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Evidence for Intramolecular Self-Cleavage of Picornaviral Replicase Precursors

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It has previously been shown that when encephalomyocarditis viral RNA is translated in cell-free extracts of rabbit reticulocytes, it synthesizes a virus-coded protease, p22, which is derived by cleavage of a precursor protein, C. Protein C is shown here to be cleaved by two different mechanisms, which were distinguished by their sensitivity to dilution. One mechanism was sensitive to dilution; the other was not. The biphasic cleavage behavior was unchanged by diluting incubation mixtures with untranslated reticulocyte extract instead of buffer, suggesting that both types of cleavage were mediated by virus translation products. It is proposed that the dilution-sensitive cleavage of protein C is due to a virus-coded protease, probably p22 itself, and that the dilution-independent cleavage is due to intramolecular self-cleavage of protein C.

Translation of encephalomyocarditis (EMC) RNA in extracts from rabbit reticulocytes proceeds primarily from a single origin to form a large polyprotein product (molecular weight, 240,000) from which functional viral proteins are derived by an extensive array of proteolytic cleavage reactions (4, 18). The initial processing events occur while the polyprotein is still nascent on ribosomes and generate a set of primary products called A1 (molecular weight, 108,000), F (molecular weight, 38,000), and C (molecular weight, 84,000) (3, 4).

Protein C is then further cleaved into several smaller polypeptides, including one designated p22, which has been shown (6, 7, 15, 21) to catalyze cleavage of capsid precursor A1 to immature capsid promoters ($\varepsilon\gamma\alpha$) thus: A1 $\rightarrow$ A $\rightarrow$ (B?) $\rightarrow$ (D1 + α) $\rightarrow$ ($\varepsilon\gamma\alpha$).

It has been proposed that p22 is also responsible for processing of protein C (15).

$$C \overset{H}{\nearrow} D \overset{E}{\nearrow} p22$$

However, such a mechanism leaves unexplained the process by which precursors C and D are cleaved before p22 has been generated initially. These first C cleavage reactions might, for example, be mediated by a second virus- or host-coded protease. An alternate possibility is that these molecules are self-cleaving by virtue of the p22 sequences contained within them. We now present evidence in support of the latter possibility. A preliminary account of this work was reported previously (17).

MATERIALS AND METHODS

In vitro translation. EMC RNA-dependent protein synthesis in rabbit reticulocyte lysates was carried out exactly as previously described (15, 19, 20). The standard protein synthesis mixture contained 90 μM each of 19 unlabeled amino acids and about 1 μM [^{35}S]methionine (1,200 Ci/mmol). Incubation was for 45 min at 30°C, at which time the reactions were terminated by the addition of cycloheximide and pancreatic RNase to 5 and 10 μg/ml, respectively. Mock-translated lysate for use as diluent was treated as above, except that the reaction mixture contained 1 μM unlabeled methionine, and EMC RNA was omitted.

Gel electrophoresis. Procedures for slab gel electrophoresis have been described previously (15). All gels contained 5 M urea, which improved the separation of protein p22 from γ and ε1 from ε. Sample preparation before electrophoresis included an acetone precipitation step (15). Detection of ^{35}S-labeled protein bands by autoradiography and fluorography was previously described (15).

RESULTS

Evidence for intramolecular cleavage of proteins C and D. The rate of a bimolecular reaction between a free enzyme and its substrate is expected to be directly dependent upon the concentration of each. This is because the probability of collision, and therefore potentially productive interaction, increases as the product of their molar concentrations. Conversely, the rate of an intramolecular or self-cleaving autocatalytic reaction should be independent of concentration, since in these reactions the enzyme (active site) and substrate are contained within the same molecule.

To test whether the proteolytic cleavages of EMC proteins C and D are sensitive to concentration effects, viral proteins synthesized in vitro were allowed to process over a wide range of dilutions (Fig. 1). The starting sample was a reticulocyte lysate which had translated EMC RNA for 45 min, a period which allowed about one round of synthesis (20). Under these conditions, proteolytic processing is still at an early stage. Thus, capsid proteins were present primarily in the precursor forms A1, A, and B, plus smaller amounts of (D1, α) (Fig. 1, lane a). Similarly, protein C was a strong band, but the appearance of D, E, and p22 indicates that the agent(s) responsible for cleavage of this region was also present and active.

Samples identical to that of lane a were diluted with increasing amounts of buffer, then incubated to allow further cleavages to occur. The proteins were collected by acetone precipitation and subjected to electrophoresis for identification. Since the translation reaction was terminated by the addition of cycloheximide and pancreatic RNase, any changes in the protein band profiles relative to that of lane a are due solely to proteolytic processing during the incubation period.

Protein F, a stable primary cleavage product (4), was not processed further. Hence, differences in the intensity of the F bands reflect slight variations in the amount of sample applied to respective lanes.

The processing of capsid proteins (i.e., disappearance of A1, A, and B and the appearance of

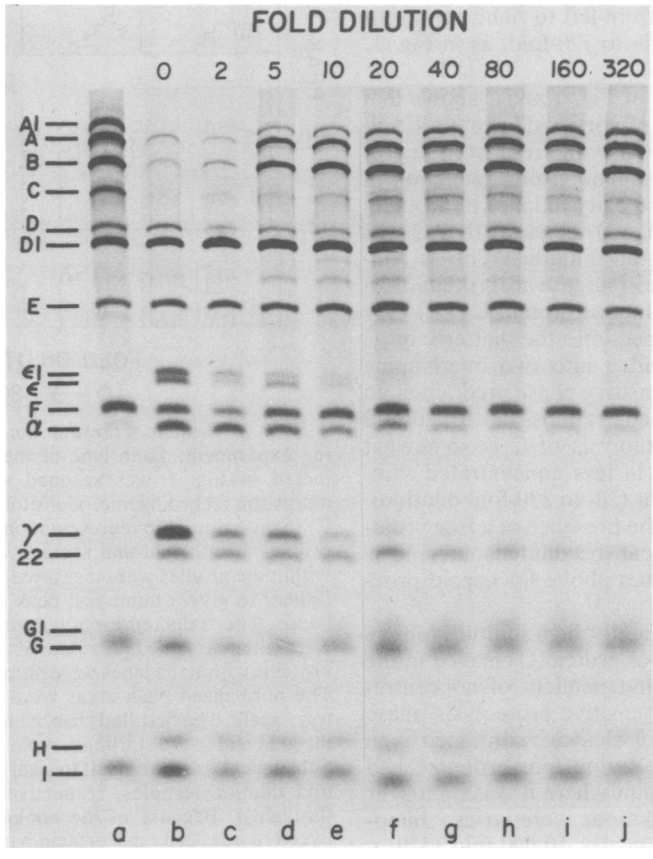

FIG. 1. Effect of dilution into buffer on in vitro proteolytic processing. In vitro EMC RNA-dependent protein synthesis in rabbit reticulocytes (120 μl) was carried out as described in the text. After the addition of cycloheximide and pancreatic RNase, a sample (10 μl) was made 1% in SDS, then chilled on ice (lane a). Identical samples were distributed to tubes containing 0, 10, 40, 90, 190, 390, 790, 1,590, or 3,190 μl of buffer (50 mM Tris-acetate [pH 7.5], 5 mM magnesium acetate, 5 mM dithiothreitol, 0.2 mM EDTA, 100 mM potassium acetate) (lanes b through j, respectively). After incubation at 30°C for 2 h, all samples were brought to 3,200 μl by the addition of buffer, then made 1% in SDS. The proteins were collected by acetone precipitation, subjected to electrophoresis on a slab gel, then visualized by autoradiography. Protein ε1, an unmapped viral protein (15), appeared and disappeared in a manner nearly identical to that of ε, α, and γ, suggesting that it also arises from the capsid region of the genome. This protein might represent a precursor form of ε.

ε, γ, and α) occurred most strongly in the undiluted sample (Fig. 1, lane b). The extent of processing of these precursors decreased progressively with dilution (lanes c through f) until, at dilutions higher than 20-fold, there was no longer any detectable change in the amount of capsid precursors relative to those in lane a. Thus, cleavage of the capsid proteins exhibited strong concentration dependence, just as expected for a bimolecular reaction in which the protease (p22) must collide with its substrate before processing can take place.

The dilution patterns of Fig. 1 are illustrated graphically in Fig. 2, where the peak areas for each protein, as determined by densitometer tracings of the autoradiogram, have been normalized to a constant value for protein F. The hatched bar at the left side of each block graph corresponds to the starting sample (Fig. 1, lane a). Subsequent bars from left to right, represent increasing dilutions (0- to 320-fold) as in Fig. 1, lanes b through j.

Careful inspection of Fig. 1 and 2 shows that proteolytic cleavage of noncapsid protein C followed a dilution response different from that of the capsid precursors. The strong reduction in the C protein band intensity in lanes b through j relative to lane a (Fig. 1) indicates that C was efficiently cleaved at all dilutions. This is confirmed by the parallel increase in protein H, a known cleavage product of protein C (14).

Upon further inspection, the pattern of C cleavage can be divided into two overlapping phases. A dilution-sensitive phase predominated at high sample concentrations (0- to 20-fold dilution), where proteolysis of C is somewhat more complete than in less concentrated samples of the experiment (20- to 320-fold dilution). This phase suggests the presence of a free protease, whose activity can be diluted out, and is analogous to that noted above for capsid processing by p22.

However, at sufficiently high dilution (above 20-fold), the fraction of protein C cleaved in the experiment became independent of concentration. This dilution-insensitive proteolysis phase indicates that protein C cleavage continued even after the free protease acting upon protein C had been diluted to the point where it was no longer effective. If these reactions were strictly bimolecular, a decline of nearly 10,000-fold (320 × 320) in the processing rate would be expected between the lowest and highest dilutions, with intermediate samples showing a progressive decline of cleavage. This was clearly not the case. The simplest interpretation of the dilution-insensitive behavior of protein C at low concentrations is intramolecular self-cleavage, by virtue of the p22 protease sequences contained within it.

In view of this proposal, it was of interest to also examine the proteolysis of protein D. Since D is both a product (of C cleavage) and a precursor (of E and p22), its processing must be monitored indirectly by following the increase of its cleavage products. At all dilutions, E and p22

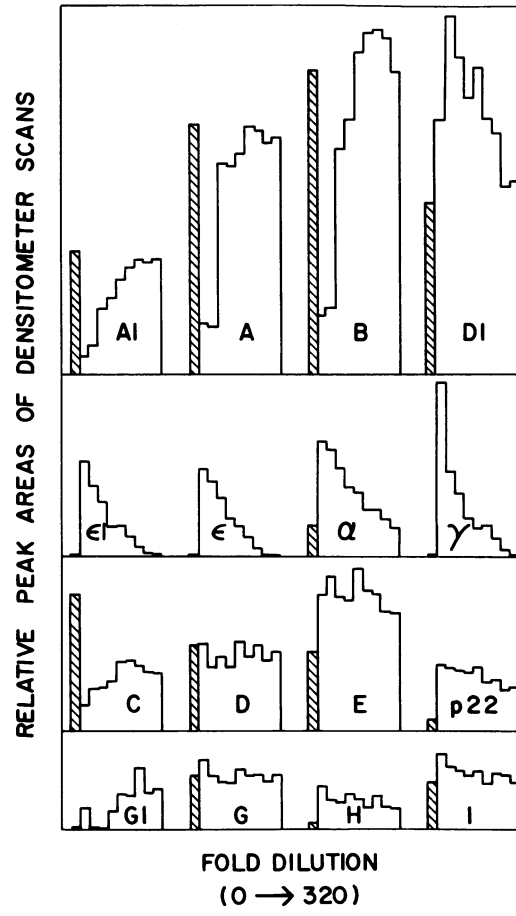

FIG. 2. Graphic representation of dilution processing experiment. Each lane of the autoradiogram depicted in Fig. 1 was scanned with a Joyce-Loebl automatic recording microdensitometer (model MKIII C) to give a graphic representation of the protein band profiles. The height and average width of every peak within the profiles were measured and combined (multiplied) to give a numerical peak area for the protein bands. The areas representing protein F for each lane were then assigned a value of one, and all other proteins within the lanes were normalized accordingly. The normalized peak areas were plotted on an arbitrary scale. The first (left) bar of each graph represents the gel band in the starting sample lane (Fig. 1, lane a). Subsequent bars, from left to right, represent 0- to 320-fold dilution samples, respectively (Fig. 1, lanes b through j). Because of the nonlinear correlation between radioactivity in a protein band and densitometer peak areas, these plots are of value chiefly in following processing trends and should not be misinterpreted as absolute quantification of the proteins.

were increased relative to the starting sample. This shows that D was also cleaved at a rate which was not strictly dependent upon concentration. Concentration-independent, efficient cleavage implies that the proteolysis of D, like that of its precursor, probably does not require a second protein and is consistent with an intramolecular, autocatalytic mechanism.

Interestingly, the concentration of protein D remained nearly constant throughout the dilution profile (Fig. 1 and 2), indicating that C and D cleavages in this experiment probably occurred at equivalent rates.

The dilution experiment was repeated several times under various incubation time, temperature, and buffer conditions. In every case, C and D cleavage showed a similar pattern of concentration independence at high dilutions, but not at low dilutions. To determine whether the dilution-sensitive protease responsible for cleaving C and D is an endogenous reticulocyte enzyme or one synthesized during translation of viral RNA, the dilution processing experiment was repeated with mock-translated lysate as diluent instead of buffer (Fig. 3). Under these conditions, the viral proteins were diluted, whereas the concentrations of reticulocyte enzymes remained constant. If the cleavage of proteins C and D was influenced by any lysate proteins, then proteolysis should be uniform and extensive at every dilution. As seen in Fig. 3, the processing pattern was the same as in Fig. 1, where the extracts were diluted into buffer. This result argues that the cleavage of C and D is not accelerated by a lysate protein; hence, the dilution-sensitive bimolecular cleavage must be mediated by a viral protease.

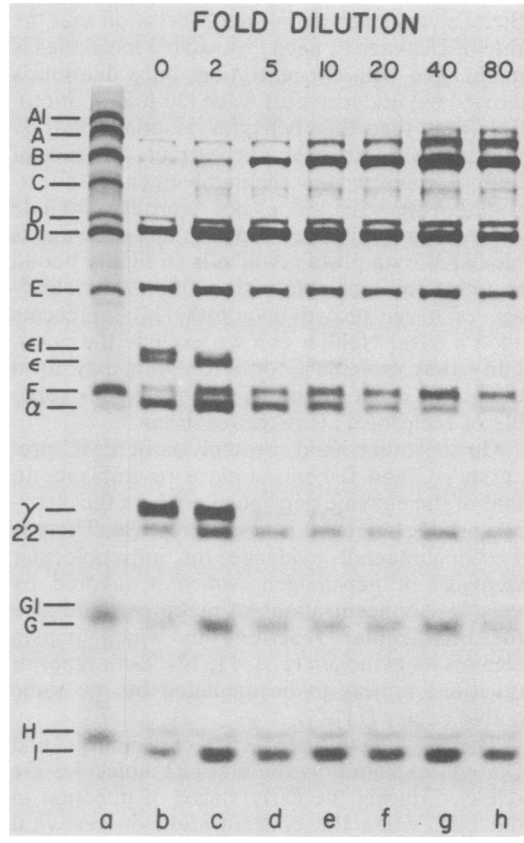

FIG. 3. Effect of dilution into mock-translated lysate on in vitro proteolytic processing. EMC RNA-dependent protein synthesis (30 μl) in rabbit reticulocyte lysates was carried out as described in the text. After the addition of cycloheximide and pancreatic RNase, a sample (1 μl) was made 1% in SDS, then chilled on ice (lane a). Identical aliquots (1 μl) were distributed to tubes containing 0, 1, 4, 9, 19, 39, or 79 μl of mock-translated lysate (lanes b through j, respectively), then overlaid with paraffin oil. After incubation at 30°C for 2 h, all samples were brought to 80 μl by the addition of mock-translated lysate, then made 1% in SDS. The paraffin oil was removed, and 6 μl from each sample (equivalent to 0.075 μl of the original lysate) was treated by an acetone precipitation step. These samples were subjected to electrophoresis on a slab gel, then visualized by fluorography.

DISCUSSION

We have shown here that the processing of EMC noncapsid proteins C and D proceeds by two independent pathways, which are distinguishable by the effects of dilution on cleavage rate. One pathway is sensitive to dilution; the other is not. We interpret the dilution-sensitive pathway as evidence for a bimolecular reaction, in which the activity of free protease is limited by the probability of productive collision with its substrate molecules, C or D. The dilution-insensitive pathway, on the other hand, appears to be best explained by intramolecular self-cleavage.

The inability of reticulocyte extracts to enhance cleavage of highly diluted viral translation products (Fig. 3) argues that the free protease is a virus-coded activity. A prime candidate for the C and D cleavage is EMC protein p22, which has already been shown to be involved in cleavage of capsid precursors (15, 21). This proposal is supported by experiments in which the processing of both capsid and noncapsid precursors is inhibited coordinately by *N*-ethyl maleimide, zinc, methyl mercuric chloride, and acetyl cystamine (3; A. C. Palmenberg, unpublished data).

The idea of a common protease operating on both replicase and capsid proteins is strongly reinforced by studies with poliovirus, where RNA sequence determinations have shown that capsid and replicase precursors homologous to EMC proteins A and C are cleaved at identical glutamine-glycine sites (9).

The proposal that proteins C and D can cleave

themselves is based on the observation that the rate of cleavage of newly translated molecules is unchanged with concentration, once dilution is carried beyond a critical value (20-fold dilution). The fact that this behavior is unaffected by diluting with reticulocyte extract, instead of buffer, argues against the involvement of a host-coded protease, but does not rigorously exclude the possibility that the apparent self-cleavage is mediated by a protein which is so tightly bound as to be undissociable with dilution. The cleavage of nerve growth factor (8, 24) represents such a case. Neither can we exclude the possibility that proteins C or D or both may form dimers or other undissociable aggregates capable of reciprocal cleavage reactions.

On the other hand, proteolysis of EMC precursors C and D bears a close resemblance to that of the enzyme pepsinogen, where the cleavage product, pepsin, is also a protease. There is well-documented evidence for intramolecular cleavage of pepsinogen, which is favored by very low concentrations of precursor, and also for bimolecular reactions in which pepsin cleaves its zymogen (1, 2, 11, 12). Both types of reactions appear to be mediated by the same active center (11).

The self-cleaving properties of proteins C and D could explain how the first p22 molecules are released during the early stages of infection in the cell, when the concentration of free viral proteins is virtually zero. Subsequent cleavages would then, presumably, be carried out by the free form of p22.

The autoproteolytic capacity of proteins C and D may also have important implications for the enzymology of picornaviral replication. One of the cleavage products, protein E, is associated with the viral RNA polymerase (14, 22). Another, protein H, contains the sequences of VPg, the viral genome-linked protein which is added to all new RNAs during, or shortly after, initiation of RNA synthesis (14, 16). A built-in protease activity would insure efficient cleavage of VPg or H or both out of protein C, especially during the early stages of replication when free viral protease is scarce.

The ability of C to cleave itself may also be relevant in attempts to purify the physiologically important forms of picornaviral replicase. It has been reported that replication complexes from Mengo, polio, and foot-and-mouth disease virus-infected cells contain only protein E or its counterpart in other viruses (5, 10, 13, 23). Earlier attempts to isolate polymerase proteins have not taken into account the potential for cleavage at very high dilutions. We suggest that failure to identify proteins C and D (or their homologs in other viruses) in these complexes does not preclude their involvement in viral replication, since their absence may be due to proteolytic interconversion of the proteins during isolation.

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