Characterization of the Large Picornaviral Polypeptides Produced in the Presence of Zinc Ion

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Zinc ion inhibits the posttranslational cleavages of human rhinovirus-1A, encephalomyocarditis virus, and poliovirus polypeptides. Each virus displayed a different susceptibility to zinc. However, in each case the cleavages of the capsid precursor and the cleavages analogous to the C → D → E conversion in encephalomyocarditis virus were most sensitive to zinc. Higher concentrations of zinc resulted in the buildup of even larger precursor polypeptides of a size between 106,000 and 214,000 daltons. The sizes of these polypeptides and the relative position of their gene loci on the viral RNA were determined. These data were used to place these polypeptides in the over-all scheme of viral protein processing.

Zinc chloride has been shown to inhibit the growth of several different types of rhinoviruses (14). The mechanism of antiviral action is to inhibit the normal cleavages by which the viral polypeptides are processed. A similar gene order and the same general pattern of protein synthesis and posttranslational cleavages are probably shared by all picornaviruses (1). This report establishes that zinc inhibits the cleavages of the viral polypeptide precursors of encephalomyocarditis (EMC) virus and poliovirus as well as human rhinovirus-1A (HRV-1A). The pattern of cleavage inhibition was determined and compared for all three viruses. The sizes and gene order of the large polypeptides produced in the presence of zinc were established and contrasted. These results provide additional insights as to the precursor-product relationships that exist in the maturation of the viral proteins.

MATERIALS AND METHODS

Materials. The source and propagation of cells and virus have been described (1). The composition of the dialysis buffer, 10× sodium dodecyl sulfate (SDS) solubilizing solution, 10% SDS-polyacrylamide gels, medium AL, and pactamycin stock solution have been described (1). The 5% SDS-polyacrylamide gels were the same as the 10% gels except that they contained 4.9% acrylamide and 0.3% (vol/vol) ethylene diacrylate. Reagent grade zinc chloride was obtained from Allied Chemical Corp. A stock solution of 5 mM ZnCl₂ in 1 mM HCl was used throughout these experiments. The L-[³H]amino acid mixture and L-[¹⁴C]amino acid mixture each containing 15 amino acids, ¹²⁵I, and

[³H]- and [¹⁴C]acetic anhydride were purchased from New England Nuclear Corp. Phosphorylase a (rabbit muscle, 2× crystallized) was obtained from Schwarz/Mann. β-galactosidase was obtained from Worthington. Myosin (rabbit skeletal muscle) was the generous gift of Richard Tregear. Pactamycin was the gift of Upjohn Laboratories.

Methods. The procedures for infection of HeLa cells, labeling viral polypeptides, pactamycin mapping, solubilizing cells, SDS-polyacrylamide gel electrophoresis and fractionation, and computer analysis of the data have been described (1).

Marker proteins were tagged with radioactive acetic anhydride by the method of R. Montelaro, University of Wisconsin (manuscript in preparation) or with ¹²⁵I by a modification of the method of Marchalonis (16).

It should be noted that the zinc concentrations reported refer to the amount of zinc added to the cell suspensions. Problems such as precipitation at higher zinc concentrations and the degree of ion uptake will be discussed elsewhere (B. Korant and B. Butterworth, manuscript in preparation).

RESULTS

Molecular weights. The solid line in Fig. 1 illustrates the normal pattern of HRV-1A polypeptides produced during a 1-h labeling period with [³H] amino acids. Some of these polypeptides are precursor molecules which mature via proteolytic cleavages to form smaller stable chains. The dashed line in Fig. 1 illustrates that, in the presence of 0.7 mM ZnCl₂, these cleavages do not occur. This results in the buildup of a set of large precursor molecules, some of which are even larger than the capsid

precursor (polypeptide 92). Polypeptides 165 and 106 are the only polypeptides in this size range that are observed without the addition of zinc (Fig. 1).

SDS-polyacrylamide gel electrophoresis is an excellent technique for determining the molecular weight of polypeptides (6, 20). However, when sizing polypeptides as large as those seen in Fig. 1, care must be exercised in choosing conditions for electrophoresis and reference proteins. Sizing of HRV-1A polypeptides on 10% gels, or using markers in the 50,000 to 100,000 molecular weight range, yields apparent molecular weights which differ greatly from more optimum conditions (Table 1). A discussion of the reliability of molecular weight values for

proteins over 100,000 is presented later (see Discussion).

The heavy chain of rabbit muscle myosin, molecular weight $200,000 \pm 10,000$ (8, 9), and phosphorylase a, molecular weight 94,000 (20), separated on 5% SDS-polyacrylamide gels, were used as a standard reference system (Fig. 2) against which the apparent molecular weights of the large polypeptides discussed here were determined (Fig. 3). The viral polypeptides are designated by their apparent molecular weights $\times 10^{-3}$, i.e., polypeptide 202 has an apparent molecular weight of 202,000. The three new major HRV-1A polypeptides produced in the presence of zinc are 214, 202, and 146.

Pactamycin mapping. The theory and pro-

FIG. 1. Cleavage inhibition of HRV-1A polypeptides by zinc. A suspension of HeLa cells (4×10^6 cells/ml) in medium AL was infected with 200 PFU of HRV-1A per cell in the presence of $5 \mu\text{g}$ of actinomycin D per ml as described (1). At 4 h and 3 min postinfection, 4 ml of the suspension was withdrawn and exposed to 0.7 mM ZnCl_2 . After 4 min, the suspension was exposed to $40 \mu\text{Ci}$ of a [^3H]amino acid mixture per ml. After a 1-h incubation at 34°C , 1 ml of the cell suspension was solubilized by the addition of 0.1 ml of $10\times$ solubilizing solution and heating in a boiling water bath for 5 min. After dialysis, a portion of the extract was electrophoresed for 22 h on 10% SDS-polyacrylamide gels (0.6 cm by 27 cm) at 8 mA/tube . Migration is from left to right (0.7 mM Zn^{2+} , dashed line). An identical sample was labeled for 1 h in the absence of added zinc, prepared and electrophoresed as above (control, solid line). The gels were divided into 1-mm fractions, solubilized, and counted in a liquid scintillation spectrometer. Each was run independently and the curves were superimposed here for comparison. The figure is a photograph of the computer analysis of the data.

cedures for pactamycin mapping have been described in detail (3). However, some modifications were necessary because of the presence of the zinc. Progressively larger concentrations of zinc resulted in the accumulation of progressively larger precursor polypeptides. It was, therefore, necessary to run the pactamycin-mapping experiment at two different zinc con-

centrations to map the whole spectrum of precursor polypeptides. Increased zinc concentrations also resulted in increased inhibition of protein synthesis. Whereas 0.1 mM ZnCl_2 inhibited protein synthesis by only 15%, 0.4 mM ZnCl_2 inhibited protein synthesis by 40%, and 1.2 mM ZnCl_2 inhibited it by 80%. When labeling for short periods, there is a high background due to label incorporated into nascent chains. It took longer for this background to decrease in the presence of zinc, which indicates a slower rate of synthesis. Therefore, the period of labeling had to be lengthened to adjust for the slower rates of protein synthesis. Figure 4a is a 10% gel which shows the differential labeling of polypeptides 165, 146, and 106 in the presence of 0.1 mM ZnCl_2 after the inhibition of initiation of protein synthesis by pactamycin. Figure 4b is a 5% gel which shows the differential effect that pactamycin has on the labeling of polypeptides 214 and 202 in the presence of 0.8 mM ZnCl_2 . After the inhibition of initiation of protein

TABLE 1. Apparent molecular weight of some large HRV-1A polypeptides under various conditions of electrophoresis

Polypeptide	Mol wt		
	10% gels; polypeptides 92, 84, 76 as markers	5% gels; polypeptides phosphorylase a and BSA as markers	5% gels; myosin and phosphorylase a as markers
202	146,000	163,000	202,000
165	129,000	141,000	165,000
106	101,000	102,000	106,000

FIG. 2. Coelectrophoresis of myosin and the large HRV-1A polypeptides produced in the presence of zinc. A sample of the extract described in Fig. 1 was mixed with myosin which had been tagged with ^{125}I and was electrophoresed for 20 h in 5% SDS-poly-acrylamide as described in Fig. 1. In other runs, phosphorylase a tagged with $[^{14}\text{C}]$ acetic anhydride migrated to the position shown by the arrow in the figure.

FIG. 3. Determination of the molecular weights of the large HRV-1A polypeptides produced in the presence of zinc. The molecular weights of the viral proteins were determined from this log molecular weight versus mobility curve using the indicated marker proteins (see Fig. 2). Note the break in the curve at phosphorylase a.

synthesis by pactamycin, those regions nearest the initiation site will be the first to be depleted of ribosomes. Polypeptides from this region will be produced in lower amounts than polypeptides translated from nearer the 3' end of the RNA. The appropriate calculations (3) yield the relative position of the corresponding gene loci for these polypeptides on the viral RNA (Fig. 4c and d). The new, large precursor polypeptides all map toward the 5' end of the RNA. This is an indication that each contains at least some, if not all, of the capsid polypeptide sequence. Probable precursor-product relationships consistent with the molecular weight and mapping data are indicated in Fig. 5.

Cleavage inhibition of EMC viral polypeptides. Table 2 illustrates that the three picornaviruses examined each exhibit a different sensitivity to cleavage inhibition by zinc ion. HRV-1A was most sensitive, followed by poliovirus and EMC virus. Figure 6 shows that 1.5 mM $ZnCl_2$ effectively prevents the maturation cleavages of the EMC viral polypeptides. This results in a pattern which has only the primary products A, C, F, and I, and two new polypeptides, 130 and 120. The new polypeptides were sized on SDS gels relative to the large HRV-1A polypeptides. Figure 7a shows the results of a pactamycin mapping experiment carried out with EMC viral-infected HeLa cells in the presence of 1.2 mM $ZnCl_2$. Polypeptide 214, which shows up in this figure, was never present in large amounts, but could be repro-

ducibly detected when high concentrations of zinc were used. The relative position of the corresponding gene loci for polypeptides 120 and 130 are shown in Fig. 7b. Polypeptide 120 maps toward the 5' end of the RNA, whereas polypeptide 130 maps toward the 3' end. Probable precursor-product relationships consistent with the molecular weight and mapping data are shown in Fig. 8.

Cleavage inhibition of poliovirus polypeptides. Figure 9 illustrates that 0.8 mM $ZnCl_2$ inhibits the posttranslational cleavages of poliovirus. The major polypeptides that accumulate under these conditions are 1a, 1b, and X. In addition, several new polypeptides can be seen (204, 137, 123, 116). These were sized on SDS-polyacrylamide gels relative to the large HRV-1A polypeptides. Figure 10 illustrates the results of a pactamycin mapping experiment performed with poliovirus-infected HeLa cells in the presence of 0.9 mM $ZnCl_2$. The major new polypeptide, 137, maps toward the right of the RNA. Polypeptide 204 was never present in large quantities, but mapped reproducibly to the left of the center of the RNA. Polypeptides 123 and 116 were reproducibly present and both seemed to map toward the 3' end of the RNA. However, the combination of a high background in this area and relatively few counts in the peaks prevented the accurate assignment of their position on the pactamycin map. Probable precursor-product relationships consistent with the molecular weight and mapping data are shown in Fig. 11.

DISCUSSION

Mechanism of zinc inhibition. One interesting aspect of this system is that the degree of cleavage inhibition varies with the zinc concentration employed. In the case of HRV-1A, there is first cleavage inhibition of the precursors 92 and 84. Increasing the zinc concentration results in the accumulation of polypeptides 165 and 146, whereas further increases produce polypeptides 214 and 202 (Fig. 4). One possible explanation for these observations is that there are several proteolytic enzymes involved in processing the viral proteins, and that each is inhibited by a different concentration of zinc. However, we favor a model in which the viral polypeptides bind zinc ion which prevents their being processed by proteolytic cleavages (Korant and Butterworth, manuscript in preparation).

Several lines of evidence suggest that the picornaviral RNA possess a single initiation site

and that each ribosome completes translation of the entire viral mRNA (3, 11). However, at the time when viral protein synthesis is maximal, polypeptide is not observed because the nascent chain is cleaved during translation (4, 10). Those polypeptides released by these nascent cleavages are termed primary products and may undergo subsequent posttranslational cleavages (1). It is probable that a particular configuration of the nascent chain is necessary for the proper nascent cleavages to occur. In the case of HRV-1A the maturation cleavages of the capsid precursor are very sensitive to zinc. Since the capsid genes are the first to be translated, it follows that in the presence of zinc the nascent chain could be improperly folded. This, in turn, could account for the inhibition of the nascent cleavage which releases the capsid precursor, thus resulting in the production of a growing chain which still contained the capsid sequence. If the nascent chain were large enough, steric

hindrance could prevent other cleavages and polypeptides in the size range of polyprotein would be produced. The observation that all of the new, large, zinc-produced HRV-1A polypeptides contain capsid sequences (Fig. 5) is consistent with this model. The cleavages that do occur retain some specificity because the polypeptides produced are of distinct sizes.

In the case of EMC virus and poliovirus, the cleavages of the capsid precursor are less sensitive than for HRV-1A (Table 2) and the primary, new polypeptide formed in the presence of zinc (EMC, 130; polio, 137) contains sequences of viral proteins translated from near the 3' end of the RNA (Fig. 8 and 11). These observations suggest that for these two viruses there is less inhibition of the nascent cleavage which releases the capsid precursor. However, the cleavage which releases F or X is inhibited. This should yield patterns which contain little protein of a size over 200,000 daltons. Fig. 7a and Fig. 10a show this to be the case.

Molecular weights. SDS-polyacrylamide

HRV-1A

FIG. 5. Proposed position of the zinc-produced HRV-1A polypeptides in the overall scheme of viral protein processing. The lateral position represents the relative location of the corresponding gene locus on the viral RNA (the 5' end of the RNA is to the left). Line lengths are proportional to molecular weight. The vertical position represents proposed precursor product relationships or alternative cleavage forms. Polypeptide 214 was produced in small amounts and its position should be considered tentative. Data for the smaller polypeptides is from reference 1.

TABLE 2. Analysis of polypeptide profiles after a 1-h labeling period with [3 H]amino acids

Virus	Concn (mM) ^a of zinc at which cleavage of the capsid precursor is inhibited by one-half	Concn (mM) of zinc at which polypeptides larger than the capsid precursor begin to be produced
HRV-1A	0.05	0.05
Polio	0.05 ^b	0.4
EMC	0.4	0.8

^a All concentrations of zinc refer to the amount of zinc added to the cell suspension. The actual intracellular zinc concentrations remain unknown because of a lack of information about zinc solubility in the medium and the degree of ion uptake by the cells.

^b It should be noted that these values are for Spinner culture in which the medium contains no serum. Using the cell monolayer conditions for plaque assay poliovirus showed no inhibition at 0.1 mM ZnCl₂ (14).

FIG. 4. Pactamycin map of HRV-1A polypeptides. At 3 h and 50 min postinfection an HRV-1A infected-cell suspension was exposed to 0.1 mM ZnCl₂. After 10 min a 2-ml sample was withdrawn and exposed to 90 μ Ci of a [3 H]amino acid mixture per ml for 20 min. The entire sample was then solubilized and electrophoresed on a 10% SDS-polyacrylamide gel as described in Fig. 1 (panel a, control, solid line). An identical sample was withdrawn from the same suspension at the same time, exposed to 0.2 μ M pactamycin for 4 min, exposed to 90 μ Ci of a [3 H]amino acid mixture per ml for 16 min, solubilized, and electrophoresed as above (panel a, pactamycin, dashed line). A duplicate experiment was performed as described above except that the suspension was exposed to 0.8 mM ZnCl₂ and the samples were run on 5% SDS-polyacrylamide gels (panel b, control, solid line; panel b, pactamycin, dashed line). In all cases the gels were run separately and superimposed for comparison. The pactamycin-control ratios were calculated as described (3). The viral polypeptides have been ordered from left to right according to increasing values of the pactamycin-control ratio so that the 5' end of the RNA is to the left (panel c, pactamycin map for the gel in panel a; panel d, pactamycin map for the gel in panel b).

FIG. 6. Cleavage inhibition of EMC viral polypeptides by zinc. An EMC virus-infected cell suspension was exposed to 1.5 mM ZnCl_2 at 3 h and 43 min postinfection. After 6 min the suspension was exposed to 4 μCi of a [^{14}C]amino acid mixture per ml and incubated for 1 h. The suspension was then solubilized and electrophoresed on 10% SDS-polyacrylamide gels as described in Fig. 1 (1.5 mM Zn, dashed line). An identical sample was labeled in the same manner but without the addition of zinc (control, solid line). The gels were run independently and have been superimposed here for comparison.

gels have been useful in sizing polypeptides in the 100,000 to 10,000 molecular weight range (6, 20). However, several difficulties are encountered when this technique is used to size polypeptides as large as those discussed in this paper. The first is the lack of well characterized marker polypeptides in this size range. Gamma globulin molecules and aggregates of bovine serum albumin have been used (12). With these, care must be taken to prevent the reduction of the disulfide bonds which hold the subunits together. In addition, because the geometry of these aggregated molecules is not the same as a single polypeptide chain, their usefulness as reference proteins may be limited. In other cases anomalous mobility is observed. We found that β -galactosidase (molecular weight 135,000) (20) migrates with an apparent molecular weight of 106,000. The use of β -galactosidase as a marker may thus explain why differing values for the poliovirus capsid precursor

(polypeptide 1a) have been reported (1, 10, 17).

A second difficulty is that extrapolating from calibrations in the 50,000 to 100,000 molecular weight range may lead to large inaccuracies in the estimated size of polypeptides in the 200,000 molecular weight range. For example, in two different reports from the same laboratory, each referring to the same set of markers, the molecular weight of the poliovirus polypeptide NCVP-OO was reported as 210,000 in the first case (10) and as 270,000 in the second (5).

The new, large, zinc-produced polypeptides were sized on 5% SDS-polyacrylamide gels using myosin and phosphorylase a as markers. In all three virus systems, cleavage inhibition by high concentrations of ZnCl_2 produced a polypeptide whose apparent molecular weight was greater than 200,000. These polypeptides are approaching a size range large enough to contain all of the viral gene products. Various

FIG. 7. Pactamycin map of EMC viral polypeptides. The experiment described in Fig. 4 was performed with an EMC virus-infected cell suspension. In this case the suspension was exposed to 1.2 mM $ZnCl_2$ at 3 h and 40 min postinfection. The cell extracts were run on 5% SDS-polyacrylamide gels (panel a, control, solid line; panel a, pactamycin, dashed line). The relative position of the genes for the viral polypeptides on the viral RNA were calculated as described in Fig. 4 (panel b).

other techniques have been used to produce viral polypeptides in this size range (10, 12, 13, 17). However, the molecular weight uncertainties of both the protein and RNA are great enough to cast doubt on any claim of the detection of a single polypeptide which represents the complete translation product of the viral mRNA.

HRV-1A. It has been proposed that polypeptides 92, 47 and 38 (as alternative cleavage forms), and 84 are primary products of HRV-1A RNA translation (1, 15). This is supported by the observation that these polypeptides become predominant when posttranslational cleavages are inhibited (Fig. 4a). Polypeptide 106 which maps with 92 is probably analogous to the EMC polypeptide A. According to the proposed cleavage scheme for HRV-1A (Fig. 5), conditions which result in the buildup of polypeptides 165 and 146 should show patterns in which the amounts of 92, 47, and 38 are decreased while 84 remains high. Figure 4b confirms this prediction.

EMC virus. The maturation cleavages of the EMC viral polypeptides are the least sensitive

to zinc ion (Table 2). It has been proposed that A, F, C, and possibly I are primary products of the translation of the EMC viral mRNA (2). This is substantiated by the fact that these are the polypeptides that are accumulated when the posttranslational cleavages are prevented (Fig. 6). Polypeptide 120 maps near the 5' end of the RNA but is not large enough to be an A-F intermediate. The small polypeptides G and I map just to the right of the capsid polypeptide (3) and it is possible that polypeptide 120 is an A-I or an A-G combination. Polypeptide 130 is the size and maps in the position of an F-C intermediate. However, the molecular weight data are not accurate enough to exclude the possibility that it is something like an F-D intermediate.

Poliovirus. It has been proposed that 1a, X, and 1b are primary products in the translation of the polioviral mRNA (1, 11, 18). This is supported by the observation that these polypeptides become predominant when maturation cleavages are inhibited by zinc ion (Fig. 9). Polypeptide 137 is the major new species observed in the presence of zinc. It maps as an X-1b combination, but the molecular weight data are not accurate enough to exclude the possibility that it is another combination of polypeptides translated from near the 3' end of the viral RNA. Polypeptides 123 and 116 are reproducibly observed in small amounts but cannot yet be assigned a position in the cleavage scheme. Other techniques have been used to produce large polioviral polypeptides in the size range as those accumulated in the presence of zinc (5, 7, 10, 13, 19). Although the different techniques employed in each laboratory make any correlation difficult, it appears as though 204 corresponds to NCVP-OO and 137 corre-

FIG. 8. Proposed position of the large, zinc-produced EMC viral polypeptides in the overall scheme of viral protein processing. Polypeptide 214 was produced in small amounts and its position should be considered tentative. Data for the smaller polypeptides is from reference 1.

FIG. 9. Cleavage inhibition of poliovirus polypeptides by zinc. A poliovirus-infected cell suspension was exposed to 0.8 mM ZnCl_2 at 3 h and 44 min postinfection. After 10 min the suspension was exposed to 40 μCi of a [^3H]amino acid mixture per ml and incubated for 1 h. The suspension was then solubilized and electrophoresed on 10% SDS-polyacrylamide gels as described in Fig. 1 (0.8 mM ZnCl_2 , dashed line). An identical sample from the same suspension was labeled for 1 h without the prior addition of zinc (control, solid line). The gels were run independently and have been superimposed here for comparison.

sponds to NCVP-OB (5).

Zinc has proved to be a useful tool in revealing more about the cleavages and intermediates that occur between polyprotein and the smaller, functional products. However, the mechanism of the cleavage inhibition is yet to be understood. The ability of zinc to prevent the maturation cleavages of several antigenically different picornaviruses is yet another example of the

FIG. 10. Pactamycin map of poliovirus polypeptides. The experiment described in Fig. 7 was performed with a poliovirus-infected cell suspension. In this case the suspension was exposed to 0.9 mM ZnCl_2 (panel a, control, solid line; panel a, pactamycin, dashed line). The relative gene order is shown in panel b. The pactamycin-control ratio values are the average of three different sets of gels from three independent cell extracts. The average deviation from the mean values shown was 0.06. The maximum deviation (polypeptide X) was 0.13.

FIG. 11. *Proposed position of the large, zinc-produced polioviral polypeptides in the overall scheme of viral protein processing. Data for the smaller polypeptides is from reference 1.*

similarities in protein-processing that are shared by these viruses.

The incorporation of amino acid analogues to prevent maturation cleavages results in altered polypeptides. An advantage of the use of zinc to accumulate precursors is that the primary structure of the polypeptides is unchanged. In addition, choosing the correct zinc concentration allows the maturation cleavages to be frozen at almost any point desired. These two observations make zinc extremely useful to those wishing to accumulate and further characterize polypeptide intermediates in the viral processing scheme.

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