ENCEPHALOMYOCARDITIS VIRUS-SPECIFIC POLYPEPTIDE p22 IS INVOLVED IN THE PROCESSING OF THE VIRAL PRECURSOR POLYPEPTIDES

A. E. GORBALENYA, Yu. V. SVITKIN, Yu. A. KAZACHKOV and V. I. AGOL

Institute of Poliomyelitis and Viral Encephalitides, USSR Academy of Medical Sciences, Moscow Region 142782 and *Moscow State University, Moscow 117234, USSR*

Received 14 September 1979

1. Introduction

Picornavirus-specific proteins are formed by post-translational cleavage of high-molecular-weight precursor polypeptides [1], which appears to be accomplished by a set of unidentified proteases of both cellular and viral origin [2]. The viral origin of at least one of these enzymes is best demonstrated by its synthesis in cell-free systems under the direction of the viral RNA [3–5]. The kinetics of the appearance of the protease activity in such systems strongly suggests that the enzyme under question is encoded in the central part of the encephalomyocarditis (EMC) virus genome [4–6]. On the other hand, it has been reported that it is an EMC virus capsid protein, polypeptide γ, encoded in the 5′-end-adjacent part of the genome, that is responsible for the virus-specific proteolytic activity [7]. The significance of the latter finding has, however, been doubtful since there exists another virus-specific polypeptide, p22 or p23, which is apparently coded for by the central region of the viral RNA molecule and which migrates, upon electrophoresis, just slightly ahead of γ and, therefore, could easily be taken for this capsid protein in poorly resolving gels [4,8–10].

Here we present evidence that the polypeptide p22 is in fact involved in the cleavage of the EMC virus precursor polypeptides.

2. Methods

2.1. *Fractionation of extracts from virus-infected cells*

Krebs-2 cells infected with EMC virus at a multiplicity of 10–100 p.f.u./cell were incubated with ^{14}C-labeled *Chlorella* hydrolysate (10 μCi/ml) between 3.5 and 4.5 h infection at 37°C. S_{30} extracts from the infected cells [11] were centrifuged in a Beckman SW-50.1 rotor at 50 000 rev./min for 4 h at 4°C and the postribosomal supernatant thus obtained was chromatographed consecutively on DEAE-52 cellulose (Whatman) and phosphocellulose (Sigma) columns essentially as in [7], the only significant modification being the use of a step-wise rather than a gradient elution procedure (see figure legends).

2.2. *Protease activity assay*

A mixture of [^{35}S]methionine-labeled high-molecular-weight precursor polypeptides to be used as a substrate in the protease activity assay, was synthesized in EMC virus RNA-programmed Krebs-2 cell-free system [11] during a 30 min incubation at 30°C. After addition of cycloheximide to 100 μg/ml final conc. and glycerol to 10%, the samples were frozen and stored at −40°C or −70°C until used. In the experiment presented in fig.1, the substrate was partially freed from ribosome-associated uncompleted polypeptides by centrifugation (105 000 × g, 60 min). For the assay, 10 μl substrate preparation was usually mixed with an equal volume of a fraction to be tested for protease activity and the mixture was incubated at 30°C for a time indicated in the figure legends.

2.3. *Slab-gel electrophoresis*

Samples to be analyzed were precipitated with cold 5% trichloroacetic acid (TCA), washed off with 5% TCA and then with acetone, dissolved in a dissociation buffer [12] and subjected to electrophoresis

in SDS-containing 10% or 12.5% polyacrylamide slab gels. In the experiment presented in fig.1, the reaction was stopped directly by the addition of the dissociation buffer and electrophoresis was carried out in a gradient (8–20%) polyacrylamide slab gel.

3. Results

To characterize the pattern of cleavage that should be expected from the action of the protease(s) sought, the high-molecular-weight precursor polypeptide substrate synthesized in vitro upon a short incubation with EMC virus RNA was incubated with an extract from the virus-infected cells. Figure 1 shows that the shortest (5 min) incubation of the substrate–enzyme mixture resulted in the appearance of the following polypeptides: D1, α, G, and two smaller polypeptides with an app. M_r of 14 000 and 12 000, p14 and p12, respectively. Upon a somewhat longer exposure of the substrate to the extract from infected cells, capsid polypeptides ε and γ were formed. The quantity of capsid polypeptides appeared to slowly increase, while the quantities of p14 and p12 decrease during the incubation. In general, the pattern of cleavage indicates that:

(i) The substrate contained precursor polypeptides, preA and A, corresponding to the 5′-end-adjacent part of the EMC virus genome;

(ii) The protease(s) present in the extracts from virus-infected cells were able to cleave these precursors to capsid polypeptides (ε, α and γ), non-capsid polypeptide G and small polypeptides p14 and p12.

The first steps of purification of the virus-specific protease(s) were by ion-exchange chromatography essentially as in [7]. The results of DEAE-cellulose chromatography of ^{14}C-labeled virus-specific polypeptides present in the extract from infected cells are shown on the left panel of fig.2. Aliquots of each fraction were assayed for protease activity and the results obtained are shown on the right panel of fig.2. It should be noted that both the substrate and the enzyme preparations were radioactively labeled in our experiments. However, a significant difference in the specific radioactivities of ^{14}C- and ^{35}S-labeled amino acids incorporated in proteins of the enzyme and the substrate preparations, respectively, allowed us to

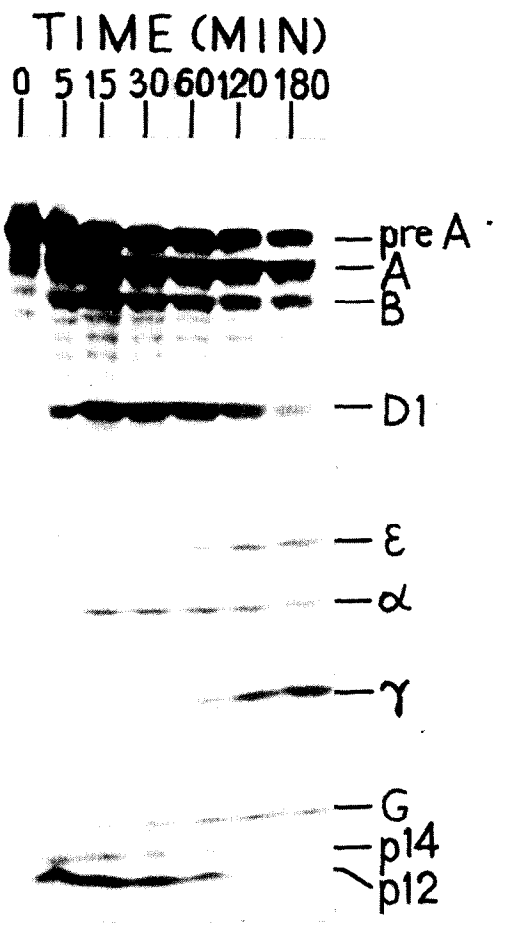

Fig.1. Kinetics of the cleavage of precursor polypeptides by extracts from EMC virus-infected Krebs-2 cells. A preparation of [^{35}S]methionine-labeled substrate polypeptides was mixed with 0.5 vol. S_{30} from infected cells and incubated at 30°C. At time intervals indicated aliquots were withdrawn and analyzed in an SDS-containing gradient (8–20%) polyacrylamide slab gel.

follow the substrate ^{35}S-label redistribution without any appreciable interference from the ^{14}C-label present in the virus-specific polypeptides of the enzyme preparations. In fact, the quantity of ^{14}C-labeled material present in the protease activity assays was 1–2 orders of magnitude (fig.2) or ⩾2 orders of magnitude (fig.3) lower than that used for the detection of virus-specific polypeptides in chromatographic fractions.

Judging by the appearance of stable cleavage

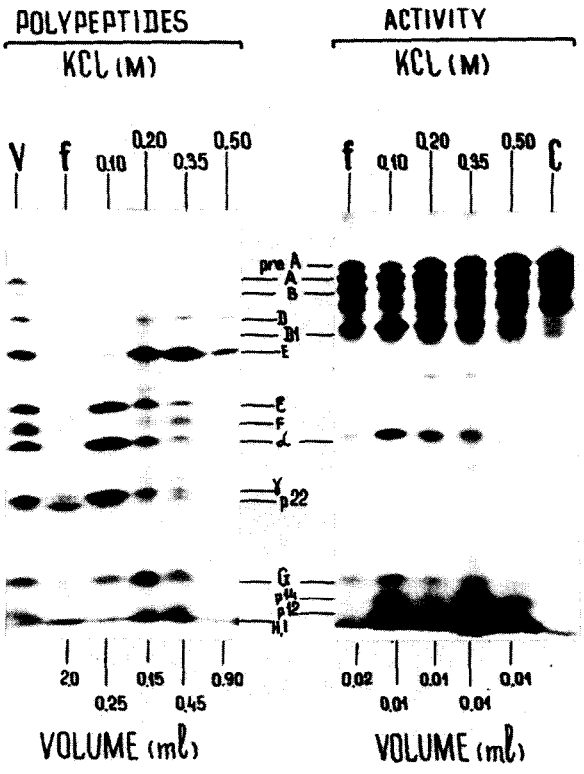

Fig.2. Fractionation on DEAE-cellulose of the postribosomal supernatant from infected cells and assay of the fractions obtained for protease activity. 10 ml of a postribosomal supernatant (see Methods) were diluted 10-fold with buffer A (0.01 M Tris–HCl (pH 7.5), 0.01 M 2-mercaptoethanol, 10% glycerol) containing 0.01 M KCl and applied onto a DEAE-cellulose column (6 ml) equilibrated with the same solution. The column was washed off with 35 ml 0.01 M KCl in buffer A and then eluted consecutively with 15–25 ml portions of buffer A containing increasing concentrations of KCl as indicated over the electrophoregram. Radioactivity in the acid-insoluble material in aliquots of the flow-through (f) and 3 ml fractions was determined and fractions of each eluate with maximal radioactivity were pooled. A volume indicated below the electrophoregram was withdrawn from each pool and the proteins were subjected to electrophoresis (left panel of fig.). Slot V contained ^{14}C-labeled extract from the virus-infected cells. Aliquots of the same chromatographic eluates, 10 μl each, except the flowthrough (f) where a 20 μl portion was used, were mixed with 10 μl aliquots of the ^{35}S-labeled substrate polypeptides and were incubated at 30°C for 4 h. The results of an electrophoretic analysis of products formed upon this incubation are presented on the right panel of the figure. Slot C contained the control substrate incubated without any additions. Electrophoresis was performed in an SDS-containing 10% polyacrylamide slab gel.

products, polypeptides α and G, the highest proteolytic activity seemed to be present in a fraction eluted from DEAE-cellulose with 0.1 M KCl. However, it should be kept in mind that the flowthrough material, which was considerably diluted, also displayed a marked activity. This material was relatively enriched in p22. Therefore, the 0.1 M KCl eluate and the flowthrough material were subjected to further purification by phosphocellulose chromatography.

As shown in fig.3, there was a clear-cut correlation between the protease activity and the presence of p22. In both cases (fig.3A,3B), p22 was especially abundant in the 0.25 M K-phosphate eluate and it was this fraction that displayed a maximal proteolytic activity. It is noteworthy that the material not retained by phosphocellulose and containing significant amounts of γ was essentially incapable of performing the cleavage; neither was there a correlation between the enzyme activity and the presence of polypeptide G (see fig.3B). It may be noted that the active fractions obtained by chromatography appeared to fail to bring about the formation of significant amounts of polypeptides ε and γ, although small quantities of these polypeptides could well be overlooked.

4. Discussion

The results of this study strongly suggest that virus-specific polypeptide p22 is involved in the formation of capsid proteins as well as polypeptide G from their precursor(s). The unpublished preliminary observations of another group also suggest that p22 is a virus-coded protease [5].

It should be admitted that preparations of p22 used were somewhat contaminated with trace amounts of some other virus-specific polypeptides and unknown amounts of host cell proteins. However, the protease activity in chromatographic fractions correlated with the presence of none of virus-specific polypeptides, except p22. Consecutive chromatography of EMC virus-specific polypeptides on DEAE-cellulose, Sepharose-6B and phosphocellulose in the presence of 8 M urea resulted in preparations of p22 lacking detectable amounts of other virus-specific polypeptides; these preparations displayed a protease activity after removal of urea (data not shown). As far as cellular contaminants are concerned, it should be noted that

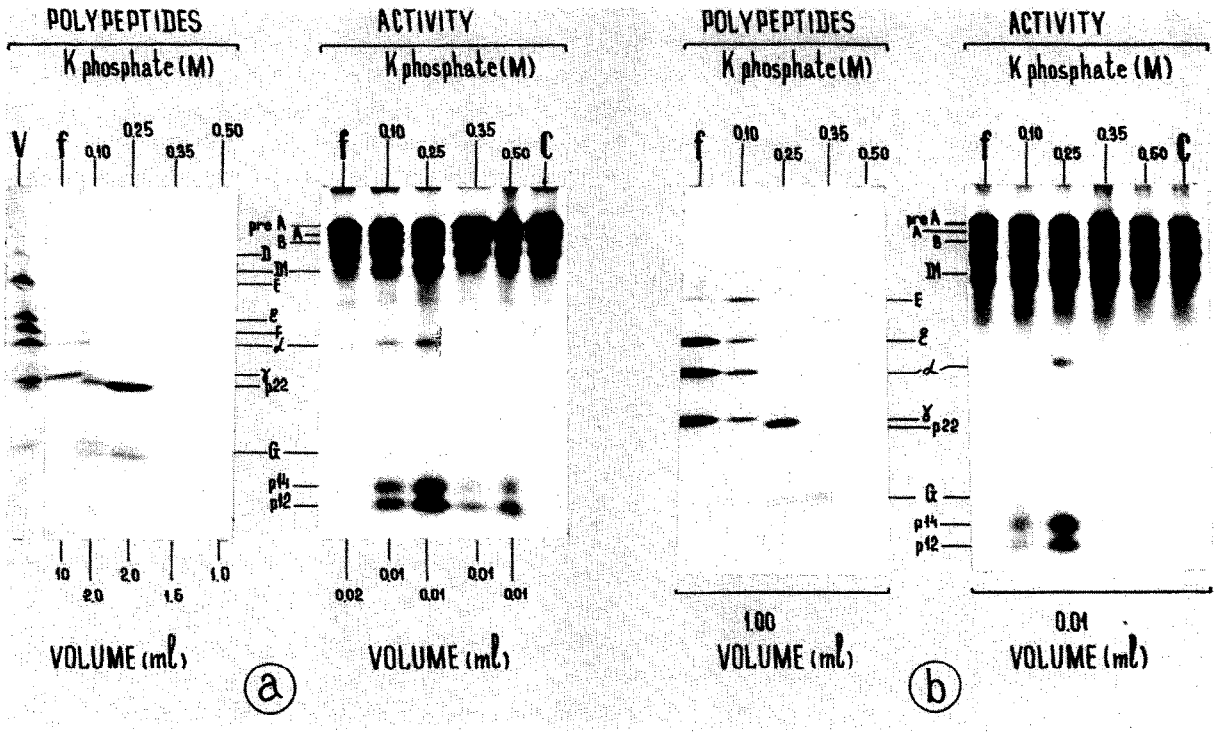

Fig.3. Fractionation on phosphocellulose of the flowthrough material (a) and the 0.1 M KCl eluate (b) obtained after DEAE-cellulose chromatography. Before chromatography, the 0.1 M KCl eluate was diluted with buffer A (see legend to fig.2), so that KCl was final conc. 0.03 M. The chromatography was performed on a phosphocellulose column (1 ml) equilibrated with buffer B (0.05 M K-phosphate (pH 6.8), 0.0005 M 2-mercaptoethanol, 10% glycerol). The unadsorbed material was washed off with 0.03 M KCl in buffer A and the elution was performed with 7–10 ml portions of buffer B with increasing concentrations of K-phosphate as indicated over the electrophoregram. For the protease activity assay, 10 μl aliquots of each fraction were used, except for the flowthrough material (f) in panel (a) where a 20 μl aliquot was used. Electrophoresis was performed in an SDS-containing 12.5% polyacrylamide slab gel. For other explanations see legend to fig.2.

extracts from uninfected cells fail to accomplish the cleavage of preA (A) [3,4]. This important fact, however, cannot be regarded as a rigorous disproof of the involvement of some cellular protein(s) in the formation of EMC virus capsid polypeptides and polypeptide G from their precursors. The cleavage system might be composed of more than one enzyme and activator protein(s) of viral and host origin. In this respect, the apparent absence of polypeptides ϵ and γ from the p22-mediated cleavage products may be of some interest. Certainly, this absence may simply be due to the low concentration of p22 present in the assay system and a relatively low susceptibility of the corresponding cleavage sites to the proteolytic action of p22; indeed ϵ and γ are the last to appear upon the precursor cleavage by extracts from infected cells (fig.1). Alternatively, the failure of p22 to form ϵ and γ may be due to a demand for an additional protein(s) involved in the cleavage. The solution of this problem requires the availability of more purified and concentrated preparations of p22.

Acknowledgements

We are greatly indebted to Dr Roland R. Rueckert who made available to us a preprint of his paper prior to publication. We also thank T. Smetanina and N. Sokolova for technical assistance.

References

[1] Rueckert, R. R. (1976) in: Comprehensive Virology (Fraenkel-Conrat, H. and Wagner, R. eds) vol. 6, pp. 131–213, Plenum, New York.
[2] Butterworth, B. E. (1977) Curr. Top. Microbiol. Immunol. 77, 1–41.
[3] Pelham, H. R. B. (1978) Eur. J. Biochem. 85, 457–462.
[4] Agol, V. I., Chumakov, K. M., Dmitrieva, T. M., Svitkin, Yu. V. (1979) in: Soviet Sci. Rev. (Biology), Overseas Publ. Assoc., Amsterdam, in press.
[5] Shih, D. S., Shih, C. T., Zimmern, D., Rueckert, R. R. and Kaesberg, P. (1979) J. Virol. 30, 472–480.
[6] Svitkin, Yu. V., Gorbalenya, A. E., Kazachkov, Yu. A., Agol, V. I. (1979) FEBS Lett. 108, 6–9.
[7] Lawrence, C. and Thach, R. F. (1975) J. Virol. 15, 918–928.
[8] Butterworth, B. E. and Rueckert, R. R. (1972) Virology 50, 535–549.
[9] Medvedkina, O. A., Scarlat, I. V., Kalinina, N. O. and Agol, V. I. (1974) FEBS Lett. 39, 4–9.
[10] Medvedkina, O. A., Scarlat, I. V., Kalinina, N. O. and Yatsina, A. A. (1977) in: Virus Inhibitors and the Mechanisms of their Action, pp. 129–136, Zinatne, Riga.
[11] Svitkin, Yu. V. and Agol, V. I. (1978) FEBS Lett. 89, 7–11.
[12] Gorbalenya, A. E., Chumakov, K. M. and Agol, V. I. (1978) Virology 88, 183–185.