JOURNAL OF VIROLOGY, Apr. 1987, p. 986–991
0022-538X/87/040986-06$02.00/0

Vol. 61, No. 4

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

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Received 2 September 1986/Accepted 3 December 1986

Infection of cells with poliovirus results in the complete shutoff of host protein synthesis. It is presumed that proteolysis of the p220 component of the cap-binding protein complex that is required for the translation of host mRNAs is responsible for the shutoff phenomenon. In this paper, we show that when cells are infected with poliovirus in the presence of guanidine or 3-methylquercetin, both inhibitors of poliovirus replication, complete cleavage of p220 occurs by 3.5 h postinfection. However, under these conditions only 55 to 77% of host protein synthesis is suppressed. Results obtained with extracts prepared from poliovirus-infected cells were similar to those obtained in vivo. These results suggest that complete inhibition of host protein synthesis after poliovirus infection requires at least one event in addition to proteolysis of p220. Thus, proteolysis of p220 is probably necessary but not sufficient for total suppression of host protein synthesis after poliovirus infection.

Poliovirus infection of HeLa cells results in a precipitous shutoff of host cell protein synthesis, concomitant with the selective translation of viral mRNA (for recent reviews, see references 13 and 34a). The proposed mechanism for the shutoff phenomenon is based on the finding that poliovirus RNA, unlike most eucaryotic cellular mRNAs, is naturally uncapped. It has been well documented that the cap structure if important for efficient translation of eucaryotic mRNAs and that cap-binding proteins (CBPs) interact with the cap structure (m^7GpppN) during translation initiation (for a recent review, see reference 34). Since poliovirus RNA is naturally uncapped (20, 29), it was suggested that poliovirus RNA bypasses the requirement for a cap structure and cap-binding protein for translation (17, 19, 33). It was further suggested that specific inactivation of a protein that recognizes the cap structure and that is required for translation of host mRNAs but not poliovirus RNA could be responsible for the shutoff phenomenon (33).

Proteins which can bind to the cap structure have been purified from several sources (see, e.g., references 11, 18, 23, 37, and 39). Originally, a 24-kilodalton (kDa) polypeptide that cross-linked to the oxidized cap structure of reovirus mRNA in a cap-specific manner was identified and subsequently purified by affinity chromatography (36, 37). In addition, a high-molecular-weight complex (termed CBP II, CBP complex, or eIF-4F) was purified in several laboratories (11, 18, 39). This complex is composed of the 24-kDa polypeptide, a 50-kDa polypeptide (which is similar if not identical to eIF-4A) (11, 18), and a 220-kDa polypeptide (p220).

Etchison et al. (15) first described a specific modification of the CBP complex after poliovirus infection. Using an immunoblot assay, they showed that an antibody raised against a 220-kDa polypeptide present in eIF-3 preparations cross-reacted with the p220 subunit of the CBP complex. However, in extracts prepared from poliovirus-infected cells, the antibody did not react with a 220-kDa component but rather with two or three polypeptides having molecular weights of approximately 110,000 to 130,000 (15). Consequently, it was proposed that poliovirus infection leads to the proteolytic cleavage of the p220. Lee et al. (23), recently isolated the CBP complex from mock- and poliovirus-infected cells and demonstrated that the CBP complex from infected cells contained the proteolytic cleavage products of p220 but not intact p220, thus providing direct evidence for the existence of a modified CBP complex in poliovirus-infected cells. However, the activity of the modified CBP complex was not tested.

The mechanism by which poliovirus induces the cleavage of p220 is unknown. Proteolysis of p220 by the well-characterized poliovirus protease 3C has been ruled out (22, 25). More recently, poliovirus protein 2A was identified as a protease that cleaves two Tyr-Gly amino acid pairs in the poliovirus polypeptide precursor (40). In addition, a poliovirus mutant with a mutation in the 2A protein lacked the ability to mediate the selective inhibition of host synthesis (5). This implicated protein 2A in the shutoff of host protein synthesis via a proteolytic event. However, a direct involvement of protein 2A in cleavage of p220 could not be demonstrated (26).

Partial inhibition (50 to 70%) of host protein synthesis after poliovirus infection occurs early in infection when no appreciable amounts of poliovirus RNA or proteins are synthesized (13). Moreover, a reduction of about 50 to 70% in host protein synthesis also occurs in the presence of guanidine, which drastically inhibits poliovirus replication (2, 3, 21, 31, 38). These findings led to the conclusion that very low levels of the putative protease activity are required to elicit the shutoff phenomenon. However, the maximum extent of shutoff of host protein synthesis occurs when high levels of poliovirus RNA and proteins are synthesized. Consequently, events other than cleavage of p220 might be involved in the repression of host protein synthesis. To examine this possibility, we studied the effect of guanidine and 3-methylquercetin (3-MQ), both inhibitors of poliovirus replication, on poliovirus-induced p220 proteolysis and inhibition of host protein synthesis. We report that poliovirus infection in the presence of guanidine or 3-MQ led to complete degradation of p220. However, a significant level

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of host protein synthesis (23 to 45% of control) occurred under these conditions. The significance of these findings in terms of the mechanism of shutoff of host protein synthesis after poliovirus infection is discussed.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells were grown in suspension in Joklik modified minimum essential medium supplemented with 10% calf serum. Cells were infected with poliovirus type 1 (Mahoney strain) at a multiplicity of infection of 100 PFU per cell.

3-MQ and guanidine hydrochloride. 3-MQ was a generous gift from Lucia Van Hoof (University of Antwerp, Antwerp, Belgium) and was used at final concentration of 10 μg/ml. Guanidine hydrochloride was obtained from Sigma Chemical Co. and used at a final concentration of 1.5 mM.

RNA isolation and analysis. HeLa cells were lysed in buffer containing 140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris hydrochloride (pH 8.6), and 0.5% Nonidet P-40 as described by Maniatis et al. (28). Cell extracts were treated with proteinase K and phenol extracted, and the RNA was precipitated with ethanol (16). Dot-blot analysis with ^{32}P-labeled nick-translated poliovirus genome (a kind gift of V. Racaniello) was performed as previously described (6).

Western blot analysis. HeLa cells were extracted with PLB solution (0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) for 10 min on ice as described by Bernstein et al. (5). Chromatin was removed from extracts by centrifugation in a microfuge for 5 min. Extracts from equal number of cells were loaded on an SDS-polyacrylamide gel (6.5% acrylamide, 0.09% bisacrylamide). Proteins were transferred to nitrocellulose paper (Schleicher & Schuell) at 25 V (1 A) for 1.5 h in transfer buffer (20% methanol, 25 mM Tris, 190 mM glycine). Blots were prehybridized in TBS (10 mM Tris hydrochloride [pH 8.0], 140 mM NaCl) containing 5% skim milk powder and 0.05% NaN_3 at room temperature for 1 h. Blots were probed with a rabbit anti-p220 polyclonal antibody (23) diluted 1:1500 in prehybridization solution. After overnight incubation at room temperature, blots were washed once in TBS for 10 min, three times in TBS containing 0.05% Nonidet P-40 for 15 min each, and once in TBS for 10 min. Blots were incubated at room temperature for 1 h in the presence of ^{125}I-labeled protein A (New England Nuclear Corp.) diluted to 0.1 μCi/ml in 50 mM Tris hydrochloride (pH 8.0) containing 140 mM NaCl. Blots were washed extensively and exposed against Fuji X-ray film with Dupont Cronex Hi-plus intensifying screens.

Cell-free translation. Preparation of cell extracts from poliovirus- and mock-infected HeLa cells and translation in these extracts were performed as described previously (24), except that the temperature of incubation was 30°C.

RESULTS

Guanidine has previously been shown to inhibit poliovirus replication in cells infected in culture (2, 3, 21, 31, 38). Despite this drastic inhibition, there is still a significant suppression of host protein synthesis. This observation led to the hypothesis that the protein(s) responsible for the shutoff of host protein synthesis might be translated from the poliovirus parental RNA and that the low level of protein synthesized might be sufficient to elicit the shutoff phenomenon (2, 3, 21, 31). It has been stated that the rate of host protein synthesis shutoff in the presence of guanidine is the same as in its absence (31). This notion is potentially misleading, since the incorporation of radioactive amino acids into protein in the absence of guanidine is directed by both cellular mRNA and poliovirus RNA. However, this might not be the case when cells are infected in the presence of guanidine, because the 20 to 30% residual protein synthesis is probably host mRNA directed. We are not aware of a study showing the pattern of proteins synthesized in guanidine-treated, poliovirus-infected cells. We therefore compared the patterns of protein synthesis in HeLa cells infected with poliovirus in the presence and absence of guanidine (Fig. 1). Treatment of mock-infected cells with guanidine had no significant effect on the extent or pattern of cellular protien synthesis (Fig. 1; compare lane 2 with lane 1). The pattern of proteins synthesized 3.5 h after poliovirus infection demonstrates that most if not all of host protein synthesis is suppressed (for example, actin, which is indicated by a vertical arrowhead in lane a, is totally absent in lane 3). When poliovirus infection was done in the presence of guanidine, no detectable amounts of poliovirus proteins were synthesized, but a significant number of cellular proteins were expressed, although at a reduced level compared with that in mock-infected cells (lane 4). However, the relative intensities of several bands were different from those in mock-infected cells (e.g., the synthesis of the proteins of approximate molecular weights 55,000 and 92,000 was not inhibited as much as that of the proteins of approximate molecular weights 35,000 and 68,000). [^{35}S]methionine in poliovirus-infected cells was incorporated exclusively into poliovirus proteins (compare lane 3 with lane 1). The striking finding in this experiment is that although no cellular proteins were synthesized in poliovirus-infected cells (lane 3), a significant level (23% of control as determined by the aver-

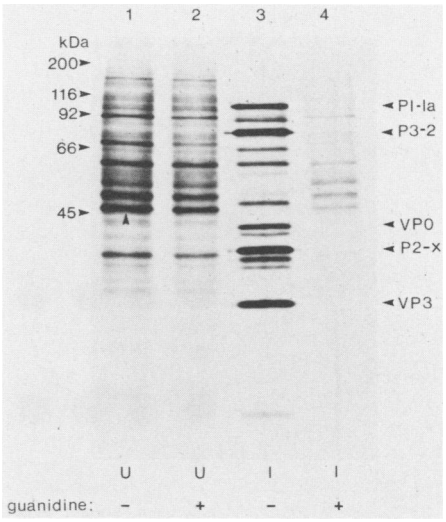

FIG. 1. Effect of guanidine on protein synthesis pattern in poliovirus-infected cells. HeLa cells were infected with poliovirus in the presence or absence of guanidine. Proteins were labeled with [^{35}S]methionine (50 μCi/ml) at 3.5 h after infection (time zero postinfection is considered to be the time at the end of virus absorption). Labeling was for 30 min at 37°C in a total volume of 1 ml. Cells were extracted with PLB solution, and samples representing equivalent number of cells were resolved on a 10% SDS-polyacrylamide gel and autoradiographed as described in Materials and Methods. Abbreviations: U, mock-infected cell extracts; I, infected cell extracts. Guanidine was added where indicated in the figure.

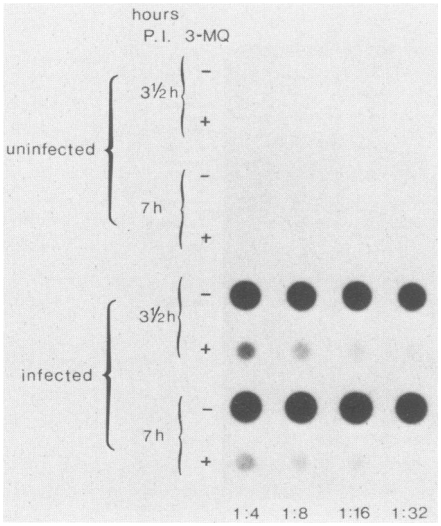

FIG. 2. Effect of 3-MQ on poliovirus RNA replication. HeLa cells were infected with poliovirus, and RNA was purified at 3.5 and 7 h postinfection as described in Materials and Methods. RNA isolated from equivalent number of cells was blotted at twofold dilutions and then hybridized with ^{32}P-labeled poliovirus cDNA (pVR 104), and the dried blots were autoradiographed. 3-MQ was added at time zero.

age obtained from scanning several bands by laser densitometry) of cellular protein synthesis was sustained in poliovirus-infected, guanidine-treated cells (compare lane 4 with lane 2). The level of host protein synthesis remained relatively constant till very late times after infection (36 h).

To further investigate this pattern of expression, we used 3-MQ, another inhibitor of poliovirus replication that has been recently described (9, 41). We confirmed that this compound inhibits the synthesis of poliovirus RNA, as recently reported by Castrillo et al (9). Figure 2 represents a dot-blot analysis of poliovirus RNA at 3.5 and 7 h after poliovirus infection, in the presence or absence of 3-MQ. The extent of inhibition of poliovirus RNA synthesis was more than 99% as measured by densitometry scanning of a shorter X-ray exposure of the blot.

Treatment of HeLa cells with 3-MQ had no effect on protein synthesis (Fig. 3, compare lane 2 with lane 1). Poliovirus infection totally abolished host protein synthesis when measured at 3.5 h postinfection (lane 3; note that the samples in this lane and in the following lanes contained 20 times more material than those in lanes 1 and 2), and at 23 h postinfection, no protein synthesis could be detected as a result of complete cell lysis (lane 4). Addition of 3-MQ to cells immediately following virus adsorption completely prevented the synthesis of poliovirus proteins, as judged by the absence of several prominent poliovirus proteins; for example, VP3, which is indicated by an arrowhead, is totally absent (Fig. 3, lane 5). However, despite the absence of poliovirus protein synthesis, infection caused a shutoff of host protein synthesis, in an analogous fashion to that observed in the presence of guanidine. In the present case, the shutoff was not complete (ca. 55% inhibition, as determined by [^{35}S]methionine incorporation) and host protein synthesis continued for a relatively long period (23 h) at a reduced level (lane 6). The cells eventually died after 48 to 60 h. The results obtained with guanidine and 3-MQ demonstrate that a significant level of host protein synthesis, constituting about 35% of the normal rate of protein synthesis, is maintained in poliovirus-infected cells treated with these compounds. These findings raise several pertinent questions. What is the effect of poliovirus- infection in the presence of guanidine on cap-dependent translation of host mRNA? What is the effect of guanidine treatment on the cleavage of the p220 component of the CBP complex? Why is there a difference in the level of host protein synthesis in the presence and absence of guanidine or 3-MQ in poliovirus-infected cells?

To answer these questions, we examined the fate of p220 after infection. Cells were infected with poliovirus in the presence or absence of guanidine or 3-MQ, and cell extracts were probed with a polyclonal anti-p220 antibody at 3.5 h postinfection (Fig. 4). Extracts prepared from mock-infected cells contained three closely migrating polypeptides in the region of 220 kDa, which were collectively termed p220 (lane 1). We and others (23, 26) have observed that the p220 can be resolved into several polypeptides, and we found by comparative peptide mapping that all are structurally related (I. Edery and N. Sonenberg, unpublished results). Extracts from poliovirus-infected cells had no p220 whatsoever; instead, they contained three faster-migrating polypeptides that are thought to be proteolytic cleavage products of p220 (lane 2) (13, 15, 22, 23, 25, 26). We then examined the effect of 3-MQ on poliovirus-induced proteolysis of p220. Treatment of mock-infected cells with 3-MQ had no effect on p220 (compare lane 4 with lane 3). In this experiment, some cleavage products are visible in mock-infected cells: this has been reported previously (5, 13, 34a) and might indicate that proteolysis of p220 is part of a normal physiological process. Cleavage of p220 in poliovirus-infected cells was not prevented by the addition of 3-MQ (Fig. 4; compare lane 6 with lane 5). It is apparent that all of the p220 was cleaved in the presence of 3-MQ, since considerably longer exposures of

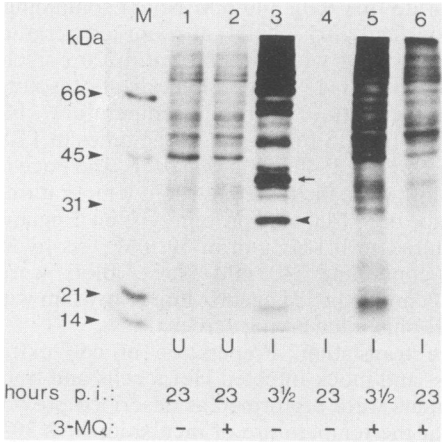

FIG. 3. Effect of 3-MQ on protein synthesis in poliovirus-infected cells. HeLa cells were mock or poliovirus infected in the presence or absence of 3-MQ as indicated in the figure. Proteins were labeled with [^{35}S]methionine (50 μCi/ml in a final volume of 1 ml) for 30 min at 37°C at different times postinfection. Following labeling, proteins were extracted with PLB buffer, and samples from equivalent number of cells (except for lanes 1 and 2, in which 20-fold less material relative to the other lanes was taken for analysis) were resolved on a 10% SDS-polyacrylamide gel as described in Materials and Methods. Abbreviations: U, mock-infected; I, poliovirus-infected extracts; 3-MQ addition of 3-MQ at time zero where indicated; hours p.i., time postinfection at which cells were labeled with [^{35}S]methionine. The arrowhead and the arrow in lane 3 point to poliovirus proteins VP3 and VP0, respectively.

the blot in Fig. 4 and other similar experiments never revealed any detectable p220 after poliovirus infection. This negates the possibility that a very small amount of intact p220 is responsible for the translation of host mRNAs in 3-MQ-treated poliovirus-infected cells, unless the amount of intact p220 which is required for translation of host mRNAs is undetectable by our procedure. However, we consider the latter possibility to be very unlikely. Similar results were obtained with guanidine: its addition to mock-infected cells had no effect on p220 (lanes 7 and 8) or on poliovirus-induced proteolysis of p220 (lanes 9 and 10). Thus, it is clear that despite the complete cleavage of the p220 component of CBP complex during poliovirus infection, poliovirus-induced shutoff of host protein synthesis is not complete in the presence of two different inhibitors of poliovirus replication.

These results suggest that total inhibition of host protein synthesis as a consequence of poliovirus infection is mediated by an event or events different from and in addition to the cleavage of p220. One such event could be the competition between cellular mRNAs (which are already translated at a reduced efficiency, because of the inactivation of the CBP complex) and the vast excess of poliovirus RNA at 3.5 h postinfection. However, a strong argument against this possibility is that most capped mRNAs, such as globin, are not translated in extracts prepared from poliovirus-infected cells which were micrococcal nuclease treated to degrade poliovirus RNA (Fig. 5; compare lane 7 with lane 5).

To determine whether the effects of guanidine which were observed in the above in vivo system could be mimicked in an in vitro translation system, we prepared extracts from mock- and poliovirus-infected cells that had been treated with guanidine. Cell extracts were treated with micrococcal nuclease and programmed with poliovirus, globin, and vesicular stomatitis virus mRNAs. Translational efficiency of poliovirus RNA was similar in extracts prepared from mock- and poliovirus-infected cells whether treated or untreated with guanidine (Fig. 5, lanes 1 to 4; note, however, that

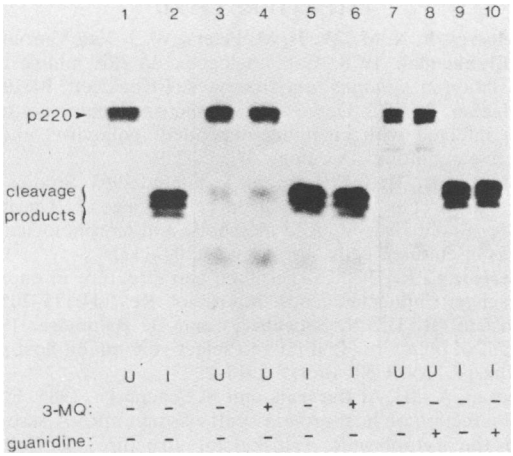

FIG. 4. Effect of 3-MQ and guanidine on p220 proteolysis in poliovirus-infected cells. HeLa cells were infected with poliovirus in the presence or absence of 3-MQ or guanidine. Cells were extracted with PLB buffer 3.5 h after infection. Samples representing equivalent number of cells were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting analysis as described in Materials and Methods. Lanes: 1 and 2, postmitochondrial extracts (S-10); 3 to 10, cell extracts prepared by PLB extraction. Abbreviations: U, mock-infected extracts; I, poliovirus-infected extracts. 3-MQ or guanidine was added where indicated in the figure.

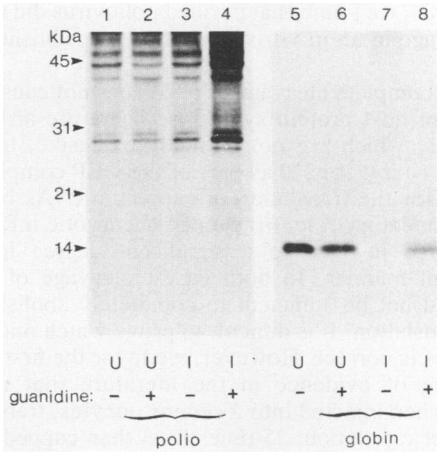

FIG. 5. In vitro translation in HeLa cell extracts. HeLa cells were mock or poliovirus infected in the presence or absence of guanidine as indicated in the figure. Cell extracts (prepared 3.5 h after infection) were made and in vitro translation of the specified amounts of exogenous mRNA was performed as described in Materials and Methods. Samples of 12.5 μl were analyzed on a 12.5% SDS-polyacrylamide gel. Abbreviations: U, mock-infected cell extracts; I, poliovirus-infected cell extracts. Lanes: 1 to 4, extracts programmed with poliovirus mRNA (0.5 μg); 5 to 8, extracts programmed with globin mRNA (0.5 μg). The top part of the X ray is not shown.

translation in poliovirus-infected, guanidine-treated cells was 2.5-fold greater than in the other lanes, but this was not a reproducible result). Globin mRNA, which translated efficiently in mock-infected cell extracts (lane 5) and did not translate in poliovirus-infected cell extracts, (lane 7), as expected of a capped mRNA, could be translated at 49% of the control level in extracts prepared from poliovirus-infected, guanidine-treated cells (Fig. 5; compare lane 8 with lane 6). We obtained similar results by using vesicular stomatitis virus mRNAs which are capped (data not shown). Consistent with previous results, vesicular stomatitis virus mRNAs did not translate in extracts prepared from poliovirus-infected cells. However, translation of vesicular stomatitis virus mRNA was evident (ca. 36% of control) in extracts prepared from poliovirus-infected, guanidine-treated cells (data not shown). These results show that the in vitro translation system reproduces the in vivo observations and thus excludes the possibility of secondary events (that are not translational) to explain the guanidine effect on the incomplete shutoff of host protein synthesis after poliovirus infection.

DISCUSSION

Several conclusions can be drawn from the results presented here. First, cleavage of p220 in poliovirus-infected cells occurs even under conditions when poliovirus replication is severely depressed. Consequently, proteolysis of p220 is most probably mediated by low levels of a poliovirus protein synthesized from parental RNA. This finding rules out the possibility that cleavage of p220 occurs as a result of virus replication and therefore indicate that it is a primary event occurring after poliovirus entry into the cell. An alternative explanation is that the input virus itself mediates the degradation of p220. The latter possibility is unlikely, because it was demonstrated that inhibition of protein synthesis requires a functional viral genome (7, 19, 31). Consis-

tent with this, we found that purified poliovirus did not effect p220 cleavage in an in vitro incubation (unpublished observations).

Second, complete cleavage of p220 does not cause the full inhibition of host protein synthesis. There are at least two possibilities, which are not mutually exclusive, to explain this result: (i) intact p220 as part of the CBP complex is not obligatory for the translation of capped mRNAs but rather plays a stimulatory role; (ii) capped eucaryotic mRNAs can be translated in vivo to a significant degree in a cap-independent manner. In both cases, cleavage of p220 by itself would not be sufficient to completely abolish capped mRNA translation. It is difficult to prove which one of these possibilities is correct. However, we favor the first possibility because of evidence in the literature that uncapped mRNAs, when injected into *Xenopus* oocytes, translate at a much lower rate (about 25 times less) than capped mRNAs when injected into oocytes (10, 27), the rate of degradation could not explain the low translational activity of uncapped mRNAs (10). Cap analogs had no effect on protein synthesis when injected into *Xenopus* oocytes (1), but it was not determined whether the cap analogs were degraded or inactivated (1). Eucaryotic mRNAs can be translated to some degree in vitro in the absence of a cap structure or in the presence of cap analogs (for a review, see reference 4). The degree of cap-independent translation varies among differnt mRNAs and is dependent on the ionic strength and temperature (4, 42). We have postulated that differential cap-dependent translation is a reflection of the degree of secondary structure that a mRNA can assume at its 5′ terminus, which is consistent with the idea that the CBP complex mediates the unfolding of the 5′ secondary structure of the mRNA (12, 30, 32, 35).

If indeed the cleavage of p220 is not sufficient to abolish host mRNA synthesis, then to explain the total inhibition of host protein synthesis after poliovirus infection, one must invoke one or more events apart from the cleavage of p220 and inactivation of the CBP complex. This other event occurs only when poliovirus replication and subsequent virus directed protein synthesis take place; i.e., it could be due to a second virally encoded function or to changes in membrane permeability that occur with virus replication and subsequent increase in intracellular Na^+ concentration. It was argued that these changes favor poliovirus RNA over cellular mRNA translation (8). The latter possibility, however, appears unlikely in light of the data in Fig. 5, showing that translation of globin mRNA is reduced in an in vitro translation system from poliovirus-infected cells in which the Na^+ concentration is very low, because the extracts were dialyzed against a buffer lacking Na^+. The possibility that excess amounts of poliovirus RNA compete with host mRNAs (which are already inefficiently translated because of CBP complex inactivation) for the translational machinery is also unlikely, as shown above.

The involvement of a second poliovirus function is also consistent with several previously unexplained observations. In the original report describing the proteolysis of p220 in poliovirus-infected cells, it was observed that proteolysis preceded inhibition of protein synthesis by about 30 min (15). This can now be explained to be a result of the low levels of poliovirus RNA at the early stages of infection, in analogy to the situation occurring when guanidine or 3-MQ is present during infection. In addition, it was observed with one particular batch of virus that infection (12 PFU per cell) led to complete cleavage of p220, but inhibition of endogenous host protein synthesis was minimal. Moreover, these cell extracts translated capped mRNAs such as globin and tobacco mosaic virus at an efficiency similar to that of mock-infected extracts (J. Pelletier and I. Edery, unpublished observations). These findings support the contention that proteolysis of p220 is by itself not sufficient to abolish cap-dependent translation. On the other hand, there is evidence that the integrity of p220 is required for the function of the CBP complex. Etchison et al. (14) showed that the CBP complex from poliovirus-infected cells was inactive in an in vitro reconstituted translation system, indicating that the cleavage of p220 inactivates the CBP complex. The proteolysis of p220 is most probably a necessary event for the inhibition of host protein synthesis after poliovirus infection. This conclusion is based on the report of Bernstein et al. (5), who demonstrated that infection of CV-1 cells with a poliovirus mutant defective in the shutoff of host protein synthesis did not cause the proteolysis of p220. They concluded that the selective shutoff of host protein synthesis after poliovirus infection correlated with the cleavage of p220.

In summary, we conclude that cleavage of p220 is most probably necesary (5) but not sufficient for the complete inhibition of cellular protein synthesis following poliovirus infection. Thus, it is plausible that a second poliovirus-mediated event, in addition to cleavage of p220, is necessary to fully suppress cellular protein synthesis.

ACKNOWLEDGMENTS

We thank E. Ehrenfeld for constructive criticism of the work and N. Parkin, J. Pelletier, and I. Edery for useful comments on the manuscript. We thank Isaac Edery for the kind gift of anti-p220 antibodies and helpful discussions during the course of the work, Lucia Van Hoof for the kind gift of 3-MQ, and Dolores Harrison for valuable assistance.

This research was supported by grants from the Medical Research Council of Canada to N.S. N.S. is a recipient of an MRC scientist award of the Medical Research Council of Canada. A.-M.B. was a recipient of a Fonds de Recherche en Santé du Québec Fellowship.

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