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Virus-specified protease in poliovirus-infected HeLa cells

(poliovirus replication/cell-free protease assay/protein cleavage)

B. KORANT*, N. CHOW*†, M. LIVELY‡, AND J. POWERS‡

*Central Research and Development Department, E. I. du Pont de Nemours and Co., Inc., Wilmington, Delaware 19898; and ‡School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332

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ABSTRACT Previous studies have shown that primary cleavages in nascent picornavirus precursors are accomplished by cellular proteases. This study has characterized the enzyme in infected cells that produces the capsid polypeptides by secondary cleavages of viral precursors. The kinetics of the production of protease activity correlate with the time course of virus protein synthesis, and the new enzyme has characteristic pH and temperature optima. Guanidine and cycloheximide, which are inhibitors of virus RNA and protein synthesis, prevent production of the protease. As determined by introduction of amino acid analogs into the protease or inhibition by a leucyl chloromethyl ketone, the enzyme is synthesized at a time of infection when host cell proteins are not produced, and the enzyme copurified with a 40,000-dalton virus polypeptide present in the cytoplasm of infected cells. Wild-type levels of protease activity are produced by viral mutants that are defective in coat protein synthesis. The conclusion is that a nonstructural poliovirus gene product participates in protein cleavages that produce the viral coat proteins.

To comprehend the complex process of viral replication, it is crucial to know the origin of the enzymes involved (for review, see ref. 1). With picornavirus protein cleavage, the enzyme(s) that produces the capsid polypeptides is of particular interest. The simplest possibility is that host proteases are used to cleave the virus-coded substrates. However, this reduces the ability of the virus to control the reactions and, in fact, there are several pieces of evidence that support the existence of a virus-coded enzyme.

Cells infected by polio or related viruses contain in their cytoplasm greater quantities of protease activity than do uninfected cells (2) and the induced activity parallels the time course of infection (3) and the amount of virus used to initiate infection (4). Extracts of infected cells are able to carry out cleavages of viral precursors leading to production of capsid polypeptides or fragments of them (5), whereas extracts of uninfected cells lack these activities (2, 3, 5, 6).

There have been several reports of protease activities associated with purified virions of animal viruses, including picornaviruses, a myxovirus, a papovavirus, and the RNA tumor virus Rous sarcoma (4). This study has examined the origin and properties of an induced protease in poliovirus-infected HeLa cells.

METHODS

Cell Culture and Virus Production. Culture conditions for HeLa O cells, replication of poliovirus, and labeling of virus proteins have been described (2). The use of chemical inhibitors to block virus protein processing was reported (2, 5).

Sodium Dodecyl Sulfate ($NaDodSO_4$) Gel Electrophoresis. Gel electrophoresis of virus polypeptides in 8–18% polyacrylamide slab gels containing $NaDodSO_4$ was performed as described by Laemmli (7).

Proteolysis Assays. Cell-free assay of proteolysis was monitored in two ways. Cells were swollen in 0.01 M NaCl/0.01 M Tris, pH 7.2, and homogenized mechanically. After removal of the intact nuclei by low-speed centrifugation, the cytoplasm was mixed with an aliquot of radiolabeled virus precursor polypeptides. Protein cleavage was monitored by either $NaDodSO_4$ gel electrophoresis of the products (2, 5) or their release from an insoluble support of Sepharose beads (8). The substrate was prepared by labeling infected cells with [^{35}S]methionine in the presence of an inhibitor of virus protein processing. The labeling was usually continued for 60 min at 35°C, and then cells were disrupted as described above. The substrate was then added to an assay directly or was treated with 0.5% deoxycholate (5) and coupled to activated Sepharose or polyacrylamide beads (8). Monitoring of solubilized protein fragments was by

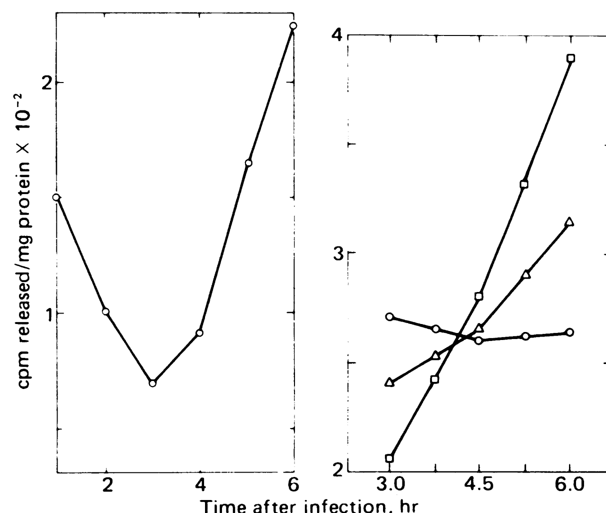

FIG. 1. (*Left*) Time course of protease induction in poliovirus-infected HeLa cells (multiplicity of infection, 50 plaque-forming units per cell). Substrate was [^{35}S]methionine-labeled viral precursor proteins, accumulated by treatment of poliovirus-infected HeLa cells with 0.1 mM iodoacetamide. Under these conditions, the substrate predominantly cleaved is the capsid protein precursor NCPVla (5). In the assay shown and in subsequent figures, substrate (specific activity, 10^6 cpm/mg) was mixed with $\approx 1 \times 10^7$ infected cell equivalents and incubated for 2 hr at 37°C. The assay was terminated by addition of $NaDodSO_4$ to 1% and heating to 100°C. The resultant radioactive products were analyzed by $NaDodSO_4$ gel electrophoresis (2, 5) and by determination of solubilized radioactivity originating from the processed substrate (8). (*Right*) Effect of multiplicity of infecting virus on protease activity in poliovirus-infected HeLa cells. Preparation of extracts and assay was as in *Left*. □, 100 plaque-forming units per cell; △, 10; ○, 1.

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; CLCK, carbobenzoxyleucyl chloromethyl ketone.

† Present address: Department of Life and Health Sciences, University of Delaware, Newark, DE 19711.

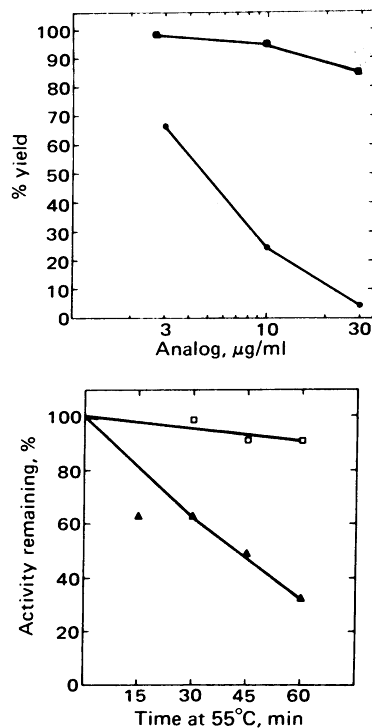


FIG. 2. (*Upper*) HeLa cells were infected with poliovirus and, after 3 hr of infection, one-half of the culture was treated with several levels of a mixture of azaleucine, fluorophenylalanine, and azatryptophan. After an additional 3 hr, the cells were harvested and extracts were tested for protease activity (■) as in Fig. 1 or assayed for infectious virus (●). (*Lower*) Extracts from untreated and analog-treated cells (each analog, 30 μg/ml) were heated at 55°C for up to 60 min. All samples were then chilled and assayed for protease activity at 35°C, as in Fig. 1. □, Untreated extracts; ▲, analog-treated cell extracts.

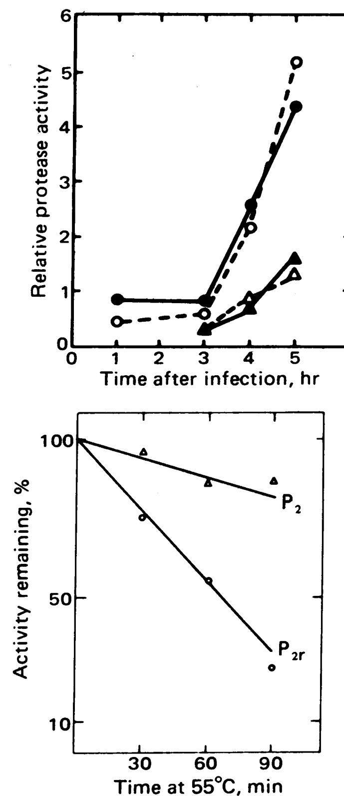


FIG. 3. (*Upper*) Protease activities of poliovirus type 2 and a mutant P2r, assayed in cell-free extracts (3×10^6 cell equivalents). ●, P2 protease, P2 substrate; ○, P2 protease, P2r substrate; ▲, P2r protease, P2 substrate; △ P2r protease, P2r substrate. (*Lower*) Heat inactivation of protease in extracts of HeLa cells infected with P2 (△) or P2r (○).

liquid scintillation counting or by autoradiography using Cronex 4 x-ray film.

Synthesis of Carbobenzoxyleucyl Chloromethyl Ketone (CLCK). This has been described (9); it was accomplished by the mixed anhydride procedure, yielding a yellow oil (reviewed in ref. 10).

RESULTS

Requirements for Production of Poliovirus Protease. The time course of induction of protease activity in poliovirus-infected HeLa cells is shown in Fig. 1 *left*. There was first a decline in an endogenous cellular protease activity, which reached a minimum at about 2.5 hr after infection (4). Thereafter, as infection proceeded, there was a 5-fold or greater increase in the rate and amount of protease activity, assayed on virus precursor proteins. The rate of new enzyme production and the amount of enzyme activity produced were positively correlated with the multiplicity of infecting virus (Fig. 1 *right*).

Production of the new protease in poliovirus infection appears to require viral RNA synthesis (4) because the activity is not present in guanidine-treated cells [2 mM guanidine has no effect on cell-free protease assays (unpublished result)]. Protein synthesis is also necessary for production of the protease, because cycloheximide blocks its appearance (4). An additional distinguishing characteristic of the new protease is a neutral pH optimum, which discriminates the protease from the major lysosomal proteases, present in uninfected cells (6).

Cycloheximide prevented protease activity from increasing in infected cells (data not shown). This indicates that protein synthesis is required for production of the enzyme. When amino acid analogs were added to poliovirus-infected cells at mid-cycle of replication, virus yields were greatly diminished; by comparison there was a virtually normal yield of protease (Fig. 2 *upper*). However, the protease produced was heat sensitive, compared to the enzyme from untreated infected cells (Fig. 2 *lower*). This indicates that the enzyme contained analogs and therefore had been synthesized at a time after infection when host protein synthesis was blocked by the virus.

A poliovirus type 2 mutant has been described (11) that lacks regulation of viral RNA metabolism. Cells infected with the mutant maintained a high rate of viral RNA synthesis for an abnormally extended time, relative to the parent virus (11). Extracts of cells infected with this virus displayed a lower protease activity than did those of wild-type-virus-infected cells (Fig. 3 *upper*). Moreover, the protease produced by the mutant virus was more temperature sensitive than that of its parent (Fig. 3 *lower*). These results indicate that the protease is itself coded for by the viral genome because it is unlikely that a viral mutant would induce an altered cellular enzyme. An additional negative control was provided by assay of extracts of vesicular stomatitis virus-infected HeLa cells. The infection by this strongly cytopathic but genetically unrelated virus produced no evidence of protease activity as monitored on poliovirus substrate (not shown).

Specificity of Poliovirus Protease. The specificity of poliovirus protease may be inferred from end group analyses of processed virus capsid polypeptides. The enzyme cleaves peptide bonds involving a carboxyl group donated by leucine or glutamic acid. This permitted use of a synthetic protease inhibitor terminating in a leucyl chloromethyl ketone residue. The effect of such an inhibitor on poliovirus protein processing is shown in Fig. 4. When the infected cells were treated with

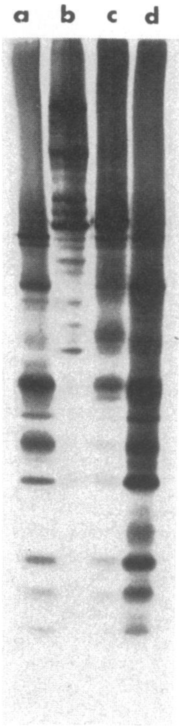
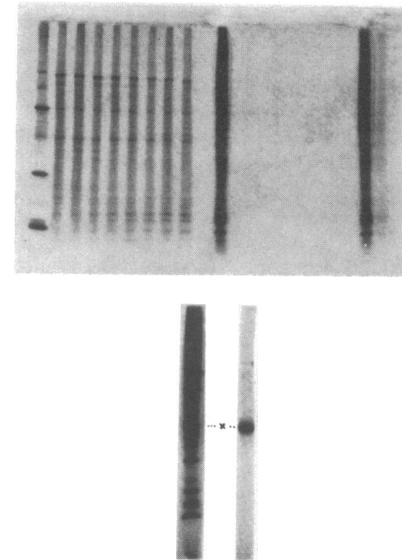


FIG. 4. $NaDodSO_4$ gels of poliovirus cytoplasmic polypeptides. Infected cells were labeled with [^{35}S]methionine, in the presence or absence of the CLCK. After an appropriate chase, cells were lysed, and the proteins were resolved and autoradiographed. Lanes: a, virus control; b, addition, during labeling, of 0.1 mM CLCK; c, as in b but excess CLCK removed and reaction chased for 60 min in medium containing excess unlabeled L-methionine and cycloheximide (100 μg/ml); d, as above, but no cycloheximide present during the chase.

inhibitor at 0.1 mM, normal processing of virus coat precursor polypeptides was rapidly blocked and high molecular weight polypeptides accumulated. Although chloroketone protease inhibitors react irreversibly, virus-specific cleavage restarted when excess inhibitor was removed by sufficient washing (Fig. 4, lane c). However, reversal of the effect required protein synthesis (Fig. 4, lane d). This indicates that the enzyme is continuously synthesized in infected cells, after synthesis of host protein is abolished.

Assignment of Proteolytic Activity to Poliovirus Protein. The protease was prepared from poliovirus-infected cells by homogenization followed by chromatography on DEAE-Sephadex and a second purification on $NaDodSO_4$/hydroxylapatite column. The protease-containing fractions were pooled and resolved by electrophoresis in a polyacrylamide gel containing $NaDodSO_4$. The protease activity had a mobility corresponding to a molecular weight of approximately 40,000 (Fig. 5). Autoradiography of the protease fraction to establish the virus protein contribution indicated that, of the poliovirus proteins, only NCVPX was present. The possibility that the NCVPX preparation was significantly contaminated with VP1, the largest virus capsid polypeptide, was examined by assaying protease levels in cells infected with one of two poliovirus mutants. These mutants contained deletions in the structural protein gene, and neither produced significant levels of capsid polypeptides (13). With both mutants, there was production of a full yield (compared to a nondefective virus) of protease (Fig. 6). This supports the view that there is no contribution of a coat polypeptide to the virus-specific protease, as assayed under *in vitro* conditions.



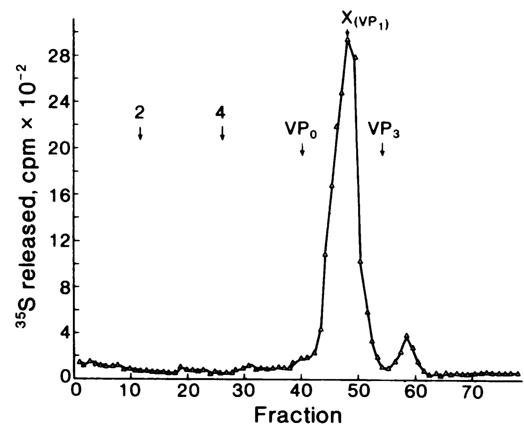


FIG. 5. HeLa cells were infected for 6 hr with poliovirus type 2 and then homogenized; the cytoplasm treated with 0.5% deoxycholate. The sample was adjusted to pH 7.2 and loaded onto a DEAE-cellulose column. The column was eluted with an increasing gradient of sodium chloride. Protease-containing fractions were pooled and rechromatographed on a hydroxyapatite column in $NaDodSO_4$ (12). Protease fractions were again pooled and electrophoresed on a polyacrylamide slab gel. Contribution of cellular proteins was monitored by Coomassie blue staining (*Top*) of the fractions from the ion exchange column (lanes 2–9) and from the $NaDodSO_4$/hydroxyapatite column (lanes 12–16). Lane 1 contains protein standards, and lower 12 and 19 resolve total HeLa cell cytoplasm. The viral protein contribution was detected by autoradiography of the dried gel (*Middle*). (*Bottom*) Electrophoretic separation and detection of poliovirus protease on a $NaDodSO_4$/12% polyacrylamide gel. Unlabeled infected extracts were mixed with a small amount of ^{3}H-labeled poliovirus proteins, and the mixture was resolved in the gel. Fractions of the gel were eluted and assayed for protease activity on ^{35}S-labeled virus precursor proteins. Makers were poliovirus polypeptides NCVP 2, 4, and X and capsid polypeptides VP_0, VP_1, and VP_3.

DISCUSSION

The evidence presented indicates that a poliovirus gene product acts as a protease on virus precursor polypeptides. An alternative possibility, that a virus protein activates a cellular protease, is inconsistent with the data presented.

The data presented, which support a direct catalytic role for a poliovirus protein in the proteolytic reactions producing coat polypeptides, are the following. (*i*) The kinetics of production of the activity parallel the synthesis of viral proteins, and the yield of enzyme correlates with the quantity of infecting virus. (*ii*) Inhibitors that block virus-specific RNA synthesis or protein

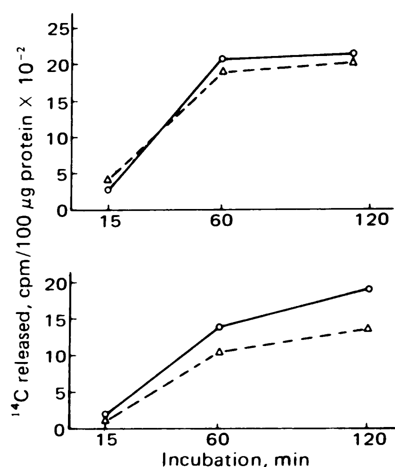


FIG. 6. Protease activity detected in extracts of HeLa cells infected with either polio type 1 Mahoney (O) or defective-interfering mutants (Δ; type 2 in *Upper*; type 3 in *Lower*) supplied by A. Nomoto and E. Wimmer.

synthesis prevent the production of the proteolytic activity. (*iii*) Poliovirus infection of HeLa cells leads to total inhibition of cellular protein synthesis, and we took advantage of this in two experiments. First, incorporation of amino acid analogs into proteins synthesized after the mid-cycle of infection led to lability of the protease at high temperature. Second, reversal of the effect of CLCK, which had blocked virus protein processing, was observed only if virus-specific protein synthesis was permitted. These results indicate that synthesis of the enzyme is occurring in infected cells, where polysomes are all virus-specific. Analysis of a viral mutant that displayed altered kinetics of RNA synthesis indicated production of relatively small amounts of an altered, temperature-sensitive protease. This is support for the role of the viral genome in coding for the enzyme, although an explanation for the pleiotropic effect on RNA synthesis is lacking. A possible explanation is that, with the protease defect, maturation of capsid polypeptides is affected, leading to an alteration in the regulation of the size of the replicating RNA pool (11).

An earlier report suggested a role for a picornavirus capsid polypeptide in the viral proteolytic reactions (3). This interpretation is in conflict with our results in several ways. First, a level of amino acid analogs that abolished native coat protein function (Fig. 2) did not decrease the enzymatic activity of the protease produced, unless it was subsequently heated. Also, purification led to isolation of a nonstructural viral polypeptide, NCVPX, along with the enzymatic activity. Finally, defective poliovirus mutants, which did not produce stable coat polypeptides, displayed wild-type levels of protease on a viral substrate. A recent study on the encephalomyocarditis virus protease was also able to disconnect it from the structural polypeptides (14). Moreover, in these experiments, the protease was actually synthesized in a cell-free reaction, coded by the viral RNA. This is convincing evidence for the viral origin of the enzyme.

A study of the T4 bacteriophage protease indicated a specificity of the enzyme for a hexapeptide sequence in the viral precursor (15). Part of the specificity of the poliovirus enzyme involves leucine or glutamic acid residues, but more extended regions are certainly involved because of the limited proteolytic action on virus precursors and the inability to cleave cellular proteins (unpublished data). A more complete description of the requirements for proteolysis could lead to a new approach to viral inhibitors, directed at the protease function.

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