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Inhibition by Zinc of Rhinovirus Protein Cleavage: Interaction of Zinc with Capsid Polypeptides[1]

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Zinc ions rapidly inhibit virus production in HeLa cells infected with human rhinovirus type 1A and lead to the accumulation of human rhinovirus type 1A precursor polypeptides. The degree to which cleavage of these precursors is inhibited is directly dependent on the quantity of cell-associated zinc. Proteolysis resumes after the removal of zinc-containing medium, and the accumulated viral precursors are cleaved predominantly to stable virus polypeptides. The precursors stabilized at the lowest zinc levels are those that contain capsid protein sequences. Furthermore, added zinc is bound to human rhinovirus type 1A capsids and prevents them from forming crystals. Zinc-resistant mutants display antigenic alterations in coat proteins. These results suggest that zinc complexes with rhinovirus coat proteins and alters them so that they cannot function as substrates for proteases or as reactants in the assembly of the virus particles.

Zinc ions play a role in many enzymatic reactions, including some catalyzed by proteases (20). With certain enzymes (e.g., ribonuclease), zinc may act as an inhibitor (23). The proteolytic reactions observed in tissue culture cells infected with a variety of animal viruses are thought to be enzymatic (for a review, see reference 13), and it was reported that zinc could stabilize precursor polypeptides of picornaviruses in vivo (4, 14) and in vitro (17).

Previous reports have indicated that viral precursor polypeptide cleavage may be inhibited in infected cells by at least two mechanisms. Inhibitors may block the cleavage by direct inactivation of the proteases (9, 11, 12). As an alternative, the substrate may be altered by heat or amino acid analogues, leading to a stable precursor (7, 10). The results of this study indicate that with human rhinovirus type 1A (HRV-1A), zinc blocks intracellular proteolysis by interacting with the protein precursors, particularly those that contain coat protein sequences (4), and altering them so they are not cleaved. A useful property of this inhibition is that the substrate is not permanently damaged or denatured, and removal of zinc permits proper cleavage of the precursors.

MATERIALS AND METHODS

Cell line and virus strains. A continuous line of HeLa cells, strain O, was propagated in monolayer culture in 100-mm plastic petri dishes (Falcon Plastics) using McCoy 5A medium with 10% calf serum (Gibco) or in suspension cultures using Joklik modified Eagle medium with 7% horse serum (Gibco). HRV-1A, strain 2060, and attenuated poliovirus, type 2, were grown at 35 C in monolayer cultures of HeLa-O cells using McCoy medium. Titrations of infectivity were performed by the plaque technique with appropriate virus dilutions on HeLa cell monolayers as previously described (15).

Isolation of zinc-resistant variants of HRV-1A. Plaque-purified HRV-1A was passaged at a multiplicity of 0.1 in HeLa cell cultures in McCoy 5A medium supplemented with 0.1 mM zinc chloride. Virus that then was able to replicate in the presence of zinc was selected by plaque titrations in the presence of 0.1 mM zinc chloride. Plaque-purified variants were further characterized by the following criteria: buoyant density in cesium chloride gradients, acid lability, and serum neutralization.

Purification and crystallization of HRV-1A. Infected cell suspensions were lysed by freezing and thawing and concentrated by differential centrifugation as previously described (15). The virus suspensions were further purified by sucrose gradient centrifugation. Volumes of 0.2 to 0.5 ml of virus suspension were placed over a 5-ml gradient of 25 to 6% sucrose (wt/vol) containing 0.01 M Tris-chloride buffer (pH 7.8). Centrifugation was at 40,000 rpm for 65 min in a Spinco SW50.1 rotor at 4 C. Virus was located in the gradients by radioactivity or light scatter and further purified by cesium chloride centrifugation to a purity at which the virus would crystallize (16).

Media and solutions. $ZnCl_2$ was obtained from Fisher Scientific Co. A stock concentrate of 10 mM

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$ZnCl_2$ in 1 mM HCl was used throughout these experiments. Carrier-free $^{65}ZnCl_2$ was obtained from Amersham/Searle. Medium AL is Eagle minimal medium lacking amino acids and supplemented with HEPES buffer to 25 mM (3). The concentration of zinc in solution was measured with a Jarrell Ash 810 atomic absorption spectrophotometer.

Gel electrophoresis. The procedures for infection of HeLa cells, in vivo labeling of viral polypeptides, solubilization of cells with sodium dodecyl sulfate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and computer analysis of the data have been described (2).

RESULTS

Inhibition of rhinovirus formation by zinc ion. Plaque formation, i.e., multicycle replication of HRV-1A and some other viruses, is prevented when growth medium is supplemented with zinc ion (8, 14). To examine in greater detail the rate at which zinc ions block formation of HRV-1A infectivity, infected cultures were supplemented with 0.1 mM zinc chloride at various times after infection, and virus production in a single growth cycle was assayed. The results (Fig. 1) indicated that zinc, added at any time postinfection, almost immediately inhibited the formation of infectious virus. The zinc, however, did not directly inactivate already mature, infectious virus. Since at later times of infection a pool of capsid protein and viral RNA is present in infected cells (1), the rapid inhibition of virus formation by zinc suggested that zinc might be interacting with one of the components which functions directly in virion formation.

Order of sensitivity to zinc of HRV-1A protein cleavages. Figure 2 illustrates that increased zinc concentrations resulted in the accumulation of progressively larger HRV-1A protein precursors. Figure 3 shows the amounts of various viral polypeptide precursors and products accumulated during a 1-h labeling period as a function of zinc concentration. Those post-translational cleavages that generated the capsid polypeptides were the most sensitive to zinc (note the marked inhibition of γ production at low levels of zinc [Fig. 3]). In fact, levels as low as 0.01 mM $ZnCl_2$ affected the amount of β produced, and generation of the other capsid polypeptides was significantly inhibited at 0.05 mM $ZnCl_2$. Zinc concentrations in the range of 0.1 mM yielded polypeptide profiles that consisted predominantly of the primary products 92, 84, 47, and 38 (2). Further increases in the zinc concentration inhibited those nascent cleavages that separate the capsid precursor (polypeptide 92) and other noncapsid precursors from the growing polypeptide chain and resulted in the buildup of the larger polypeptides 146 and 202. There was no accumulation of polypeptide 165 with increasing zinc concentrations.

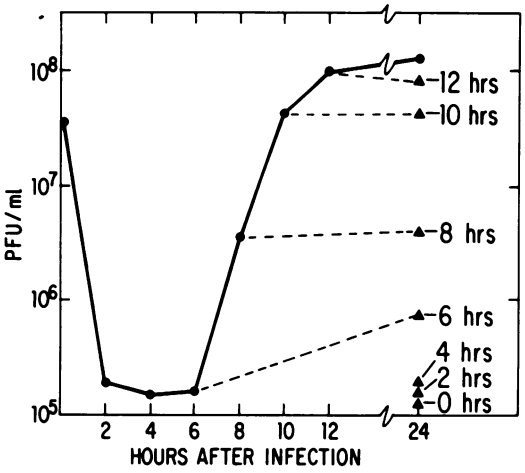

FIG. 1. *One-step growth curve of HRV-1A in HeLa cells with and without zinc ions. HRV-1A (10 PFU/cell) was added to HeLa cell monolayers in petri dishes. After 1 h at 35 C to allow for virus adsorption, the plates were thoroughly drained and washed with growth medium to remove unadsorbed virus. McCoy medium, with no zinc supplement, was added to all the cultures, and they were placed in an incubator at 35 C in an atmosphere of air-CO_2 (5%). At varying times thereafter samples were removed and assayed for infectivity. At the same time zinc chloride was added to duplicate cultures to bring the final concentration to 0.1 mM. All samples were then titered for infectivity after a total of 24 h. Infectivity (●) and infectivity after the addition of zinc at times indicated (▲) were assayed at 24 h.*

Interaction of zinc ions with HRV-1A capsids. Figure 3 illustrates the marked sensitivity of capsid precursor cleavage to inhibition by zinc ion. To test the possibility that zinc ions were stabilizing some of the HRV-1A precursors by reacting with capsid sequences, direct binding of $^{65}Zn^{2+}$ to viral capsids was assayed. Figure 4 indicates that zinc ions did bind to HRV-1A virions (Fig. 4A). Zinc ions did not bind as avidly to poliovirus (Fig. 4B), which is less susceptible to inhibition by zinc (14). Only 2 mol of zinc was bound per mol of virion in the case of HRV-1A; however, the sucrose gradient was probably sufficiently contaminated with nonradioactive zinc to partially block binding of ^{65}Zn to the virions.

Further evidence of direct interaction of zinc with the viral capsids was obtained using the ability of purified HRV-1A to crystallize under appropriate conditions (16). Approximately 5 mg of purified HRV-1A was divided into three

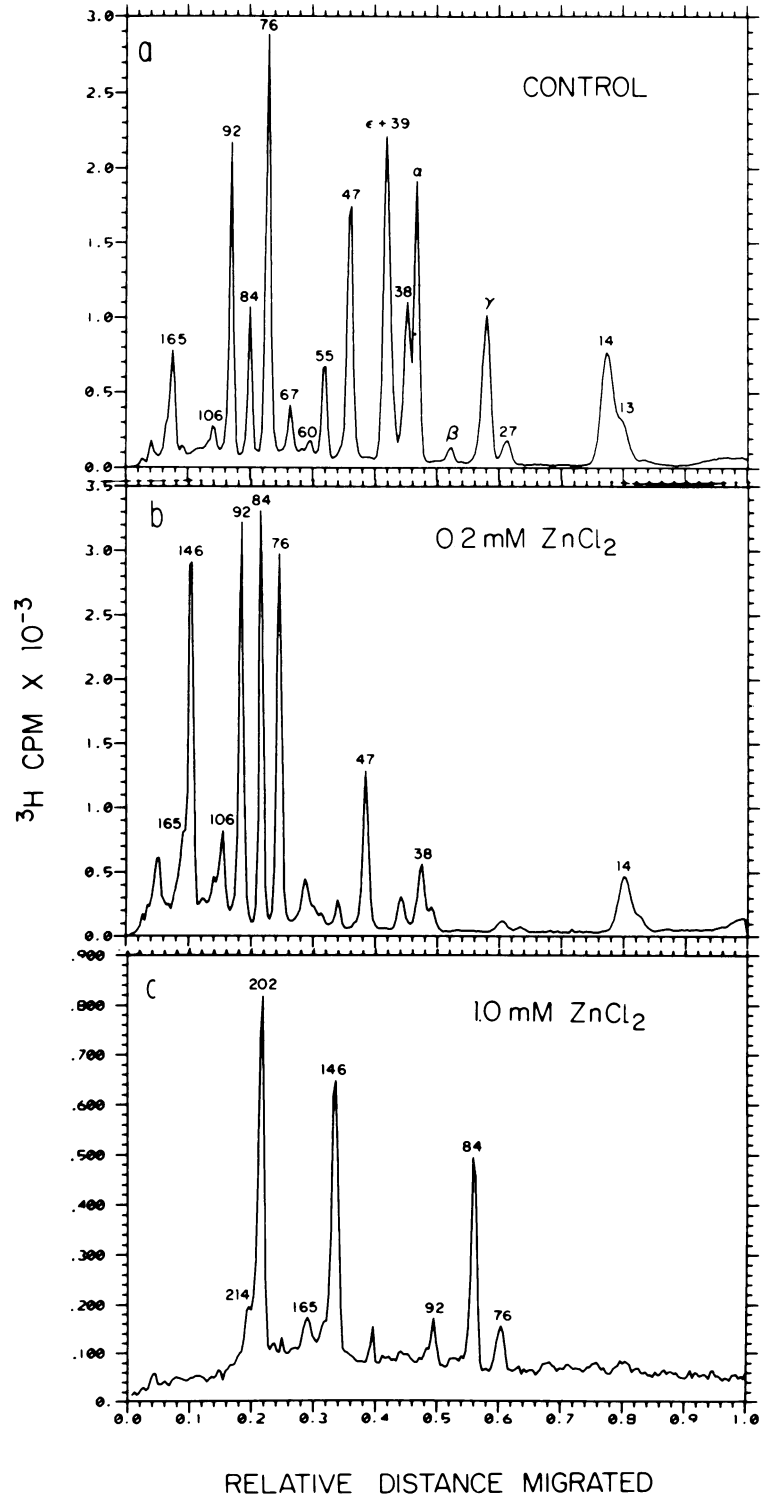

FIG. 2. *Inhibition of cleavage of HRV-1A polypeptides by different zinc concentrations. All infected cell suspensions were prepared as described. At 4 h postinfection, 4-ml portions from an HRV-1A-infected cell suspension were exposed to various concentrations of $ZnCl_2$. Four minutes thereafter each aliquot was exposed to 48 μCi of a ^3H-labeled amino acid mixture per ml. After a 1-h incubation at 34 C in a shaking water bath, a 1-ml sample was withdrawn, added to 100 μl of a 10× solubilizing solution, and heated for 5 min in a boiling water bath. After dialysis each sample was run on 10 and 5% gels as described. Shown here are the 10% gel electropherograms for the control and 0.2 mM $ZnCl_2$ samples (a and b) and the 5% gel for the 1.0 mM $ZnCl_2$ sample (c).*

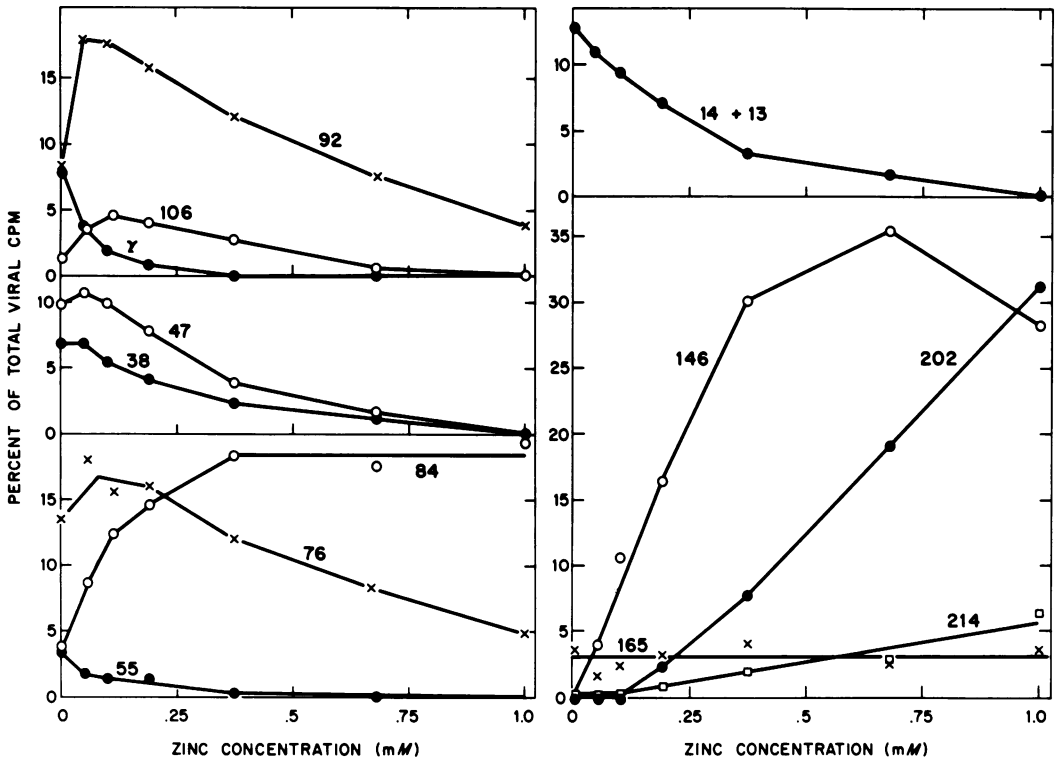

FIG. 3. *Degree of cleavage inhibition versus zinc concentration. The amount of each viral polypeptide in each pattern from Fig. 2 was determined and expressed as a percentage relative to the total viral counts per minute. This percentage is plotted versus the zinc concentration. It should be noted that the abscissa scale represents the total amount of zinc added under the precise conditions specified.*

portions. Sufficient zinc was added to bring the final concentration of zinc to 0, 1 μM, and 0.1 mM. Virus to which no zinc had been added crystallized after 3 days (Fig. 5a). By comparison, virus in 0.1 mM zinc formed instead amorphous clusters, which grew extensively after prolonged incubation (Fig. 5b). Concentrations of zinc as low as 1 μM prevented crystals of the virus from forming. Since the purified virus was approximately 10^{-7} M, as few as 10 zinc ions per virion altered the capsid structure sufficiently to prevent crystallization.

Characterization of zinc-resistant variants derived from HRV-1A indicates alterations in capsid protein structure (Table 1). Compared with HRV-1A, the two zinc-passaged isolates described here differed in their susceptibility to neutralization by immune serum to HRV-1A. Zr_2 was fully resistant to the serum, whereas Zr_1 was partially susceptible. Note that replication of Zr_1 was still somewhat inhibited by zinc. Both variants were still sensitive to acid (Zr_2 less so than HRV-1A or Zr_1) and had buoyant densities nearly equal to that of HRV-1A (~1.38 g/ml). The latter properties clearly indicate that both variants remained rhinoviruses. The results indicate that selecting for zinc resistance leads to isolation of viruses with altered capsid properties; in the specific examples given in Table 1, the variants exhibited altered neutralizing antigens.

In light of the complete resistance of Zr_2 to anti-HRV-1A serum, it is possible that it may represent a contaminating rhinovirus, with a distinct serotype. Of the ten rhinovirus strains we examined, only HRV-5 was resistant to zinc ion (14). Zr_2, when treated with anti-HRV-5 serum, was unaffected (data not shown). Therefore, the serotype of Zr_2 is not known.

Necessity of cell-associated zinc for cleavage inhibition. It was possible that the zinc was effecting some kind of cellular change that resulted in the cells' inability to cleave the viral proteins. To test this possibility, an infected cell suspension was exposed to 0.4 mM $ZnCl_2$ and 0.02 μCi of $^{65}ZnCl_2$ per ml. The cells were washed three successive times by sedimenting and suspending in fresh medium. The amount

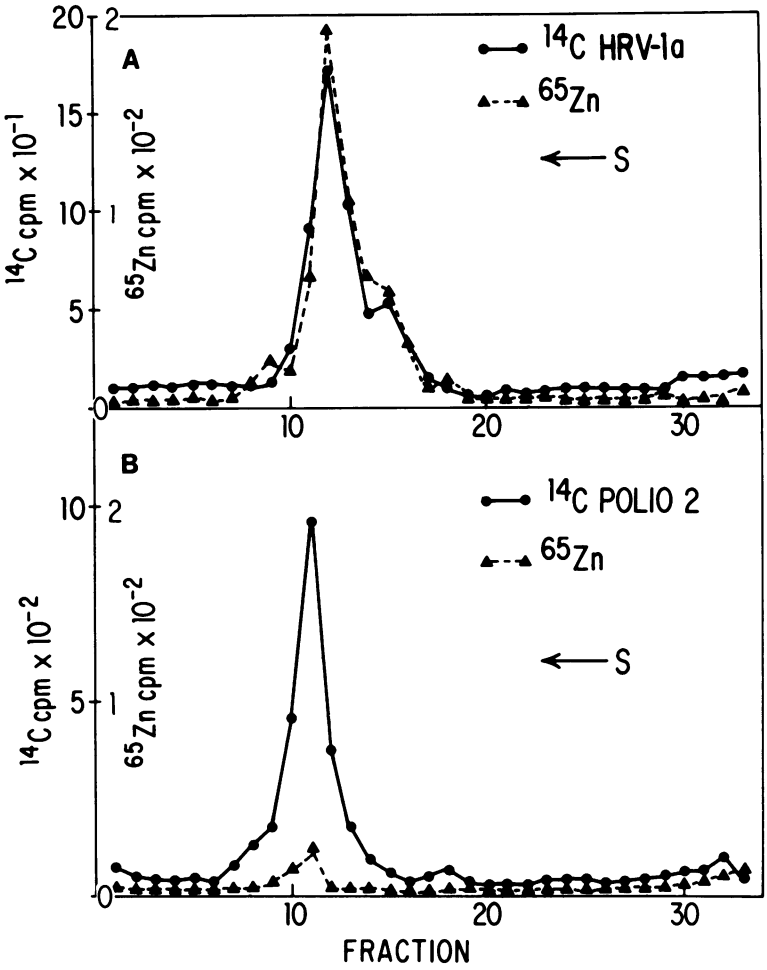

FIG. 4. *Sucrose gradient centrifugation of purified poliovirus or HRV-1A in solutions containing ^{65}Zn. Sucrose gradients were formed as described, except that ^{65}zinc (10^5 counts/min) was added to the solutions prior to gradient formation and was therefore distributed equally throughout the gradients. Purified poliovirus and HRV-1A capsids, both labeled with ^{14}C-labeled amino acids, were centrifuged through the ^{65}zinc-containing sucrose gradients. Concentrations of virus were estimated based on absorbance at 260 nm of 1 for 10^{13} virus particles (15), and the samples were adjusted prior to centrifugation to contain equal amounts of virus particles (approximately 10^{12} per gradient). Centrifugation was at 4 C for 65 min at 35,000 rpm (see Materials and Methods). Gradients were fractionated from the bottom, and ^{14}C (●) and ^{65}Zn (▲) were determined by liquid scintillation spectrometry (sedimentation right to left). (A) HRV-1A; (B) poliovirus. Background ^{65}zinc counts per minute (2,400) in each of the gradient fractions were subtracted to obtain the base line of zero used for plotting.*

of zinc present was monitored by measuring the radioactive ^{65}Zn. One wash reduced the cell-associated zinc to 21% of its starting value, the second wash reduced it to 6%, and the third wash reduced it to 3% (data not shown). After each washing procedure a portion of cells was removed and exposed to ^{3}H-labeled amino acids, and the viral protein profile was determined. The patterns showed that the degree of cleavage inhibition was correlated directly with the amount of cell-associated zinc. There was no lasting effect of the zinc; when the ion was removed the normal cleavage activity was restored. This is consistent with a model where the zinc itself is participating directly in the cleavage inhibition.

Reversibility of zinc inhibition. During a normal infection, the primary products are always cleaved from the growing chain before translation of the viral RNA is completed, so

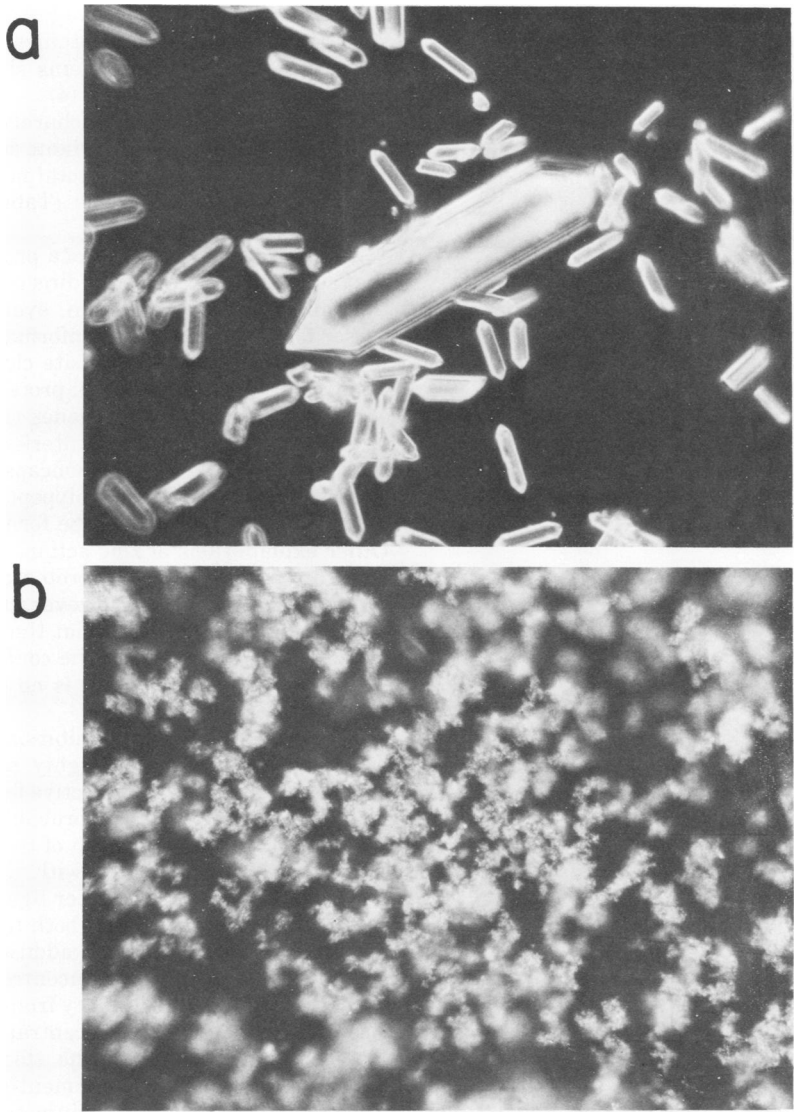

FIG. 5. *Effect of zinc on crystallization of HRV-1A. Purified HRV-1A was dialyzed in 0.15 M ammonium formate and divided into three portions (see Materials and Methods). To two of the portions zinc was added. All samples were placed at 4 C and photographed after several days. (a) No added zinc; (b) zinc added to 0.1 mM. All photography was darkfield at ×150.*

that polyprotein (molecular weight, ~200,000) is not observed (6). However, there is evidence that appropriate cleavages can result, even after completion and release of the precursor polypeptides (7, 11). To further test those results, precursors 202, 146, 92, and 84 were accumulated by labeling in the presence of $ZnCl_2$. The zinc and ^3H-labeled amino acids were then washed from the cells, and the cells were incubated for an additional 1 h to see if the precursors would cleave. Figure 6a and b show the normal flow of radioactivity from the precursor to the product polypeptides. The zinc-accumulated precursor polypeptides failed to cleave if the zinc was not removed (Fig. 6c and d). When the zinc was removed, the precursors were cleaved and gave rise to a normal pattern of stable polypeptides (Fig. 6e and f). The cleavages took place after translation was completed, supporting the view that cleavage is not an inherent translational event. Furthermore, production of correct stable polypeptides from

TABLE 1. *Properties of zinc-resistant variants selected from HRV-1A*

Virus designation	Density (g/ml)[a]	% Inhibition in 0.1 mM zinc[b]	% Inactivation at pH 5[c]	% Neutralization by anti-1A serum[d]
HRV-1A	1.38	99.9	99.95	99.9
Zr_1	1.38	50–90	99.95	95
Zr_2	1.38	<10	99	<10

[a] CsCl density gradient centrifugation as in reference 15.

[b] Plaque-purified virus was used to infect HeLa cells at a multiplicity of infection of 0.5 to 1. Zinc was added to a final concentration of 0.1 mM at 120 min postinfection; virus yield was measured 17 h later.

[c] Crude virus suspensions were diluted 1:100 into pH 5.0 sodium acetate buffer containing 0.1 M NaCl at 25 C. Infectivity was assayed after 60 min.

[d] Serum neutralization was determined as previously described (22).

the large proteins accumulated in zinc provides additional evidence that they are, in fact, the biological precursors of the viral proteins. However, about 50% of the nondialyzable counts were lost during the chase period, indicating that a percentage of the polypeptides were possibly degraded to dialyzable fragments.

DISCUSSION

We propose that normal proteolytic processing of HRV-1A polypeptides is inhibited by the binding of zinc to the rhinoviral protein precursors, particularly in regions containing the capsid sequences. The fact that the degree of cleavage inhibition is proportional to the amount of intracellular zinc present suggests that the zinc itself participates directly in the cleavage inhibition. The observation that the inhibition is reversible by a simple washing procedure (Fig. 6) supports the same conclusion and implies that cleavage is not an inherent translational event. The pattern of zinc action in which there is immediate inhibition of the formation of infectious virus (Fig. 1) suggests that zinc may be interacting with one of the precursors of virion formation.

Several lines of evidence support the idea of a high-affinity zinc binding site in the rhinovirus coat protein sequence. Figure 4 demonstrates the direct binding of ^{65}Zn to HRV-1A virions, and HRV-1A replication is greatly inhibited by zinc. There was little binding to poliovirions, and poliovirus replication is little affected by zinc (14). Zinc concentrations above 1 μM inhibit the crystal formation of HRV-1A virions (Fig. 5), also indicating an effect of zinc on the capsid polypeptides. Figure 3 clearly shows that the capsid polypeptide cleavages are the most sensitive to zinc. Mapping studies on the zinc-induced large HRV-1A proteins show that all contain the capsid sequence (4).

In addition, isolation and characterization of zinc-resistant rhinovirus variants selected from HRV-1A indicate partial or total nonidentity of capsid-neutralizing antigens (Table 1) of the resistant viruses.

It is consistent with the data presented here that sufficient zinc will bind directly to a site in the rhinovirus capsid protein, even in its precursor form, and alter its conformation so that it cannot undergo the requisite cleavage reactions. Since initiation of viral protein synthesis occurs in the coat protein genes (2, 5, 19), an altered coat precursor may interfere with subsequent cleavages of the noncapsid polypeptides, and much larger polypeptides, up to about 2×10^5 daltons, may be formed (Fig. 2). Other explanations of zinc action, particularly at high zinc levels, such as protease inhibition, cannot be ruled out. However, taken as a whole, these data suggest that the mechanism of action of zinc is to alter the configuration of the capsid protein so that it is no longer processed normally.

The degree of cleavage inhibition by zinc under defined conditions is highly reproducible. However, the nature of the active form of zinc is not clear, and the system is probably a complex one. It is probable that much of the zinc added to the medium complexes with amino acids, phosphate, proteins, and other ligands. In particular, added serum affects both the solubility and activity of zinc. Prior addition of horse serum to medium AL to a concentration of only 0.05% increased zinc solubility from 0.03 mM to 0.31 mM. Higher serum concentrations further increased zinc solubility. The standard infection procedure involves sedimenting the cells from the Eagle growth medium that contains 7% horse serum and suspending the cell pellet directly in medium AL for infection. The amount of horse serum carried over was sufficient to keep any added zinc in solution. The addition of 0.5% horse serum to the infected cell suspension did result in somewhat greater zinc activity (data not shown).

The addition of $ZnCl_2$ to a concentration of 0.4 mM to medium AL followed by sedimentation of the resulting zinc phosphate precipitate gives a supernatant which contains only 0.03 mM zinc. Yet, this supernatant was almost as active in inhibiting the viral protein cleavages as the control in which the zinc was added directly to the cell suspension (data not shown).

Proteolytic processing of viral proteins may be interrupted by amino acid analogues, pro-

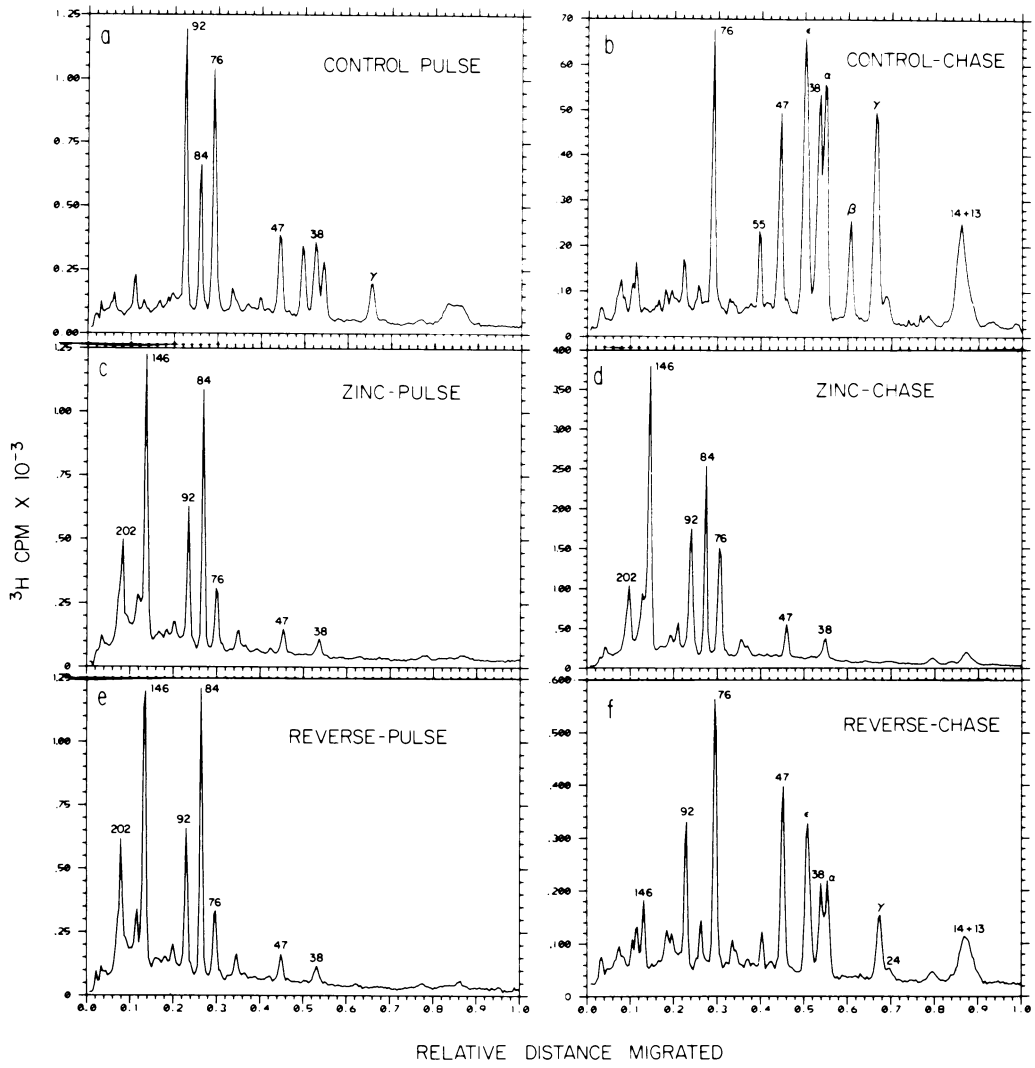

FIG. 6. *Cleavage of the viral precursor polypeptides after removal of zinc. At 3 h and 50 min postinfection, 5 ml of an HRV-1A-infected cell suspension was exposed to 35 μCi of a ^{3}H-labeled amino acid mixture per ml for 30 min. A 0.5-ml sample was removed, solubilized, and analyzed electrophoretically (a, control pulse). Two duplicate samples were also labeled and analyzed as the control except that they were exposed to 0.6 mM $ZnCl_2$ during the labeling period (c, zinc-pulse; e, reverse-pulse). The cells were then washed two times by diluting to 50 μl with medium AL, sedimenting the cells, and suspending to the original 4 × 10^6 cells/μl in fresh medium AL to remove the zinc and unincorporated ^{3}H-labeled amino acids. However, the zinc-chase sample was washed with medium that contained 0.6 mM $ZnCl_2$. After a 60-min incubation, a sample of each was solubilized and analyzed electrophoretically (b, control-chase; d, zinc-chase; f, reverse-chase).*

tease inhibitors, and high temperature (13). However, the use of zinc can provide several distinct advantages over these. Undesirable side effects such as cellular toxicity and inhibition of protein synthesis (18) are associated with most physical and chemical treatments. In addition, the effects are often irreversible. By comparison, up to 0.2 mM zinc only slightly inhibited protein synthesis and there was retention of cell viability up to 0.1 mM zinc (data not shown). The reversibility of zinc inhibition may be its most useful feature. Normal intracellular cleavages of precursors after zinc removal indicate that the polypeptide substrates have remained native (Fig. 6). This permits studies of synchronized cleavage of large quantities of labeled precursors within infected cells.

Zinc ions can act to inhibit various aspects of

the replication of viruses other than picornaviruses. Susceptible viruses include representatives of the arbovirus (M. Bracha and M. Schlesinger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S90, p. 228), rhabdovirus (L. Everhart and B. Korant, manuscript in preparation), oncornavirus (21), and herpesvirus (8) groups. Further detailed studies may indicate a common peptide sequence participating in the replication of otherwise dissimilar viruses.

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