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Amino acid substitutions within the matrix protein of type D retroviruses affect assembly, transport and membrane association of a capsid

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The functional roles of the matrix (MA) protein in the assembly and maturation of retroviruses was investigated with a series of MA mutants of Mason-Pfizer monkey virus (M-PMV), an immunosuppressive type D retrovirus. The mutants we describe here were generated by the introduction of random point mutations within the MA coding domain by use of sodium bisulphite mutagenesis. Studies of these mutants show that the MA protein plays a critical role in three different, sequential events in the final stages of type D retrovirus replication: (i) folding of the *gag* gene-encoded precursor polyproteins into a stable conformation for capsid assembly in the cytoplasm of infected cells; (ii) capsid transport from the site of assembly to the plasma membrane; and (iii) capsid association with, and extrusion of the membrane during virus budding. The mutants described here interfere with or block M-PMV replication at each of these stages. Large numbers of preassembled capsids accumulate within the cytoplasm of transport-defective mutant-infected cells, suggesting that transport of M-PMV capsids to the plasma membrane is an active and specific intracellular targeting process. The initial association of the capsid with the membrane may depend upon this intracytoplasmic transport process but additional protein-lipid interactions that involve the MA protein are required for membrane extrusion around the preformed capsids; in cells infected with the budding-defective mutant, assembled capsids accumulate under the inner surface of the cell plasma membrane, and are retarded in their release from the infected cell.

Key words: matrix protein/Mason-Pfizer monkey virus/sodium bisulphite mutagenesis/virus particle assembly

Introduction

In most enveloped RNA viruses, a protein is commonly found in association both with the lipoprotein envelope and with the internal nucleocapsid; it has been designated the matrix (MA) or membrane (M) protein. The mediating interaction of the MA protein between two structural units in mature virus particles points to an essential role in viral assembly, although its exact role in the virus cycle has not been elucidated. In early studies of type B and C retroviruses (Bolognesi *et al.*, 1978; Cardiff *et al.*, 1978; Marcus *et al.*, 1978) the MA proteins of retroviruses were determined to be located external to the inner ribonucleoprotein core and in Rous sarcoma virus, it was shown the pp19 (MA) could be cross-linked to membrane lipid (Pepinsky and Vogt, 1979). Recently Gelderblom *et al.* (1987) showed in immuno-electron microscopic studies of human immunodeficiency virus that its matrix protein, p17, lines the inner surface of virus envelope and is a component of an envelope-associated icosahedral capsid.

The MA protein of a retrovirus is synthesized as a component of the *gag* gene-encoded precursor polyprotein (Gag polyprotein), and is invariably located at the amino-terminus of this precursor (Bolognesi *et al.*, 1978; Cardiff *et al.*, 1978; Gelderblom, *et al.*, 1987; Marcus *et al.*, 1978; Pepinsky and Vogt, 1979). Since Gag polyproteins are processed to the mature capsid proteins shortly after budding from the plasma membrane, the MA protein might be expected to play a key role in its precursor form in the assembly of an immature capsid and in its membrane association during virus budding. After cleavage from the precursor in the mature virion, it is postulated to form the envelope-associated outer shell of the polyhedral capsid that surrounds the inner ribonucleoprotein core.

In most mammalian retroviruses the MA protein is cotranslationally modified by addition of myristic acid to an amino-terminal glycine residue of the Gag polyprotein immediately after removal of the terminal methionine (Bradac and Hunter, 1986b; Henderson *et al.*, 1983, 1988; Ratner *et al.*, 1985; Rhee and Hunter, 1987; Schultz and Oroszlan, 1983, 1984; Towler and Gordon, 1988). On the basis of the membrane-binding function of the MA protein, it has been suggested that myristic acid is necessary for a stable protein-lipid interaction at the cell membrane. Indeed, with Moloney murine leukemia virus (MuLV), a mammalian type C virus in which capsid assembly occurs simultaneously with budding at the plasma membrane, it was shown that myristylation of the Gag polyprotein $Pr65^{gag}$ was required for the molecule to associate with membrane (Rein *et al.*, 1986; Schultz and Rein, 1989). It may also serve similar functions in HIV morphogenesis since in the absence of myristylation, no membrane associated viral structures were found (Göttlinger *et al.*, 1989). Therefore, myristic acid-mediated membrane association of a Gag polyprotein is an essential step in assembly and budding of type C morphogenic retroviruses. In contrast, our previous studies of Mason-Pfizer monkey virus (M-PMV), the prototype of type D retroviruses, showed that myristylation is not required for preassembly of a capsid which occurs in the cytoplasm of M-PMV-infected cells (Rhee and Hunter, 1987). Instead this modification plays a crucial role in mediating the intracytoplasmic transport of the type D virus capsid from the site of assembly within the cytoplasm to the site of budding on the plasma membrane. We have recently described a point mutant of M-PMV, with an altered MA protein, which shows a changed morphogenesis and assembles capsids at the membrane in a manner similar to a type C retrovirus (Rhee and Hunter, 1990a). This result indicates that the major difference between type D and type C retroviruses is the intracellular site to which capsid

precursors are targeted for assembly. Thus it is possible that myristic acid might also play a transport function in type C retroviruses. Importantly, this result indicates that M-PMV will provide an ideal system for defining the functional roles of retroviral *gag*-gene encoded proteins since in this virus the processes of capsid assembly, intracellular transport and budding are distinct. We focus here on molecular genetic studies of the MA protein.

Previously the MA protein of M-PMV was demonstrated to play an important structural role in the correct folding of Gag polyproteins, which in turn stabilizes the molecules and allows intermolecular interactions for self-assembly into an intracytoplasmic capsid (Rhee and Hunter, 1990b). Internal truncations of 7–84 amino acids in the MA protein appeared to induce a conformational change in the Gag polyprotein, which resulted in their rapid degradation. These results may reflect the contribution of the MA protein of M-PMV to protein–protein interactions in the cytoplasm and presumably also protein–lipid interactions at the plasma membrane. We have here extended these studies of the M-PMV MA protein with a series of mutants, generated by sodium bisulphite mutagenesis, which have amino acids randomly substituted within the MA coding region. Expression of these mutant genomes in both HeLa cells and COS cells has allowed us to define at least three steps involved in the late phase of type D retrovirus replication. The MA protein appears to play key roles in the sequential events of: (i) folding a stable Gag precursor which can be efficiently assembled into an intracytoplasmic capsid; (ii) transport of capsids to the plasma membrane; and (iii) virus capsid-induced membrane extrusion during the initial stages of budding from the membrane. Mutants defective in each step have been characterized and are described here.

Results

MA mutants of M-PMV with amino acid substitutions within the MA protein domain

The *gag* gene of M-PMV encodes a Gag polyprotein of 78 kilodaltons (kd), $Pr78^{gag}$, which is proteolytically cleaved by the viral protease to yield the six capsid proteins of mature virion (p10, pp16-18, p12, p27, p14 and p4) (Bradac and Hunter, 1984; Henderson *et al.*, 1985). Because it is in an analogous position to the matrix proteins of other retroviruses and is myristylated, p10 has been designated the matrix protein of M-PMV (Bradac and Hunter, 1986b). To introduce point mutations randomly within the p10 coding region, we carried out sodium bisulphite mutagenesis on a fragment of the *gag* gene subcloned into a single stranded bacteriophage as described in Materials and methods (Figure 1). Sodium bisulphite specifically deaminates cytosine bases in single-stranded DNA (Kai *et al.*, 1974; Shapiro *et al.*, 1973), which allows a C to T transition on the p10 coding strand of the *gag* gene. After mutagenesis, the mutated region from 150 clones was sequenced (Sanger *et al.*, 1977), and the mutant fragments were cloned into an M-PMV expression vector pSHRM15 (Rhee *et al.*, 1990). A total of eleven MA mutant genomes were constructed and were studied to determine the effects of the mutations on M-PMV replication (Table I). Substituted amino acids and their positions in each mutant are indicated as a designation of the mutant; e.g. in mutant P43L/S81F a proline residue at position 43 and a serine at position 81 within the MA protein were substituted by leucine and phenylalanine, respectively.

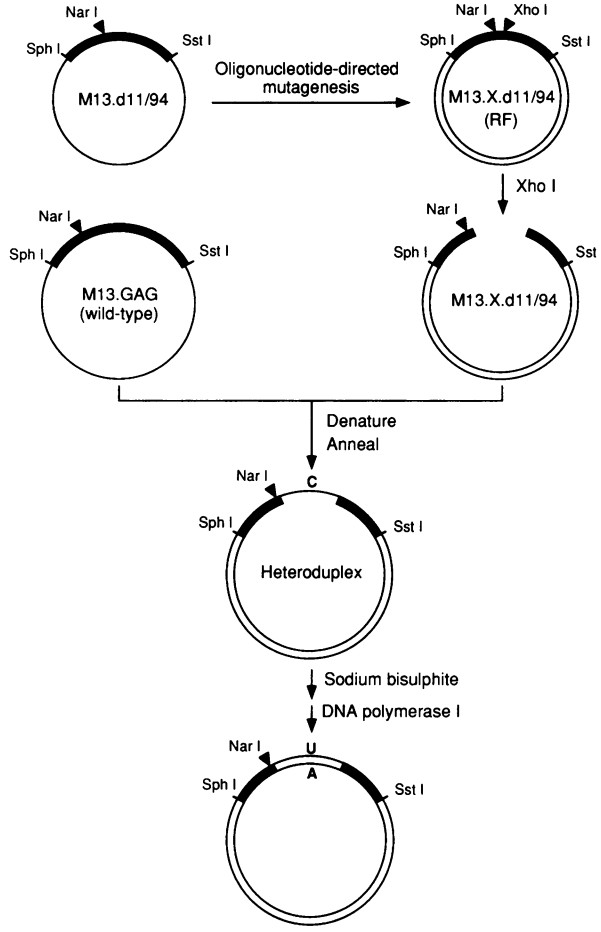


Fig. 1. A schematic diagram of the mutagenesis method. The *Sph*I–*Sst*I fragment from pMPMV6A/7 was cloned into M13mp19 to give M13.GAG (wild-type; shaded box). 252 nucleotides corresponding to the MA cloning region were deleted from M13.GAG and an *Xho*I site was inserted at the site of deletion by oligonucleotide-directed mutagenesis to give M13.d11/94 and M13.X.d11/94, respectively. Replicative form (RF) DNA of M13.X.d11/94 was cut with *Xho*I and hybridized with single-stranded DNA of M13.GAG to generate circular heteroduplexes with a single-stranded region in the M-PMV fragment. This gapped DNA was treated with sodium bisulphite to mutate unpaired cytosine residues (C) to uracil residues (U); the gap was then filled with DNA polymerase I in the presence of all four deoxynucleotide triphosphates to give the mutant double-stranded circular DNA. Details of the procedures used are described in Materials and methods.

Synthesis and processing of viral proteins in MA mutant-infected cells

To determine whether mutant Gag polyproteins are synthesized and processed into mature structural proteins in a wild-type manner, viral proteins of MA mutants were expressed in COS-1 cells following transfection of mutant viral genomes. In the initial study, cells were pulse-labelled for 20 min or pulse-labelled and then chased in complete medium for 4.5 h when both cell-associated and released-virus polypeptides were analysed (Figure 2). M-PMV-infected cells produce two major polyproteins, Gag polyprotein $Pr78^{gag}$ and the envelope precursor glycoprotein $Pr86^{env}$, and two minor, Gag-related molecules of 95 and 180 kd (Bradac and Hunter, 1984, 1986a). The latter

Table I. Summary of the phenotypes of MA mutants of M-PMV

Mutants	Mutations	Precursor stability[a]	Capsid assembly[b]	Virus release[c]	Cell-free invectivity[d]
Wild-type		+++	+++	+++	+++
Wild-type like mutants:					
T21I	T(21)→I	+++	+++	+++	+++
T41I	T(41)→I	+++	+++	+++	+++
R57C	R(57)→C	+++	+++	+++	+++
Stability/Assembly-defective mutants:					
P43L	P(43)→L	+	+	−	NA
P72S	P(72)→S	+	+	−	NA
P43L/S81F	P(43)→L S(81)→F	+	+	−	NA
P43S/T69I/A79V	P(43)→S T(69)→I A(79)→V	+	+	−	NA
Transport-defective mutants:					
A18V	A(18)→V	+++	+++	−	NA
A79V	A(79)→V	+++	+++	+	−
T69I	T(69)→I	+++	+++	+++	+
Membrane association-defective mutant:					
T41I/T78I	T(41)→I T(78)→I	+++	+++	++	+

Data are from four separate experiments. Cells expressing wild-type or mutant genomes were labelled with [^{3}H]leucine for 20 min and chased for the indicated period as described in Materials and methods. The amount of each viral protein immunoprecipitated with the indicated antibodies was quantified by counting the radioactivity of the band in a liquid scintillation counter.

[a]Stability of Gag polyproteins was determined as the amount precipitated after a 2 h chase relative to that after a pulse-labelling (Figure 2): +++, ≥70% processed to mature capsid proteins; +, ≤30%.

[b]The efficiency of intracytoplasmic capsid assembly was determined as the amount incorporated into capsid relative to that of total labelled Gag polyproteins during the pulse labelling (Figure 4): +++, 30−50%; +, 10−15%.

[c]Extracellular virions in the media of the cells were immunoprecipitated after a 12 h chase and compared with those of wild-type-infected cells (Figure 3): +++, ≥90%; ++, ≥60%; +, ≥30%; −, no viruses.

[d]Infectivity of viruses released from COS-1 cells transfected with mutant genomes (Figure 6): +++, fully infectious: +, poorly infectious; −, non-infectious; NA, not applicable.

molecules are the precursors of the viral protease and of the RNA-dependent DNA polymerase (reverse transcriptase) which are presumably synthesized via a frame-shifting mechanism as a Gag−Pro fusion protein and a Gag−Pro−Pol fusion protein, respectively (Bradac and Hunter, 1984; Sonigo *et al.*, 1986). While the three Gag-containing polyproteins are processed by the viral protease to the mature, structural and non-structural proteins shortly after virus budding, the Env precursor Pr86^{env} is cleaved by a host-derived protease into two cell-associated glycoproteins, gp70 and gp22, probably late in the Golgi complex (Bradac and Hunter, 1986a and b; Hunter, 1988). After virus release, the transmembrane (TM) glycoprotein gp22 is further processed by the viral protease to the virion-associated gp20 (S.S.Rhee, B.A.Brody, M.A.Sommerfelt, and E.Hunter, in preparation). This type of processing of Gag polyproteins, envelope precursor glycoproteins and of virion-associated glycoproteins was observed in cells transfected with a wild-type genome. Bands of viral polyproteins (Pr78^{gag}, Pr86^{env}, and Pr95) were detected in the lysates of cells pulse-labelled for 20 min (Figure 2A, lane 1). After a 4.5 h chase, bands of p27 and gp22—the major capsid protein and the cell-associated TM protein, respectively—appeared as processing products of the polyproteins (Figure 2A and B, lane 2). In extracellular virions that were released from pulse-labelled cells during the 4.5 h chase, a characteristic pattern of mature viral proteins were observed (Figure 2C, lane 1).

In cells transfected with mutant genomes of T21I (Figure 2A and B, lanes 3 and 4) and of T41I and R57C (data not shown), Gag polyproteins were processed to capsid proteins in a wild-type manner, and mutant virions were detected in the cuture media as with wild-type virus (Figure 2C, lane 2). Thus these amino acid substitutions have no discernable effect on M-PMV capsid assembly and maturation. In contrast, while the Gag polyprotein of mutant P43L was synthesized at a normal level (Figure 2A, lane 5), none was processed to mature proteins (Figure 2A and B, lane 6). During the chase period most of the molecules were turned over, and no virions were released into the medium (Figure 2C, lane 3). Identical phenotypes were observed in mutants P72S, P43L/S81F, and P43S/T69I/A79V which have in common the change of a proline residue (data not shown). In mutant A18V-infected cells (Figure 2A and B, lanes 7 and 8) a significant proportion of mutant Gag polyproteins remained unprocessed after a 4.5 h chase. No mature protein products were observed and no viral proteins were detected in the culture medium (Figure 2C, lane 4). The Gag polyproteins of three mutants, A79V, T691 and T41I/T78I, were processed during the 4.5 h chase into capsid proteins but with a lower efficiency than that observed with wild-type virus (Figure 2A and B, lanes 9−14). In addition, fewer virus particles were found in the culture medium (Figure 2C, lanes 5−7).

Kinetics of processing and turnover of mutant Gag polyproteins

The pulse−chase experiments described above showed that the processing of Gag polyproteins was altered in several of the MA mutants. However, because they represented a single time point, they did not provide information on the efficiency and kinetics of this processing event. To investigate this in more detail, we pulse-labelled COS-1 cells expressing the wild-type and mutant genomes, and determined the rate with which the labelled Gag polyproteins were processed into capsid proteins and incorporated into released virus. The results are shown in Figure 3.

The processing of wild-type Gag polyproteins was detected after a 1 h chase by the presence of a faint band of cell-associated p27 (Figure 3A, wild-type, lane 2); increasing amounts of p27 were detected following 2 h (lane 3) and 4 h (lane 4) chases. Half of the newly synthesized Gag polyproteins appear to be processed to mature proteins within 1.0−1.5 h in COS-1 cells (Figure 3B), which is approximately twice the rate we have previously observed in HeLa cells (Rhee and Hunter, 1990b). After a 12 h chase, ~95% of the precursors were processed (Figure 3A, wild-type, lane 5). Most of the cleavage products are not cell-associated but are found as virion-associated proteins, released into the culture medium during the chase of pulse-labelled cells. The amount of virus-associated cleavage products increased during the 4 h chase (Figure 3C, wild-type, lanes 1−3) and release was essentially complete by this time point; no signifi-

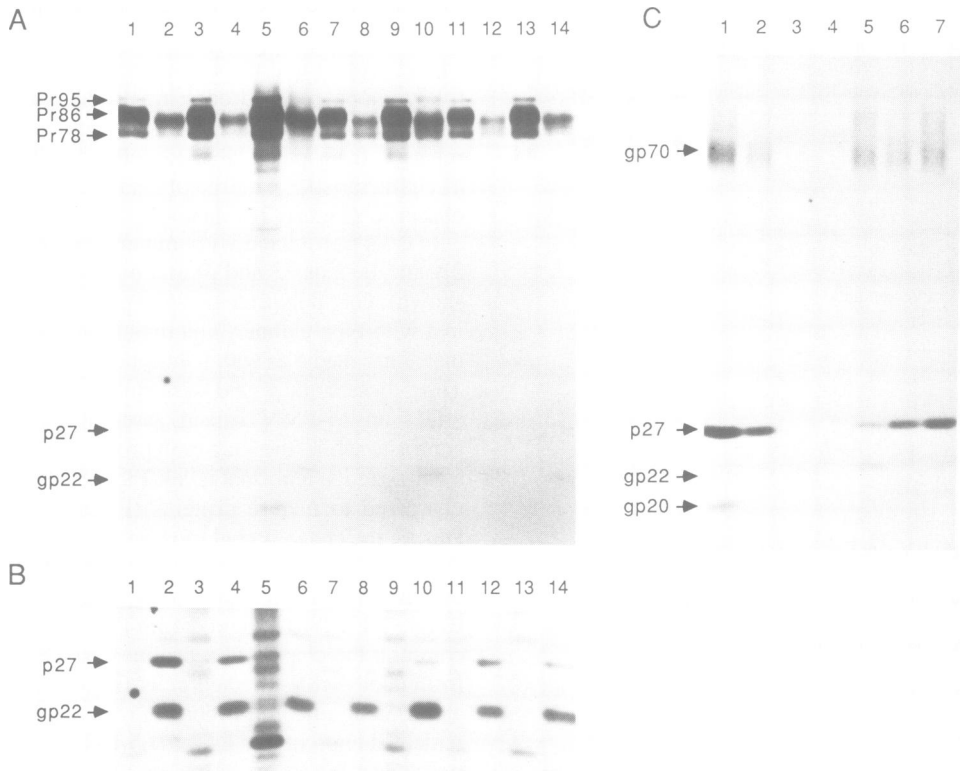


Fig. 2. Immunoprecipitation of cell- and virion-associated viral proteins. (**A** and **B**) To investigate the biosynthesis and processing of the Gag and Env precursors, $Pr78^{gag}$ and $Pr86^{env}$, COS-1 cells transfected with either wild-type pSHRM15 or mutant genomic DNAs were pulse-labelled with [^{3}H]leucine for 20 min (lanes 1, 3, 5, 7, 9, 11 and 13) and chased for 4.5 h (lanes 2, 4, 6, 8, 10, 12 and 14). Virus specific proteins in the cell lysates (cell-associated) were immunoprecipitated with anti-MPMV antiserum and analysed by SDS−PAGE. Lanes 1 and 2, wild-type; lanes 3 and 4 mutant T21I; lanes 5 and 6, P43L; lanes 7 and 8, A18V; lanes 9 and 10, A79V; lanes 11 and 12, T69I; lanes 13 and 14, T41I/T78I. A longer autoradiographic exposure of the same gel is shown in panel B to emphasize the processed products p27 and gp22 of the Gag and Env precursor polyproteins, respectively. (**C**) Extracellular virions were pelleted from the culture fluids of pulse−chased cells, and virion-associated proteins were then immunoprecipitated with anti-MPMV antiserum. The positions of p27, the major capsid protein, and gp70 and gp22/20, the envelope glycoproteins, are shown. Lane 1, wild-type; lane 2, mutant T21I; lane 3, P43L; lane 4, A18V; lane 5, A79V; lane 6, T69I; lane 7, T41I/T78I.

cant increase was detected following a 12 h chase (lane 4). Therefore, in wild-type genome expressing cells, the majority of the newly synthesized Gag polyproteins is assembled into virions and released into the medium within 4 h.

The Gag polyproteins of A18V were stably expressed in the cells without being released into the culture medium and without being processed to capsid proteins (Figure 3A and B). The Gag polyproteins of this mutant were turned over with a half-life ($t_{1/2}$) of 3.5 h, and even after a 12 h chase, 15% of the newly synthesized polyproteins were still present within the cells (Figure 3A, A18V lane 5). In contrast, Gag polyproteins of the P43L mutant and other proline mutants were turned over rapidly (Figure 3A and B). In cells transfected with the mutant P43L genome, $<10\%$ of the pulse-labelled molecules remained after a 4 h chase without any evidence of their being processed into mature capsid proteins (Figure 3A, P43L lane 4). The unstable Gag polyprotein of mutant P43L thus has a $t_{1/2}$ of $\sim$1 h in COS-1 cells. For mutant P43L/S81F, the same unstable phenotype was observed (data not shown). However, while the Gag precursors of mutants P72S and P43S/T691/A79V were turned over without being processed into virion proteins, they had a longer half-life than those of P43L (data not shown).

In mutant A79V genome-expressing cells, pulse-labelled Gag polyproteins turned over slowly, with similar kinetics to the polyprotein of mutant A18V (Figure 3A, A79V lanes 1−5). However, in this case, a small proportion of the precursors was processed to mature proteins, but was only detected after a 12 h chase (lane 5). Much reduced levels of virus particles were detected in the medium after a 12 h chase (Figure 3C, A79V lanes 1−4). It should be noted that labelled viral glycoproteins could be detected in pelleted virus before labelled Gag proteins could be observed (Figure 3C, lane 3), and that the ratio of labelled p27 to glycoprotein seen in virions after a 12 h chase was significantly lower than that observed in wild-type virions. These results suggested that the radiolabelled A79V mutant Gag polyproteins were released into virions with slower kinetics than the radiolabelled glycoproteins; the latter are presumably incorporated into virions comprised of capsid proteins synthesized prior to the pulse.

Two mutants T691 and T41I/T78I synthesized and processed Gag polyproteins and released virions into the culture medium. However, processing and virion release occurred with slower kinetics than those observed with the wild-type M-PMV. After a 4 h chase, approximately half of the mutant Gag polyproteins remained unprocessed (Figure 3A, T691 and T41I/T78I lanes 4), while $\sim$80−90% of wild-type molecules were processed at this time (Figure 3A, wild-type, lane 4). However, the mutant polyproteins

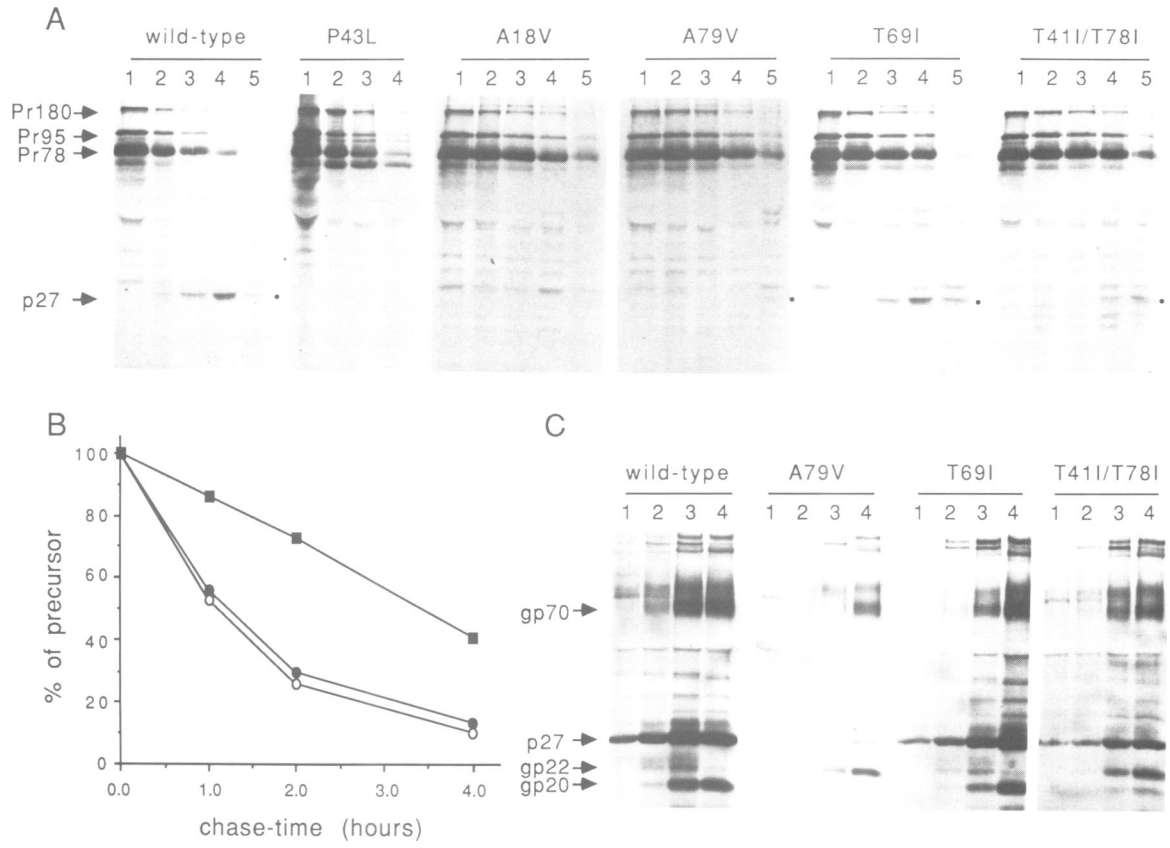


Fig. 3. Kinetics of processing and turnover of mutant Gag polyproteins. (**A**) To determine the rate of mutant Gag polyprotein processing or turnover, COS-1 cells were transfected with wild-type pSHRM15 or mutant genomic DNAs and pulse-labelled (lane 1) with [^{3}H]leucine for 20 min. After 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), and 12 h (lane 5) chases, cells were lysed, and Gag polyproteins and the p27 capsid protein were immunoprecipitated with anti-p27 antiserum. Some cellular proteins of 18–50 kd which were non-specifically precipitated in the pulse could be seen also in the chases of some of the immunoprecipitates. Similar bands were observed following immunoprecipitation of mock transfected COS cells (data not shown). The position of the p27 protein band is denoted by a • adjacent to the band. (**B**) The graph shows the relative amount of Gag polyproteins remaining at each time point in wild-type (open circles), P43L (closed circles), and A18V (closed squares)-infected cells. (**C**) The culture fluids of the cells expressing wild-type or mutant (A79V, T69I, or T41I/T78I) genomes were harvested after 1 h (lane 1), 2 h (lane 2), 4 h (lane 3), and 12 h (lane 4) chases and virions were pelleted to determine the efficiency and the rate with which virus and incorporated proteins were released. Viral proteins in the pellets were immunoprecipitated with anti-M-PMV antiserum.

were processed (rather than being degraded) following a 12 h chase since an increasingly intense p27 band could be detected in the cell lysates (Figure 3A, T69I and T41I/T78I lanes 4 and 5) and increasing amounts of virion proteins were released into the medium during this chase period (Figure 3C, T69I and T41I/T78I lanes 1–4).

Kinetics of an intracytoplasmic capsid assembly of MA mutants

It was of interest to determine whether in the case of mutants A79V, T69I, and T41I/T78I, slow processing of Gag polyproteins resulted from the slow assembly of intracytoplasmic capsids. In addition, the proline substitution mutants (P43L and P72S) which expressed unstable Gag polyproteins, were examined to determine whether mutant polyproteins were transiently assembled into a capsid prior to degradation. To determine the rate and extent of capsid formation, pulse-labelled polyproteins were fractionated into free and capsid-associated forms by sedimentation analysis after various times of chase (Figure 4) (Rhee and Hunter, 1990b); free molecules remain in the soluble fraction and those incorporated into capsids are recovered in the pellet fraction. These experiments were performed with HeLa cell lines which contain an integrated copy of each MA mutant proviral DNA.

As observed previously (Rhee and Hunter, 1990b), in cells expressing a wild-type genome, newly synthesized Gag polyproteins were found predominantly in the soluble fraction after the pulse-label (Figure 4, wild-type, lane 1); ~30% of the molecules appeared to have been incorporated into capsids during this time (Figure 4, wild-type, lane 2). Progressively more of the pulse-labelled precursors were assembled into intracytoplasmic immature capsids (wild-type, lanes 3–6) as the length of the chase increased ($t_{1/2}$ = 1.0–1.5 h). After a 3–4.5 h chase the amount of polyprotein in both fractions decreased, consistent with the release of virions from the cell. Mature virions are sensitive to detergent lysis and so increasing amounts of p27 could be detected in the soluble fractions during these later chase periods (wild-type, lanes 7–10). Intracytoplasmic capsids of three slow processing mutants, A79V, T69I, and T41I/T78I, were assembled with similar efficiency to wild-type in the initial phase of capsid assembly (data shown of mutant T69I in Figure 4, T69I lanes 1–6). However, mutant polyproteins continued to accumulate in the pellet fractions during the 3 and 4.5 h chases, while those in the soluble fractions decreased (T69I lanes 7–10). These results indicate that soluble forms of the polyproteins were assembled into capsids with normal kinetics but that the capsids were released at a slower rate.

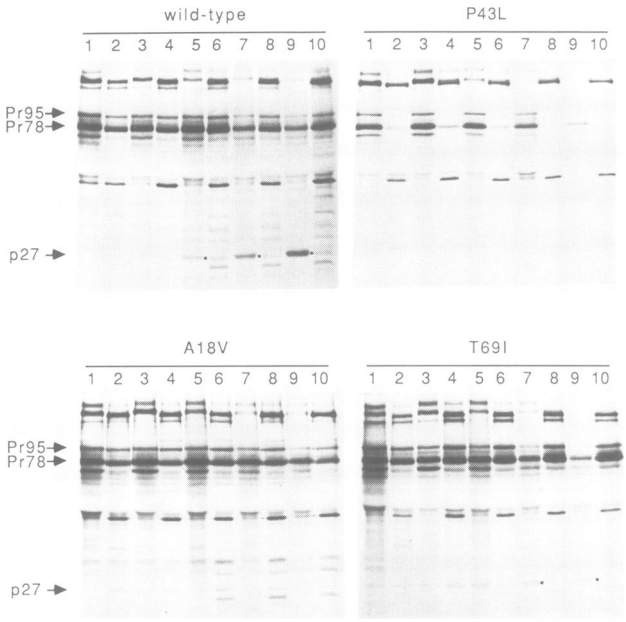


Fig. 4. Kinetics of intracytoplasmic capsid formation. HeLa cell lines, expressing wild-type or mutant genomes, were pulse-labelled with [^{3}H]leucine for 20 min (lanes 1 and 2) and then chased for 0.5 h (lanes 3 and 4), 1.5 h (lanes 5 and 6), 3.0 h (lanes 7 and 8), and 4.5 h (lanes 9 and 10). Cells were lysed in Triton X-100 lysis buffer, and assembled capsids were pelleted by centrifugation as described in Materials and methods. Gag polyproteins in the soluble (free molecules; lanes 1, 3, 5, 7 and 9) and pellet fractions (capsid-associated molecules; lanes 2, 4, 6, 8 and 10) were immunoprecipitated with anti-p27 antiserum.

The Gag polyprotein of mutant P43L, which was degraded rapidly ($t_{1/2}$ of 1.0 h), were recovered mostly in the soluble fraction during the pulse-labelling period (Figure 4, P43L lanes 1 and 2), and even after chase periods as long as 4.5 h (P43L lanes 3–10). Only 10–15% of the molecules formed pelletable capsids during the 20 min pulse-labelling period, and no increase was observed in the fraction following 0.5–4.5 h chases (P43L lanes 3–10). Similar results were observed in the other three unstable proline mutants P72S, P43L/S81F and P43S/T69I/A79V (data not shown). Therefore, amino acid substitutions at proline residues in the MA protein appear to both destabilize the Gag polyprotein and interfere with the process of M-PMV capsid assembly.

In contrast to the proline mutant (P43L), mutant A18V, which in pulse–chase experiments yielded stable, unprocessed Gag polyproteins, assembled these molecules into capsids with kinetics similar to those of wild-type (Figure 4, A18V lanes 1—10). These data suggest that this single amino acid substitution in the MA protein has no effect on assembly of Gag polyproteins into capsids, but prevents their transport to or release from the plasma membrane.

Electron microscopic analysis of MA mutants

The experiments described above pointed to stages in the virus replication cycle that might be defective in the different mutants; mutants with unstable Gag polyproteins, i.e. P43L, appeared to be defective in polyprotein stability and capsid assembly, whereas mutants in which processing of Gag polyproteins was slow or absent but in which assembly occurred normally, i.e. A18V or T69I, appeared defective in the processes of capsid transport or budding. To identify more definitively the block in replication, electron microscopic studies of COS-1 cells transfected with each mutant genome were carried out (Figure 5). In wild-type M-PMV genome-transfected cells (Figure 5A), preassembled capsids were observed scattered throughout the cytoplasm and could be seen at the plasma membrane in the process of budding. Extracellular immature virions were found adjacent to the cells.

Thin sections of mutant P43L-infected cells (Figure 5B and C) showed intracytoplasmic inclusions that are composed of partially assembled capsids. Only a few capsids with complete protein shells could be observed, confirming the results of sedimentation analysis that only a small proportion of this mutant Gag polyprotein was assembled into capsids that were stable and pelletable under mild detergent conditions. Partially formed mutant capsids were presumably disrupted under these conditions so that the polyprotein remained in the soluble fractions. Both complete and incomplete capsids differed in appearance from wild-type capsids, suggesting that these structures are undergoing rapid proteolysis. Mutant capsids in the process of assembly accumulated in distinct regions of the cytoplasm, indicating that M-PMV Gag polyproteins are transported to a specific site in the cytoplasm where capsid assembly occurs. The proline mutation allowed mutant peptides to be transported to and concentrated at this assembly site, but inhibited normal capsid assembly through a structural effect of the mutation and/or by triggering rapid degradation of the mutant molecules.

In mutant A18V-infected cells (Figure 5D and E), large intracytoplasmic accumulations of assembled capsids were seen and no particles in the process of budding and release from the cells were observed. This observation supports the biochemical data that mutant Gag polyproteins were stable and incorporated into capsids, but no particles were released. Therefore, a valine substitution for alanine in the MA protein appears to block the plasma membrane transport (PM transport) of capsids. Similar accumulations were also found in the cells infected with mutant A79V which exhibited slow processing of Gag polyproteins and greatly reduced levels of released virus (Figure 5F). However, in this mutant a few capsids could be seen in the process of budding, indicating that this mutation results in capsids being transported to the plasma membrane at very low efficiency. Most appear to be arrested at the site where they are assembled and are eventually degraded in the cells. In contrast, in mutant T691-infected cells numerous capsids were found both distributed through the cytoplasm and in the process of budding at the plasma membrane (Figure 5G), suggesting that mutant capsids were transorted out of the site of assembly but were moved through the cytoplasm with slower kinetics than those of wild-type M-PMV.

Thin sections of mutant T41I/T78I-infected cells showed large numbers of preassembled capsids lining the cytoplasmic side of the plasma membrane with no accumulations at the cytoplasmic assembly site (Figure 5H and I). This situation is quite distinct from that of the transport-defective mutant T69I-infected cells, even though both mutants exhibited similar kinetics for Gag polyprotein processing and virus release. The lack of an accumulation of T41I/T78I mutant capsids in the cytoplasm indicates that capsids are transported to the plasma membrane with normal kinetics. The electron

micrographs show that capsids accumulated under the plasma membrane at an early step in the extrusion process, thus in this mutant virus the rate-limiting step in replication is the process of virus budding itself. Therefore the MA protein of M-PMV must play an important role in capsid–membrane association during this process.

Infectivity of MA mutant viruses

Since virions were released from cells expressing the genomes of the transport-defective mutants A79V and T69I, and the membrane association-defective mutant T41I/T78I, we determined whether they were infectious. COS-1 cells were transfected with mutant DNAs, and culture fluids containing the viruses released from the cells were harvested. Levels of viral proteins and reverse transcriptase (RT) activity in the medium were determined, and HeLa cells were infected with equivalent amounts of RT-containing medium. The spread of infectious virus through the HeLa cells was monitored by RT assays at various days post-infection. With wild-type virus the release of RT-containing virions could be detected at 6 days post-infection and a rapid increase in enzyme activity was observed at days 8 and 10 post-infection. RT activities increased in the medium of the cells infected with wild-type-like mutant viruses at a similar rate (data not shown). Thus single amino acid substitutions at positions 21 (mutant T21I), 41 (T41I) and 57 (R57C) within the MA protein have no effect on type D virus replication. In contrast, no RT activity was detected in the culture fluids of transport-defective mutant A79V virus-infected cells. This demonstrates that while RT-containing mutant virions are assembled and released from genome-transfected cells, they are non-infectious. The T69I and T41I/T78I mutant viruses showed a much slower rate of RT activity increase and the levels remained low, indicating a much reduced infectivity.

Discussion

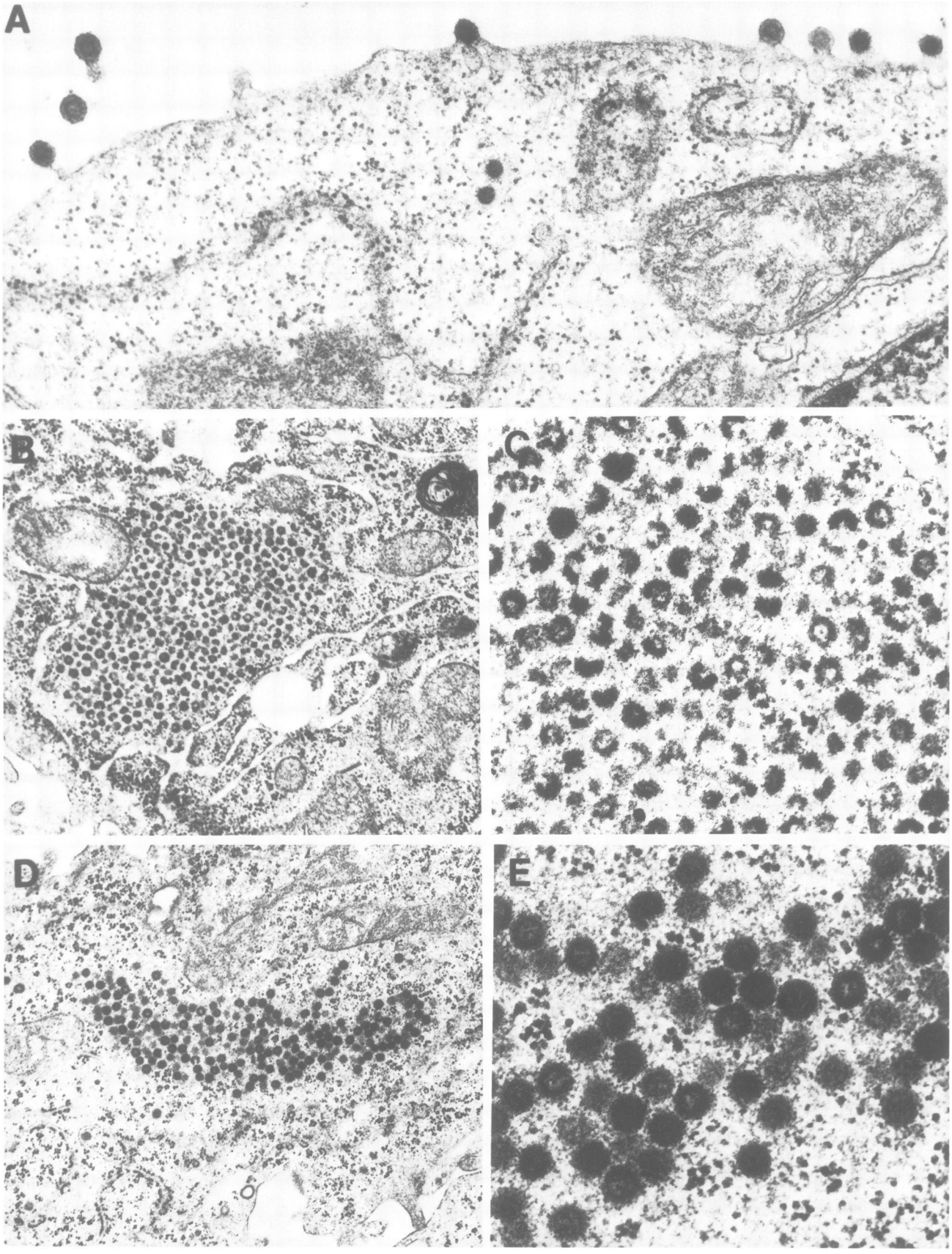
The late phase in the retrovirus life cycle includes a series of events which depend upon the machinery of the host cell; in most cases it begins with the synthesis and modification of the viral proteins, and ends with budding of a viral nucleocapsid from the plasma membrane. The capsids are assembled from the *gag*-gene encoded precursor polyproteins within the cytoplasm or at the plasma membrane, depending on the retrovirus type. In both type C and type D retroviruses the *gag*-gene products appear to contain intrinsic information which specifies the site of capsid self-assembly. The presence of viral envelope glycoproteins on the membrane are not required for virus budding since in their absence capsids can be assembled and released from the cells in a manner equivalent to that seen with wild-type virus (Kawai and Hanafusa, 1973; Linial *et al.*, 1980; Rhee *et al.*, 1990). However, detailed mechanisms responsible for the process of capsid assembly and budding are not understood for either morphogenic group. The studies of MA mutants of M-PMV, that we present here, define new roles for the MA protein in retrovirus morphogenesis.

The data presented here show that the MA protein serves a critical role in the multistep processes of virus replication. At least three processes seem to require a functional MA protein: stable polypeptide folding prior to intracytoplasmic capsid assembly, capsid transport to the plasma membrane, and virus budding from the membrane. In addition MA protein appears to play a role in a postbudding cleavage of the TM glycoprotein gp22 to produce gp20, which is accomplished by the viral protease, since this process is defective in some of the mutants described here (S.S.Rhee, B.A.Brody, M.A.Sommerfelt, and E.Hunter, in preparation). Previously we showed that a native MA protein is required to fold the Gag polyproteins into a conformation that confers intracytoplasmic stability and allows the intermolecular interactions involved in self-assembly to occur (Rhee and Hunter, 1990b). Mutant Gag polyproteins with internal deletions of 7–84 amino acids within the MA protein were synthesized and modified with myristic acid but were so unstable that half of the newly synthesized molecules disappeared, without being processed into mature capsid proteins, within 1 h. Furthermore, these mutant Gag polyproteins were assembled with very low efficiency into capsids in the cytoplasm which were neither released from, nor accumulated in the cells. The same phenotype was observed in stability/assembly-defective mutants, P43L, P72S, P43L/S81F, and P43S/T69I/A79V (Table I). There are five proline residues within the p10 coding region of M-PMV, and two of them at positions 43 and 72 were independently substituted to leucine or serine in this group of mutants. Mutant Gag polyproteins of the proline mutants were rapidly turned over with a half-life similar to that of the wild-type virus in COS-1 cells, but in the absence of specific cleavage to mature proteins. Thus, proline residues within the MA region appear to play a critical role in the folding process of the M-PMV Gag polyprotein and it is possible that proline turns are important for initiating or stabilizing the folded conformation of the polyprotein. Interestingly in both cases where polypetides had a serine substitution for a proline residue, they were somewhat more stable than those with a leucine substitution. Since serine also favours turns in a polypeptide chain, the introduction of a serine residue instead of a leucine at the proline positions may result in a smaller effect on the folded structure, which in turn might render intermediate stability to the molecules.

Pulse–chase experiments coupled with cell fractionation studies indicate that the structural alteration in these unstable mutant molecules not only triggered a protein degradation mechanism but also interfered with the assembly process itself such that few capsids were completed. Electron microscopy, however, showed that in mutant P43L virus-infected cells, partially assembled capsids could be observed accumulating in the cytoplasm. The accumulations of partially or rare completely assembled capsids in these mutant virus-infected cells indicate that these mutant Gag polyproteins of M-PMV retain the ability to be transported to a region within the cytoplasm where they can initiate the assembly process. Nevertheless, the ragged appearance of the capsid structure, coupled with evidence for their rapid turnover, suggests that both precursors and capsids are tagged for degradation and that there is a race between the assembly and degradation mechanisms.

In type D retroviruses the nascent Gag polyproteins appear to be targeted to (or their transport is arrested at) a defined site within the cytoplasm where they self-assemble into immature capsids. It is this intracytoplasmic targeting/retention signal that differentiates type C and D retrovirus assembly since we have shown that a single point mutation within the MA protein allowed the M-PMV Gag polyproteins to be transported directly to the plasma membrane where

capsids were assembled through a morphogenic process similar to that of type C retroviruses (Rhee and Hunter, 1990a). The proline mutations we describe here provide something of a paradox: they have no effect on the process of nascent Gag polyprotein targeting/retention and self-assembly since partially assembled capsids are found accumulating in discrete areas of the cytoplasm, yet at the same time they tag the molecules for rapid degradation.



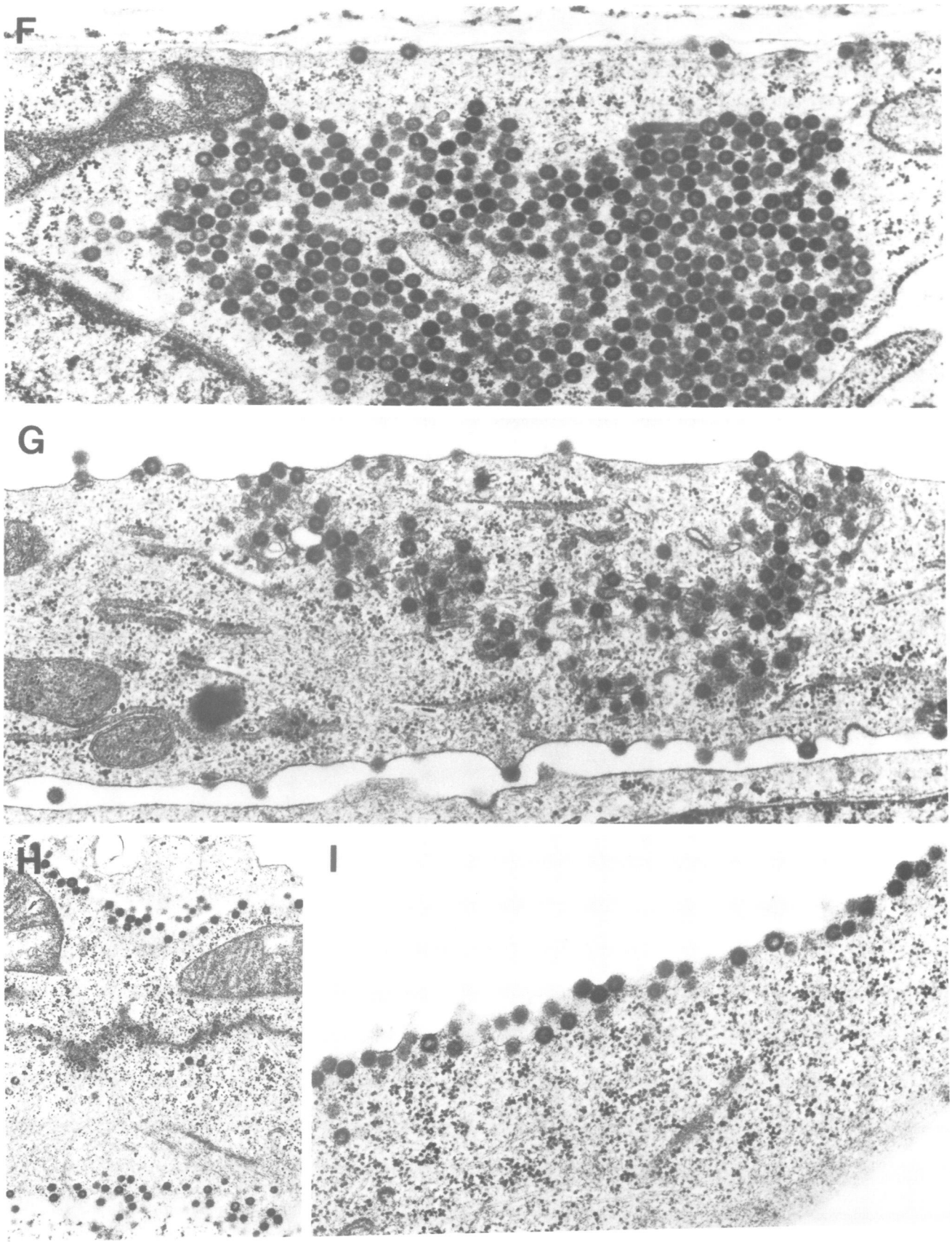


Fig. 5. Electron micrographs of COS-1 cells expressing wild-type and mutant viral genomes. Thin sections of COS-1 cells which had been transfected with wild-type genome or mutant genomes were examined under the electron microscope to determine the stages blocked in mutant virus morphogenesis. Panel **A**, a section of wild-type-infected cells; panels **B** and **C**, mutant P43L; panels **D** and **E**, A18V; panel **F**, A79V; panel **G**, T69I; panels **H** and **I**, T41I/T78I. Magnifications: panels A, F, G and I, ×43 000; panels B, D and H, ×24 000: panels C and E, ×95 000.

In wild-type virus-infected cells, virus capsid inclusions are not seen; instead capsids are found scattered through the cytoplasm and in the process of budding from the plasma membrane. This suggests that in the wild-type virus, capsid transport is closely linked with capsid assembly. In contrast, we interpret the results obtained with mutants A18V and A79V to mean that in these mutants, initiation of the transport process from the assembly site is the rate-limiting step in the replication process. As with the mutants with proline changes, the capsids accumulate in discrete areas of the cytoplasm that are depleted of ribosomes. Because partially formed capsids, apparently in the process of assembly, can be seen in these regions (Figure 5D), we would argue that they reflect a discrete assembly site for the virus. Mutant A79V is biologically somewhat leaky (Table I) and a few capsids can be observed by electron microscopy in the process of budding. Given the fact that A79V precursor proteins are assembled into capsids as rapidly as those of wild-type M-PMV, the 12 h lag before labelled capsid proteins can be detected in mutant virus particles suggests that intracellular transport of assembled capsids is very inefficient and subject to a significant delay following assembly. It remains possible that the capsid accumulations are merely the aggregation of biologically inactive structures, and because these studies employ non-conditional replication mutants of M-PMV, we cannot prove that this is not the case. Nevertheless, because we have shown that a mutation in the matrix protein can redirect assembly of M-PMV capsids from an intracytoplasmic site to the plasma membrane (Rhee and Hunter, 1990a), and that different point mutations, as well as mutations which block myristylation of the matrix protein (Rhee and Hunter, 1987), result in a similar 'transport-defective' phenotype, we favour the interpretation of a defect in a specific transport function.

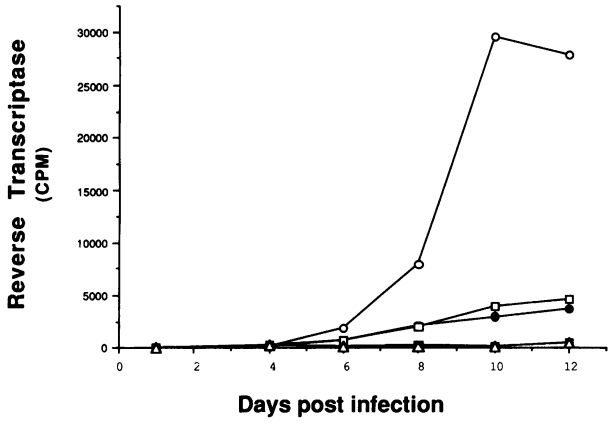


Fig. 6. Determination of the infectivity of mutant virions. To determine the infectivity of mutant viruses released from COS-1 cells transfected with wild-type or mutant genomes, virus-containing culture media were harvested and equivalent amounts of RT-containing medium were used to infect HeLa cells. Culture medium from these infected HeLa cells was assayed for RT activity at days 1, 4, 6, 8, 10 and 12 post-infection. Wild-type virus-infected cells (open circles) show a rapid increase in RT activity 6–10 days after infection, reflecting the rapid spread of infectious viruses through the culture. In contrast, mutant virions of T691 (closed circles) and T41I/T78I (open squares) showed much lower RT activities at each time point and its increase was significantly slower than that of wild-type viruses. In mutant A79V virus-infected cells (closed squares), no RT activity above that detected with the uninfected cells (open triangles) was observed.

In cells expressing the mutant T69I, numerous capsids were found dispersed through the cytoplasm rather than accumulated within a discrete area. Since again mutant capsids were shown to be assembled with normal kinetics, a single amino acid change of threonine to isoleucine in the MA protein appears to alter the rate of capsid transport through the cytoplasm itself; a slower rate of capsid transport would explain why more capsids were found in the cell and why pulse-labelled Gag polyproteins were released in extracellular virions at a slower rate than wild-type polyproteins. It should be noted, however, that amounts of labelled virus proteins equivalent to that observed with the wild-type are eventually found in released virus, indicating that all of the assembled capsids are ultimately transported and released.

We previously reported that myristylation of the Gag polyprotein plays a role in the PM transport of the type D virus capsid. In the absence of myristylation, preassembled capsids accumulated within the cytoplasm of mutant-infected cells (Rhee and Hunter, 1987). The transport mutants A18V and A79V, described here, show that myristylation itself is required, but not sufficient, to function as the primary PM transport signal since the capsids of these mutants are assembled from normally myristylated precursor molecules (data not shown). In the picornaviruses and papovaviruses where capsid proteins are also modified by myristic acid, this hydrophobic residue has been located by crystallographic methods within the interior of the capsid (Hogle *et al.*, 1985; Paul *et al.*, 1987; Rossman *et al.*, 1985; Schmidt *et al.*, 1989). Therefore, it seems possible that myristic acid addition may result in a conformational change that is necessary to render the capsid transport-competent. Mutants A18V and A79V accumulate capsids within similar intracytoplasmic assembly areas as the myristylation-defective mutant we described previously (Rhee and Hunter, 1987); it therefore seems likely that a single amino acid change of alanine to valine at position 18 or 79 within the MA protein might also prevent the acquisition of transport competency either by interfering with such a conformational change or by altering a putative PM transport signal itself.

A late event in virion assembly of M-PMV in infected cells is the budding of a intracytoplasmically-targeted capsid from the plasma membrane. Type D and B retroviruses are interesting in this regard since they must effectively drag the membrane around the preassembled capsid as they are extruded from the cell. Studies of mutant T41I/T78I demonstrate that the MA protein domain in the Gag polyprotein is required for this critical capsid–membrane interaction. Mutant capsids appeared to be assembled and transported to the inner surface of the membrane at a normal rate; however, they accumulate under the membrane and are released inefficiently. Few capsids were observed in the late stages of the budding process, suggesting that the two amino acid substitutions in the MA protein interfered in some way with capsid interactions with the membrane. Since mutant T41I, with a significant change at position 41, showed no differences in virus replication from wild-type virus, the change of threonine at position 78 is probably responsible for this reduced capsid–membrane interaction. This threonine residue is located within a long stretch of uncharged amino acids which we have previously shown, with a 7 amino acid deletion mutant (d79/85), to be structurally important for precursor stability (Rhee and Hunter, 1990b). Therefore, it is possible that the threonine

substitution could induce a conformational change which results in the altered interaction.

The studies of the MA mutants we present here allow us to define several events in type D retrovirus morphogenesis. In M-PMV-infected cells, Gag polyproteins synthesized and modified (with myristic acid) as cytosolic precursor polyproteins are targeted to a specific cytoplasmic region where the molecules are retained to a sufficiently high concentration for self-assembly into an immature capsid. Preassembled capsids then become transport-competent and are rapidly transported by the intracytoplasmic transport machinery of the cell to the plasma membrane where virus budding occurs. The MA protein of M-PMV has multiple functional roles in these complex processes. In addition its native structure in the precursor may contribute to the stability of the precursor molecules and to the kinetics of some processes that are very sensitive to changes in the tertiary structure of the Gag polyprotein itself.

Materials and methods

DNAs

M13.GAG is a mutagenesis vector containing a 1.1 kbp *Sph*I–*Sst*I fragment from an infectious M-PMV proviral clone, pMPMV6A/7 (Barker *et al.*, 1985). This fragment encompasses the 3′ end of the left long terminal repeat (LTR) and half of the *gag* gene, including the entire coding sequence of the MA protein. M13.d11/94 contains the *Sph*I–*Sst*I fragment with a 252 bp deletion within the MA protein coding domain. This deletion clone was constructed by oligonucleotide-directed mutagenesis on single-stranded M13.GAG viral DNA (Rhee and Hunter, 1990b). A unique *Xho*I site was inserted at the site of deletion in M13.d11/94 to give M13.X.d11/94 by using oligonucleotide-directed mutagenesis (Zoller and Smith, 1984).

Sodium bisulphite mutagenesis

Sodium bisulphite mutagenesis was carried out as previously described with some modifications (Everett and Chambon, 1982; Shortle and Nathans, 1978). The replicative form DNA of M13.X.d11/94 was cut with *Xho*I and 2.5 μg of this linear double-stranded DNA was mixed with 1.75 μg of single-stranded DNA of M13.GAG in 25 μl of annealing buffer [0.2 NaCl, 1.0 mM EDTA, 10 mM Tris–hydrochloride (pH 7.5)]. After paraffin oil was layered above the mixture, the tube was incubated in a boiling water bath for 5 min and then incubated at 65°C for 14 h. The mixture was allowed to cool slowly to room temperature, and the DNA was ethanol precipitated. The precipitated DNA was resuspended in 15 mM NaCl, 1.5 mM sodium citrate (pH 7.0) and treated with 3 M sodium bisulphite at 37°C for 1 h. The reaction was terminated by diluting the mixture in 6 volumes of 60 mM NaCl, 2.4 mM EDTA, 0.24 M Tris (pH 9.2) and precipitating the DNA four times with isopropanol. This process completed the deamination reaction and removed the bisulphite from the modified DNA. The single-stranded region of the mutated heteroduplex was then filled in at 16°C for 4 h with 1.5 U DNA polymerase I in the presence of all four deoxynucleotide triphosphates at a concentration of 100 μM each. The unpaired bases at the extremities of the gap derived from the *Xho*I insertion in M13.X.d11/94 were removed by the exonuclease activities of the holoenzyme. The modified DNA was transfected into the M13 host TG-1 and viral DNA from the resultant plaques was sequenced (Sanger *et al.*, 1977). After mutagenesis, a *Nar*I–*Sst*I fragment was excised from the replicative form of mutant phage and substituted for the wild-type fragment in an M-PMV expression vector pSHRM15 (Rhee *et al.*, 1990). This plasmid contains a infectious M-PMV genome, and a hygromycin resistance gene under control of the SV40 early promoter.

Cells, immunoprecipitation, and gel electrophoresis

To establish cell lines containing an integrated copy of the mutant proviral DNA, HeLa cells were transfected by the calcium phosphate precipitation method (Graham and Van der Eb, 1973; Stow and Wilkie, 1976) with viral DNAs linearized with *Fsp*I. Resistant cell colonies were selected in medium containing 250 U/ml of the antibiotic hygromycin B and screened to determine whether they expressed viral structural proteins (Rhee and Hunter, 1987).

For the transient expression of viral proteins in COS-1 cells, the cells were transfected with viral DNAs (5 μg/35 mm plate) by the modified calcium phosphate precipitation method (Chen and Okayama, 1987). At 48 h after transfection, cells were pulse-labelled for 20 min with [^{3}H]leucine (0.8 mCi/ml, 157 Ci/mmol; DuPont Co.) and chased for the indicated period in complete growth medium (Rhee and Hunter, 1987). Cells were lysed in lysis buffer A [1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris (pH 7.5)], and cell-associated viral proteins were immunoprecipitated with goat anti-MPMV antiserum (Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, MD; Bradac and Hunter, 1984). Radiolabelled, extracellular virus particles were pelleted from the culture medium of the pulse–chase-labelled cells by centrifugation for 10 min at 80 000 r.p.m. in a Beckman TLA 100 rotor at 4°C, and the virus pellet was lysed in lysis buffer B [0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.05 M Tris–hydrochloride (pH 7.5)]. Virion-associated viral proteins were immunoprecipitated with antiserum against M-PMV proteins as described above.

Virus polypeptides immunoprecipitated with virus-specific antibodies were separated on a 10% resolving gel by SDS–PAGE (Bradac and Hunter, 1984). The amount of [^{3}H]leucine-labelled protein was quantified by counting the radioactivity of bands excised from the dried gel in a liquid scintillation counter (Wills *et al.*, 1984).

Fractionation of Gag polyprotein

Gag polyproteins were fractionated into free and capsid-associated forms as described previously (Rhee and Hunter, 1990b). HeLa cell lines were pulse-labelled, chased for the indicated period as described above, and were lysed in 1 ml of Triton X-100 lysis buffer [0.5% Triton X-100, 0.25 M sucrose, 1.0 mM EDTA, 0.14 M NaCl, 10 mM Tris (pH 7.5)] at room temperature for 1 h. After the lysates were centrifuged at 80 000 r.p.m. for 10 min in a Beckman TLA100 rotor at 4°C, viral proteins in the supernatant fraction and in the pellet fraction were separately immunoprecipitated with rabbit anti-p27 antiserum.

Electron microscopy

COS-1 cells were transfected with viral DNAs, and at 48 h post-transfection they were fixed at room temperature for 1 h with 1% glutaraldehyde (Compans *et al.*, 1966). After washing in phosphate-buffered saline, they were postfixed with 1% osmium tetroxide and then embedded in an epoxy resin mixture. Thin sections of the cells were stained with uranyl acetate and lead nitrate. All preparations were examined in a Philips 301 electron microscope.

Cell-free infection and RT assay

Culture fluids were harvested for 24 h from the COS-1 cells which had been transfected 48 h previously with proviral DNAs. They were clarified by centrifigation 10 000 r.p.m. for 10 min in a Beckman JA20 rotor at 4°C, then equivalent amounts of RT-containing medium were used to infect HeLa cells in the presence of 2.0 μg/ml of polybrene (hexadimerthrine bromide; Sigma) at 37°C for 1.5 h. On days 1, 4, 6, 8, 10 and 12 post-infection, culture fluid was harvested from the cells, and assays for RT were carried out (Rhee and Hunter, 1987; Sacks *et al.*, 1978). A 7.5 μl portion of the disrupted virus was incubated at 37°C for 1 h with 30 μl RT mixture containing poly(rA)–oligo(dT) as synthetic RNA template–DNA primer (Boehringer Mannheim Biochemicals) and [^{3}H]TTP (57 Ci/mmol; Amersham Corp.). The reaction was terminated with a final concentration of 50 mM sodium pyrophosphate on ice, and the mixture was spotted onto an NA45 Anion-exchange membrane (Schleicher & Schunell, Inc.). Membranes were washed thoroughly in 0.5 M phosphate buffer (pH 6.5). The extent of DNA synthesis by RT activity was measured by counting the amount of radioactivity on the dried membrane in an aqueous scintillation cocktail (Budget Solve; Research Products Int. Corp.)

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