JOURNAL OF VIROLOGY, July 1991, p. 3903–3905 Vol. 65, No. 7
0022-538X/91/073903-03$02.00/0
Copyright © 1991, American Society for Microbiology

Cap-Binding Complex Protein p220 Is Not Cleaved during Echovirus 22 Replication in HeLa Cells

BETH-ANN G. COLLER,[1] STEVEN M. TRACY,[1,3]* AND DIANE ETCHISON[2]

Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198-6495,[1] and Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66103[2]

Received 14 February 1991/Accepted 11 April 1991

Previously we demonstrated that echovirus 22 is an atypical enterovirus which does not shut off host cell protein synthesis. We extend these findings by showing that echovirus 22 does not cleave p220, part of the cellular cap-binding complex necessary for cap-dependent translation, suggesting a biology more consistent with cardioviruses than enteroviruses.

Echovirus 22 (EV22), a member of the enterovirus genus in the family *Picornaviridae*, has been associated with serious human illnesses, including encephalitis, paralysis, and myocarditis (6, 10, 14). Several reports have suggested that EV22 displays characteristics atypical of the enterovirus paradigm best typified by the polioviruses and group B coxsackieviruses (3, 8, 17–21). Our laboratory extended these findings by conducting an extensive molecular and biologic characterization of EV22 which demonstrated that EV22 does not exhibit detectable nucleotide identity with other members of the enterovirus genus (2). At the antigenic level, EV22 appears enteroviruslike: EV22 proteins share an enteroviral group-specific antigen recognized by enterovirus-immune T cells which is not shared by the cardioviruses. Determinations of the S value for denatured EV22 RNA and the density in CsCl are consistent with enteroviral biophysical characteristics (2, 16). However, EV22 also shares several important biological characteristics with the cardioviruses (2). EV22 fails to shut off host cell protein synthesis efficiently in infected cells and acts as an efficient message in rabbit reticulocyte lysate in vitro translation systems (2). Other studies have demonstrated evidence for the presence of an extensive 5′ hairpin structure in the viral RNA (16, 17). Limited sequence data suggest the 3′ end of the genome is significantly diverged from other enteroviral sequences and may as well form a hairpin structure (1).

Extensive studies on the polioviruses have shown that the shutoff of host cell protein synthesis by these viruses is accomplished through cleavage of a component of the mRNA cap-binding complex, protein 220 (p220), resulting in abrogation of translation of capped messages (7, 11, 12, 15). The viral protease 2A has been shown to be responsible for the cleavage, although 2A does not cleave p220 directly (11, 12). In contrast, the cardioviruses do not directly shut off host cell protein synthesis by cleaving p220, but are believed to outcompete host cell message for the translational machinery (9, 12). On the basis of our previous study which showed that EV22 does not shut off host cell protein synthesis and acts as an efficient message in in vitro translation systems, we hypothesized that EV22, like the cardioviruses, would not cleave p220.

EV22 (Omaha) and coxsackievirus B3 (CVB3) have been describe elsewhere (2). HeLa cells were plated in 6- or 24-well plates and infected with EV22 or CVB3 at a multiplicity of infection of 100 50% tissue culture infective doses per cell. After 1 h at 37°C, the medium was replaced either with fresh medium (6-well plates) or with minimal essential medium lacking cysteine and methionine (GIBCO) supplemented with 5% fetal bovine serum (24-well plates). At 3, 5, 7, and 9 h postinfection (p.i.), 30 μCi of [^{35}S]cysteine-methionine (>1,000 Ci/mmol; Tran^{35}S-label; ICN) was added to each well of the 24-well plate. One hour after addition of label, the medium was removed, and the cells were harvested by addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing sample buffer (13). Unlabeled infected cells were harvested from the six-well plates at the same time points by the same method. Uninfected cell controls were harvested as described for the infected cells. The cell lysates were heated at 100°C for 5 min and stored frozen at −20°C.

^{35}S-labeled samples were analyzed for shutoff of host cell protein synthesis by SDS-PAGE and fluorography as previously described (2). Cleavage of p220 was assayed by Western immunoblot analysis using a monoclonal antibody specific for p220 as previously described (5). Briefly, 15% of the unlabeled cell lysates was applied to an 8% SDS-PAGE gel, and following electrophoresis, the proteins were electrophoretically transferred to nitrocellulose. The nitrocellulose was incubated with a 1/1,000 dilution of anti-p220 monoclonal antibodies (4) and then with alkaline phosphatase-conjugated goat anti-mouse antibodies (Pierce Chemical Co.). The immunoblot was developed by using nitroblue tetrazolium according to standard techniques.

The results of the shutoff experiment (Fig. 1) confirmed our previous findings (2) that EV22 does not efficiently shut off host cell protein synthesis even late in infection (9 hours p.i.), although prominent viral proteins are evident by 5 h p.i. This is in contrast to the marked shutoff demonstrated during infection by the prototypic enterovirus CVB3, in which shutoff is apparent by 3 hours p.i. The overall loss in proteins detected at late time points in infection in both the EV22- and CVB3-infected cells is likely due to massive cell death late in infection and correlates with a loss in total protein as determined by Coomassie blue staining (data not shown). While EV22 infection progresses more slowly than CVB3 infection, we have previously demonstrated that even very late in infection (12 and 17 h p.i.), when EV22-infected cells exhibit advanced cytopathic effects, EV22 does not shut off host cell protein synthesis (data not shown). Thus, the lack of shutoff exhibited by EV22 is not simply due to

* Corresponding author.

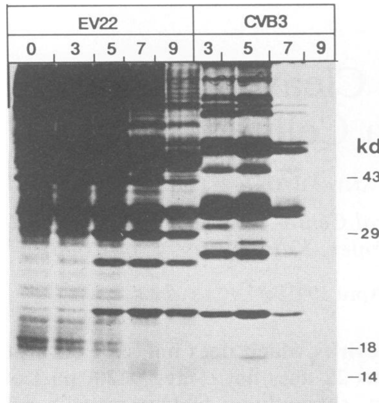

FIG. 1. SDS-PAGE analysis of host cell protein synthesis shutoff by EV22 and CVB3. HeLa cells were infected with EV22 or CVB3, pulsed with [^{35}S]cysteine-methionine at 3, 5, 7, and 9 h p.i., and harvested after 1 h of labeling. Equivalent aliquots of each sample were analyzed on 12.5% SDS-PAGE gels by fluorography. Numbers above the lanes represent the hours postinfection, with time 0 representing uninfected cells. Locations of molecular size markers in kilodaltons (kd) are indicated. The fluorograph was exposed for 2.5 h at room temperature.

differences in the infection rate between CVB3 and EV22 but is the result of a true biologic difference.

The immunoblot (Fig. 2) demonstrated that the cap-binding complex component p220 is not cleaved in EV22-infected HeLa cells. This is in contrast to poliovirus type 1 (PV1)- or CVB3-infected cells, in which only p220 cleavage products can be detected by 3 to 4 hours p.i. The loss of signal for either p220 (EV22) or p220 cleavage products (CVB3) is likely due to a loss in total protein due to cell death late in infection.

These data suggest that EV22 is the only human picornavirus known which, by not cleaving p220, apparently fails to shut off host cell protein synthesis. These results are consistent with other data which individually and cumulatively

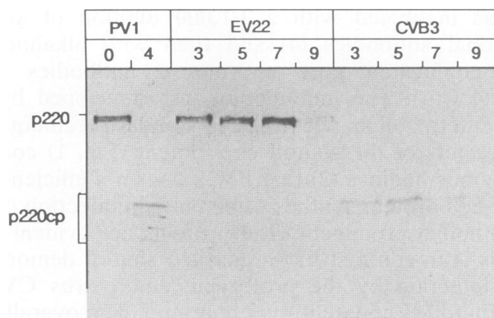

FIG. 2. Immunoblot of cell lysates from EV22-, CVB3-, and PV1-infected cells probed with anti-p220 monoclonal antibodies. Cell lysates obtained from infected cells at 3, 5, 7, and 9 h p.i. (EV22 and CVB3) or 4 h p.i. (PV1) were electrophoresed on 8% SDS-PAGE gels, and the proteins were electrophoretically transferred to nitrocellulose. The blots were probed with anti-p220 monoclonal antibodies, and the results were visualized by using alkaline phosphatase-conjugated secondary antibody and nitroblue tetrazolium. Numbers above the lanes represent the hours postinfection, with time 0 representing uninfected cells. Locations of p220 and p220 cleavage products (p220cp) are indicated.

suggest that EV22 is not a true enterovirus, but may be a recombinant virus having characteristics of both the cardiovirus and enterovirus genera or perhaps the prototype of a new picornavirus genus. EV22 shares a group-specific T-cell epitope with the enteroviruses (2). However, while the enterovirus-immune T cells recognize and respond to EV22 as an antigen in in vitro proliferation studies whereas cardioviruses are not similarly recognized, the recognition is delayed relative to other enteroviruses. Such data suggest that the antigenic site may be significantly diverged as well (2). Thus, the classification of EV22 as an enterovirus must now be viewed as tenuous. The complete nucleotide sequence analysis of the EV22 genome will soon be available from other laboratories. To date only limited sequence from the 3′ terminus is available (1). Complete analysis of the nucleotide sequence will permit a precise classification of this interesting virus through comparison with other sequenced picornaviral genomes.

We thank Raymond Cologna for excellent technical assistance and Glyn Stanway for communicating unpublished data.

This work was supported in part by grants from the Public Health Service (HL40303 [S.M.T.] and AI24356 [D.E.]).

ADDENDUM IN PROOF

The complete nucleotide sequence of EV22 (Harris) has been determined (T. Hyypia and G. Stanway, personal communication).

REFERENCES

1. **Auvinen, P., and T. Hyypia.** 1990. Echoviruses include genetically distinct serotypes. J. Gen. Virol. **71**:2133–2139.
2. **Coller, B. G., N. M. Chapman, M. A. Beck, M. A. Pallansch, C. J. Gauntt, and S. M. Tracy.** 1990. Echovirus 22 is an atypical enterovirus. J. Virol. **64**:2692–2701.
3. **Crowther, D., and J. L. Melnick.** 1961. Studies on the inhibitory action of guanidine on poliovirus multiplication in cell cultures. Virology **15**:65–74.
4. **Etchison, D., and J. R. Etchison.** 1987. Monoclonal antibody-aided characterization of cellular p220 in uninfected and poliovirus-infected HeLa cells: subcellular distribution and identification of conformers. J. Virol. **61**:2702–2710.
5. **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.
6. **Figueroa, J. P., D. Ashley, D. King, and B. Hull.** 1989. An outbreak of acute flaccid paralysis in Jamaica associated with echovirus type 22. J. Med. Virol. **29**:315–319.
7. **Holland, J. J.** 1964. Enterovirus entrance into specific host cells and subsequent alteration of cell protein and nucleic acid synthesis. Bacteriol. Rev. **28**:3–13.
8. **Jamison, R. M.** 1974. An electron microscopic study of the intracellular development of echovirus 22. Arch. Gesamte Virusforsch. **44**:184–194.
9. **Jen, G., and R. E. Thach.** 1982. Inhibition of host translation in encephalomyocarditis virus-infected L cells: a novel mechanism. J. Virol. **43**:250–261.
10. **Koskiniemi, M., R. Paetau, and K. Linnavuori.** 1989. Severe encephalitis associated with disseminated echovirus 22 infection. Scand. J. Infect. Dis. **21**:463–466.
11. **Kräusslich, 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.
12. **Kräusslich, H.-G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57**:701–754.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the

assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

14. **Maller, H. M., D. F. Powars, R. E. Horowitz, and B. Portnoy.** 1967. Fatal myocarditis associated with echovirus, type 22, infection in a child with apparent immunological deficiency. J. Pediatr. **71**:204–210.
15. **Rose, J. K., H. Trachsel, K. Leong, and D. Baltimore.** 1978. Inhibition of translation by poliovirus: inactivation of a specific initiation factor. Proc. Natl. Acad. Sci. USA **75**:2732–2736.
16. **Seal, L. A., and R. M. Jamison.** 1990. A structural model for the genome of echovirus 22. Arch. Virol. **111**:45–61.
17. **Seal, L. A., and R. M. Jamison.** 1984. Evidence for secondary structure within the virion RNA of echovirus 22. J. Virol. **50**:641–644.
18. **Shaver, D. N., A. L. Barron, and D. T. Karzon.** 1961. Distinctive cytopathology of echoviruses type 22 and 23. Proc. Soc. Exp. Biol. Med. **106**:648–652.
19. **Tamm, I., and H. J. Eggers.** 1962. Differences in the selective virus inhibitory action of 2-(alpha-hydroxybenzyl) benzimidazole and guanidine HCl. Virology **18**:439–447.
20. **von Zeipel, G.** 1980. Most echoviruses reach higher titers in RD than GMK-AH1 cells, regardless of their passage history. Arch. Virol. **63**:143–146.
21. **Wigand, R., and A. B. Sabin.** 1961. Properties of echo types 22, 23, and 24 viruses. Arch. Gesamte Virusforsch. **11**:224–247.