ml column of ssDNA cellulose. The column was washed with buffer containing 0.1 M NaCl, 10 mM Tris–HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF and then processed with portions of the same buffer containing NaCl in concentration of 0.25, 0.4, 0.6, 0.8, and 1 M NaCl. Ten-microliter portions from the fractions were analyzed by SDS-13% PAGE followed by Coomassie staining. The yield of fragments in each fraction was determined by using ImageQuaNT software (Amersham Biosciences).

MALDI/TOF analysis

LEF-3 (20 μg) was digested with the sequencing grade modified trypsin (1 μg) in a mixture (15 μl) containing 25 mM Tris, pH 7.5, 16.7% glycerol, 67 mM NaCl, and 2 mM DTT. The digestion was carried out at 25 °C. 3-μl aliquots were taken from the mixture at 5, 10, 20, 30, and 40 min and were immediately frozen in liquid nitrogen. For MALDI *tof-tof analysis*, the samples were mixed in a 1:6 ratio of a-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA, and 0.5 μl was applied to the sample plate. The crystallized sample material was rinsed with deionized water to remove impurities. Molecular mass analysis was performed by matrix-assisted laser desorption/ionization time of flight mass spectrometry using a Proteomics Analyzer ABI 4700 TOF/TOF mass spectrometer (Applied Biosystems Inc.) with an accelerating voltage of 20 kV.

Other methods

SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (1970). LEF-3 concentration in the purified samples was determined spectrophotometrically using an extinction coefficient E_{280} in 6 M guanidium hydrochloride of 0.89 M^{-1} cm^{-1} or, in diluted samples, by SDS-PAGE followed by optical densitometry of the gels stained with Coomassie brilliant blue. Bovine serum albumin (BSA) loaded in different amounts on separate lanes of the same gel was used for generation of the calibration curve. For quantitative analysis, the stained gels were analyzed with ImageQuaNT software (Amersham Biosciences). Light scattering of LEF-3 samples was analyzed by using a Fluorescence Spectrophotometer SLM 8000C (SLM Instruments, Inc.) in a cell with 5-mm path at the excitation and emission wavelength of 400 nm. LEF-3 samples (0.8 mg/ml) were in a buffer containing 2 mM Tris, pH 7.5, 0.2 M NaCl, 2% glycerol, 0.04 mM EDTA, and 1 mM DTT. The heating was carried out at a rate of 0.2 °C/min. The fluorescence spectra of LEF-3 were recorded on a Cary Eclipse Fluorescence Spectrophotometer (Varian) in a sub-micro cell of 40 μl at the excitation wavelength of 280 or 292 nm and the emission interval of 300 to 450 nm. LEF-3 (50 μg/ml) was in a buffer containing 2 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM DTT.

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