Degradation of Cellular Proteins during Poliovirus Infection: Studies by Two-Dimensional Gel Electrophoresis

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Picornaviruses encode for their own proteinases, which are responsible for the proteolytic processing of the polyprotein encoded in the viral genome to produce the mature viral polypeptides. The two poliovirus proteinases, known as proteins 2A and 3C, have a selectivity for cleavage of the tyrosine-glycine (YG) and glutamate-glycine (QG) pairs, respectively, present in the poliovirus polyprotein (3, 5, 13, 17, 20–23, 25, 27). However, not all YG or QG pairs that occur in this polyprotein are recognized and cleaved by proteinases 2A and 3C, suggesting that the folding of this molecule plays a part in regulating which cleavages take place (4, 17, 20, 21). In fact, these two proteinases are thought to be very selective in the recognition of their substrates, since proteins from cardioviruses or even other viral proteins are not degraded when mixed-infection experiments are carried out (1, 2, 7, 9). Previous studies indicated that cellular proteins are not substrates for the poliovirus-encoded proteinases and are apparently not degraded during viral infection (14). There is only one cellular protein known to be degraded during poliovirus replication, the p220 polypeptide that forms part of the cap-binding complex (11, 26). However, neither of the poliovirus proteinases seems to be directly involved in its degradation (16–19). Infection of BHK cells with foot-and-mouth disease virus seems also to degrade the cellular protein histone 3 (12). However, a thorough study of the possibility that cellular proteins are substrates of the poliovirus proteinases is still lacking.

Two-dimensional (2-D) gel electrophoretic analyses of translation initiation factors from mock-infected and poliovirus-infected cells reveals no differences (10), despite the fact that at least two virus-coded proteinases are expressed during infection. In order to understand to what extent cellular proteins are affected by poliovirus infection, we have analyzed the stability of cellular polypeptides by 2-D gel electrophoresis. The results presented in this contribution suggest that several, but not many, cellular proteins are specifically degraded in poliovirus-infected cells and that the kinetics of this reduction do not correlate with the virus-induced shutoff of host translation.

MATERIALS AND METHODS

HeLa cells and viruses. HeLa cells, mycoplasma free, were grown as monolayers in Dulbecco modified Eagle medium containing 10% newborn calf serum and antibiotics (10,000 IU of penicillin and 50 mg of streptomycin per ml). Poliovirus type 1 Mahoney, encephalomyocarditis virus (EMC), Semliki Forest virus (SFV), and vesicular stomatitis virus (VSV) were grown in HeLa cells.

Conditions of infection and protein labelling. HeLa cells were plated in 96-well Linbro plates. Before the cells reached confluency, they were labeled with [35S]methionine as previously described (24) by adding 50 µl of methionine-free medium containing 1 mCi of [35S]methionine per ml (1,450 Ci/mmol; The Radiochemical Centre, Amersham, England) and incubating them at 37°C. After the labeling period, the medium was removed, and cells were infected with poliovirus, EMC, SFV, or VSV (50 PFU per cell). After 1 h at 37°C, the medium was removed and replaced by fresh medium. At the times indicated in the figure legends, the cells were harvested in lysis buffer (9 M urea, 0.1 M dithiothreitol, 2.8% Nonidet P-40, 2% ampholytes [pH 7 to 9]), and the proteins were analyzed by 2-D gel electrophoresis.

Analysis of proteins by 2-D gel electrophoresis. The procedure for 2-D gel electrophoresis has been described in detail elsewhere (6). Briefly, the first-dimension separations of acidic proteins (isoelectric focusing) were performed on polyacrylamide gels (230 by 1.5 mm; 4%, wt/vol) containing 4% ampholytes (3.2% at pH 5 to 7 and 0.8% at pH 3.5 to 10). The first dimension of basic protein nonequilibrium gel electrophoresis was done exactly as described previously.
(6). The second dimension was done in a 15% acrylamide gel (25 by 25 cm) and electrophoresed at room temperature overnight. Gels were processed for fluorography, dried, and exposed at −70°C.

Quantitation of proteins. Proteins were quantitated in a densitometer. The area of each peak corresponding to the spot on the film was compared with that of a sample from noninfected control cells.

RESULTS
To determine whether gross modifications of cellular proteins occur during poliovirus infection, HeLa cells were labeled with [35S]methionine for 12 h and infected in the absence of radioactive label. At 2, 4, 6, and 8 h postinfection (p.i.), cells were collected and the proteins were analyzed by one-dimensional polyacrylamide gel electrophoresis. When compared with the uninfected control HeLa cells collected at the same times (results not shown), no protein showed any modification. We conclude from this experiment that no gross modifications in cellular proteins that can be detected by standard one-dimensional gel electrophoresis take place after poliovirus infection.

The possibility that minor cellular proteins are degraded during infection was examined by means of 2-D gel electrophoresis. For this purpose, HeLa cells were labeled to a high specific activity with [35S]methionine for 12 h. Afterwards, the cells were infected with poliovirus, and the proteins were analyzed by 2-D gels every hour after infection. Figure 1 shows the pattern of cellular proteins at 0 and 5 h p.i. Almost no differences were seen between the two gels, indicating that, in general, cellular proteins were not targets for the two poliovirus-coded proteases. Moreover, no new polypeptides were observed in the regions where the major viral proteins appeared, suggesting that the [35S]methionine label present in cellular polypeptides is not used by poliovirus to make its own proteins. Nevertheless, the most striking finding was that a few cellular proteins clearly disappeared after poliovirus infection and were not present in the infected cells after 5 h of infection (Fig. 1B). The disappearance of those proteins is very reproducible, since this experiment was repeated four times with the same results. The disappearance of these proteins could have been due to protein modification (methylation, phosphorylation, etc.) after poliovirus infection, although the most likely possibility is that they were degraded by poliovirus proteases.

To determine the kinetics of cellular protein degradation, the proteins present in the three regions depicted in Fig. 1 were analyzed every hour p.i. and compared in Fig. 2, 3, and 4. Also, poliovirus-infected cells in the presence of an inhibitor of translation such as cycloheximide or an inhibitor of poliovirus RNA replication such as 3-methylquercetin were included as controls. The degradation of these proteins in HeLa cells infected with other viruses, such as EMC, VSV, and SFV, has also been analyzed. The controls for protein synthesis in the presence of the inhibitors and also the proteins synthesized in cells infected with these viruses were analyzed (results not shown). They indicated that the inhibitors were acting adequately and that the viruses were in fact replicating in these cells.

Figure 2 shows the degradation of proteins in the region labeled 1 in Fig. 1A. Proteins 2, 3, and 6 showed similar degradation kinetics, and they almost disappeared by 3 h p.i. Protein 4 suffered a higher level of degradation, but these four proteins were all clearly apparent at 2 h p.i. (Fig. 2D). Proteins 1, 5, 7, and 8 decreased from 4 h p.i., but they were still seen at 5 h p.i. Two new spots labeled a and b were apparent from 3 h p.i. These new polypeptides probably corresponded to the degradation products of cellular proteins and not to virus-coded polypeptides, since none of the major viral proteins migrated in this region (results not shown).

In poliovirus-infected HeLa cells treated with cycloheximide (Fig. 2L) and collected 6 h p.i., a doublet that appeared in the control gel did not appear (Fig. 2K). The disappearance of this spot was not connected with viral gene expression, since cycloheximide was present from the beginning of poliovirus infection. It is possible that this degradation was coupled with virus internalization. The level of this polypeptide was reduced in control poliovirus-infected cells, and it disappeared in the infected cells treated with 3-methylquercetin (Fig. 2N). In this instance, two other proteins were absent that were not degraded in control infected cells. Other viruses, such as EMC, SFV, and VSV, did not produce the degradation of these proteins even at 6 h p.i. Only VSV infection induced the disappearance of protein 4.

The region of the gel marked 2 (Fig. 1) is shown in more detail in Fig. 3. A protein labeled 9 completely disappeared at 5 h after poliovirus infection, but it was still present at control levels at 4 h. This protein was one of the clearer markers of polypeptide degradation in poliovirus-infected cells because it was very abundant and appeared in a region of the gel in which the proteins were very clearly resolved. The disappearance of polypeptide 9 was dependent on virus replication, since it did not take place in the presence of cycloheximide or 3-methylquercetin. None of the viruses tested induced the degradation of this protein. Concomitant with the degradation of this protein, two additional polypeptides, labeled c and d, appeared in the gel. The possibility exists that at least one of these polypeptides was related to protein 9, since c and d had lower molecular masses and migrated in a region of the gel of similar pi.

There were also clear reductions of some basic proteins (nonequilibrium gel electrophoresis), all located in the region labeled 3 (Fig. 4). These polypeptides were present 2 h p.i., and some clearly began to disappear at 3 h. By 5 h p.i., none of the polypeptides labeled 1, 2, 3, 4, or 5 were seen in the gels. Two additional proteins, labeled a and b, appeared at 3 h p.i.; the relationship of these two polypeptides (a and b) to polypeptides 1 to 5 was unclear, because the molecular masses of a and b were higher than those of 1 through 5. We have been unable to detect the disappearance of basic proteins of Mₛ higher than those of a and b that could account for the generation of these two proteins.

In parallel experiments we analyzed the inhibition of host protein synthesis induced by poliovirus. There was a clear inhibition of host translation from 1 to 2 h p.i., and the bulk of poliovirus protein synthesis took place between 3 and 5 h p.i. (Fig. 5B, C, and D). We compared the reduction of two representative cellular proteins, acidic protein 9 and basic protein 4 (Fig. 5A and C). The degradation of protein 4 was clear at 5 and 6 h p.i., whereas protein 9 was already very much reduced at 4 h p.i. Nevertheless, neither of them was significantly reduced by 2 h p.i., when a great inhibition of cellular translation had occurred; rather, they disappeared once the bulk of viral proteins had been synthesized.

DISCUSSION
Significant advances in the identification and characterization of the action of poliovirus proteases 2A and 3C have
FIG. 1. 2-D gel electrophoresis of polypeptides from HeLa cells labeled with \(^{35}\text{S}\)methionine (1 mCi/ml) for 12 h. After labeling, cells were infected with poliovirus and harvested at 0 h p.i. (A) or 5 h p.i. (B). IEF, Isoelectric focusing; NEPHGE, nonequilibrium gel electrophoresis; SDS, sodium dodecyl sulfate. Boxed-in areas indicate corresponding areas in Fig. 2 through 4.
FIG. 2. 2-D gel electrophoresis of HeLa cell proteins corresponding to area 1 in Fig. 1. All cells were labeled with [35S]methionine (1 mCi/ml) for 12 h. (A) Control. (B) After labeling, cells were incubated for 7 h at 37°C with cold medium containing 2% newborn calf serum. (C to G) Cells were infected with poliovirus and harvested in lysis buffer at 0 (C), 2 (D), 3 (E), 4 (F), and 5 (G) h.p.i. (H to J) Cells were infected with EMC (H), SFV (I), or VSV (J) and harvested in lysis buffer at 6 h.p.i. (K and L) Cells were mock infected (K) or infected with poliovirus (L) in the presence of 5 x 10^{-5} M cycloheximide and at 6 h.p.i. were harvested in lysis buffer. (M and N) Cells were mock infected (M) or infected with poliovirus (N) in the presence of 3-methylquercetin (20 μg/ml) and harvested in lysis buffer at 6 h.p.i.
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In the presence of 3-methyladenine (3MA) and thymidine (TdR) and harvested at 6 h.p.i. in BS buffer, (A) Cells were mock infected (M) and harvested in BS buffer at 6 h.p.i. (B) Cells were mock infected (M) and harvested at 10 h.p.i. (C) Cells were mock infected (M) and harvested at 20 h.p.i. (D) Cells were mock infected (M) and harvested at 30 h.p.i. (E) Cells were mock infected (M) and harvested at 60 h.p.i. In the presence of cycloheximide (0.5 μg/ml) and thymidine (0.5 μg/ml), (A) Cells were mock infected (M) and harvested at 6 h.p.i. (B) Cells were mock infected (M) and harvested at 10 h.p.i. (C) Cells were mock infected (M) and harvested at 20 h.p.i. (D) Cells were mock infected (M) and harvested at 30 h.p.i. (E) Cells were mock infected (M) and harvested at 60 h.p.i.

Fig. 1. 2D gel electrophoresis of HeLa cell proteins corresponding to area 3 in Fig. 1. All cells were incubated for 12 h with 30 mM 3-methyladenine (3MA) and then were harvested (A) 30 min, (B) 60 min, (C) 120 min, (D) 240 min, (E) 360 min, (F) 480 min, (G) 600 min, (H) 720 min, (I) 960 min, (J) 1200 min, (K) 1440 min, (L) 1680 min, (M) 1800 min.
occurred during the last few years (3, 4, 13, 17, 20–23, 25, 27). These two proteins play a key role in the tailoring of poliovirus-coded proteins, since they arise from a single precursor polyprotein (17, 20, 24). A striking fact is the high selectivity of these two proteases. Thus, protein 2A apparently recognizes only 2 of the 10 tyrosine-glycine pairs present in the poliovirus polyprotein (17, 27), whereas protease 3C can recognize 9 of the 13 glutamine-glycine pairs present in this precursor (3, 13, 17, 22, 23, 25). No other viral protein has been reported to be cleaved by these poliovirus proteases, since in mixed-infection experiments, the proteins of the accompanying viruses migrate as in the singly infected controls (1, 7, 9). It has been suspected that some cellular polypeptides are substrates for the poliovirus proteases (17, 24, 26). In that case, the degradation of cellular proteins could contribute to the acute cytopathic effect induced by poliovirus (7, 26). However, analysis of the initiation factors from poliovirus-infected HeLa cells by high-resolution gel electrophoresis showed no degradation of initiation factors (10). Other groups found degradation of a p220 protein that forms part of the cap-binding complex eIF-4F (26). Nevertheless, neither of the two poliovirus proteases is directly responsible for the cleavage observed in p220 (eIF-4F) (16, 17, 19, 20). In this report we show that a number of cellular proteins appeared to be degraded after poliovirus infection. This degradation was specific for poliovirus and did not take place with a related virus such as EMC, nor with SFV or VSV, suggesting that degradation is not the by-product of cell damage induced by viral infection. The possibility that poliovirus proteases are directly or indirectly involved in this degradation remains to be demonstrated. The inhibition of this degradation by cycloheximide or 3-methylquercetin supports the idea that viral gene expression is required.

Another point of interest is the precise timing of cellular poly peptide degradation. No exact correlation exists between the kinetics of poliovirus-induced shutoff of host translation and the degradation of these cellular proteins. The degradation of the polyprotein p220 during poliovirus infection has been widely documented (26). Normally, to analyze the p220 degradation, the cells are collected at late times, well after the shutdown of translation has occurred (15, 26). We should emphasize that during the preparation of these lysates, degradation of polypeptides by poliovirus proteases present in high quantities cannot be avoided (7, 15). This degradation was less likely to occur in our system, since the cells were immediately lysed in the presence of 9 M urea and were frozen at −70°C. The kinetics of the degradation of the polypeptides described in this article clearly indicates that the polypeptides are not related to the poliovirus-induced shutoff of protein synthesis (7). The possibility that the degradation of these polypeptides might contribute to the development of the cytopathic effect needs to be investigated. However, we have to keep in mind that from 3 to 4 h p.i. the cell membrane started to be affected (7, 8), and this phenomenon is more likely to contribute to the cytopathic effect that starts to develop in our system from 6 h p.i. (7).

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LITERATURE CITED


