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The Leader Peptide of Theiler's Murine Encephalomyelitis Virus Is a Zinc-Binding Protein

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The leader (L) peptide is located in the amino-terminal part of the polyprotein of members of the *Cardiovirus* (which includes Theiler's murine encephalomyelitis virus) and *Aphthovirus* genera of picornaviruses. Although the function of L is unknown, strain DA of Theiler's murine encephalomyelitis virus with a mutation of L produces a cell-specific restricted infection. We now report that the DA L peptide is a metalloprotein and that zinc binds to a Cys-His motif that is conserved among cardioviruses.

Theiler's murine encephalomyelitis virus (TMEV) is a group of mouse picornaviruses of one serotype generally classified as belonging to the *Cardiovirus* genus (9). TMEV strains have a leader (L) peptide which is composed of 228 nucleotides located in the amino-terminal part of the polypeptide. It is a highly acidic protein (10) with a putative zinc-binding motif, Cys-His-Cys-Cys (CHCC) (6). The L peptide is present in other members of the *Cardiovirus* genus, which includes encephalomyocarditis virus, and the *Aphthovirus* genus of picornaviruses, but not in other genera. In foot-and-mouth disease virus, an aphthovirus, L is a protease which cleaves itself from the rest of the viral polypeptide and also mediates cleavage of a cap-binding factor, p220 (a component of eIF-4A) (3, 13, 18). The function of L in cardioviruses, however, is unknown.

We have previously shown that strain DA of TMEV can productively infect BHK-21 cells despite a complete deletion of the L coding region (6); however, a low multiplicity of infection of L929 cells with this mutant DA virus produces a restricted infection with production of little infectious virus. Of interest is the fact that DA L has a putative CHCC motif that is conserved among TMEV strains and also among other cardioviruses; the conservation of this motif occurs in the case of encephalomyocarditis virus despite an overall amino acid identity of less than 50% between TMEV L and encephalomyocarditis virus L. DA virus with a mutation in the coding region of this putative zinc-binding motif, DAFSSC:L(3S,5L) virus (which has a change of the Cys at codon 3 to Ser and a change of the His at codon 5 to Leu), also displayed a restricted viral infection in L-929 cells (6), demonstrating that this region was critical for the spread of the virus. In the present report, we describe the zinc-binding characteristics of DA L.

To test the zinc-binding ability of DA L, we made use of the following mutant plasmid constructs: pDAFSSC:L(3S,5L) (see above), pDAFSSC:LΔ2-7 (deletion of L amino acid residues 2 through 7), and pDAFSSC:LΔ39-46 (deletion of L amino acid residues 39 through 46, which is located carboxylterminally to the Cys-His motif). The details of construction of these three mutants have been previously described (6). Virus derived from each of these three constructs showed a cell type restriction in spread (6).

In order to obtain proteins to test for zinc binding, we ligated the respective open reading frames of wild-type or mutant DA L to the bacterial maltose-binding protein (MBP) gene, *malE*, in the fusion protein expression system pMAL-c (New England Biolabs). For the wild-type protein MBP-L, the pDAFSSC viral fragment from nucleotide 1001 to 1332, which includes the L coding sequence, was blunt ended, ligated to *Bam*HI linkers, digested with *Bam*HI endonuclease, and then ligated in frame into the *Bam*HI site of pMAL-c. The same fragments from pDAFSSC:L(3S,5L), pDAFSSC:LΔ2-7, and pDAFSSC:LΔ39-46 were blunt ended, and then each was separately ligated to the *Eco*RI-digested, blunt-ended pMAL-c vector. Figure 1 displays a schematic diagram of the control protein (MBP) and the mutated fusion proteins, MBP-L(3S,5L), MBP-LΔ2-7, and MBP-LΔ39-46. The fusion proteins were expressed and purified according to the manufacturer's protocol. As a positive control, we used papillomavirus E7 protein, which has been shown to bind zinc specifically (1, 11).

We analyzed the zinc-binding activity of these various fusion proteins according to the method described by Barbosa et al. (1). Proteins were separated on a duplicate set of sodium dodecyl sulfate–12.5% polyacrylamide gels; one of the gels was dried after staining with Coomassie blue (Fig. 2B), and the other was transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.). The blot was incubated in renaturing buffer (100 mM Tris, 50 mM NaCl, 10 mM dithiothreitol; pH 6.8) for 1 h with three changes of buffer. All buffers were adjusted to pH 6.8, which has been shown to increase the level of stringency for zinc binding (1, 14, 16). Labeling buffer (100 mM Tris, 50 mM NaCl; pH 6.8) was flushed with N_2 to remove dissolved oxygen and thus avoid oxidation of blotted proteins. The filter was rinsed in labeling buffer twice and then incubated with 15 μM $^{65}ZnCl_2$ in labeling buffer for 30 min. The filter was then rinsed twice in washing buffer (100 mM Tris, 50 mM NaCl, 1 mM dithiothreitol; pH 6.8) and washed for 1 h with three changes of buffer and exposed to XAR film (Kodak) (Fig. 2A).

Two protein products were demonstrated in some of the lanes of the stained gel (Fig. 2B, lanes 1 to 5). The protein products of more rapid electrophoretic mobility are presumed to correspond to products of partial proteolytic cleavage that occurred during growth of the bacteria or protein purification. The protein products of slower electrophoretic mobility correspond to the wild-type and mutated L fusion proteins. Because different insertion sites of the pMAL-c vector were used to construct the L fusion proteins, the wild-type MBP-L fusion protein contains a shorter additional sequence and conse-

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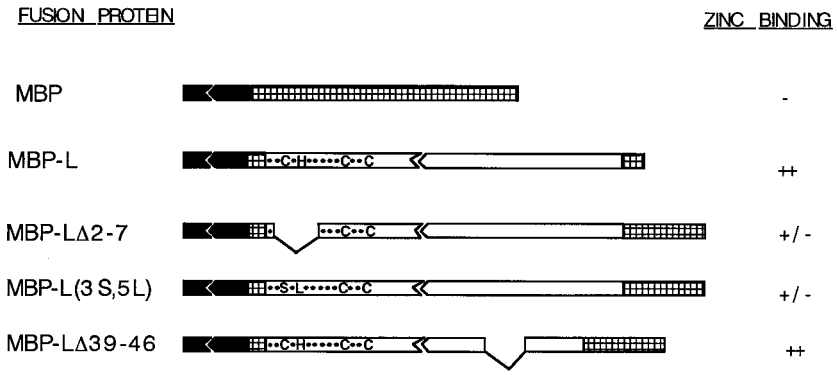

FIG. 1. Schematic diagram of fusion protein constructs and their zinc-binding activities (based on data from Fig. 2). The constructs have a domain consisting of the bacterial MBP (closed box) followed by the wild-type or mutant DA L sequence (open box) and/or amino acid sequences not related to either MBP or viral L (crosshatched box). The first 14 amino acid residues at the amino terminus of L, which include the CHCC motif, are indicated by dots and uppercase letters. Note that the Cys at codon 3 and the His at codon 5 in MBP-L(3S,5L) are changed to Ser and Leu, respectively. The locations of the deletions in the other two mutations are indicated.

quently migrates faster (Fig. 2B, lane 2) than do the mutated fusion proteins MBP-LΔ2-7 (Fig. 2B, lane 3) and MBP-L(3S,5L) (Fig. 2B, lane 4) and has approximately the same mobility as the other mutated fusion protein with a larger deletion at the L coding sequence, MBP-LΔ39-46 (Fig. 2B, lane 5).

Figure 2A shows the results of the zinc binding (which are summarized in Fig. 1). The E7 protein positive control bound zinc (Fig. 2A, lane 6), whereas the bacterial negative-control fusion protein MBP did not (Fig. 2A, lane 1). The wild-type MBP-L fusion protein bound zinc with high affinity (Fig. 2A, lane 2). In contrast, both the MBP-L(3S,5L) and the MBP-LΔ2-7 mutated fusion proteins, which have a disruption in the Cys-His motif, showed a markedly reduced labeling with ^{65}Zn (Fig. 2A, lanes 3 and 4). After a prolonged exposure of the filter, a very low level of ^{65}Zn labeling was detected for these two mutated fusion proteins. The duplicate Coomassie blue-stained gel shows that for each mutated L fusion protein approximately three to four times the amount of the wild-type fusion protein was loaded (Fig. 2B, lanes 3 to 5 versus lane 2). Thus, the reduced binding for the mutant L fusion proteins is not due to an unequal and decreased loading of these proteins compared with the wild type. The residual low level of zinc binding of these two mutants despite the disruption of the Cys-His motif may be related to the remaining cysteine residues (Cys-11 and Cys-14) that retain a low zinc-binding ability. A similar reduction rather than disappearance of zinc binding has been reported for the mutated zinc-binding motif of the vaccinia virus A2L gene (5).

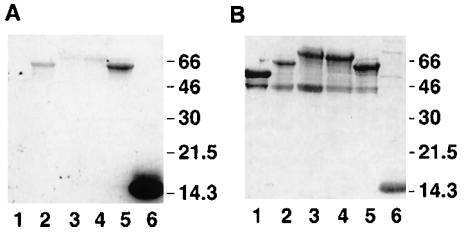

FIG. 2. Identification of zinc binding of wild-type and mutated L fusion proteins. Proteins were separated on a duplicate set of sodium dodecyl sulfate–12.5% polyacrylamide gels by electrophoresis, and one of the gels was transferred to a nitrocellulose membrane. (A) Autoradiogram of the nitrocellulose blot after renaturation and labeling with $^{65}ZnCl_2$ (15 μM) as described in the text. Molecular size markers are indicated on the right. Lanes: 1, MBP; 2, MBP-L; 3, MBP-LΔ2-7; 4, MBP-L(3S,5L); 5, MBP-LΔ39-46; 6, papillomavirus E7 protein. (B) Coomassie blue stain of the duplicate gel.

The mutated fusion protein MBP-LΔ39-46 showed significant labeling with ^{65}Zn, presumably because the deletion is in the middle of the L coding sequence, i.e., carboxy terminal to the zinc-binding motif. The mutated fusion protein MBP-LΔ39-46 showed approximately three to four times the amount of ^{65}Zn-labeling as the wild type fusion protein MBP-L (Fig. 2A, lane 5 versus lane 2); this binding probably reflects that fact that three to four times more of this mutated fusion protein was loaded on the gel (Fig. 2B, lane 5 versus lane 2).

The significance of the zinc-binding property of TMEV L remains unclear. It is of interest to note that putative zinc-binding motifs have been identified in two proteins of members of the *Enterovirus* genus of picornaviruses. Yu and Lloyd (19) noted a conserved CCCH motif in the 2A protease of poliovirus; site-directed mutagenesis studies suggested that these residues are critical for maintaining the active conformation of the 2A protease and supporting its catalytic activity. Sommergruber et al. (17) subsequently showed that 2A protease of rhinovirus bound zinc and that the zinc was critical for the formation of an active enzyme. Pfister et al. (12) reported in a recent abstract that poliovirus 2C has a putative zinc binding motif and binds zinc; 2C is believed to play a role in viral RNA synthesis and viral assembly.

The sequence of the CHCC zinc-binding domain of DA L (Ck--HgypDvCPIC) (capital letters represent identity with gp32, except for "I," which is a conservative change to V in gp32; dashes represent absent amino acid residues compared with gp32) bears some similarity to a region of bacteriophage gene *32* protein; in addition, both gp32 and TMEV L have an acidic domain (4, 6, 10). gp32 functions at an autoregulatory translational level by binding to a predicted pseudoknot structure near the 5′ end of its own mRNA (8); cooperativity of binding to the pseudoknot and translational repression are both dependent on zinc binding (2, 15). The above observations raise the possibility that TMEV L could regulate its synthesis, and therefore the synthesis of the polyprotein, by binding its RNA (perhaps to a pseudoknot, which has been predicted to occur in picornavirus RNA [7]), i.e., the production of L (and therefore viral polyprotein) in a cell continues to increase until there is binding of a sufficient amount of L to the viral genomic RNA to terminate translation of L (and viral proteins). Experiments to test these predictions are in progress.

In summary, we have characterized TMEV L as a metalloprotein capable of binding zinc at a CHCC motif. Although information concerning the biological significance of zinc binding of L awaits future study, our results suggest that the zinc-binding motif is critically important in the host cell-specific restricted infection found after mutation of L (6). A disruption of the Cys-His motif may disturb the autoregulation of L and thereby produce a phenotype similar to that seen following a complete deletion of L.

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