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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

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One of the characteristics of picornavirus infection of cells in tissue culture is a specific inhibition of utilization of host cell mRNA for protein synthesis. In this study we show that human rhinovirus 14 is similar to poliovirus in that the inhibition of host cell translation that occurs during infection correlates with the proteolytic cleavage of an M_r 220,000 subunit of the cap-binding protein complex.

A number of picornaviruses have been shown to inhibit translation of host cell mRNA during the infectious process (for a review see reference 3). Infection by one of these viruses results in a decrease in the rate of synthesis of host proteins, an effect which precedes substantial synthesis of viral proteins. The mechanism of picornavirus-induced inhibition of host cell mRNA translation involves discrimination between capped cellular mRNA and uncapped viral mRNA, although structural features of the mRNA other than the cap may also be involved. mRNA discrimination is apparently accomplished by more than one mechanism. Poliovirus (a member of the *Enterovirus* genus) alters the ability of protein synthesis initiation factors to initiate translation on mRNAs containing 5′-end cap structures (11, 13). The result of this alteration is that uncapped viral message is utilized exclusively in poliovirus-infected cells by about 2.5 h after infection. Infection with encephalomyocarditis virus (a member of the *Cardiovirus* genus), on the other hand, results in a competitive utilization of viral message at the expense of host cell message (6, 9, 10, 12). As viral mRNA accumulates, it outcompetes cellular mRNA for one or more of the protein synthesis initiation factors.

In poliovirus-infected cells, only one of the protein synthesis initiation factors, the cap-binding protein complex (CBC), has been shown to be both structurally and functionally altered (4, 5). The CBC is a multi-subunit complex which can be purified by virtue of its ability to bind to mRNA cap structure analogs (21). Affinity chromatography on m^7GTP-Sepharose results in purification of a complex consisting of three or four polypeptides (M_rs of 220, 49, and 28 kilodaltons and possibly one of 73 kilodaltons) (2, 7). Two of the subunits of CBC chemically cross-link to labeled mRNA caps in vitro, and this cross-linking activity is abolished when crude initiation factors from poliovirus-infected cells are used (13, 19, 20). CBC activity that has been determined by a fractionated translation assay can be identified in sucrose gradients of crude initiation factors from uninfected cells, but it is not present among those from poliovirus-infected cells (4). Finally, the ability of infected cell lysates to translate capped mRNA in vitro can be restored by the addition of purified CBC (7). These results indicate that the alteration in poliovirus-infected cells involves inactivation of capped mRNA recognition by CBC.

Structural studies of CBC by immunoblot analysis have demonstrated that the M_r 220-kilodalton subunit (p220) is proteolytically degraded in poliovirus-infected cells (4). Degradation occurs early after infection and correlates in time with the inhibition of cellular mRNA translation. Crude initiation factors from infected cells contain an activity which can degrade intact p220 in preparations from uninfected cells; thus, there is a viral or virally induced protease which specifically degrades p220. Degradation of the p220 subunit of CBC is presumably responsible for inactivation of capped mRNA recognition in infected cells.

In this study we investigated the effect of another picornavirus, human rhinovirus 14 (HRV-14), a member of the *Rhinovirus* genus, on HeLa cell protein synthesis. Like poliovirus, HRV-14 infects human cells, but the pathogenicities and growth properties of these two viruses are somewhat different. HRV-14 causes upper respiratory disease, whereas poliovirus produces gastrointestinal disease with occasional central nervous system involvement, and HRV-14 grows optimally at a lower temperature than does poliovirus. The results of this study show that, like poliovirus, HRV-14 degrades the p220 subunit of CBC and inactivates the ability of initiation factors to utilize capped mRNA in vitro.

To analyze the effect of HRV-14 infection on cellular protein synthesis, samples of infected cells were pulse labeled for 15 min at different times after infection. Figure 1 shows an autoradiograph of pulse-labeled infected cell lysates which demonstrates that at early times after infection only host proteins are synthesized. As infection proceeds, the relative proportion of viral to host proteins synthesized increases, until by 6 h after infection virtually all newly synthesized proteins are viral. HRV-14 is therefore similar to other picornaviruses in that it specifically inhibits the synthesis of cellular proteins.

Since poliovirus inhibition of cellular protein synthesis is accompanied by the degradation of the CBC subunit (p220), we wanted to determine whether a similar effect occurs after HRV-14 infection. Separate samples of the infected cells analyzed in Fig. 1 were harvested, and their postnuclear supernatants were analyzed by immunoblotting with a monoclonal antibody preparation directed against p220. This

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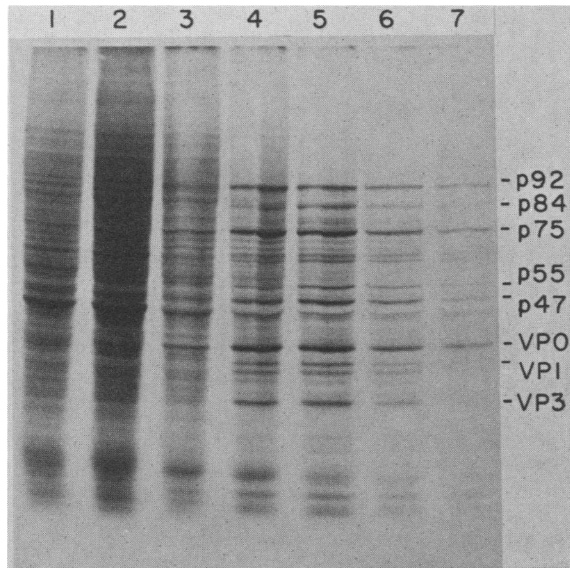

FIG. 1. Autoradiograph of mock- and HRV-14-infected HeLa H1 cells. Suspension cultures of HRV-sensitive HeLa H1 cells were propagated as described by Medappa et al. (16). Cells were infected at a multiplicity of 100 PFU of HRV-14 per cell. At the times indicated below, 5-ml samples from a mock-infected culture of HeLa H1 cells (lane 1) or an HRV-14-infected culture (lanes 2 to 7) were transferred to 25-ml flasks containing 100 μCi of [^{35}S]methionine and incubated for 15 min at 35°C. Labeled cells were chilled on ice, collected by centrifugation at 600 × g, and suspended in 1 ml of distilled water. After disruption of the cells with three freeze-thaw cycles, the proteins were acetone precipitated, dried under vacuum, and suspended in electrophoresis sample buffer. Portions of 10 to 20 μl from each sample were run on a 7.5 to 15% polyacrylamide gradient gel (14), and labeled proteins were identified by autoradiography. Labeling times began at 0, 1.5, 3, 4, 5, 6, or 7 h postinfection (lanes 1 to 7, respectively). The identification of virion capsid proteins VP0, VP1, and VP3 were confirmed by electrophoresis of purified HRV-14 virions and RNA-free protein shells (data not shown). The major noncapsid viral proteins were identified by their apparent molecular weights (14).

antibody probe was developed against a crude CBC preparation (D. E. Etchison and J. R. Etchison, manuscript in preparation). Figure 2 shows that the antibody reacts with a set of polypeptides of approximately M_r 220,000 in uninfected HeLa S3 (Mock polio) as well as HeLa H1 (Mock HRV) cells, but only with smaller polypeptides, presumably cleavage products, in poliovirus-infected cells. As many as three polypeptides with apparent molecular weights of approximately 220,000 can be resolved by immunoblotting of both crude cell lysates and purified CBC with this monoclonal antibody, and these are collectively referred to as p220. In HRV-14-infected cells, intact p220 is present 0 and 1.5 h after infection but disappears entirely by 3 h after infection. The cleavage products, which appear by 3 h after infection, are virtually identical in size with those present in poliovirus-infected HeLa S3 cells; however, there are some differences in the distribution of various size classes. Panel B of Fig. 2 shows the decrease in the rate of protein synthesis in the HRV-14-infected cells analyzed above. As shown, the decrease in the rate of protein synthesis slightly lags the degradation of p220, an effect which was also seen in poliovirus-infected cells (4).

To show that CBC is inhibited in HRV-14-infected cells,

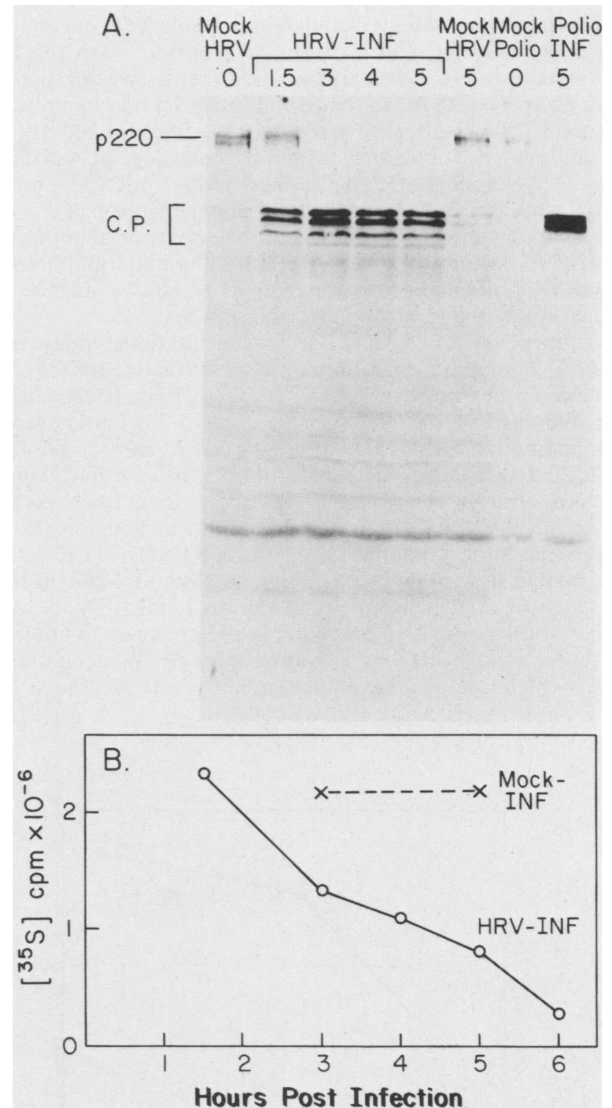

FIG. 2. p220 immunoblot analysis and rate of protein synthesis determined at different times after infection of HeLa H1 cells with HRV-14. Postnuclear supernatants of the mock- or HRV-14-infected cells described in the legend to Fig. 1 were prepared, and approximately 0.15 A_{280} units were applied to a 10% polyacrylamide gel. The proteins on the gel were electrophoretically transferred to nitrocellulose sheets and immunoblotted with antibody by procedures described previously (5, 17). The immunoblots were first incubated with a 1/5,000 dilution of ascites fluid collected from mice injected with hybridoma cells secreting immunoglobulin G (IgG) (κ chain-type) antibodies directed against the p220 subunit of CBC (see the text for a more detailed description). After the nitrocellulose paper was washed with antibody blotting buffer, a second incubation was performed with goat anti-mouse IgG, IgA, and IgM antibodies (Cappel Laboratories, Cochranville, Pa.). The immunoblot was again washed, and a third incubation was performed with ^{125}I-labeled rabbit anti-goat IgG antibodies. The washed immunoblot was dried and exposed to Kodak X-Omat SB5 film (panel A). Lanes: Mock HRV 0 and Mock HRV 5, postnuclear extracts from HeLa H1 cells harvested 0 and 5 h after mock infection; HRV-INF, extracts of HRV-14-infected HeLa H1 cells harvested 1.5, 3, 4, and 5 h after infection; Mock Polio 0, extract of uninfected HeLa S3 cells harvested 0 h after mock infection; Polio INF 5, extract of HeLa S3 cells harvested 5 h after infection with 50 PFU of poliovirus 1 (Mahoney strain) per cell. C.P. refers to cleavage products. Panel B shows the [^{35}S]methionine incorporation in 200-μl samples of mock- or HRV-14-infected cells as described in the legend to Fig. 1.

uninfected (mock-infected) and infected cells were harvested 4 h after infection, and a ribosomal high-salt wash (RSW) containing protein synthesis initiation factors was prepared from each. The RSW from infected (inf-RSW) and uninfected (uninf-RSW) cells were separately analyzed for activity in a fractionated translation system consisting of purified ribosomal subunits, tRNA, capped globin mRNA, and a cytoplasmic pH 5 fraction. Figure 3 shows that translation of capped globin mRNA is stimulated by increasing amounts of uninf-RSW but not by inf-RSW. Thus, the initiation factors in HRV-14-infected cells are defective and are unable to initiate translation on capped globin mRNA.

The inf-RSW preparation was further fractionated into a 0 to 40% ammonium sulfate-precipitated fraction (RSW A, containing eucaryotic initiation factor 3 [eIF3], eIF4B, CBC, and one-third of the eIF4A) and a 40 to 70% ammonium sulfate fraction (RSW B, containing eIF2, eIF4C, eIF4D, eIF5, and two-thirds of the eIF4A) (1; unpublished data). Each fraction was analyzed separately in a fractionated globin mRNA translation assay containing an active RSW A or B preparation from uninfected HeLa S3 cells. Inf-RSW A was unable to stimulate translation of globin mRNA in the presence of control RSW B. In contrast, inf-RSW B was active in the corresponding assay in the presence of control RSW A. This result was expected since RSW A contains CBC which, as we have shown above, is degraded in HRV-14-infected cells. We next attempted to restore the RSW A assay with various purified or partially purified initiation factors (Table 1). Purified factors which contained no detectable p220, CBC-free eIF3, and CBP I (the M_r 24,000 to 28,000 subunit of CBC) were unable to stimulate translation in the presence of HRV-inf-RSW A. Among the factors which contained p220, however, the only one which was able to significantly restore activity was a relatively crude eIF3-CBC fraction from uninfected HeLa S3 cells. This preparation consisted of pooled eIF3 and p220 antigen-containing fractions of a sucrose gradient of uninf-RSW A. Translation reactions containing inf-RSW A and eIF3-CBC incorporated as much as 250-fold more tritiated leucine than control reactions lacking eIF3-CBC, a level of activity which approached that of uninf-RSW A alone. Restoration by purified CBC preparations, on the other hand, was surprisingly inconsistent. One such preparation, CBC-PC, was purified by its ability to stimulate translation of globin mRNA in the presence of purified initiation factors in an assay described previously (4). In some experiments CBC-PC stimulated translation by about 40% in the presence of HRV-inf-RSW A, whereas in other experiments it failed to stimulate translation at all. Its activity in the presence of poliovirus-inf-RSW A was similarly low (56% stimulation). Increasing amounts of CBC-PC improved the activity only slightly (data not shown). Another CBC preparation, CBP II, was purified by cap-affinity chromatography. It was also inactive.

Although the failure of purified CBC preparations to consistently restore globin mRNA translation by HRV-inf-

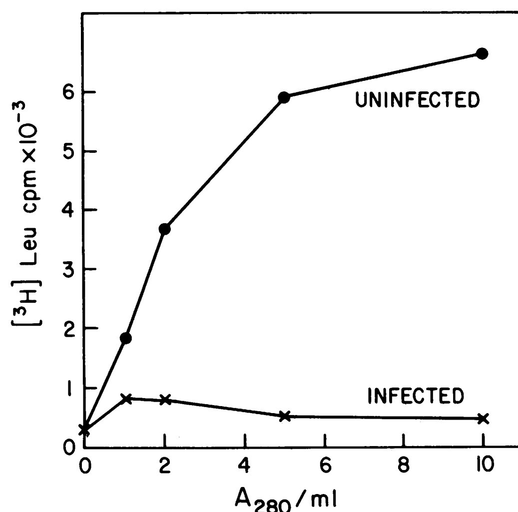

FIG. 3. Stimulation of translation of globin mRNA in a fractionated translation system by crude initiation factors from uninfected and HRV-infected cells. Mock- or HRV-14-infected cells were harvested 5 h after infection, washed with phosphate-buffered saline, and suspended in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.5)–10 mM potassium chloride–1.5 mM magnesium acetate. After 10 min on ice the cells were Dounce homogenized and centrifuged for 15 min at 10,000 × g. The postnuclear supernatants were brought to 10% glycerol and 7 mM β-mercaptoethanol, sealed in ampoules, and frozen at −70°C until use. RSW was prepared as described previously (8). Increasing amounts of uninfected or infected RSW were added to a fractionated translation system described previously (4). The reaction mixture included ribosomal subunits from purified rat liver, tRNA, globin mRNA, and a cytoplasmic fraction of pH 5. Tritiated leucine incorporation was determined as acid-precipitable counts per minute. No additional sources of initiation factors were added except those present in the RSW.

TABLE 1. Stimulation of globin mRNA translation by purified initiation factors in the presence of various sources of RSW A[a]

Expt	Addition	$[^3H]$Leucine incorporation (cpm)	% Stimulation
1	HRV-inf-RSW A	697	
	HRV-inf-RSW A+CBC-PC	974	40
	HRV-inf-RSW A+eIF3-CBC	2,676	284
	Unif-RSW A	2,913	
2	HRV-inf-RSW A	1,670	
	HRV-inf-RSW A+CBP I	1,775	6
	HRV-inf-RSW A+eIF3	1,772	6
	HRV-inf-RSW A+CBP II	1,494	0
	HRV-inf-RSW A+CBC-PC	1,669	0
3	Polio-inf-RSW A	1,631	
	Polio-inf-RSW A+CBC-PC	2,543	56
	Polio-inf-RSW A+eIF3-CBC	3,633	123

[a] Translation assays were performed essentially as described previously (4). Reaction mixtures (20 μl) contained RSW B (0.01 A_{280} units) from uninfected HeLa S3 cells and HRV-inf-RSW A (0.02 and 0.03 A_{280} units in experiments 1 and 2, respectively) or 0.02 A_{280} units of poliovirus-infected (Polio-inf) or uninfected RSW A as indicated. Purified initiation factors added as indicated were CBC-PC (0.22 μg), eIF3-CBC (6.0 μg), CBP I (0.04 μg), CBP II (0.24 μg), and CBC-free eIF3 (1.8 μg). All factors were added at levels which were determined to be optimal in assays with purified factors. CBC-free eIF3 was purified by published procedures (1), except that the absence of p220 was monitored by immunoblotting. eIF3-CBC was prepared as pooled fractions from a sucrose gradient of uninf-RSW A. Fractions containing both eIF3 and p220 antigens were pooled, and the sample was concentrated by 40% ammonium sulfate precipitation. CBC-PC was purified by the same protocol as that used for eIF3 except that CBC activity as determined by an assay described previously (4). Fractions containing CBC activity were pooled and chromatographed on DEAE-cellulose and then on phosphocellulose (PC). CBP I and CBP II were purified by cap-affinity chromatography (21).

RSW A was unexpected, we reasoned that this might reflect the presence of a high level of the viral or virally induced p220 protease in inf-RSW A preparations. To test this possibility, HRV-inf-RSW A was incubated with CBC-PC and with eIF3-CBC preparations under translation conditions (60 min, 30°C). Proteins were separated on a polyacrylamide gel, transferred to nitrocellulose paper, and incubated with anti-p220 monoclonal antibody as described in the legend to Fig. 2. The majority of the p220 present in the CBC-PC preparation which was unable to consistently restore globin translation was degraded under these conditions (data not shown). The eIF3-CBC preparation which was able to restore globin translation contained a greater concentration of p220 antigen than the CBC-PC preparation, and only part of the p220 was degraded by incubation with HRV-inf-RSW A. In a control incubation with poliovirus-inf-RSW A, p220 was degraded to a reduced degree, indicating that the poliovirus-induced p220 protease may be less active than the HRV-14-induced protease. Separate studies on the poliovirus-induced p220 protease indicate that its activity is somewhat unstable to freezer storage (unpublished data); thus, comparative and quantitative studies will require fresh infected cell material and were not attempted here.

The results presented here indicate that HRV-14 affects cellular protein synthesis by a mechanism which is similar to that of poliovirus. Infection by these viruses results in the proteolytic cleavage of the p220 subunit of the CBC initiation factor, a complex which has been demonstrated elsewhere to be involved in the recognition of mRNA cap structures. The effect of p220 cleavage correlates with the inhibition of cellular protein synthesis by poliovirus or HRV-14 infection and with the inability of the initiation factors to function on capped globin mRNA in vitro. We were unable to conclusively demonstrate that CBC was able to restore globin mRNA translation by initiation factors from HRV-infected cells, possibly because of the presence of active p220 protease in the crude initiation factor preparations.

In separate studies, other viruses have been analyzed for their effects on p220 (R. Duncan and J. Hershey, manuscript in preparation; J. Mosenkis, S. Daniels-McQueen, S. Janovec, R. Duncan, J. Hershey, J. A. Grifo, W. C. Merrick, and R. E. Thach, submitted for publication). Virus systems tested include vesicular stomatitis virus-, encephalomyocarditis virus-, mengovirus-, and vaccinia virus-infected HeLa cells, as well as reovirus-infected mouse L cells. All of these viruses have some effect on mRNA discrimination, but none of them affect the integrity of the p220 subunit of CBC. Thus, encephalomyocarditis virus (a cardiovirus) inhibits protein synthesis by a different mechanism than do poliovirus (an enterovirus) and HRV-14 (a rhinovirus).

It has recently been found that poliovirus protease 3C (standard picornaviral nomenclature; 18), which is involved in the cleavage of viral protein precursors into their final products, is not the mediator of p220 cleavage (R. E. Lloyd, D. Etchison, and E. Ehrenfeld, in press). It is possible, however, that another viral protein may be the p220 protease or that 3C or another viral protein may be responsible for activating a cellular protease. Nucleotide sequence studies show that at the amino acid level HRV-14 is very similar to poliovirus (R. J. Colonno, personal communication) and that both HRV-14 and poliovirus are less related to encephalomyocarditis virus (A. C. Palmenberg, personal communication). It is likely that the degree of relatedness of these picornaviruses is reflected in their differential abilities to cleave p220.

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