Mass Spectrometric Identification of Leucine Zipper-like Homodimer Complexes of the Autoantigen L7*

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The eucaryotic protein L7, originally isolated from rat liver cells, associates in the cytoplasm with the large subunit of ribosomes and to interact specifically with as yet unknown cognate sites of mRNA, thereby inhibiting cell-free translation (Neumann, F., Hemmerich, P., von Mikecz, A., Peter, H., and Krawinkel, U. (1995) Nucleic Acids Res. 23, 195–202). The N-terminal region of protein L7 contains a sequence motif similar to the leucine zipper domain of eucaryotic transcription factors, which promotes dimerization through α-helical coiled coil formation. Using electrospray-ionization mass spectrometry as a method of molecular specificity, we have directly identified the dimeric complexes comprising the leucine zipper-like region of protein L7 and have determined the dissociation constant of L7 homodimers in an affinity binding assay. We also demonstrate the high content of α-helicity of the dimer by circular dichroism spectra and computer-based structure elucidation and show that the leucine zipper region of protein L7 is fully sufficient to mediate the inhibition of cell-free mRNA translation. A structural basis for the function of L7 to regulate translation is discussed. From the present results we conclude that L7 interacts with double stranded mRNA in a similar fashion as leucine zipper proteins with specific cognate sites on double stranded DNA.

The eucaryotic protein L7, originally isolated from rat liver cells, associates in the cytoplasm with the large subunit of ribosomes (1–3). Like other riboproteins, L7 has been identified as a potent autoantigen in systemic autoimmune diseases such as systemic lupus erythematosus, mixed connective tissue disease, and progressive systemic scleroderma, whereas a defined biological function has remained as yet unknown. In systemic lupus erythematosus, both quantitative and qualitative changes of the anti-L7 autoantibody response are correlated with acute disease manifestation, thus indicating that this protein is involved in as yet unknown pathomechanisms (4–6).

The identification of the structural and functional properties of the autoantigen L7 therefore has become of considerable interest in order to elucidate the molecular basis of autoimmune processes (7, 8).

The N-terminal region of protein L7 contains a sequence motif which is similar to the "basic region leucine zipper" (BZIP) domain of eucaryotic transcription factors (9). In the canonical BZIP domain, the leucine zipper region promotes homo- or heterodimerization through coiled coil formation, and the BZIP dimer interacts with specific target sequences on DNA (10, 11). We have previously shown that the basic domain of protein L7 interacts specifically with as yet uncharacterized cognate sites on mRNAs, thereby inhibiting their cell-free translation (12), and that L7 is capable of forming homodimers according to cross-linking and gel electrophoresis studies (9), thus suggesting that L7 interacts with mRNA as a dimer. Upon stable transfection of protein L7 into Jurkat T-lymphoma cells, the synthesis of at least two proteins associated with the nucleus is suppressed (12). These results provide strong support for the hypothesis that protein L7 is involved in translational regulation.

The development of "soft ionization" methods of mass spectrometry, particularly matrix-assisted laser desorption-ionization (MALDI-MS) and electrospray-ionization (ESI-MS), has enabled precise molecular mass determinations of polypeptides up to large (>100 kDa) proteins (13, 14). In addition to the already established applications to the determination of primary structures and post-translational modifications of proteins, the capability of ESI-MS to analyze directly specific noncovalent interactions and supramolecular complexes of biomacromolecules has been of particular interest (15–17). Several types of noncovalent protein complexes have been identified by ESI-MS in recent work including dimeric and trimeric complexes of DNA-binding proteins (18, 19). In the present study we have identified by direct ESI-mass spectrometric determination the homodimer complex of the BZIP-like region of L7 and demonstrate the dimerization domain as the predominant structure mediating the inhibition of cell-free translation. Moreover, the mass spectrometric results were fully ascertained by binding studies and determination of the dissociation constant of the L7 dimers.

MATERIALS AND METHODS

Cloning, Expression, and Analysis of Recombinant L7 Proteins—Fusion proteins comprising the human L7 sequence (7) either completely (HisL7) or the amino acid positions 2–49 (HisL7H) with an N-terminal oligo-His-tag (MRGSHHHHHHH) and an oligopeptide linker were expressed in Escherichia coli strains BL21(DE3) and BLR (DE3) (Novagen, Madison, WI) (see Fig. 1a). To produce HisL7 the NdeI-Xhol fragment of the human L7 cDNA clone pHUL7–14 (9) was ligated into expression vector pRSET-A (Invitrogen, Heidelberg, FRG). HisL7H is derived from the 140-base pair BamHI-HindIII fragment of human L7–14 in pRSET-A. Recombinant plasmids were amplified in E. coli, and the integrity and orientation of their cDNA inserts were verified by nucleotide sequencing. HisL7H was isolated from bacterial lysates by affinity chromatography on Ni2+-chelate columns under denaturing conditions as described (20, 21) and further purified by high pressure liquid chro-

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‡ The abbreviations used are: BZIP, basic region leucine zipper; MS, mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; MALDI-MS, matrix-assisted laser desorption.

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matography on a C4 reversed phase column. Its primary structure was ascertained by molecular weight determination using MALDI-MS and by mass spectrometric peptide mapping analysis (22, 23) after trypsin and Staphylococcus aureus V8-protease digestion (see Table I).

HisL7 was purified from inclusion bodies (24) according to the following protocol. The bacteria were pelleted, re-suspended in 10 volumes of lysis buffer I (4 M urea, 2.5% Triton X-100), lysed by freezing and re-thawing, and washed three times in 100 volumes lysis buffer I. Inclusion bodies were lysed in lysis buffer II (6 M guanidinium/HCl, 50 mM β-mercaptoethanol, 40 mM Tris acetate, pH 7.5), and the purity and concentration of HisL7 was estimated by SDS-polyacrylamide gel electrophoresis. Final purification of HisL7 was carried out by Ni2+-chelate affinity chromatography with buffers containing 10 M β-mercaptoethanol. Renaturing was performed by dialysis of the eluate against 2 M urea, 20 mM Tris acetate, pH 7.5, 5 mM β-mercaptoethanol. Finally, the protein was desalted by gel filtration and kept in 20 mM Tris acetate, 1.4% n-octylglucoside, pH 7.5.

Standard techniques of cloning, nucleic acid, and protein analysis were performed as described (25). Protein concentrations in binding and translational inhibition analyses were determined photometrically (26). N-terminal sequences of both proteins were additionally verified by automated Edman sequence determination.

Mass Spectrometry and Spectroscopic Analysis—MALDI-MS analyses were carried out with a Bruker Reflex II time-of-flight mass spectrometer (Bruker-Franzen, Bremen, FRG) equipped with a UV-nitrogen laser (337 nm) and a dual channel plate detector. Protein solutions (1 μl) were mixed directly on the sample target with 1 μl of matrix solution consisting of 10 M α-cyano-4-hydroxycinnamic acid dissolved in acetoni-trile/trifluoroacetic acid (3:1) and spectra were acquired after evaporation of the solvent and processed with the Bruker Reflex II data system. Peptide mapping analyses were performed both by MALDI-MS and by 252-Cf-plasma desorption MS with peptide mixtures by trypsin and S. aureus V8-protease digestion, as described previously (23).

ESI-MS was performed with a Vextec-A20L quadrupole mass spectrometer (Vextec, Houston, TX), by generating the electrospray with a voltage of 2 kV at the inlet needle and a temperature of approximately 45 °C in the spraying region (27). HisL7H was dissolved at a concentration of (8 μM) in 9 volumes of 2 M ammonium acetate/l volume of methanol, pH 6.5, and pumped with a Harvard microinfusion pump (Harvard, Franklin, MA) at a flow rate of 3 μl/min through an unmodified fused silica capillary into the ion source. A “declustering” voltage (ΔCS) of 5–20 V between the inlet nozzle and the repeller electrode (18) was applied for desolvation of microdroplets, and spectra were recorded with a scan rate of 7 s using a Teknivent (Houston, TX) vector II data system.

CD spectra were determined with a Jasco J-500 C spectropolarimeter at 20 °C at a scan speed of 1 min/m. Proteins were dissolved at a concentration of 19 μM in 20 mM phosphate buffer containing 46 mM n-octylglucoside. Secondary structure prediction was performed with the program “coils” as described (28), using the algorithm described by Parry (29). The width of the chosen window was 14 amino acids.

Determination of Dissociation Constants and Inhibition of Cell-free Translation—Apparent dissociation constants (Kd) of L7 homodimers were determined essentially as described (30, 31) by measuring the binding of [35S]methionine-labeled protein L7 to immobilized HisL7 or HisL7H, respectively. Different amounts of fusion protein denatured in 8 M urea were immobilized on Ni2+-chelate-agarose in 20 M minicolumns (Promega, Madison, WI). The column was renatured and extensively washed with phosphate-buffered saline, and the coupling efficiency was monitored photometrically. The columns were then loaded at room temperature either with full-length labeled human L7 or with [35S]methionine-labeled luciferase as a specificity control in a binding buffer containing 20 mM Tris acetate, 10% glycerol, 5 M β-mercaptoethanol, 100 mM NaCl, 20 mM imidazole, pH 7.5. After washing with fifteen volumes of binding buffer, the fractions of ligand retained on the column and eluted with the wash were determined by SDS-polyacrylamide gel electrophoresis and autoradiography. At a concentration of 25 μM, the bound protein retained 96% of the ligand in one batch volume or 47% in fifteen volumes. Thus, the apparent Kd is estimated as 0.5 μM of the minimum concentration of immobilized HisL7 or HisL7H required to retain the radiolabeled ligand efficiently. The inhibition of translation of luciferase mRNA by HisL7 and HisL7H was determined in a reticulocyte lysate as described previously (12).

RESULTS

Expression and Structural Characterization of oligo-HisL7 Fusion Proteins—Human ribosomal protein L7 carries a basic sequence within a BZIP-like domain that interacts with cognate sites on mRNAs (9, 12). This domain differs from canonical leucine zippers in that it has glutamine and positively charged amino acids instead of β-branched amino acids at the a position of the repeating seven-residue pattern (abedefg), where leucine dominates at position d. Hydrophobic residues at positions a and d provide an interface for the dimerization of BZIP domains through formation of parallel coiled coiled (10, 32–37).

For detailed structural analysis the entire human protein L7 and its partial sequence comprising the BZIP-like domain (residues 2–49) were expressed in E. coli as fusion proteins with an N-terminal oligo-His-tag and an additional linker peptide due to the vector employed. (HisL7 and HisL7H; Fig. 1a). The correct sequence alignments and primary structures were ascertained for both proteins by (i) direct molecular weight determinations using MALDI-MS and ESI-MS yielding precise values (31,262 and 12,986 Da) in accordance with the sequence molecular masses; (ii) MALDI-MS and plasma desorption-MS molecular masses of peptide fragments after trypsin and S. aureus V8-protease digestion; and (iii) Edman sequence determinations of the N-terminal oligo-His-tag residues (MRGSHH-HHHH). Relevant mass spectrometric peptide mapping analyses of the BZIP domains in HisL7H are summarized in Table I, providing overlapping and complete primary structure coverage.

Although the BZIP-like domain of L7 deviates from the structure of an ideal zipper, an α-helical conformation and a


92% probability to homodimerize as a coiled coil nevertheless are predicted by computer-based structure simulations for this region (28) (Fig. 1a). Furthermore, a CD spectrum confirmed the predominantly \( \alpha \)-helical conformation of the B2P-like region; an \( \alpha \)-helical content of 75% was determined for HisL7 (Fig. 1b).

**ESI Mass Spectrometric Identification of the HisL7H-Homodimer Complex—**The identification of several noncovalent protein complexes including leucine zippers has been reported recently (18, 19, 38, 39). In the desolvation process of charged macromolecules, a small (\( \approx 10-100 \) V) potential difference between the ESI capillary tip and a skimmer-type repeller electrode has been shown to be of critical importance for the analysis of noncovalent complexes (counter electrode skimmer potential, \( \Delta CS \)); hence leucine zipper-type homodimers are characterized by all odd charged ions. ESI spectra of HisL7H at \( \Delta CS \) of 10 and 20 V are shown in Fig. 2. The spectrum at low declustering potential (Fig. 2a) provides the direct identification of the homodimer complex by its precise molecular mass of 25,072 Da derived from the 15+, 17+, and 19+ charged ions, in addition to the multiply charged ion series of the monomer of higher abundance. The ions of the dimer are completely removed in the spectrum at \( \Delta CS = 20 \) V, indicating facile dissociation of the complex (Fig. 2b). Relative abundances of complex ions were found increased at higher concentration (\( >10 \mu M \)) and decreased at higher capillary temperature (data not shown); no quantitative formation of dimers was obtained yet as previously observed for canonical leucine zippers due to their facile dissociation at the polar capillary surface. (18, 19). No ions indicative of higher oligomerization states were detected. Furthermore, these data were fully consistent with results of cross-linking experiments for cell-free translated complete protein L7 (9), which did not contain a His-tag an a linker peptide. We conclude that protein L7 forms homogeneous dimers through noncovalent, coiled-coil-like interactions between the regions.

**Table I: Primary structure characterization of fusion protein HisL7H by MALDI-MS molecular mass determination and mass spectrometric peptide mapping analysis of tryptic and V8-protease fragments**

| Sequence | Tryptic peptide* | Molecular mass cDNA | MH+ ions (m/z 2F) |
|----------|-----------------|---------------------|------------------|
| 1-113    | 12986           | 12987*              |
| 3-23*    | T2*             | 2278                | 2278             |
| 34-45    | T5              | 1335                | 1336             |
| 52-64    | T7/10           | 1465                | 1463             |
| 64-73    | T10/14          | 1290                | 1291             |
| 74-80    | T15/19          | 940                 | 940              |
| 88-100   | T23/26          | 1368                | 1367             |
| 100-113  | T27/29          | 1458                | 1461             |
| 1-41*    | V1*             | 4674                | 4678             |
| 42-50    | V2/4            | 1023                | 1025             |
| 51-60    | V5/6            | 1124                | 1126             |
| 61-71    | V7              | 1391                | 1390             |
| 72-103   | V8              | 3894                | 3893             |

* Tryptic peptides (T) and S. aureus V8-protease peptides (V) are denoted consecutively from the N terminus. Peptides marked by asterisks are comprising the oligo-His-tag and linker sequences.

**Dissociation Constants of L7 Dimers—**The mass spectrometric identification of the B2P-like dimer complexes was ascertained by quantitative determination of the dissociation constants for the dimerization, utilizing a binding assay with the oligo-His-tagged polypeptides immobilized on a Ni\( ^{2+} \)-chelate column. Immobilized HisL7H were incubated with \( ^{35} \)S-labeled L7 or with radiolabeled luciferase as a control ligand. The column was washed with a total of 15 batch volumes of binding buffer, and the fractions of column-bound and eluted ligand were determined for increasing dilutions of HisL7 and HisL7H (Fig. 3). At a starting concentration of \( 2.5 \times 10^{-5} \) M, a lowest concentration of approximately \( 25 \times K_d \) was found that provided nearly complete retention of liquid phase L7; hence \( K_d \) values of 40 and 120 nM were estimated for HisL7 and HisL7H, respectively. No retention of the control ligand luciferase was observed on the HisL7 or HisL7H column, thus demonstrating the specific interaction with L7.

**Inhibition of Cell-free Translation by HisL7 Proteins—**We have previously shown that the interaction of L7 with L7mRNA is mediated by the B2P-like region at a 2:1 molar ratio and specifically inhibited cell-free translation of mRNA (12). Both HisL7 and HisL7H inhibited the translation of luciferase mRNA in a reticulocyte lysate with comparable dose-response curves (Fig. 4), thus establishing the functional integrity of these proteins. The B2P-like region of L7 as represented by HisL7H was found to be as efficient as the complete protein, with 50% translational inhibition obtained at concentrations of 130 (HisL7) and 190 nM (HisL7H), respectively. According to the \( K_d \) values determined above, approximately 70% of L7 should exist in the dimer form at this concentration. Furthermore, the dose-response curves of HisL7 and HisL7H determined here were nearly identical to those...
obtained previously for different recombinant fusion proteins and mRNA preparations (12).

DISCUSSION

Protein L7 binds to a distinct yet currently unknown conformation on a number of mRNAs and inhibits their cell-free translation. It has been assumed that both functions require a dimeric form of the protein (9, 12). In the present study we have demonstrated the mass spectrometric identification of a defined region of L7 mediating the formation of homodimers and quantified the strength of intermolecular interactions.

ESI-MS has only been introduced recently and successfully employed as a sensitive method to identify oligomerization states of leucine zippers in solution (18, 38). The ESI spectra in this study demonstrate that a polypeptide comprising the BZIP-like region of L7 form specific noncovalent dimers with properties characteristic of coiled coil leucine zippers but without any evidence for the formation of stable trimers observed for several "classical" BZIP polypeptides (18, 19). Although the dimeric form of HisL7H should predominate at the concentrations employed, no quantitative detection of dimers was achieved, which is consistent with the previously observed lability of leucine zippers at the present ESI-MS conditions. However, the dissociation constants of approximately 40 nM for the dimer of the complete L7 protein and 120 nM for dimers of L7 fragments comprising the BZIP-like region are very comparable with Kd values for canonical BZIP proteins such as the yeast GCN4 leucine zipper.² The Kd values estimated here are based on several assumptions (30, 31) with a significant potential source of error in the uncertainty of which fraction of the bound ligand remains functional upon immobilization. Hence, the difference between the Kd values determined here for HisL7 and HisL7H might not be significant, and both polypeptides are indistinguishable in their capability to inhibit cell-free translation.

In conclusion, the BZIP-like region of protein L7 is shown to be fully sufficient in mediating the formation of homodimers and inhibiting cell-free translation. The latter function depends on specific mRNA-binding, which is likely to depend on homodimerization through coiled coil formation, as suggested by the high probability for coiled coil formation predicted by computer-based structure simulation. Moreover, the high (approximately 75%) α-helical content of L7 suggests significant α-helicity for the BZIP-like region. Further supportive evidence is provided by results showing that (i) L7 binds to L7mRNA at a 2:1-molar ratio (12); (ii) the dissociation constant of the L7-mRNA complex (approximately 50 nM) is nearly identical to the Kd of the HisL7 and HisL7H homodimers, suggesting that the dimerization may be the rate-limiting step in the formation of the protein-mRNA complex; and (iii) efficient inhibition of cell-free translation of L7-mRNA is observed at inhibitor concentrations at which the L7 dimer should predominate in solution. Thus, although it is yet unknown whether dimers comprising the BZIP-like region of L7 are built of α-helices that are parallel and in register like canonical leucine zippers, we speculate that L7 interacts with a double stranded region on mRNA in a similar fashion as BZIP proteins with their cognate sites on double stranded DNA (36, 37).

FIG. 3. SDS-polyacrylamide gel electrophoresis/autoradiography binding assay of ³⁵S-labeled L7 protein to immobilized HisL7 (a) and HisL7H (b). Fractions E or W denote the relative amounts of ligand retained on the column or eluted with the washing fraction, respectively, using luciferase as a specificity control.

FIG. 4. Inhibition of cell-free translation of luciferase mRNA by HisL7 and HisL7H. The concentration of the reporter mRNA is 30 nM. Inhibitor concentrations range from 0 to 900 nM.

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² H. R. Bosshard, personal communication.
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