

Leader Protein of Foot-and-Mouth Disease Virus Is Required for Cleavage of the p220 Component of the Cap-Binding Protein Complex

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Suppression of host protein synthesis in cells infected by poliovirus and certain other picornaviruses involves inactivation of the cap-binding protein complex. Inactivation of this complex has been correlated with the proteolytic cleavage of p220, a component of the cap-binding protein complex. Since picornaviral RNA is not capped, it continues to be translated as the cap-binding protein complex is inactivated. The cleavage of p220 can be induced to occur in vitro, catalyzed by extracts from infected cells or by reticulocyte lysates translating viral RNA. Expression of polioviral protease 2A is sufficient to induce p220 cleavage, and the presence in 2A of an 18-amino-acid sequence representing a putative cysteine protease active site correlates with the ability of different picornaviruses to induce p220 cleavage. Foot-and-mouth disease virus (FMDV) infection induces complete cleavage of p220, yet the FMDV genome codes for a 2A protein of only 16 amino acids, which does not include the putative cysteine protease active site. Using cDNA plasmids encoding various regions of the FMDV genome, we have determined that the leader protein is required to initiate p220 cleavage. This is the first report of a function for the leader protein, other than that of autocatalytic cleavage from the FMDV polyprotein.

Infection of cells by poliovirus, or certain other picornaviruses, results in cessation of host protein synthesis, accompanied by a selective translation of viral RNA (18). Although multiple factors are probably required for the complete inhibition of host protein synthesis (2), one important mechanism involves the inactivation of the cellular cap-binding protein complex (19). This complex is required for the formation of ribosome initiation complexes with capped mRNAs (reviewed in reference 16). Picornavirus mRNA, which is not capped, is presumed to initiate its translation via a cap-independent mechanism. One component of the cap-binding protein complex is a 220,000-dalton polypeptide, p220, which is cleaved to lower-molecular-mass products in poliovirus-infected cells (5). The precise function of p220 is not understood, but it appears to be required for activity of the cap-binding protein complex (12). The protein responsible for cleavage of p220 in infected cells is unknown. Neither of the two known proteases of poliovirus, 2A and 3C, has been shown to directly cleave p220, but it is possible that viral infection activates a host protease which results in p220 degradation (8, 10, 13). A poliovirus mutant, containing a single amino acid insertion in the 2A sequence, fails to cleave p220 during infection, suggesting involvement of the 2A protease in p220 cleavage (1). Furthermore, p220 was completely cleaved when HeLa cell lysates were mixed with reticulocyte lysates expressing poliovirus 2A protease in vitro (8). Recently, foot-and-mouth disease virus (FMDV) was found to cleave p220 (11), an

unexpected finding because this virus contains a 2A protein of only 16 amino acids (14, 20).

Both FMDV and cardioviruses code for a leader protein which is present at the amino terminus of the viral polyprotein (15). For FMDV the leader protein is removed from its precursor by autocatalytic cleavage (17). Using cDNA plasmids encoding various regions of the FMDV genome, we have identified a requirement for the leader protein to initiate p220 cleavage. This is the first report of a role for the leader protein, other than that of autocatalytic cleavage (17).

The construction of recombinant plasmids containing segments of the FMDV genome under the control of a T7 promoter for RNA polymerase has been reported previously (20). The plasmids were linearized downstream from the FMDV insertions by digestion with *Ssp*I (except where stated) and transcribed in vitro, and the resulting transcripts were translated in rabbit reticulocyte lysates containing either [³⁵S]methionine or 67 μM nonradiolabeled methionine (20). The radiolabeled lysates were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the synthesis of the expected FMDV-specific proteins was observed as previously described (20). Equivalent unlabeled lysates were evaluated for the ability to degrade p220 when mixed with HeLa cell lysate. The HeLa cell lysate provided a source of p220 that was reactive with antiserum raised in rabbits by using p220 degradation products from poliovirus-infected HeLa cells as antigen (12). As reported, this antiserum reacts very poorly with rabbit reticulocyte p220 (12). After 2 h of translation, 2 volumes of nonradiolabeled reticulocyte lysate were mixed with 1 volume of HeLa cell postmitochondrial cytoplasmic extract (S10), and the mixture was incubated for 20 h at room temperature. HeLa S10 was prepared by lysis of freeze-

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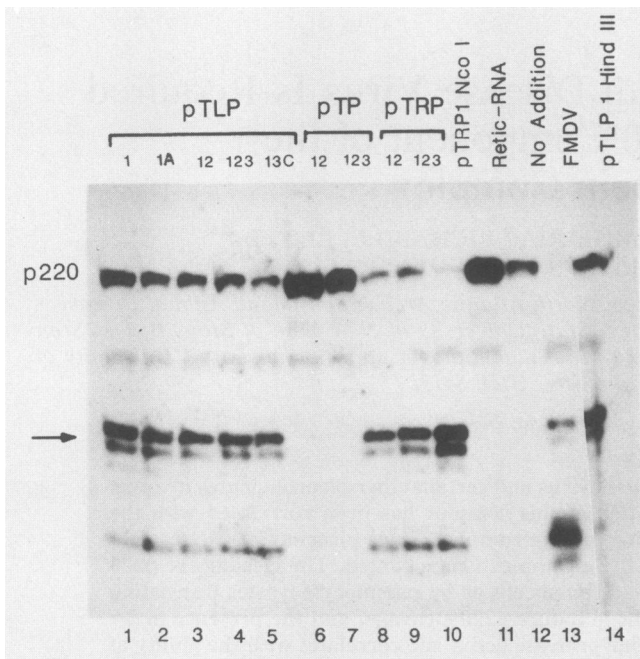


FIG. 1. Effect of in vitro-synthesized FMDV proteins on p220. Reticulocyte translation mixtures expressing FMDV proteins were mixed with extracts of uninfected HeLa cells. Cleavage of HeLa p220 was assayed by SDS-PAGE and immunoblotting as described in the text. Intact p220 is marked, and the arrow indicates some p220 cleavage products. The polypeptide, found in all lanes, migrating between p220 and its cleavage products is a contaminant reactive with p220 polyclonal serum (11). Transcripts used to program the reticulocyte translation mixtures are described in the text.

thawed cells in 2 volumes of hypotonic buffer (10 mM KCl, 2.5 mM dithiothreitol, 1.2 mM magnesium acetate, 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.5]) followed by centrifugation at $10,000 \times g$. SDS-PAGE on 7.5% slab gels (9) followed by immunoblotting (3) was used to assay p220 and its immunoreactive degradation products. Proteins transferred to nitrocellulose were detected by using a 1:250 dilution of anti-p220 rabbit serum followed by 2×10^7 cpm of ^{125}I -labeled staphylococcal protein A per ml. Dilutions of antiserum and protein A and blocking and washing of the transfer membrane were carried out with 3% nonfat dry milk-0.5 M NaCl-10 mM Tris hydrochloride (pH 8.5). Autoradiography was performed for 72 h at -70°C with BB-5 film (Eastman Kodak Co.) and an intensifying screen (Cronex Lightning-Plus; Du Pont Co.).

The effect on p220 of FMDV proteins in the reticulocyte lysates is shown in Fig. 1. The coding capability and translation of plasmid-derived transcripts have been reported (20) and are summarized in Table 1. Briefly, all pTLP plasmids contain the coding sequence for the leader protein followed by sequences for the P1, P2, and P3 regions of the FMDV genome (all P3 sequences are terminated at a *Bam*HI site in the polymerase 3D gene). The clone designated pTLP13C contains a deletion of the region between *Xho*I sites in the 2B and 3B genes. The pTP plasmids lack the coding region for the leader proteins; pTRP plasmids contain 400 bases of 5' noncoding sequences, as well as the coding region for the major and minor initiation sites, yielding the leader and alternate leader proteins of FMDV (20).

Reticulocyte lysates containing FMDV leader protein (pTLP series) or alternate leader proteins (pTRP series) all

TABLE 1. Coding capability of FMDV cDNA clones

Clone	Viral polypeptides encoded ^a
pTLP1.....	L, P1*
pTLP1A.....	L, P1, 2B*
pTLP12.....	L, P1, P2
pTLP123.....	L, VP0, VP1, VP3, 2A, 2B, 2C, 3A, 3B, 3C, 3D*
pTLP13C.....	L, VP0, VP1, VP3, 2A, 2B*, 3B*, 3C, 3D*
pTP12.....	P1, P2
pTP123.....	VP0, VP1, VP3, 2A, 2B, 2C, 3A, 3B, 3C, 3D*
pTRP12.....	L'/L, P1, P2
pTRP123.....	L'/L, VP0, VP1, VP3, 2A, 2B, 2C, 3A, 3B, 3C, 3D*
pTRP1 (<i>Nco</i> I).....	L'/L, VP0*
pTLP1 (<i>Hind</i> III).....	L*

^a Symbols: *, truncated polypeptide; L', alternate leader protein.

resulted in cleavage, whereas lysates lacking leader protein (pTP series) failed to cleave p220 (Fig. 1, lanes 1 to 9). The HeLa S10 lysate incubated without additions showed no conversion of p220 to degradation products, demonstrating the stability of p220 for the duration of the experiment (lane 12). HeLa S10 lysate incubated with 2 volumes of reticulocyte lysate without added exogenous RNA resulted in no loss of p220, demonstrating that FMDV-specific proteins, rather than endogenous reticulocyte proteases, were required for p220 cleavage (lane 11). The degradation of p220 by proteins derived from translation of purified FMD virion RNA in reticulocyte lysates is shown in lane 13. Similar cleavage of p220 is observed in FMDV-infected bovine kidney cells, indicating that p220 cleavage is not merely an artifact of the reticulocyte-HeLa assay system (11). We have observed that translation of lysates programmed with FMD virion RNA is more efficient than translation with any of the synthetic RNA transcripts (data not shown). Therefore, the incomplete cleavage of p220 by lysates programmed with transcripts from the various recombinant plasmids may simply reflect this observation.

The results demonstrate that FMDV leader protein is required for p220 cleavage. To determine the minimum FMDV coding capacity to give p220 cleavage, we prepared transcripts from plasmids pTRP1 and pTLP1 linearized at restriction endonuclease sites in or near the leader gene rather than the usual *Ssp*I site.

Plasmid pTRP1 was cut at an *Nco*I site which is 32 codons downstream of the leader coding region. Transcription and translation of the plasmid linearized at the *Nco*I site resulted in synthesis of only alternate leader or leader proteins, which comigrated on SDS-PAGE with proteins present in FMDV-infected cells (data not shown). This indicates that the leader proteins are processed free from the additional 32 amino acids encoded at the 3' end of this construction. Plasmid pTLP1 was linearized at a *Hind*III site which is 3 codons upstream of the carboxy terminus of the leader protein. Transcription and translation of this plasmid generated a single protein comigrating on SDS-PAGE with the leader protein in FMDV-infected cells (data not shown). The non-radiolabeled reticulocyte lysates directed by both pTRP1 (*Nco*I)- and pTLP1 (*Hind*III)-derived transcripts resulted in the cleavage of p220 (Fig. 1, lanes 10 and 14). This demonstrates that the 16,000-dalton leader protein lacking 3 amino acids at the carboxy terminus (derived from pTLP1 cut at *Hind*III) is the only FMDV protein required to induce p220 cleavage.

The requirement for the FMDV leader protein to induce the cleavage of p220 distinguishes this virus from other picornaviruses. For poliovirus, in vitro (8) or in vivo (1) expression of wild-type 2A protease is required for induction of p220 cleavage. However, 2A protease does not copurify with the p220 cleavage activity from poliovirus-infected cells (13). These data suggest that poliovirus protease 2A indirectly induces p220 cleavage via alteration or activation of a cellular protease. In addition, anti-2A serum inhibited subsequent p220 cleavage if added during in vitro translation of 2A sequences, but did not inhibit p220 cleavage if added after activation of the p220-specific protease had occurred (8). Picornavirus 2A proteins can be divided into two classes based on the presence or absence of an 18-amino-acid sequence which shares homology with a putative active-site cysteine protease sequence in 3C, a protease common to all picornaviruses (13). Picornaviruses which contain 2A homology with the putative 3C protease active site, such as enteroviruses (11) and rhinoviruses (4), induce p220 cleavage. However, two cardioviruses, encephalomyocarditis virus and Theiler's encephalomyelitis virus, which contain 2A proteins lacking the putative 3C active site, fail to cleave p220 (11). FMDV 2A consists of only 16 amino acids, which lack homology with the 3C active site, yet FMDV induces p220 cleavage (11). This inconsistency is resolved here by the demonstration that the leader, rather than 2A, is responsible for the induction of p220 cleavage by FMDV. The amino acid sequence of the FMDV leader protein lacks the 3C homology defined above. However, two different blocks of conserved sequences between the leader and 3C were identified by Strebel and Beck (17). The cardioviruses also contain leader proteins, yet they fail to cleave p220. Apparently, the leader proteins of these viruses have either lost or not evolved the capacity to induce cleavage of p220.

FMDV, like poliovirus, very rapidly and efficiently inhibits host cell protein synthesis (6; M. J. Grubman, unpublished observations); in contrast, infection of cells by cardioviruses such as encephalomyocarditis virus results in a slow decline in host cell polypeptide synthesis (7). It appears that the ability of poliovirus and FMDV to cleave p220 may be responsible for their rapid inhibition of host cell protein synthesis.

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LITERATURE CITED

- Bernstein, H. D., N. Sonenberg, and D. Baltimore. 1985. Poliovirus mutant that does not selectively inhibit host cell protein synthesis. *Mol. Cell. Biol.* **5**:2913-2923.
- Bonneau, A.-M., and N. Sonenberg. 1987. Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. *J. Virol.* **61**:986-991.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* **112**:195-203.
- Etchison, D., and S. Fout. 1985. Human rhinovirus 14 infection of HeLa cells results in the proteolytic cleavage of the p220 cap-binding complex subunit and inactivates globin mRNA translation in vitro. *J. Virol.* **54**:634-638.
- Etchison, D., S. C. Milburn, I. Edery, N. Sonenberg, and J. W. B. Hershey. 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. *J. Biol. Chem.* **257**:14806-14810.
- Grubman, M. J., M. Zellner, and J. Wagner. 1987. Antigenic comparison of the polypeptides of foot-and-mouth disease virus serotypes and other picornaviruses. *Virology* **158**:133-140.
- Jen, G., B. M. Detjen, and R. E. Thach. 1980. Shutoff of HeLa cell protein synthesis by encephalomyocarditis virus and poliovirus: a comparative study. *J. Virol.* **35**:150-156.
- Krausslich, H. G., M. J. H. Nicklin, H. Toyoda, D. Etchison, and E. Wimmer. 1987. Poliovirus proteinase 2A induces cleavage of eucaryotic initiation factor 4F polypeptide p220. *J. Virol.* **61**:2711-2718.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Lloyd, R. E., D. Etchison, and E. Ehrenfeld. 1985. Poliovirus protease does not mediate cleavage of the 220,000-Da component of the cap binding protein complex. *Proc. Natl. Acad. Sci. USA* **82**:2723-2727.
- Lloyd, R. E., M. J. Grubman, and E. Ehrenfeld. 1988. Relationship of p220 cleavage during picornavirus infection to 2A proteinase sequences. *J. Virol.* **62**:4216-4223.
- Lloyd, R. E., H. G. Jense, and E. Ehrenfeld. 1987. Restriction of translation of capped mRNA in vitro as a model for poliovirus-induced inhibition of host cell protein synthesis: relationship to p220 cleavage. *J. Virol.* **61**:2480-2488.
- Lloyd, R. E., H. Toyoda, D. Etchison, E. Wimmer, and E. Ehrenfeld. 1986. Cleavage of the cap binding protein complex polypeptide p220 is not effected by the second poliovirus protease 2A. *Virology* **150**:299-303.
- Robertson, B. H., M. J. Grubman, G. N. Weddell, D. M. Moore, J. D. Welsh, T. Fischer, D. J. Dowbenko, D. G. Yansura, B. Small, and D. G. Kleid. 1985. Nucleotide and amino acid sequence coding for polypeptides of foot-and-mouth disease virus type A12. *J. Virol.* **54**:651-660.
- Rueckert, R. R., and E. Wimmer. 1984. Systematic nomenclature of picornaviral proteins. *J. Virol.* **50**:957-959.
- Shatkin, A. J. 1985. mRNA cap binding proteins: essential factors for initiating translation. *Cell* **40**:223-224.
- Strebel, K., and E. Beck. 1986. A second protease of foot-and-mouth disease virus. *J. Virol.* **58**:893-899.
- Summers, D. F., J. V. Maizel, and J. E. Darnell. 1965. Evidence for virus-specific noncapsid proteins in poliovirus-infected cells. *Proc. Natl. Acad. Sci. USA* **54**:505-513.
- Tahara, S. M., M. A. Morgan, and A. J. Shatkin. 1981. Two forms of purified m⁷G-cap binding protein with different effects on capped mRNA translation in extracts of uninfected and poliovirus-infected HeLa cells. *J. Biol. Chem.* **256**:7691-7694.
- Vakharia, V. N., M. A. Devaney, D. M. Moore, J. J. Dunn, and M. J. Grubman. 1987. Proteolytic processing of foot-and-mouth disease virus polyproteins expressed in a cell-free system from clone-derived transcripts. *J. Virol.* **61**:3199-3207.