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Structural Role of the Matrix Protein of Type D Retroviruses in Gag Polyprotein Stability and Capsid Assembly

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To obtain a better understanding of the role of the *gag* gene-encoded matrix (MA) protein in the assembly and maturation of type D retroviruses, we have made five mutants with specific in-frame deletions within the p10-coding region by the use of oligonucleotide-directed mutagenesis. The changes in the Gag polyprotein made by these mutations resulted in almost identical phenotypes. In cells expressing mutant genomes, the mutant Gag polyproteins were synthesized and modified with myristic acid in a normal manner. However, they were so unstable that the bulk of the newly synthesized polyproteins was degraded within 1 h without being processed into mature structural polypeptides. In contrast, wild-type polyproteins have a processing half-life of 3.0 to 3.5 h. The mutant Gag polyproteins were assembled with very low efficiency into capsids in the cytoplasm of the mutant-infected cells. Moreover, the few capsids that formed were neither released from nor accumulated in the cells. These results suggest that the matrix protein plays an important role in guiding the correct folding of the Gag polyprotein, which is presumably crucial for both stabilizing the molecule and facilitating the intermolecular interactions that occur during assembly of immature capsids.

In the late stages of retrovirus replication, a capsid shell is assembled from the viral *gag* gene products (Gag polyproteins) and buds from the plasma membrane of an infected cell. Shortly after budding, or late in the budding process itself, the virus undergoes final maturation as the Gag polyproteins of the immature capsid are proteolytically cleaved to the internal structural proteins of the mature virus. In the mature virion, the viral envelope surrounds the capsid, which consists of an envelope-associated outer shell and an inner ribonuclear-protein core composed of genomic RNA and structural protein subunits (20, 22). In most mammalian retroviruses, the Gag polyproteins are cleaved to yield at least three internal structural proteins arranged in the order NH_2–membrane-associated matrix (MA) protein–major capsid (CA) protein–nucleocapsid (NC) protein–COOH. This generalized scheme varies in various retroviruses, but these defined functional domains appear to be located in the same relative positions in all Gag polyproteins. Although there has been progress in understanding the processes of retroviral capsid assembly and maturation, the exact roles of the internal structural proteins in these events are poorly understood.

Studies with several retroviruses have suggested that the amino-terminal hydrophobic protein of the *gag* gene product associates with the virus envelope (6, 19, 25). Therefore, this protein has been designated as the retroviral MA protein by analogy with the lipid-associated M protein of vesicular stomatitis virus and other negative-stranded viruses (25). The MA protein of a retrovirus presumably functions in mediating interactions between the other internal structural proteins and the cell membrane. Indeed, immunoelectron microscopic studies by Gelderblom et al. (14) showed that the MA protein of human immunodeficiency virus, p17, is a component of the envelope-associated icosadeltahedral capsid that lines the inner surface of the virus envelope. Because of its association with the membrane, the MA protein has also been postulated to interact with the virus envelope glycoproteins at the plasma membrane during budding (6, 21). This concept is supported by chemical cross-linking studies; with Rous sarcoma virus, Gebhardt et al. (13) showed that the MA protein, p19, could be cross-linked to the transmembrane glycoprotein, gp37. However, the nature of the interaction between these two proteins has not been defined, and it is not clear whether such interactions are essential for glycoprotein incorporation into virions.

We focus here on molecular genetic studies of the MA protein of Mason-Pfizer monkey virus (M-PMV), which is the prototype of the type D retroviruses (9, 12). In M-PMV-infected cells, the Gag polyproteins are synthesized as cytoplasmic polypeptides which are assembled within the cytoplasm into an immature capsid before transport to the plasma membrane. Our previous studies (26) on the role of myristylation of the Gag polyprotein of M-PMV showed that this modification plays a crucial role in mediating capsid transport from the site of assembly within the cytoplasm to the site of budding on the membrane. In the absence of myristylation, preassembled capsids accumulated within the cytoplasm of infected cells. Although these results demonstrated a requirement for myristic acid addition, the fact that several myristylated viral and cellular proteins are not membrane bound argued against myristic acid per se being the primary transport signal (1, 5, 7, 10, 23, 33). It therefore seemed possible that the signals for capsid transport could be located in the p10 (MA) protein of M-PMV, since this protein is likely to be exposed on the exterior of the intracytoplasmic capsid.

As an initial approach to obtain a better understanding of the role of this protein in virus assembly and replication, five in-frame deletions within the p10-coding region were constructed by the use of oligonucleotide-directed mutagenesis. All five mutations resulted in the synthesis of highly unstable Gag polyproteins which were inefficiently assembled into capsids. The latter were neither transported to the plasma membrane nor released from the infected cell. These results

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point to an important role for the MA protein in directing the correct folding of Gag polyprotein, which is in turn crucial for both stabilizing the polyprotein and allowing the intermolecular interactions of the polyproteins to assemble an immature but transportable capsid.

MATERIALS AND METHODS

Oligonucleotide-directed mutagenesis and cells. To generate in-frame deletions in the MA protein of M-PMV, p10, oligonucleotide-directed mutagenesis was carried out with various synthetic oligonucleotides as previously described (40). To perform the mutagenesis, a 1.1-kilobase-pair *Sph*I-*Sst*I fragment from an infectious M-PMV proviral clone, pMPMV6A/7 (2), was subcloned into M13mp19. This fragment contains the 3′ end of the left long terminal repeat and half of the *gag* gene including the entire coding sequence of p10. After mutagenesis, a 0.8-kilobase-pair *Nar*I-*Sst*I fragment was excised from the replicative form of mutant bacteriophage and substituted for the wild-type fragment in the original plasmid. The presence of the mutations was confirmed by dideoxy sequencing of the double-stranded DNA (27).

To establish the cell lines containing integrated wild-type or mutant virus DNA, semiconfluent monolayers of HeLa cells were cotransfected with either wild-type or mutant virus DNA and pPB3 by the calcium phosphate precipitation method (15, 32). pPB3, which expresses the hygromycin resistance gene, was constructed and kindly provided by P. Bird, Health Sciences Center, Dallas, Tex. Resistant cell colonies were selected in medium containing 250 U of the antibiotic hygromycin B per ml and screened to determine whether they expressed viral structural proteins (26).

Radiolabeling, immunoprecipitation, and gel electrophoretic analysis of virus proteins. Confluent monolayers of HeLa cell clones were incubated for 1 h in leucine-free Dulbecco modified Eagle medium and then pulse-labeled for 20 min at 37°C in leucine labeling medium containing [^{3}H]leucine (0.8 mCi/ml; 57 Ci/mmol; Amersham Corp.) with occasional rocking. Pulse-labeled cells were chased for 0.5 to 4.5 h in complete growth medium. Fatty acid labeling was carried out for 2 h at 37°C in [9,10-^{3}H]myristic acid labeling medium (0.5 mCi/ml; 55 Ci/mmol; Amersham Corp.) as described previously (26).

Radiolabeled viral proteins were immunoprecipitated with rabbit anti-p27 or goat anti-M-PMV antiserum (Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, Md.) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (3). To quantitate the amount of [^{3}H]leucine-labeled viral proteins, each band was excised from the dried gels after fluorography and its radioactivity was measured in a liquid scintillation counter (39).

Western blot (immunoblot) analyses of virus and capsid. Viruses released from the culture fluids of wild-type and mutant cell clones were pelleted through a 24% sucrose cushion by centrifugation at 27,000 rpm in a Beckman SW28 rotor for 2 h (26). For capsid preparation, cell monolayers were washed twice in TNE buffer (10 mM Tris hydrochloride [pH 7.5], 0.15 M NaCl, 1.0 mM EDTA) and lysed in Triton X-100 lysis buffer (0.25 M sucrose, 1.0 mM EDTA, 10 mM Tris hydrochloride [pH 7.5], 0.14 M NaCl, 0.5% Triton X-100). Nuclei were removed by centrifugation at 10,000 rpm in a Beckman JA20 rotor for 10 min. Capsids were pelleted from the clarified supernatants through a 30% sucrose cushion by centrifugation at 30,000 rpm in a Beckman SW41 rotor for 100 min (26). Virus and capsid pellets were dissolved directly in PAGE sample buffer and subjected to SDS-PAGE. Proteins in polyacrylamide gels were electrophoretically transferred to nitrocellulose at 200 V for 1 h. After transfer, nitrocellulose blots were saturated in BLOTTO (5% nonfat dry milk in phosphate-buffered saline) (18) and then incubated with rabbit anti-p27 or anti-gp70 antiserum for 16 h at 4°C (8, 26). Bound antibodies were detected by incubating blots with ^{125}I-labeled protein A (0.06 mCi/ml; 2 to 10 mCi/mg; Du Pont Co.) for 1 h at 37°C.

Fractionation of Gag polyproteins. Gag polyproteins were fractionated into free and capsid-associated forms as described previously (26) with some modification. Cells were pulse-labeled for 20 min with [^{3}H]leucine (0.8 mCi/ml), chased for the indicated period as described above, and then lysed in 1 ml of Triton X-100 lysis buffer by 1 h of incubation at room temperature. Nuclei were removed from the lysates by centrifugation in a microcentrifuge for 5 min at 4°C. The entire supernatant was then centrifuged at 80,000 rpm for 10 min in a Beckman TLA100 rotor at 4°C. The fraction of free Gag polyproteins (supernatant) was adjusted to 0.1% SDS, and capsid-associated Gag polyproteins (pellet) were suspended 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]). Viral proteins were immunoprecipitated with anti-p27 antiserum and analyzed by SDS-PAGE as described above.

RESULTS

MA protein deletion mutant *d*11/94 synthesizes truncated, unstable Gag polyproteins. To address the possible role of MA protein in the processes of capsid preassembly and transport as well as budding and maturation of M-PMV, a mutant which deleted most of the MA (p10)-coding sequences was constructed. In this mutant, *d*11/94 (Fig. 1), the entire coding region of p10 was deleted except for the amino-terminal 10 amino acids, which we postulated would be required for myristic acid addition, and the carboxy-terminal 6 amino acids, which we predicted would retain the cleavage site for viral protease. The deletion thus results in a Gag polyprotein truncation of 84 amino acids.

The mutated *gag* gene fragment (*Nar*I-*Sst*I fragment) was used to replace the corresponding wild-type sequences in the M-PMV expression vector pMPMV6A/7 (26). Initial experiments in which the mutant genome was transfected into susceptible target cells showed that it was no longer infectious (data not shown). The mutant DNA was therefore transfected into HeLa cells along with a hygromycin-resistance expression plasmid (pPB3) to establish the mutant genome-expressing cell line, *d*11/94, as described in Materials and Methods. Cell line 6A/7, which expresses the wild-type M-PMV genome, was established in a similar manner (26).

In order to determine whether mutant Gag polyproteins are synthesized and processed into mature structural proteins, a pulse-chase experiment was carried out (Fig. 2A). In virus-infected cells, the M-PMV Gag polyprotein, Pr78^{gag}, is cleaved to yield the six nonglycosylated internal structural proteins (p10, pp24-16, p12, p27, p14, and p4) of the mature capsid shortly after virus budding (3, 16). This processing was observed in the wild-type 6A/7 cells (Fig. 2A, lanes 1 and 3). In cells pulse-labeled for 20 min, three *gag*-related precursors (Pr78^{gag}, Pr95, and Pr180) can be identified (lane 1). Pr95 and Pr180, presumably synthesized via a frameshifting mechanism, are the precursors of the viral protease and

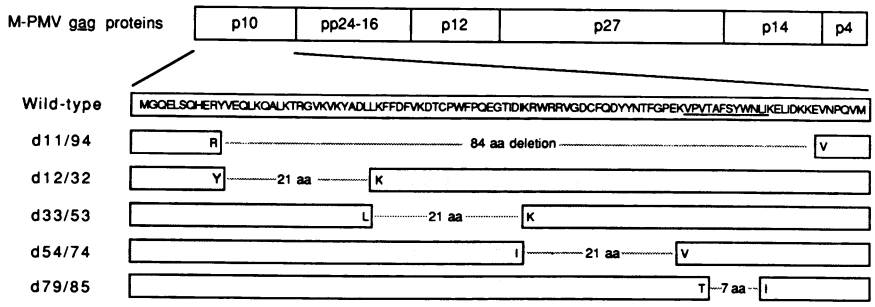

FIG. 1. Schematic representation of the p10 deletion mutants of M-PMV. The arrangement of the structural proteins within the *gag* gene-encoded polyprotein is schematically presented with the amino acid sequences of wild-type p10 (MA) protein by using single-letter amino acid codes. The amino acid sequences were deduced from the nucleic acid sequences (31). Sizes and positions of amino acids deleted in each mutant are indicated as a designation of the mutant (i.e., mutant *d*11/94 has an internal deletion of 84 amino acids [aa] from amino acid residue 11 to 94). A stretch of uncharged amino acids near the carboxy terminus of p10 is underlined.

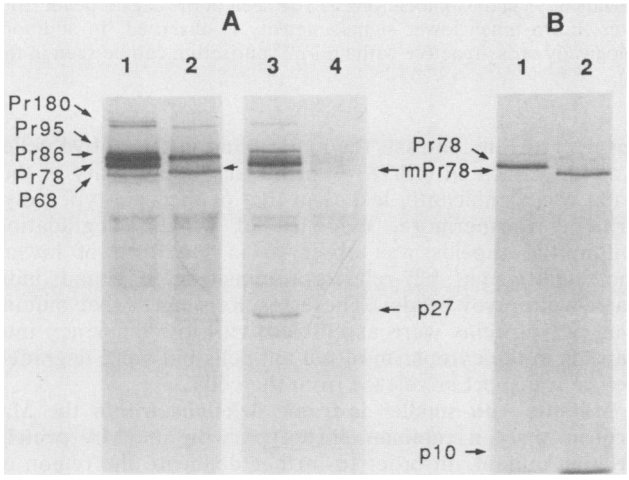

FIG. 2. Immunoprecipitation of M-PMV proteins labeled with [^{3}H]leucine or [^{3}H]myristic acid. Cells of lines 6A/7 and *d*11/94, expressing the wild-type and 84-amino-acid deletion mutant viral genomes, respectively, were pulse-labeled with [^{3}H]leucine (A) or labeled for 2 h with [^{3}H]myristic acid (B). Radiolabeled viral proteins were immunoprecipitated with goat anti-M-PMV antiserum to identify the *gag* and *env* gene products of M-PMV, which were then separated by SDS-PAGE (12% polyacrylamide). (A) Three *gag*-related precursor polyproteins (Pr180, Pr95, and Pr78^{gag}) and the *env* precursor glycoprotein (Pr86^{env}) can be seen in [^{3}H]leucine-labeled wild-type-infected cells (lane 1). In addition, an internal initiation product of the *gag* gene (P68) is detectable (lane 1). Truncated forms of Pr180 and Pr78^{gag} can be seen in mutant-infected cells (mPr78^{gag}; lane 2). The truncated counterpart of Pr95 comigrates with the *env* gene product Pr86^{env} in [^{3}H]leucine-labeled cells (lane 2) but is clearly seen in [^{3}H]myristic acid-labeled cells (panel B, lane 2). After a 4.5-h chase, a new band corresponding to the major internal structural protein p27 of the virion can be seen only in wild-type 6A/7 cells with residual unprocessed precursor polyproteins (lane 3). In contrast, no processing products of the *gag* polyproteins are observed in mutant *d*11/94 cells (lane 4), but a drastic reduction in the intensity of the mPr78^{gag} band can be seen (lane 4). (B) [^{3}H]myristic acid-labeled Pr95 and Pr78^{gag} in wild-type 6A/7 cells (lane 1) and their truncated counterparts in mutant *d*11/94 cells (lane 2) are seen. In 6A/7 cells, an additional radiolabeled protein corresponding to the matrix protein of M-PMV p10 is visible (lane 1).

of the reverse transcriptase, respectively (3, 31). In addition, an intense band corresponding to the glycoprotein precursor, Pr86^{env}, can be seen. After a 4.5-h chase, a band of 27 kilodaltons corresponding to the major capsid protein, p27, could be detected in the lysates of 6A/7 cells as a processing product of the polyproteins (Fig. 2A, lane 3). While a significant proportion of the polyproteins was processed during this chase period, approximately 30% remained unprocessed (lane 3). Thus, the wild-type Gag polyprotein has a processing half-life on the order of 3 h (see below).

In contrast, in mutant *d*11/94 cells, the truncated proteins (Fig. 2A, lane 2), mPr95 and mPr78^{gag}, were smaller by an amount consistent with an 84-amino-acid deletion and comigrated with the glycoprotein precursor Pr86^{env} and an internal initiation product of the *gag* gene, P68 (26). After a 4.5-h chase, no band corresponding to p27 could be detected even though the radiolabeled mutant Gag polyproteins were lost during the chase period (lane 4).

These results suggested that the truncation of p10 triggered a rapid degradation of the entire Gag polyprotein. However, to rule out the possibility that this molecule was being rapidly assembled into virions, processed, and released from the cell, culture supernatants were centrifuged at 27,000 rpm in a Beckman SW28 rotor for 2 h through a 24% sucrose cushion. The pelleted virion proteins were separated by SDS-PAGE and immunoblotted with anti-p27 or anti-gp70 antiserum (Fig. 3A). Intense bands corresponding to the major capsid protein p27 and envelope glycoprotein gp70 were seen in supernatants from the wild-type 6A/7 cells, but no evidence of any viral proteins could be seen in mutant *d*11/94 cell supernatants. This result was supported by assays for reverse transcriptase (RT)-containing particles in the medium of wild-type and mutant infected cells. High levels of RT activity were present in 6A/7 culture fluids, whereas no RT activity could be detected in the culture fluids of mutant cells (data not shown). Thus, it can be concluded that the mutant polypeptides are rapidly degraded, since they neither accumulate within the cells nor are released into the culture medium.

Destabilization of the mutant Gag polyprotein is not due to lack of myristylation. Since the deletion in *d*11/94 left only 10 amino-terminal amino acids of p10, it was possible that the unstable phenotype resulted from a combination of the truncation and a lack of myristylation. Except for an amino-terminal glycine residue, there is no clear consensus sequence which operates as a myristylation signal among all of the known myristylated proteins (30). However, recent

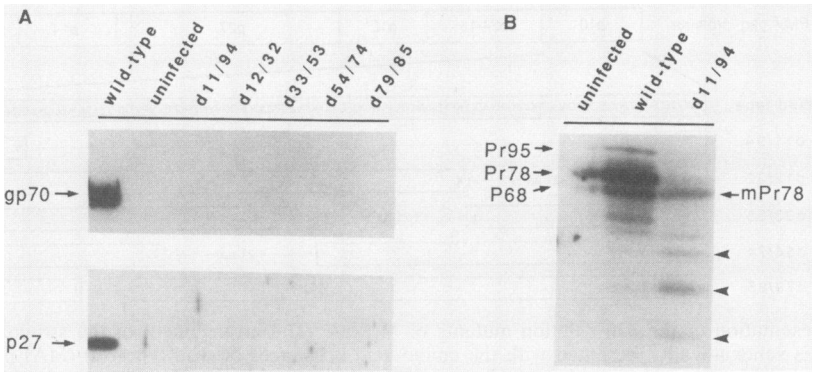

FIG. 3. Western blot analysis of released virus and intracellular capsids. (A) To determine whether the mutant genome-expressing cells released virions into the culture media, virus particles were pelleted by centrifugation from the culture fluids of wild-type and mutant cells through a 24% sucrose cushion. Viral proteins in the pellets were separated by SDS-PAGE and immunoblotted with anti-p27 and anti-gp70 antisera. Strong signals can be seen for both p27 and gp70 only in the lane corresponding to wild-type cell culture medium. No evidence of virion release could be observed in any of the mutant cell clones. (B) To determine whether the unstable mutant Gag polyproteins can be assembled into capsids in mutant *d*11/94 cells, crude intracellular capsids were prepared by pelleting 0.5% Triton X-100 lysates of equivalent numbers of wild-type 6A/7 and *d*11/94 cells through a 30% sucrose cushion. Capsid proteins in the pellets were detected as described above with rabbit anti-p27 antiserum. A major band of 78 kilodaltons and minor bands of 95 and 68 kilodaltons can be seen in the capsid pellet from 6A/7 cells. The truncated mPr78^{gag} can be detected in *d*11/94 cell lysates, but a much lower signal intensity is observed. In addition, degradation products of mutant Gag polyproteins (◄) which are immunologically cross-reactive with anti-p27 antiserum can be seen in the mutant capsid pellet.

studies with the in vitro myristylation of synthetic peptides by Towler et al. (35–37) suggest that the signal for myristylation exists in the primary sequence of amino acids near the amino terminus of the protein. These in vitro observations have been supported by in vivo studies of p60^{src} (4, 24). We therefore determined whether the mutant Gag polyprotein retained sufficient signal sequences to be myristylated.

Cell lines *d*11/94 and 6A/7 and uninfected HeLa cells were labeled for 2 h at 37°C in [^{3}H]myristic acid-labeling medium (0.5 mCi/ml), lysed, and then immunoprecipitated with anti-p27 antiserum. In M-PMV-infected cells, all three *gag*-containing precursor polyproteins (Pr180, Pr95, and Pr78^{gag}) (3) are modified with myristic acid through an amide linkage to the amino-terminal glycine residue (26, 28). [^{3}H]myristic acid-labeled Pr78^{gag} and Pr95 and their truncated counterparts can be clearly seen in both 6A/7 and *d*11/94 cells, respectively (Fig. 2B, lanes 1 and 2). In addition, the amino-terminally myristylated matrix protein, p10, was detected in wild-type cells (lane 1), whereas in mutant *d*11/94 cells no band equivalent to mature structural proteins was observed (lane 2). Pr180 was not detected in this experiment, reflecting the fact that Pr180 is synthesized at only 5% of the level of Pr78^{gag}. These data indicated that there was no defect in myristylation in mutant *d*11/94 and therefore that the instability of the precursor proteins resulted from the truncation alone.

In mutant *d*11/94 cells capsids are assembled with very low efficiency but are degraded without being released. To determine whether the unstable mutant Gag polyproteins can be assembled into capsids, we prepared crude capsids by pelleting them from 0.5% Triton X-100 lysates of 6A/7 and *d*11/94 cells through a 30% sucrose cushion. Under these conditions, the newly synthesized polyproteins remained in the soluble fraction and preassembled capsids were pelleted (see below). The proteins in the pellets were separated on a 10% polyacrylamide gel, transferred to nitrocellulose, and probed with anti-p27 antiserum. The results of this experiment are shown in Fig. 3B. In the wild-type 6A/7 pellet, an intense band of Pr78^{gag} could be observed, along with less intense bands of Pr95 and P68, consistent with our previous results (26). In contrast, the major band in the *d*11/94 pellet was the truncated mPr78^{gag}; however, the intensity of this band was significantly less than that of the wild-type polyprotein. Furthermore, evidence of protein degradation within the capsids was observed in the form of lower-molecular-weight, p27-related proteins (Fig. 3B; bands indicated with arrowheads). These results suggest that mutant Gag polyproteins were assembled with low efficiency into capsids in the cytoplasm of mutant cells but were degraded before transport or release from the cells.

Mutants with smaller in-frame deletions within the MA protein share a common phenotype with the MA protein deletion mutant. In order to further delineate the region of the MA protein required for Gag polyprotein stability, as well as other potential MA protein functions, we constructed an additional four MA mutants with small in-frame deletions within the p10 coding domain by oligonucleotide-directed mutagenesis and established cell lines which express mutant viral genomes (Fig. 1). Mutants *d*12/32, *d*33/53, and *d*54/74 have consecutive 21-amino-acid deletions in the amino-terminal two-thirds of p10, while mutant *d*79/85 contains a 7-amino-acid deletion within a stretch of uncharged amino acids near the carboxy terminus of the protein.

To determine the kinetics of processing for the mutant Gag polyproteins, we performed a pulse-chase experiment (Fig. 4). The four mutant cell lines as well as wild-type and *d*11/94 cells were pulse-labeled with [^{3}H]leucine for 20 min and chased for 0.5 to 4.5 h. In wild-type 6A/7 cells, the processing of Pr78^{gag} was observed with a faint band of p27 after a 1.5-h chase (Fig. 4, lane 3); increasing amounts of p27 were detected after 3-h (lane 4) and 4.5-h (lane 5) chases. This result confirmed the processing half-life of 3.0 to 3.5 h determined above. In contrast, none of the mutant Gag polyproteins was processed to mature structural proteins; most of the newly synthesized mutant polyproteins, including that of *d*11/94, were degraded within 1 h. Thus, all five deletion mutations within the MA protein resulted in an identical phenotype, that of destabilizing the Gag polyprotein.

Preliminary studies from Western immunoblot analyses

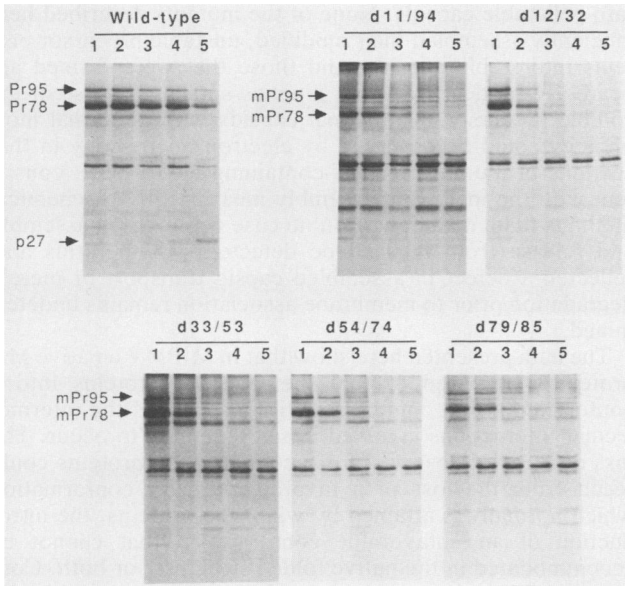

FIG. 4. Kinetics of Gag polyprotein turnover in wild-type and mutant cell lines. To determine the rate of mutant Gag polyprotein turnover, HeLa cell clones were pulse-labeled (lanes 1) with [^{3}H]leucine for 20 min and chased for 0.5 h (lanes 2), 1.5 h (lanes 3), 3.0 h (lanes 4), or 4.5 h (lanes 5). Radiolabeled viral proteins were immunoprecipitated with anti-p27 antiserum and separated by SDS-PAGE (10% polyacrylamide). In wild-type-infected cells, the pulse-labeled Gag polyprotein Pr78^{gag} (Wild-type; lane 1) is processed to mature structural proteins, which can be correlated with a faint band of cell-associated p27, during a 1.5-h chase (lane 3). Increasing intensity of the p27 band can be observed following 3.0-h (lane 4) and 4.5-h (lane 5) chases, while decreasing amounts of Pr78^{gag} are detected. In mutant genome-infected cells, an amount of pulse-labeled mutant Gag polyprotein mPr78^{gag} equivalent to the amount of wild-type Pr78^{gag} can be seen (lane 1), but no band corresponding to the processed products of Gag polyprotein is detectable in any mutant cells. Instead, a drastic decrease in the intensity of the mPr78^{gag} band can be observed following 0.5-h (lanes 2 in panels representing mutants) and 1.5-h (lanes 3) chases, and most of the mPr78^{gag} has been lost following a 3.0-h chase (lanes 4).

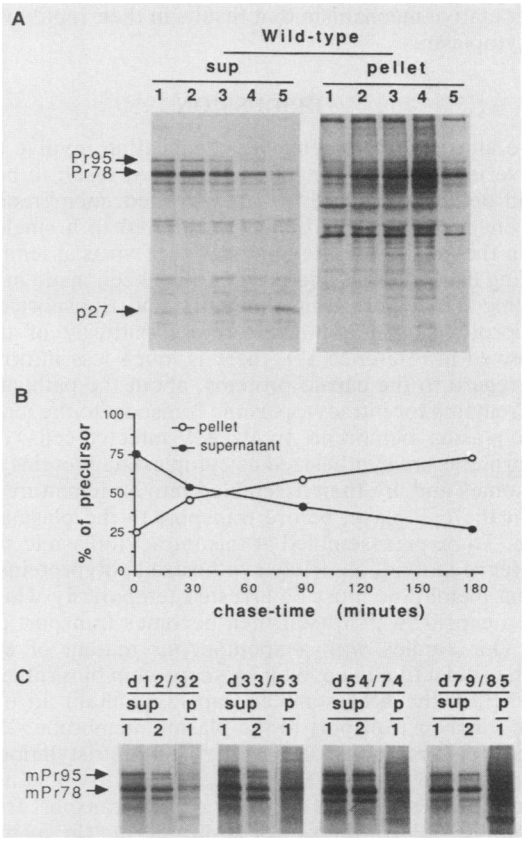

FIG. 5. Kinetics of intracellular capsid formation. To determine the rate and extent of capsid formation of mutant Gag polyproteins, wild-type and mutant cells were pulse-labeled with [^{3}H]leucine and chased as described in the legend to Fig. 4. After the indicated chase time (0.5 to 4.5 h), cells were lysed and assembled capsids were pelleted by centrifugation. Radiolabeled Gag polyproteins in the pelleted (pellet or p) and soluble (sup) fractions were immunoprecipitated with anti-p27 antiserum and separated by SDS-PAGE (10% polyacrylamide). The amount of [^{3}H]leucine-labeled Gag polyprotein was measured by counting the radioactivity of each band excised from the dried gel in a liquid scintillation counter. (A and B) After the pulse-label in wild-type 6A/7 cells, radiolabeled Pr78^{gag} can be detected in both soluble (panel A, sup lane 1) and pellet (pellet lane 1) fractions, which contain approximately 75% and 25% of the precursor, respectively (B). During the chase periods (panel A, lanes 2 to 5), increasing amounts of Pr78^{gag} can be seen in the pellet fractions, with concomitant decreases in the soluble fractions. One-half of the pulse-labeled Gag polyproteins can be found in the capsid-associated forms within 45 min (B). In addition, the major capsid protein (p27) of the virion can be seen in the supernatant fraction after a 1.5-h chase (panel A, sup lane 3), and its intensity increases during the later chase periods (sup lanes 4 and 5). In contrast, no p27 band can be observed in the pelletable fractions. (C) In the mutant cells, most of the pulse-labeled mutant Gag polyprotein mPr78^{gag} can be found in the soluble fraction (sup lane 1), and less than 10% of it is detected in the pellet fraction (p lane 1).

indicated that capsids were inefficiently formed in all of the small in-frame deletion mutants, and these preassembled forms were not accumulated in the cytoplasm (data not shown). In addition, no release of mutant capsids was observed (Fig. 3A). In order to more carefully examine the kinetics of wild-type and mutant capsid assembly, Gag polyproteins pulse-labeled for 20 min were fractionated into soluble and pelletable (capsid) forms at various times of chase (Fig. 5), as described in Materials and Methods. In wild-type 6A/7 cells, about 25% of the newly synthesized Gag polyproteins were already assembled into capsids during the 20-min pulse-labeling period (Fig. 5A; compare sup lane 1 with pellet lane 1), and increasing fractions of them were incorporated into capsids during the chase periods (Fig. 5A, pellet lanes 2 to 5). In M-PMV infected cells, half of the newly synthesized Gag polyproteins were assembled into capsids within 45 min (Fig. 5B). Interestingly, mature capsid protein, p27, was detected in the soluble fraction at later chase times (Fig. 5A, sup lanes 3 to 5). This is consistent with earlier observations that the mature retrovirus capsid is a relatively fragile structure which is easily disrupted in mild detergent concentration (34). In the mutant cells, including *d*11/94 cells, less than 10% of the pulse-labeled polyproteins were recovered in the pellet fraction during the pulse-labeling period (Fig. 5C, p lane 1), and no increase was observed during the chase periods (data not shown). Instead, mutant polyproteins were hardly detected in the pellet fraction even after a chase period as short as 30 min; this explains why accumulation of mutant capsids was not detectable in Western blot analyses. These results suggest that all of the deletion mutations constructed in the MA protein reduce the efficiency of capsid assembly and trigger

a degradative mechanism that results in their rapid loss from the cytoplasm.

DISCUSSION

The later stages of retrovirus replication involve a complex series of molecular interactions that result in both the capsid polypeptides and the glycosylated, membrane-spanning envelope proteins being transported to a single point within the cell where the final stages of virus assembly and budding can occur. While progress has been made in understanding the requirements for entry and transport of viral glycoproteins through the secretory pathway of the cell (reviewed in reference 17), there is much less information, with regard to the capsid proteins, about the pathways and requirements for intracytoplasmic transport to the inner face of the plasma membrane. In M-PMV-infected cells, the Gag polyproteins are synthesized as cytoplasmic proteins on free polysomes and are then assembled into an immature capsid within the cytoplasm before transport to the plasma membrane. To be preassembled at this intracytoplasmic site, the transfer of individual, newly synthesized polyproteins to the plasma membrane must be arrested temporarily while they form a capsid, which itself then becomes transport competent. Our studies with a spontaneous mutant of M-PMV which is defective in envelope glycoprotein biosynthesis has shown that the preassembled capsids contain an intrinsic signal for their transport to the plasma membrane (25a).

Our previous studies (26) of the role of myristylation of the Gag polyprotein of M-PMV showed that fatty acid addition plays a required role in signaling capsid transport from the site of assembly to the site of budding from the membrane. In its absence, preassembled capsids accumulated within the cell cytoplasm infected with mutant genome. Nevertheless, the fact that several viral proteins as well as cellular proteins modified with myristic acid are not targeted to the plasma membrane prompted us to investigate the role of amino acid sequences within the Gag polyprotein in the transport process. The deletion mutations within the MA-coding region described in this paper were constructed as an initial analysis of MA function in the assembly and transport processes, since this region of the Gag polyprotein would be expected to be at least partially exposed on the outer face of the preassembled capsid.

The unstable Gag polyprotein phenotype observed for each of the five mutants was unexpected, since in both the avian retroviruses (38) and in murine leukemia virus (11, 29), deletions within the *gag* gene region not affecting the CA protein primarily resulted in stable Gag polyproteins that could be assembled into modified capsid structures. Indeed, we have observed that a deletion that removes two-thirds of the p12-coding domain of M-PMV results in the synthesis of a very stable, truncated Gag precursor (unpublished data). The instability of the M-PMV MA deletion mutants did not result from a lack of myristic acid addition since even in mutant *d*11/94, in which most of the p10-coding region was deleted, myristylation still occurred with high efficiency. This result shows that, as with the *src* oncogene (4, 24), the presence of 10 amino acids at the amino terminus of $Pr78^{gag}$ is sufficient to signal this cotranslational modification.

The studies presented here show that for M-PMV the processes of capsid assembly, intracellular transport, and release from the cell are relatively slow. The initial phase of capsid assembly within the cytoplasm has a $t_{1/2}$ of approximately 45 min, and this time can be conveniently measured by the conversion of the initially soluble translation products into pelletable capsids. None of the mutants described here efficiently assembled their modified, unstable precursor proteins into stable capsids, and those that were formed appeared to be degraded with kinetics similar to those of the soluble precursor. Occasional capsids with abnormal morphology could be observed by electron microscopy in thin sections of mutant provirus-containing HeLa cells, consistent with the inefficient assembly measured by biochemical methods (data not shown). In no case could virion assembly and release from the cell be detected; whether this also reflected a defect in assembled capsid transport or merely degradation prior to membrane association remains undetermined.

The data presented here show that in M-PMV a native MA protein is required to fold the Gag polyproteins into a conformation that confers stability and allows the intermolecular interactions involved in self-assembly to occur. The loss of stability observed for the mutant polyproteins could result from the loss of a favorable ternary conformation which normally is attained by wild-type proteins, the introduction of an unfavorable conformation that cannot be accommodated in the native folded structure, or both. Consequently, this alteration in the folded precursor molecule subjects mutant Gag polyproteins to rapid protein degradation within the cytoplasm and interferes with the specific intermolecular interactions necessary for capsid assembly. The fact that all five deletions affected the stability of the Gag precursor in the same manner indicates that the structure of this MA protein is highly constrained and may reflect the requirement for a molecule capable of multiple protein-protein and perhaps protein-lipid interactions necessary for transport and release of the type D virion. Mutagenic approaches that introduce point mutations into these critical regions of the MA-coding domain may provide additional clues to the structure-function relationship of this protein.

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