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Cleavage of Mengovirus Polyproteins In Vivo

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The synthesis of mengovirus-specific proteins in vivo was studied by labeling the viral proteins with radioactive amino acids under conditions in which host protein synthesis was almost completely inhibited. Pulse-chase experiments enabled the kinetic analysis of the cleavages of certain viral protein precursors and the formation of others. The pattern of cleavages of mengovirus precursor polypeptides is similar to that of encephalomyocarditis virus. The major difference between the two viruses seems to be in the molar concentration in which the various primary products are produced. The molar ratio of the A protein, which is the precursor of the capsid proteins, to that of the primary products F and C, is approximately 1.5 to 2.0:1:1. Possible explanations for the unequal appearance of the structural and nonstructural proteins are discussed.

In many, if not all, of the picornaviruses, functional viral proteins arise by select proteolytic cleavages of one large precursor molecule which represents the entire informational content of the viral genome. Two clues led to this realization. In poliovirus-infected HeLa cells, after a short pulse of radioactive amino acids, about 14 virus-specific proteins were identified, the combined molecular weight of which considerably exceeded the apparent coding capacity of the viral genome (13, 15). Also, after a chase period with unlabeled amino acids, radioactivity in some viral peaks disappeared, whereas in others it increased (8, 13).

Extensive studies have been carried out on the in vivo cleavage pattern of poliovirus (7, 8, 13, 14) and encephalomyocarditis (EMC) virus (2, 4) precursor proteins. The number and sizes of proteins made by these two viruses are quite similar. It has not been possible to demonstrate the presence of the largest theoretically possible precursor by normal pulse-labeling experiments in cells infected by either of these two viruses. This large precursor does accumulate in poliovirus-infected HeLa cells in the presence of amino acid analogues (7, 8) or certain proteolytic enzyme inhibitors (11, 16), such as tolylsulfonylphenylalanyl chloromethyl ketone (TPCK). In contrast, the giant precursor is readily detected in coxsackievirus B1-infected HeLa cells (10) even in the absence of amino acid analogues after a short pulse with radioactive amino acids.

If picornavirus proteins arise by cleavage of one giant precursor, there should be only one site of initiation of protein synthesis. This is the case with mouse Elberfeld virus, EMC virus, and mengovirus (12), as determined by studies on the incorporation of [^{35}S]methionine from fMet-tRNA$_f^{met}$ into viral RNA-coded proteins in extracts of Ehrlich ascites cells, and with poliovirus (14, 17), as shown by kinetic analyses with pactamycin, an inhibitor of the initiation of protein synthesis. The gene order of both poliovirus (14, 17) and EMC virus (3) RNA has been determined and is, from 5′ to 3′, δ, β, γ, α, G, I, F, H, and E, as designated by Butterworth and Rueckert (3) for EMC virus proteins.

Little is known about the mechanism of cleavage of picornavirus precursor proteins. The observation that inhibitors of proteolytic enzymes also inhibit cleavage (11, 16) has been interpreted to mean that host enzymes may be responsible for at least some of the cleavages (11). Other results (6, 11), however, suggest that virus-specified enzymes may also play a role.

The original objective of this research was to isolate and characterize the enzymes involved in the cleavages of mengovirus precursor proteins. Before this program could be initiated, it was necessary to characterize the in vivo synthesized products of mengovirus so that a standard of comparison would be available for analysis of our in vitro-cleaved products. Also, it was thought essential to understand the in vivo kinetics of cleavage of the precursors before attempting to study the in vitro cleavage. Thus, this report is concerned with the in vivo aspects of mengovirus cleavage of precursors and with our attempts to demonstrate the presence of the

giant mengovirus precursor by using amino acid analogues and proteolytic enzyme inhibitors.

Our results indicate that the pattern of post-translational cleavages of mengovirus precursors is similar to that observed with other picornaviruses. But in contrast to the findings of others, mengovirus proteins are not synthesized in equal amounts as expected if all cleavages occur from one giant polyprotein. These findings suggest that their synthesis is under some sort of control.

MATERIALS AND METHODS

Growth of L cells and infection with mengovirus. L cells, strain 929, were grown as monolayer cultures on Petri dishes (100 by 20 mm) in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (complete medium). When the monolayers were nearly confluent (2×10^7 cells), they were washed two times with phosphate-buffered saline (PBS), and infected with mengovirus (small plaque strain) at a multiplicity of infection (MOI) of 20 PFU per cell. After a 30- to 45-min adsorption period at 37 C, 15 ml of complete medium containing 3 μg of actinomycin D per ml was added and the infection was allowed to progress.

Five hours after infection the medium was aspirated and the cells were washed two times with PBS. The cells were then pulsed as indicated for either 7 or 12 min with 2.5 ml of MEM, lacking amino acids, 10 μCi of a [^{3}H]amino acid mixture per ml (NET 250, New England Nuclear), and those unlabeled amino acids absent from the labeled mixture, including methionine (0.13 μM), tryptophan (0.067 μM), and cysteine (0.027 μM). After the pulse period, the mixture was aspirated, and 15 ml of MEM containing a complete complement of unlabeled amino acids and 5% FCS was added for varying periods of time (chase period) as indicated in the figures. At the end of the chase period the medium was aspirated, the cells were washed quickly with ice-cold PBS and a mixture of 0.5 ml of boiling sodium dodecyl-sulfate (SDS), and 1% 2-mercaptoethanol was added. The cells were scraped from the monolayers, the mixture was heated for 3 to 5 min at 100 C to dissociate the proteins into their constituent polypeptide chains, and the samples were analyzed by acrylamide gel electrophoresis as described in the following section.

Acrylamide gel electrophoresis of viral proteins. Urea-SDS polyacrylamide gels were prepared by the method of Summers et al. (15). The gels consisted of 7.5% acrylamide, 0.2% *N*,*N*'-bis-methylene acrylamide, 0.1% SDS, 0.5 M urea, and 0.1 M sodium phosphate (pH 7.2). Polymerization was catalyzed by *N*,*N*,*N*',*N*'-tetramethylethylenediamine, and ammonium persulfate at final concentrations of 0.05% (vol/vol) and 0.056%, respectively. The electrode buffer consisted of 0.1 M sodium phosphate, pH 7.2, containing 0.1% SDS.

Two sizes of gel columns were used (0.6 by 8 cm and 0.6 by 20 cm). The latter was necessary for obtaining good resolution of the ϵ and F, and the α and β viral proteins. The samples to be analyzed were made 15% in glycerol or sucrose and 0.01% in bromophenol blue, and approximately 200 μliters of each was layered on the gel under the electrophoresis buffer. Disc gel electrophoresis was carried out at 5 mA/gel until the tracking dye had migrated to near the bottom of the gel. In the short gels, this took approximately 10 h and in the long gels, it took approximately 18 to 20 h.

The apparent molecular weight of each polypeptide was calculated from its mobility on 7.5% acrylamide-SDS gels compared to several standards, including the following: bovine serum albumin (66,000), ovalbumin (45,000), chymotrypsin (25,000), and cytochrome *c* (12,500). The standard gels were stained with Coomassie brilliant blue to visualize the protein bands (19).

The gels containing the labeled viral proteins were cut into 1-mm sections, each section dissolved at 50 C in 0.1 ml of 30% H_2O_2, and counted in 4 ml of PCS (Amersham-Searle) in an Isocap 300 Nuclear-Chicago liquid scintillation counter.

[^{14}C]mengovirus capsid proteins. Cells were grown to confluency in a roller bottle (approximate cell number per roller bottle, 2×10^8) in MEM containing 10% FCS. The medium was removed and the cell layer was washed with PBS. The cells were infected with an MOI of 10 PFU per cell in 15 ml of MEM. After 30 min at 37 C, 30 ml of MEM containing 5% FCS was added. At 5 h, 30 μCi of a [^{14}C]amino acid mixture (CFB 104, 57 mCi/matom, from Amersham-Searle) was added, and the infection was allowed to continue for another 15 h. The virus was purified as described by Kerr and Martin (9). The capsid proteins were solubilized using SDS-mercaptoethanol and subjected to gel electrophoresis as described above.

RESULTS

Kinetics of labeling of mengovirus proteins. Our initial experiments were designed to determine the number of mengovirus proteins appearing after a short pulse with radioactive amino acids and after chase periods of varying lengths of time with unlabeled amino acids. To accomplish this, monolayer cultures of L cells which had been treated with actinomycin D to suppress host RNA and protein synthesis, were infected with mengovirus as described in Materials and Methods. After 5 h, the cultures were pulsed with a mixture of radioactive amino acids for a particular time period to label the viral proteins. The labeling was either stopped by the addition of hot SDS-mercaptoethanol (see Materials and Methods) or chased in the presence of unlabeled amino acids for varying lengths of time up to 80 min. Each sample was subjected to polyacrylamide gel electrophoresis. The nomenclature of Butterworth et al. (2) for EMC viral proteins will be used throughout this report.

The bottom panel of Fig. 1 demonstrates the

distribution of radioactivity in the viral polypeptides after a 12-min pulse period with a mixture of [^{3}H]amino acids. As can be seen, there is a considerable amount of label in the protein designated A, and less in those designated B, C, D, E, F, and G. After a 10-min chase period in medium containing unlabeled amino acids, there is a decrease in the amount of radioactivity in the A peptide and an increase in polypeptides ϵ, α, and γ. The latter constitutes part of the viral capsid because they co-electrophorese with homologous chains from purified [^{14}C]virus (data not shown). The capsid proteins of mengovirus, α, β, γ, and δ (20) migrate on these polyacrylamide gels as shown in the uppermost panel of Fig. 1. After a 20-min chase period, little of the A and B proteins remain. These experiments suggest that, as in the case of EMC virus (2, 4), the A and probably the B polypeptides are precursors of the capsid proteins.

After 40 to 80 min of incubation in chase medium, the ϵ polypeptide begins to decrease and the β increases, suggesting that ϵ is the precursor of β. In EMC virus (2), the ϵ is cleaved to β and δ. No protein corresponding to the δ capsid protein, which is shown in the uppermost panel of Fig. 1, was observed in our experiments, probably because it migrates faster than our marker dye on these gels and thus inadvertantly may have been lost. The D polypeptide disappears and the E increases, suggesting a precursor-product relationship between them (Fig. 1). Butterworth et al. (2) have shown by cyanogen bromide methods that the C, D, and E proteins of EMC virus are related, the C being the precursor of D, which in turn, is the precursor of E.

The kinetic behavior of the various mengovirus proteins can be more clearly analyzed if the data in Fig. 1 is plotted as in Fig. 2. The relative molar concentration of each viral protein peak is expressed as a function of time. Operationally, the counts per minute in each peak from Fig. 1 is first expressed as percent of total viral counts per minute. This is then divided by the molecular weight of each viral peak. Finally, the relative molar concentration is standardized to a particular protein which presumably is stable throughout the course of the pulse-chase period. In this report the data have been standardized to the F protein, a presumed primary cleavage product.

The curves in Fig. 2 illustrate clearly the precursor nature of A, B, C, D, D1, and ϵ, and the flow of label into components ϵ, α, β, γ, E, and H. The F, I, and G are the only polypeptides that do not change in amount during the chase period, although the scatter of points in F is considerable. Thus, the F, I, and G are assumed to be primary cleavage products. These preliminary data agree well with the model for the biosynthesis of EMC virus proteins proposed by Butterworth and Rueckert (4).

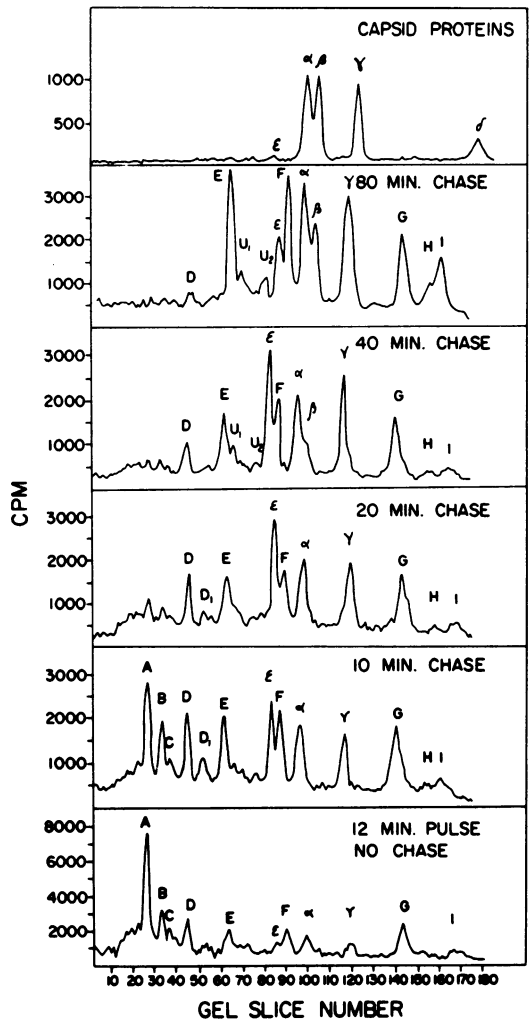

FIG. 1. *Pattern of labeling of viral proteins during pulse-chase experiments. At 5 h after infection L cell monolayer cultures were pulse-labeled for 12 min with a [^{3}H]amino acid mixture and chased with unlabeled amino acids for varying time periods as indicated in the figure. At the end of the chase period the reaction was stopped by the addition of a solution of hot SDS-mercaptoethanol. For electrophoretic analysis 200 μliters of each sample was applied to a column (0.6 by 20 cm) of 7.5% polyacrylamide gel. The specific details of these procedures are described in Materials and Methods.*

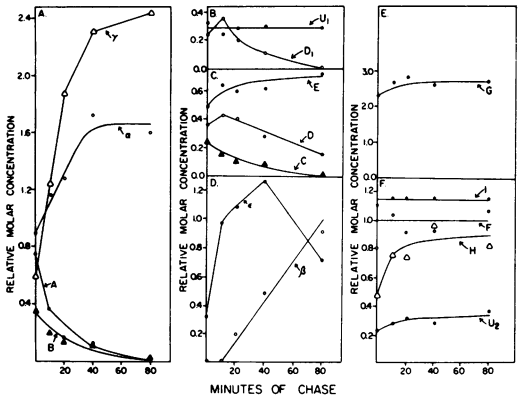

FIG. 2. *Kinetics of cleavage of mengovirus precursor proteins and kinetics of formation of stable viral proteins. The molar concentration of each component was determined as described in the text. The data used for these calculations were taken from Fig. 1. Similar analyses of data from six different experiments such as shown in Fig. 1 gave identical results. The symbols U1 and U2 stand for unknowns 1 and 2, and they may be residual host proteins.*

To determine the half-life of the A and C precursor polypeptides, pulse-chase experiments were carried out as described above, except that the pulse period with radioactive amino acids was 7 min instead of 12 min. This allowed the labeling of the primary products without extensive cleavage. The labeling period was followed by chases of varying lengths of time. A plot (Fig. 3) of the logarithm of the percent composition of either A or C as a function of the length of the chase period gives a straight line in each case, suggesting that each is cleaved with first order kinetics. The half-life of A was estimated from the slope of the curve to be 7 min, and that of C was estimated to be 9.5 min. For EMC virus, the equivalent half-lives of the A and C are 6.7 and 10 min, respectively (4).

Size, stability, and relative molar concentration of mengovirus polypeptides in infected L cells. On the basis of the pulse-chase experiments shown in Fig. 1 and 2, it was possible to determine the number of polypeptides specified by mengovirus and to estimate their size, stability, and relative molar concentration in infected cells. Approximately six unstable polypeptides and eight stable polypeptides are observed (Table 1). Had the δ not been lost, it would also be expected to be stable. The total molecular weight of the stable polypeptides including δ is 238,000, which approximates the total coding capacity of the virus, based on a molecular weight of 2.4×10^6 (20) for its genome. The labeled polypeptides U1 and U2 were found to be present throughout the pulse-chase period in a constant, low molar concentration (see Fig. 2), and may represent residual host proteins.

If Table 1 of this publication is compared to Table 1 in Butterworth et al. (2), and Fig. 2 is compared to Fig. 4 of Butterworth and Rueckert (4), it can be seen that the size, stability, and kinetic behavior of mengovirus and EMC virus proteins are very similar. The differences in the molecular weights of the larger polypeptides probably reflect errors in estimating the molecular weights of this size polypeptides on 7½%

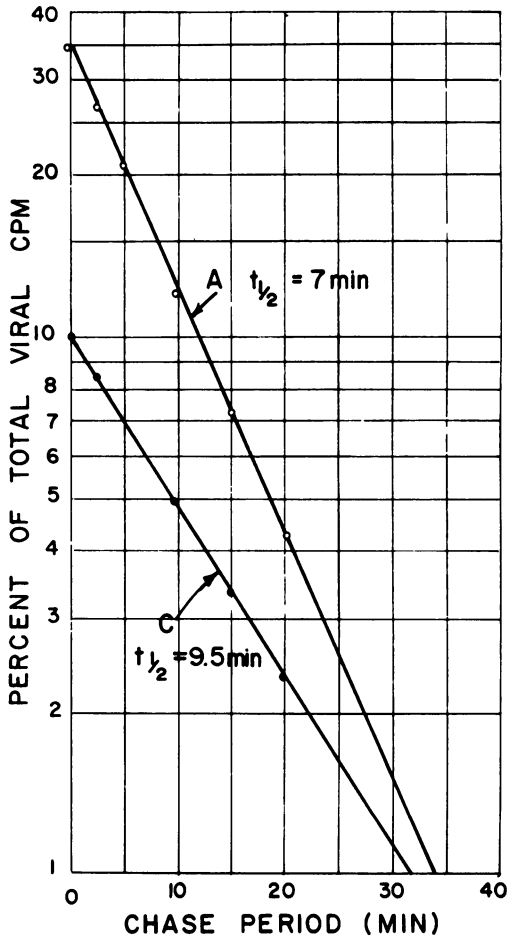

FIG. 3. *Rate of cleavage of mengovirus A and C proteins. Five hours after infection L cells were pulsed for 7 min with a mixture of [^{3}H]amino acids and chased as indicated in the figure. Samples of 200 μliters were applied to columns (0.6 by 20 cm) containing 7.5% polyacrylamide gels as described in Materials and Methods. The data in this figure represent an average of six different determinations.*

polyacrylamide gels rather than any real differences between the two viruses. The similarity of mengovirus and EMC virus in these parameters is hardly unexpected, since the two viruses are alike in most other respects such as their size, shape, and the number of capsid proteins, immunological cross-reactivity, etc.

One difference between mengovirus and EMC virus appears to be the molar concentration in which each of the primary products is present. Theoretically, if all of the functional viral proteins arise via cleavage and if there is only one site of initiation of protein systhesis, the primary products should appear in a 1:1 proportion. This is indeed the case for EMC virus. For mengovirus, however, the A protein and its cleavage products are present in amounts ranging from 1.5 to 2 times that of F and C (see calculations in Table 1). Possible reasons for this observation will be presented in the Discussion. The G is also present in relative molar concentrations greater than 1. In this case, however, it is possible that another cleavage product co-migrates with it, since the G peak is not symmetrical on these gels. It would be difficult to explain the great abundance of the A protein in this manner since its presumed cleavage products, α, β, and γ, are also present in quantities greater than 1 mol/mol of the other primary products. If each final cleavage product consisted of two proteins, the total coding capacity of the viral RNA would be exceeded.

Effect of proteolytic enzyme inhibitors and amino acid analogues on the in vivo cleavage of mengovirus precursor proteins. No protein larger than the A precursor is observed in any of these pulse-chase experiments (Fig. 1). Even after pulse periods as short as 5 min (data not shown), no polypeptide of molecular weight greater than the A precursor is observed. This is also the case for poliovirus and EMC virus-infected HeLa cells, suggesting that many of the cleavages of the primary products occur while the polypeptide is still growing on the polysomes. It also suggests that the formation of the giant precursor polypeptide is not necessary for cleavage to occur.

As mentioned earlier, in the presence of TPCK or amino acid analogues, several viral polypeptides greater than the A equivalent appear in HeLa cells infected with poliovirus (7, 8, 11, 16), including one which encompasses the entire informational content of the viral genome. To see if such a large precursor might also be detected in mengovirus-infected L cells, the effects of several proteolytic-enzyme inhibitors on the cleavage of mengovirus precursors were examined. The inhibitors included tolylsulfonyllysyl chloromethyl ketone (TLCK), a trypsin inhibitor, TPCK, a chymotrypsin inhibitor, and phenylmethylsulfonylfluoride (PMSF), a general protease inhibitor. Of these only TPCK, at 0.75×10^{-4} M, had any inhibitory effect (see Fig. 4). Higher concentrations of this inhibitor depressed protein synthesis to such an extent that little incorporation of labeled amino acids into protein was observed.

TABLE 1. *Size and stability of viral polypeptides and molar concentrations of end-product polypeptides*

Polypeptide	Apparent[a] mol wt $\times 10^{-3}$	Stability[b]	% of total[c] viral counts/min	Relative[d] molar concn.
A	110 ± 4	u		
B	99 ± 5	u		
C	95 ± 6	u		
D	80 ± 4	u	3	0.14[e]
D_1	70 ± 2	u	3	0.14
E	60 ± 3	s	11	0.64
U_1	56 ± 2	?	4	0.25
U_2	47 ± 2	?	4	0.32
ϵ	41 ± 2	u	7	0.61
F	39 ± 1	s	11	1.00
α	34 ± 1	s	13	1.34
β	31 ± 1	s	7	0.79
γ	24 ± 1	s	14	2.07
G	17 ± 1	s	11	2.39
H	12 ± 1	s	4	1.14
I	11 ± 1	s	8[f]	2.53[f]
δ	<10	s	?	?

[a] These molecular weight estimations represent an average of over 15 determinations.

[b] s, Stable (no decrease of counts during a chase); u, unstable.

[c] This represents the total viral counts per minute after a 12-min pulse and an 80-min chase (Fig. 1). The results are an average of four separate experiments.

[d] The mass of the proteins was assumed to be proportional to counts per minute. The molar concentrations were calculated by dividing the % of each polypeptide by the estimated molecular weight, and normalizing with respect to F.

[e] If the cleavage pattern of the capsid proteins is as follows: $A \rightarrow B \rightarrow D_1 \rightarrow \epsilon + \gamma + \alpha$, and $\epsilon \rightarrow \beta + \delta$, then the molar concentration of A and its cleavage products would be:

$$A + B + D_1 + \frac{(\epsilon + \beta) + \alpha + \gamma}{3} = 1.74$$

The total amount of protein C synthesized was calculated from the following relationship: C + D + E = 0.78. Thus, the molar ratio of the synthesis of A:F:C = 1.74:1:0.78, or close to 2:1:1.

[f] The molar concentration of I was higher than usual after an 80 min-chase period for reasons not readily understood. It is possible that δ migrated with I under these conditions.

To obtain better resolution of the shoulder to the left of the A precursor (Fig. 4) resulting from TPCK treatment, the sample was subjected to gel electrophoresis using two concentrations of acrylamide, 5 and 7½%, and longer gel columns. Two distinct peaks larger than the A protein are detected (Table 2). The molecular weight of the faster migrating peak, P2, has been estimated to be approximately 120,000 to 125,000, and that of the slower migrating peak, P1, has been estimated at 135,000 to 145,000.

In an attempt to ascertain the possible relationship of these two peaks to the other translational products, the relative molar concentration of each protein present after treatment with TPCK was determined. The most striking effects of TPCK (Table 2, column 2), compared with the control sample (column 1), are the decreased cleavages of A and C. Following a 12-min pulse and a 40-min chase (column 1), the molar concentrations of A and C were less than 0.1. In the presence of TPCK, the same values were 1.00 for A and 0.34 for C. The observation that the cleavage of C is stopped by TPCK, yet some D is formed, suggests that the D may sometimes appear as a primary product, or else the blockage of cleavage of the C protein is not complete.

If the cells are pretreated with TPCK for about 2 min before the addition of the labeled amino acid mixture (or pulse period), less C, D, and G are synthesized, and there is a corresponding increase in the amounts of both precursors, P1 and P2. This suggests that the larger precursor may represent the uncleaved I, F, and C region, corresponding to a molecular weight of 145,000, and the smaller precursor may represent A and its nearest neighbor, G, correspond-

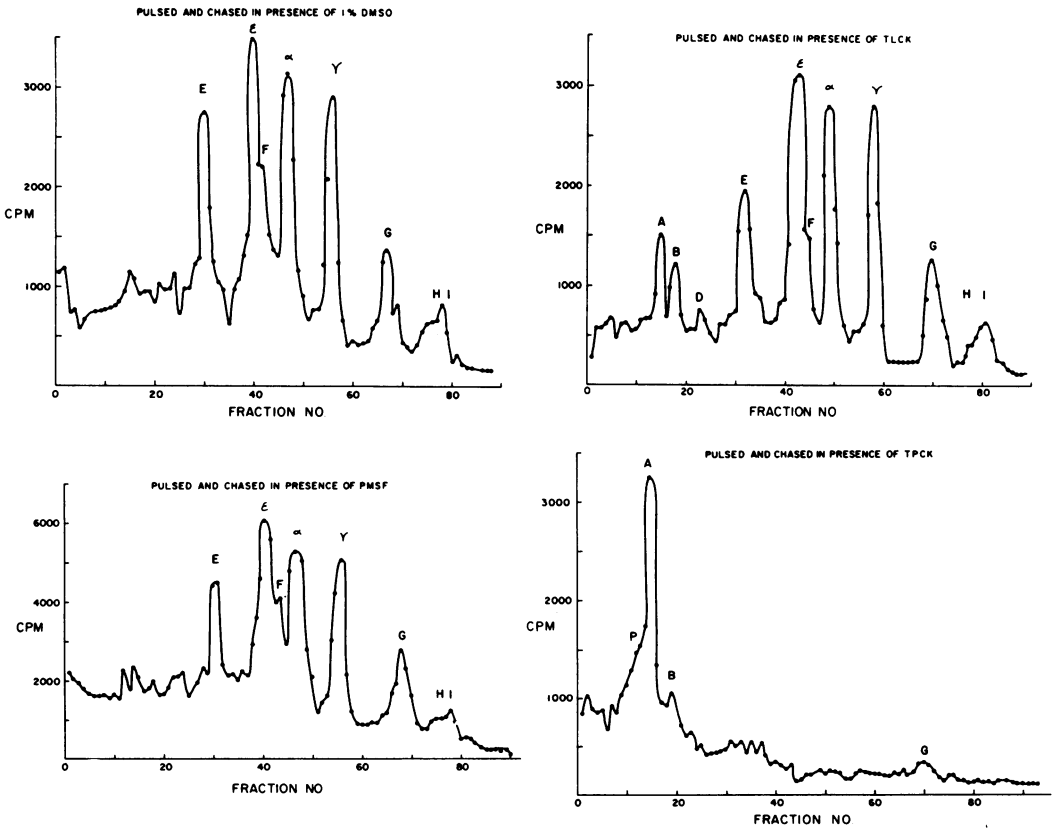

FIG. 4. *Effect of proteolytic enzyme inhibitors on the cleavage of mengovirus precursor proteins. Five hours after infection L cells were pulsed for 7 min with a mixture of [³H]amino acids, and where indicated, 1 × 10⁻⁴ M TLCK, 1 × 10⁻⁴ M PMSF, or 0.75 × 10⁻⁴ M TPCK. These inhibitors were dissolved in 100% dimethylsulfoxide (DMSO), and the final concentration of DMSO in the pulse mixture was 1%. This amount of DMSO did not affect the cleavage pattern of mengovirus proteins. The cultures were chased for 40 min in the presence of unlabeled amino acids and the particular inhibitor used, and were analyzed on short, 7.5% polyacrylamide gels (0.6 by 8 cm) as described in Materials and Methods.*

jng to a molecular weight of 124,000.

From the molecular weight estimations it is apparent that neither of the precursors obtained in the presence of TPCK correspond to the giant precursor containing all of the information of the viral genome. The actual composition of the observed precursors could only be determined by digesting them with trypsin or cyanogen bromide, and comparing their fingerprints to the individual viral proteins.

Similar experiments attempting to accumulate the giant mengovirus precursor were carried out using the amino acid analogues, *p*-fluorophenylalanine, canavanine, and azetidine-2-carboxylic acid. Depending on whether the cells are pretreated with the analogues before the pulse period with radioactive amino acids, one or two precursors accumulate, neither of which corresponds to the largest species. The data from an experiment in which the cells were not pretreated with analogues are presented in Table 2. One precursor is formed and has a molecular weight of 120,000 to 125,000. As in the case of TPCK treatment of cells, there is an accumulation to the A and C proteins and no capsid proteins are formed. With pretreatment of the cells with analogues, two precursors accumulate corresponding to the P1 and P2 proteins in the TPCK experiment, and there is a large decrease in the amount of C, D, E, F, and G formed.

p-Fluorophenylalanine alone stops the cleavage of the A protein (data not shown), but there is no accumulation of precursors (8, 18). None of the other analogues alone cause the accumulation of precursors. Azetidine-2-carboxylic acid alone slows the cleavage of A but does not stop it (data not shown and reference 5).

TABLE 2. *Molar concentration of viral polypeptides in the presence or absence of inhibitors*

Mol wt × 10^{-3}		1 12-min pulse, 40-min chase	2 12-min pulse, 40-min chase + TPCK[a]	3 12-min pulse, 40-min chase + analogues[b]
P1[c]	135 ± 10[d]	0	0.43[e]	0
P2[c]	120 ± 5	0	0.33	0.37
A	110 ± 4	<0.1[f]	1.00	1.00
B	99 ± 5	0.11	0.21	0.56
C	95 ± 6	<0.1	0.34	0.43
D	80 ± 4	0.30	0.31	0.50
D_1	70 ± 2	0.14	0	0
E	60 ± 3	0.53	0	0.53
U_1	56 ± 2	0.30	0.55	0.73
U_2	47 ± 2	0.31	0	0
ϵ	41 ± 2	1.32	0	0
F	39 ± 1	0.75	0	0.58
α	34 ± 1	1.79	0	0
β	31 ± 1	0.46	0	0
γ	24 ± 1	2.42	0	0
G	16 ± 1	2.71	0.70	2.43
H	12 ± 1	1.00	0.10	0.97
I	11 ± 1	1.21		

[a] The concentration of TPCK used was 0.75×10^{-4} M. It was present throughout the pulse and chase periods.

[b] The concentrations of analogues used were: *p*-fluorophenylalanine, 1.6 mM; canavanine, 1.8 mM; and azetidine-2-carboxylic acid, 4 mM. The analogues were present throughout the pulse and chase period.

[c] P1 and P2 are abbreviations for precursor 1 and 2.

[d] The molecular weights of the P1 and P2 were also estimated using 5% acrylamide gels, since 7½% acrylamide gels do not give accurate estimations in this range. Paramyosin (110,000) and myosin (212,000) were used as markers in addition to those mentioned in Materials and Methods.

[e] The samples were analyzed on 7½% acrylamide gels (0.6 by 20 cm) as described in Materials and Methods.

[f] The molar concentrations were calculated as described in Table 1. These results represent an average of four separate experiments. The data in column 1 were normalized to an average value of F, and in columns 2 and 3, to A.

DISCUSSION

In this report the kinetic behavior of mengovirus-specific proteins has been described. The results of pulse-chase experiments indicate that the protein designated A is the precursor of the structural proteins. The data also suggest that F, I, and G are primary products in addition to A and C, since they appear after a short pulse with radioactive amino acids and persist in the same molar concentration even after lengthy chase periods. The proteins C, D, and E also appear to be related, as indicated by the rates of degradation of C and D and the kinetics of formation of E.

These results are very similar to those obtained by Butterworth et al. (2) for EMC virus. The primary difference between the two viruses appears to be the relative proportion in which the primary products are formed. As mentioned, the A precursor appears to be synthesized in twice the amount as the primary products F and C. There are several possible explanations for these results. (i) The nonstructural proteins are more susceptible to nonspecific proteolytic degradation than the structural proteins during the isolation and electrophoretic procedures. Since our calculations of the molar concentration of each species are adjusted to the value of the protein F, which is presumably a stable product, a low value for F would tend to increase the A:F ratio. This

possibility, however, seems unlikely as the molar concentration of F remained unchanged even when the viral proteins were isolated in the presence of TPCK, TLCK, and PMSF. (ii) At the end of the A protein cistron on the viral RNA there is a nucleotide sequence that is sometimes read as a stop signal, resulting in premature termination of the A protein. This would be a difficult hypothesis to test and one would have to explain why half of the time the termination signal is recognized and the rest of the time it is not. (iii) There is more than one site of initiation of protein synthesis on mengovirus RNA, one at the beginning of the cistron coding for the A precursor and one internally. Steric hindrance at the internal initiation site might affect the efficiency with which it is initiated and thus increase the A:F or A:C ratio. As mentioned, Öberg and Shatkin (12) have determined the number of initiation sites on mengovirus and have concluded that there is only one. However, it is conceivable that the amino acid sequence at the initiation sites of both cistrons is the same. This possibility, however, does not seem very likely for it would be difficult to imagine that mengovirus, which is so similar to polio and EMC viruses, would have evolved another initiation site. (iv) The coat protein somehow acts as a repressor of the translation of the rest of the viral RNA. During assembly of the mengo virion, the capsid proteins interact with the viral RNA and thus must have an affinity for the RNA. Perhaps this interaction slows the translation of the F and C region of the viral genome. Experiments are underway in this laboratory to test this hypothesis. The interesting feature of the observation that mengovirus proteins are synthesized in unequal amounts is that it may represent a hitherto unknown control mechanism in the picornavirus family of viruses.

This report also shows that neither TPCK nor amino acid analogues cause the accumulation of the giant precursor which contains the entire informational content of the mengovirus RNA in infected L cells. Both TPCK and amino acid analogues effectively block the cleavages of A to the capsid proteins and C to D and E. Two precursors accumulate in the presence of these inhibitors, one of which, P1, may represent the combined informational content of I, F, and C, or close to the second half of the viral genome, the A protein being considered as the first half.

Preliminary experiments from this laboratory suggest that in an in vitro protein-synthesizing system from L cells using mengovirus RNA as message, the giant precursor does not accumulate and is cleaved during its synthesis, giving the primary products A, G, F, and C. Similar results have also been noted using EMC virus RNA as template (1). The in vitro-cleaved products co-migrate on acrylamide gels with authentic primary products made in vivo, suggesting that the cleavage mechanism is operating in vitro. Thus, it seems that it will be possible to examine the enzymes involved in the cleavage reactions.

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