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PROTEOLYTIC ACTIVITY OF THE NONSTRUCTURAL POLYPEPTIDE p22 OF ENCEPHALOMYOCARDITIS VIRUS

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Summary. A nonstructural polypeptide, p22, of encephalomyocarditis virus has been partially purified from extracts of virus-infected cells. Evidence is presented suggesting that this polypeptide, rather than some contaminants, possesses a proteolytic activity. Purified preparations of p22 containing no other detectable virus-specific proteins, cleave a high-molecular-weight virus-specific precursor-polypeptide into capsid polypeptides, ε, α, and γ, nonstructural polypeptide G as well as several low-molecular-weight products.

Introduction

Structural and nonstructural proteins of picornaviruses are formed from a giant precursor-"polyprotein" by a number of consecutive cleavages (1). It has recently been shown that an encephalomyocarditis (EMC) virus-specific nonstructural polypeptide with a M_r of 22,000, p22, is involved in the proteolytic cleavages that lead to the appearance of at least some capsid polypeptides as well as noncapsid polypeptide G. This conclusion was based largely on two types of experiments. Firstly, when EMC virus RNA was translated in a cell-free system, the corresponding proteolytic activity could be detected simultaneously with the synthesis of p22 (2). Secondly, partially purified preparations of p22 were shown to convert a precursor polypeptide, preA, into poly-

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peptide D1 (an intermediate in the capsid polypeptides formation), capsid protein α, non-capsid polypeptide G, and some low-molecular-weight polypeptides (3,4). However, the exact role of p22 in the formation of EMC virus capsid proteins remained unknown.

This study was aimed at answering two questions. Is p22 itself a protease or does it function as a protease activator? Is the cleavage of polypeptide D1 into capsid proteins ε and γ accomplished by p22 or by another protein? Evidence presented here strongly suggests affirmative answers to both questions.

METHODS

Purification of p22. Krebs-2 cells infected with EMC virus at a multiplicity of 10 to 100 PFU/cell were incubated with ^{14}C-labeled protein hydrolysate from 3.5 to 4.5 hr of infection at 37°. S_{30} extracts were prepared as described previously (3), but 0.1 volume of a solution containing 10% Nonidet (NP_40) and 5% deoxycholate was added after swelling the cells in a hypotonic buffer. The concentration of KCl in the S_{30} was increased up to 0.5 M and the extract was centrifuged in a Beckman SW65 rotor at 49,000 at 4° for 4 hr. The detailed procedure of purification of p22 from the resulting supernatant (S_{250}) will be described elsewhere. Briefly, it included the following consecutive steps: (i) chromatography on a DEAE-cellulose column, (ii) gel filtration on a Sepharose-6B column, (iii) chromatography on a phosphocellulose column, and (iv) chromatography on a hydroxyapatite column. Steps i through iii were carried out in the presence of 7 M or 8 M urea and all the solution used contained 0.01% or 0.1% Triton X-100. The purified preparations of p22 were concentrated with the help of Aquacide, dialysed against 30 mM tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 14 mM mercaptoethanol, 0.01% Triton X-100 (buffer A), supplemented with glycerol (10% final concentration) and stored at -70°.

Electrophoresis was performed in SDS-containing 12.5% polyacrylamide slabs as described (5). The gels were stained with Coomassie R-250, destained, and fluorographed (6).

Determination of proteolytic activity of p22 was carried out using two substrates, denatured bovine serum albumin (BSA) and polypeptide precursor of EMC virus capsid proteins. [^{125}I]BSA was kindly donated by Dr. V.N. Sidorov and additionally purified by SDS-polyacrylamide gel electrophoresis. The purified protein (sp. act. about 7 x 10^5 cpm/µg) dissolved in 30 mM tris-HCl, pH 7.5, 0.5% SDS, 1 mM dithiothreitol was denatured by heating at 95° for 5 min. The assay of proteolytic activity was a modification of the procedure descri-

bed (7). Two µl of the preparation of p22 was preincubated for 1 hr at 30° with 2 µl of either a buffer containing 30 mM tris-HCl, pH 7.5, 140 mM KCl, 60% dimethylsulfoxide (DMSO) or 6 mM $ZnCl_2 \cdot 3NH_4Cl$ prepared on the same buffer. One µl of 80 mM dithiothreitol, 80 mM KCl, 20% DMSO was then added, and after 10 min incubation at 30°, 2 µl of [^{125}I]BSA (~3,700 cpm) were introduced. Incubation was carried out at 30° for 24 hr under 25 µl of Bayol F.

Assay of the ability of p22 preparations to cleave precursors of EMC virus capsid proteins was performed essentially as described (3) but samples of smaller volumes were incubated for longer time intervals under Bayol F (4).

For the detection and identification of proteases a new technique was also worked out, which represented a reciprocal variant of a partial proteolysis method (2,8). A 7.5% polyacrylamide gel strip polymerized in the presence of highly-labeled protein substrate [^{125}I]BSA [substrate strip, see Fig. 2(c)] was laid over a vertical 20% polyacrylamide slab (resolving gel). Another strip (sample strip) was excised from the 12.5% polyacrylamide slab after 1st dimension electrophoresis of a protein mixture, in our case, a partially purified preparation of p22. After excision from the first slab, the sample strip was soaked in a buffer containing 50 mM tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA. When both the substrate and sample strips were in place over the resolving gel, not yet polymerized 7.5% polyacrylamide was added to fill the space between the strips and the resolving gel. After polymerization, 2nd-dimension electrophoresis was carried out. Protease(s) present in the sample strip entered the substrate strip, attacked [^{125}I]BSA, and radioactive peptides thus formed migrated in the resolving gel in front of BSA. These peptides were detected by fluorography.

RESULTS AND DISCUSSION

Purification of p22. In our previous study (3) the purification of p22 from the extracts of virus-infected cells was accomplished by consecutive chromatography on DEAE-cellulose and phosphocellulose. The resulting preparations were highly enriched in p22, but, in addition, contained minor quantities of other virus-specific polypeptides and unknown amounts of cellular proteins. In an attempt to achieve a better purification, we included in the protocol two additional steps (see Methods). Furthermore, urea and Triton X-100 were added to the solutions, since this modification appeared to improve the yield of p22.

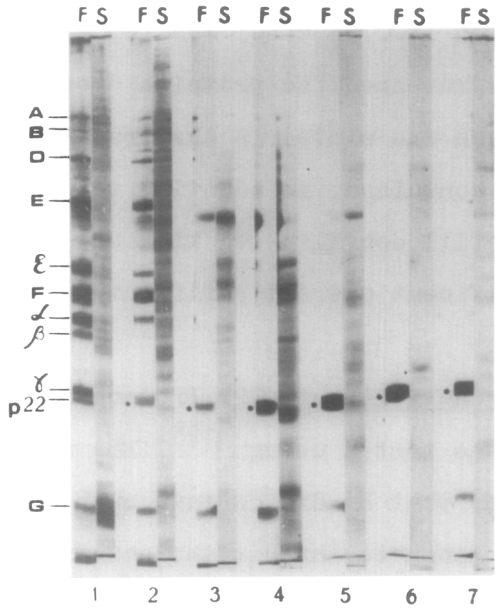

Fig. 1. Purification of p22. All the preparations were run on the same gel and photographs of portions of the fluorogram (F) and stained gel (S) are presented. 1. Non-fractionated extract of virus-infected cells; 2. S_{250}. 3. Material unadsorbed on DEAE-cellulose in 10 mM tris-HCl, pH 8.45, 14 mM mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100, 7 M urea. 4. Material enriched in p22 obtained upon gel filtration on Sepharose 6B (20 mM HEPES-KOH, pH 6.7, 50 mM KCl, 14 mM mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100, 8 M urea; buffer S). 5. Material eluted from phosphocellulose in buffer S containing 170 mM KCl. 6. Material eluted from hydroxyapatite in 10 mM HEPES-KOH, pH 6.7, 140mM KCl, 3 mM dithiothreitol, 0.01% Triton X-100; preparation H1. 7. Material eluted from hydroxyapatite in 20 mM tris-HCl, pH 7.5, 400 mM KCl, 3 mM dithiothreitol, 0.01% Triton X-100, 8 M urea; preparation H2.

The results obtained at each step of the purification procedure are presented on Fig. 1. Material which was not adsorbed on DEAE-cellulose contained two major viral proteins, p22 and G, as well as many cellular polypeptides. Gel filtration on Sepharose 6B resulted in the loss of some host contaminants, and chromatography on phosphocellulose lead to the preparation which was highly enriched with p22 but contained some G and cellular material. Upon chromatography on hydroxy-

apatite, p22 was distributed among two fractions, H1 and H2. Fraction H1 eluted at 0.14 M KCl was found to be devoid of any detectable virus-specific proteins except p22, whereas fraction H2, which was eluted in the presence of 8 M urea and 400 mM KCl, contained, in addition to p22, some G. Both fractions were still contaminated with cellular proteins, although a significant overall purification seemed to be achieved.

Proteolytic activity of p22. Proteolytic activity of the preparation H1 was tested using [^{125}I]BSA as substrate. It is seen on Fig. 2(a) that H1 did contain such an activity and that Zn^{++} ions, known to inhibit the activity of picornavirus-specific protease (9), depressed markedly proteolysis of BSA mediated by H1. Since H1 contained, in addition to p22, some cellular proteins, one might argue that it was these cellular contaminants, rather than p22, that were responsible for the cleavage of BSA. Therefore, an attempt was made to directly estimate M_r of the protease.

Another preparation of partially purified p22, H2, was subjected to electrophoresis, and the proteolytic activity of its protein constituents was investigated by a specially devised technique described under Methods. As shown in Fig. 2(b), virus-specific polypeptides p22 and G present in H2, were situated in the resolving gel, after the 2nd-dimension electrophoresis, on a diagonale, as could be expected taking into account their M_r's. However, the vertical lane coresponding to p22 additionally contained radioactive material migrating both ahead and behind of p22 (arrows). These additional spots evidently represented products of proteolysis of BSA, which could be formed only by an enzyme having a M_r

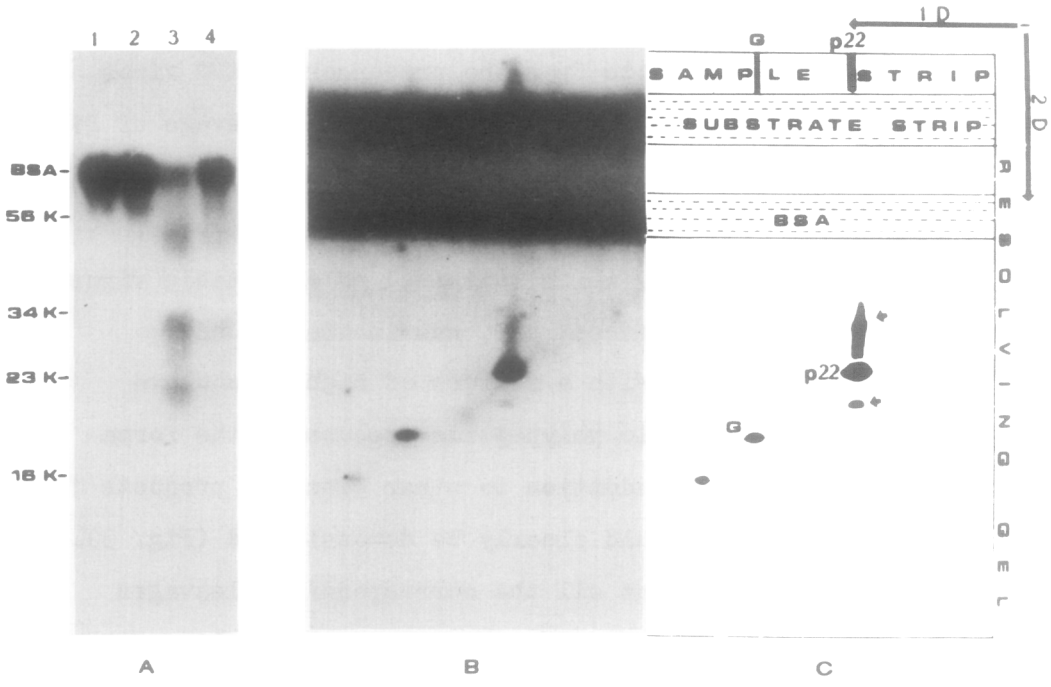

Fig. 2. Proteolytic activity of p22. A. Effect of a preparation of p22 (preparation H1; see Fig. 1) on [^{125}I]BSA. Electrophoretic pattern of reaction products. 1. Untreated [^{125}I]BSA. 2. [^{125}I]BSA incubated in the absence of H1. 3. [^{125}I]BSA incubated with H1. 4. [^{125}I]BSA incubated with H1 pretreated with $ZnCl_2$. For details see Methods. Positions of the marker proteins run on the same gel are indicated. B. Estimation of M_r of the protease present in a preparation of p22 (preparation H2). A sample of H2 was subjected to electrophoresis in a SDS-polyacrylamide slab, a strip (sample strip) was excised and the ability of protease contained in it to attack [^{125}I]BSA polymerized in the substrate strip was analyzed as described under Methods. Heavy radioactivity in the region of the substrate strip after electrophoresis is explained by covalent binding of a portion of [^{125}I]BSA to polyacrylamide(10). C. Schematic representation of panel B.

identical to that of p22. Thus, these data strongly suggest that the protease activity residues in p22 itself, rather than in contaminating cellular material.

The alternative explanation that the proteolysis is mediated by a host protein with a M_r of 22,000 seems to be highly unlikely, although cannot be excluded completely.

p22-Mediated capsid protein formation. It has been shown previously that partially purified preparations of p22 bring

about the formation of polypeptides D1, α, G and some low-molecular-weight products from the precursor of EMC virus capsid proteins (3,4). On the other hand, the cleavage of D1 into ε and γ has not as yet been registered, leaving the possibility that it may require other proteins. However, when the preparation H1, which contained no detectable virus-specific polypeptides except p22, was incubated under appropriate conditions with a mixture of high-molecular-weight EMC virus-specific polypeptide precursors, the formation of ε and γ, in addition to other cleavage products (D1, α, G, p14, p12) could clearly be demonstrated (Fig. 3). This result suggests that all the corresponding cleavages are accomplished solely by p22, although participation of some other proteins (present in the preparations of the substrate) in certain steps of the processing of the capsid protein precursor cannot as yet rigorously be ruled out.

Concluding remarks. The results presented in this paper strongly suggest that polypeptide p22 is responsible for the cleavage of the precursor of capsid proteins of EMC virus. The nature of protease(s) which cleaves polypeptides encoded in the 3'-terminal portion of the viral genome remains, however, unknown. In particular, it is important to elucidate how p22 itself is formed from its precursor. Two possibilities may be considered. (i) p22 is cleaved off from its precursor by another, possibly cellular, protease. (ii) The polypeptide sequence corresponding to p22, while being a part of the precursor molecule, possesses a proteolytic activity, permitting autocatalytic processing. Further experiments are required to solve this problem.

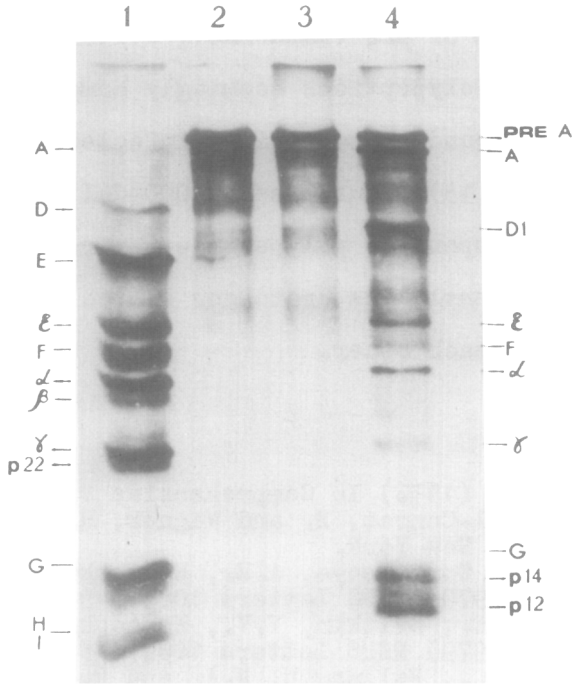

Fig. 3. Cleavage of precursor(s) of EMC virus capsid polypeptides by a preparation of p22. Electrophoretic patterns. 1. Non-fractionated extracted of [^{14}C]-labeled EMC virus-infected Krebs-2 cells. 2. [^{35}S]-labeled polypeptides synthesized upon a short incubation in a cell-free system programed with EMC virus RNA (substrate); the translation was terminated by addition of cycloheximide (3). 3. One µl of the substrate was incubated with 1 µl of buffer A for 24 hr at 30°. 4. One µl of the substrate was incubated with 1 µl of H1 for 24 h at 30°.

It was reported recently (11) that the proteolytic activity specified by another picornavirus, poliovirus, copurified with a protein having a M_r of 40,000; the authors suggested that it was polypeptide NCVPX. If this assignment is correct, either the protease activity of two picornaviruses resides in entirely different proteins, or two different proteases are encoded in the picornavirus genome. However, another possibility should be kept in mind. Upon translation *in vitro* of EMC virus RNA, a polypeptide with a M_r of about 39,000, p39, is formed (12), and this polypeptide contains

amino acid sequence of p22 (Kazachkov, Svitkin, and Agol, to be published). Polypeptides seemingly homologous to p39 and p22 are also found in poliovirus-infected cells (6a and 7c, respectively) (13). Thus, the 40,000-dalton poliovirus protease may correspond to polypeptide 6a, rather than to NCVPX, and consequently the proteases of two picornaviruses may be related to each other.

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