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Synthesis and Subcellular Localization of the Murine Coronavirus Nucleocapsid Protein

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Received June 9, 1983; accepted August 3, 1983

The synthesis and processing of the nucleocapsid protein (pp60) of the JHM strain of murine coronaviruses were examined. Pulse-chase experiments showed that pp60 was synthesized initially as a protein of approximately 57,000 in molecular weight (p57). Immunoprecipitation using mouse anti-JHMV antiserum indicated that p57 was virus specific. Immunoprecipitation with monoclonal antibodies specific for pp60 showed that p57 was antigenically related to pp60 and was not phosphorylated, while the intracellular protein that comigrated with the virion nucleocapsid protein, pp60, was phosphorylated. The p57 was found exclusively in the cytosol while the majority of pp60 was associated with the membrane fraction but pp60 was not an integral membrane protein.

Coronaviruses comprise a group of enveloped viruses which cause a variety of diseases, usually confined to their natural hosts (22). These viruses contain an RNA genome of positive polarity that is approximately $5.4 \times 10^6$ Da in size (5). Intracellular RNA replication is accomplished by a virion-induced polymerase (2) which synthesizes a full-length negative strand (8). Seven distinct mRNAs are then synthesized from a single species of negative-strand template and comprise a nested set with common 3' ends (7, 8, 16).

Purified coronavirus particles of the A59 (MHV-A59) strain contain three structural proteins, i.e., gp90/180, gp25, and pp60, while the JHM strain (JHMV) may be composed of four, containing gp65 in addition (14, 20). We have further shown that MHV contains only a single phosphorylated protein, the nucleocapsid protein (pp60). It is phosphorylated only on serine residues (19), presumably by a virion-associated protein kinase (13). Sturman et al. (21) have also shown that pp60 interacts with the smaller envelope glycoprotein (gp25) and have postulated that this interaction may be a regulatory mechanism operable during virus maturation.

Studies on the intracellular synthesis of MHV proteins by pulse-chase experiments have shown that the nucleocapsid protein, pp60, is a primary gene product (3, 12). In vitro translation indicated that the virus-specific messenger RNA No. 7 ($1.8 \times 10^6$ Da) codes for a protein with a tryptic peptide map identical, or closely related, to that of virion pp60 (9, 11, 14, 15). However, proteins of lower molecular weight are also synthesized, occasionally in excess of the virion pp60 (9, 12). It has been suggested that these proteins of lower molecular weight might be degradation products of pp60, which are generated during virus replication or during in vitro translation (11, 12). In addition, two-dimensional nonequilibrium isoelectric focusing of infected cell lysates indicated that the nucleocapsid protein was composed of multiple heterogeneously charged species which are homogeneous in size (1).

In examining the kinetics of the appearance of JHMV proteins in infected DBT cells, we noted that the region of the gel which contains the pp60 protein also contained another protein of slightly lower molecular weight (Fig. 1A). To understand the possible relationship of this protein...
Fig. 1. Viral polypeptides synthesized in JHMV-infected DBT cells. (A) Cells were infected with JHMV and labeled for 15 min prior to lysis. Equal amounts of radiolabeled lysate were analyzed by electrophoresis on a 10% linear gel. Lane 1 = 4 hr postinfection, lane 2 = 5 hr, lane 3 = 6 hr, lane 4 = 7 hr, and lane 5 = 8 hr postinfection. Lane 6 is a lysate of uninfected DBT cells. (B) Pulse-chase labeling of JHMV-infected DBT cells. At 5.25 hr postinfection, the cells were pulse-labeled for 2 min with [\textsuperscript{35}S]methionine (25 \textmu Ci/ml) (lane 2) and chased with excess cold methionine for 10 min (lane 3), 20 min (lane 4), 30 min (lane 5), and 40 min (lane 6). Uninfected control cells, lane 1, and infected cells lysed at 6 hr (lane 8) and 8 hr (lane 7) postinfection were included as controls. Lysates were analyzed on a 6-15% linear gradient gel.

(Designated p57) with viral structural proteins, we performed kinetic studies of the synthesis of the intracellular viral-specified proteins. Confluent monolayers were infected with the JHMV strain of mouse hepatitis virus at 37\textdegree C for 1 hr with an m.o.i. of approximately 1.0. Following removal of the inoculum, DMEM containing 1 \mu g/ml of actinomycin D was added to each plate. At 5 hr postinfection, the DMEM was replaced with methionine-free DMEM (MFDMEM) containing 1 \mu g/ml actinomycin D. After 15-min incubation at 37\textdegree C, the MFDMEM was replaced with 2.0 ml MFDMEM containing 1 \mu g/ml actinomycin D and 25 \mu Ci/ml of [\textsuperscript{35}S]methionine (New England Nuclear, Boston). The cells were solubilized in buffer consisting of 10 mM Tris-\text{HCl}, pH 8.8, 2% SDS, 4 M urea, and 2% 2-mercaptoethanol and heated at 56\textdegree C for 2 min. Electrophoresis of equal amounts of radioactivity from infected cells lysed at different time points after infection indicated that p57 was detectable throughout infection, although its ratio to pp60 decreases as infection proceeds (Fig. 1A). To further examine the relationship of these two proteins, we carried out pulse-chase experiments early in infection (5-6 hr) to minimize possible protein degra-
When infected cells were pulse-labeled with $[^{35}S]$methionine for 2 min and then chased with excess unlabeled methionine (20 mM) for various lengths of time, only p57 was detected within the pulse interval (Fig. 1B). Within the first 10-min chase, pp60 became detectable. This is faster than the processing of p23 into gp25 (14), since the gp25 was not detected until 20 min into the chase period (Fig. 1B). As the chase was extended, the amount of p57 decreased while pp60 increased, suggesting that p57 is the precursor protein to pp60.

The precursor-product relationship between p57 and pp60 was further tested with hyperimmune serum and monoclonal antibodies specific for JHMV. These monoclonal antibodies recognize at least four different antigenic determinants on pp60 (Flcming, J. O., in press). As shown in Fig. 2A, hyperimmune serum precipitates both pp60 and p57 suggesting that p57 is indeed a viral protein (Fig. 2A). Also, all of the monoclonal antibodies precipitated pp60. Two of these monoclonal antibodies, J3.13 and J3.14, also precipitated p57. This result shows that pp60 shares antigenic determinants with p57, but pp60 also contains additional antigenic determinants. To confirm that the intracellular form of pp60 is phosphorylated, and to determine whether p57 is also phosphorylated, immunoprecipitates from $[^{32}P]$orthophosphate-labeled cell lysates were analyzed. As can be seen in Fig. 2B, both the monoclonal antibodies J3.13 and J3.14, which precipitated both pp60 and p57 (Fig. 2A), precipitated only $[^{32}P]$labeled protein which comigrated with pp60. No $[^{32}P]$labeled p57 was detected. These results and those obtained by pulse-

![Fig. 2](image-url)
chase analysis suggest that p57 is not phosphorylated and that phosphate is added during the processing of p57 into pp60.

To gain some insight into the maturation pathways of pp60, we examined the subcellular localization of p57 and pp60. Infected cells were labeled with [3S]-methionine, suspended in RS buffer and lysed by Dounce homogenization (18). The lysates were separated into soluble (S100) and particulate (P100) fractions by centrifugation at 100,000 g for 1 hr. The S100 phase contained p57 and pp60, while the P100 membrane fraction contained only pp60 (Fig. 3). This result suggests that p57 is present exclusively in cytosol while pp60 might also be associated with cellular membranes. To rule out the possibility that this apparent membranous association is due to contamination from polysomes or nonspecific association with membranes, the P100 fraction was treated with 100 mM EDTA to disassociate pp60 from ribosomes and then with 2 M NaCl to disassociate it from membranes. Neither of these treatments released any detectable pp60 from the P100 fraction (data not shown), suggesting the possibility that pp60 was an integral membrane protein. To rigorously examine this possibility, the P100 fraction was treated with alkali which removes membrane-associated proteins but not the integral membrane proteins (4, 17). Alkali treatment at pH 12, but not pH 10 removed pp60 from the P100 fraction and released it into the S100 fraction (Fig. 3). In contrast, neither gp25 nor p120, which are integral membranous proteins (14), were affected by this treatment, indicating that pp60 is strongly associated with, but not integrated into, the host cell membranes.

These results suggest that the nucleocapsid protein, pp60, of murine coronavirus is synthesized first as a nonphosphorylated precursor protein, p57, which undergoes phosphorylation immediately after synthesis. This conversion occurs rapidly, since the synthesis of p57 in the absence of pp60 could only be detected following short (2-5 min) pulses, and large quantities of pp60 could be found after chase intervals of as short as 5 min. In addition, using 32P labeling, we could detect small amounts of phosphorylated pp60 after a 5-min pulse (data not shown). The rapidity of the phosphorylation explains the previous failure to appreciate the precursor-product relationship of these two

![Fig. 3. Alkali treatment of membranes from JHMV-infected cells. Infected cells were labeled with [3S]-methionine for 15 min at 5.25 hr postinfection as described in Fig. 1A. Cells were swollen in RS buffer (10 mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 7.4) and lysed by Dounce homogenization. The lysate was centrifuged at 100,000 g for 1 hr and the soluble (S100) and particulate (P100) fractions were analyzed. The P100 fraction was resuspended in either RS buffer, or treated for 5 min at pH 10 or 12. Membranes were centrifuged again at 100,000 g for 1 hr and the supernatant and pellets examined for pp60 by electrophoresis on 6-15% linear gradient gels. Lane 1, control lysate; lanes 2 and 8, RS buffer supernatant and pellet; lanes 4 and 5, supernatant and pellet following treatment at pH 10; lanes 6 and 7, supernatant and pellet following treatment at pH 12; lane 8, control JHM lysate.](image-url)
proteins. It should be noted that, in different in vitro translation systems carried out in different laboratories, the primary translation product of the mRNA No. 7 of MHV obtained from infected cells was found to be either p60 or p57 (9, 12). It is not clear if this discrepancy is due to the possibility that p60 and p57 were not electrophoretically separated in some studies, or whether some in vitro translation systems allowed phosphorylation of the primary gene product to take place. From the results presented here, it is more likely that p57 is the nonphosphorylated primary translation product of mRNA No. 7.

The conversion of p57 to pp60 probably involves only phosphorylation, since both proteins could be immunoprecipitated by anti-JHMV serum and by two anti-JHMV monoclonal antibodies specific for the pp60 of the purified virion. The observation that most of the monoclonal antibodies did not precipitate p57, but only pp60, suggests that phosphorylation apparently induces a drastic conformational change. This conformational change might be responsible for some regulatory functions, such as (1) controlling the rate of transcription, (2) the switching of RNA transcription to replication, or (3) influencing the association of this protein with the virion genomic RNA or the cellular membranes. It is not clear whether there is heterogeneity in the degree of phosphorylation of pp60. However, the finding that p57 was not completely converted to pp60 even after prolonged chase (Fig. 1B) suggests that some p57 might not be phosphorylated under certain conditions.

The most unexpected finding in these studies is the close association of pp60 with cellular membranes. This protein is synthesized on free polysomes (10), consistent with the current finding that p57 is present exclusively in the soluble fraction of the cytoplasm (Fig. 3). These fractions also contain pp60, suggesting that phosphorylation probably takes place in the cytosol. The majority of the pp60 is associated with the cellular membranes. However, pp60 could be removed from the membrane by harsh treatment with alkali, which removes only nonintegral membrane proteins (4, 17), while gp23 and gp90/180, which are synthesized and processed on the cellular membranes (10), remained associated with the membranes in the P100 fraction (Fig. 3). These observations indicated that pp60 is closely associated with, but not integrated into host cell membranes. The nature of this association is very intriguing. It has been shown that pp60 is linked to gp25 in mature viral particles (21). There may be a similar interaction between these two proteins in the infected cells, which could serve as the focal points for virus maturation. It raises an exciting possibility that phosphorylation of pp60 might influence this interaction and thereby influence the virus maturation process. Such an idea is presently being examined in our laboratories.

ACKNOWLEDGMENTS

We wish to thank Todd Kennell and Gabriele Olivka for excellent technical assistance and Raymond Mitchell and Alisa Young for editorial assistance in the manuscript preparation.

This work was supported in part by Grant PCM-4567 from the National Science Foundation, by Public Health Service Research Grants AI19244 and NS18146, and Grant RG 1449 from the National Multiple Sclerosis Society.

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