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Postassembly Cleavage of a Retroviral Glycoprotein Cytoplasmic Domain Removes a Necessary Incorporation Signal and Activates Fusion Activity

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Viral protease-mediated cleavage within the cytoplasmic domain of the transmembrane (TM) glycoprotein of the type D retrovirus, Mason-Pfizer monkey virus, removes approximately 16 amino acids from the carboxy terminus of the protein. To determine the functional significance of this cleavage in the virus life cycle, we introduced premature stop codons into the TM coding domain, resulting in the production of truncated glycoproteins. Progressive truncation of the cytoplasmic domain identified the carboxy-terminal third as being required for efficient incorporation of the glycoprotein complex into budding virions and profoundly increased the fusogenic capability of the TM glycoprotein. These results, together with the ability of matrix protein mutations to suppress TM cleavage, imply that this portion of the glycoprotein interacts specifically with the capsid proteins during budding, suppressing glycoprotein fusion function until virus maturation has occurred.

Budding viruses preferentially incorporate their own (virus-encoded) surface glycoproteins while excluding surface glycoproteins that are encoded by the host cell. The means by which this selective mechanism for incorporation is able to operate remain enigmatic. Because of the topological arrangement of membrane-spanning glycoproteins, it has been an assumption that the cytoplasmic domain, being exposed at the inner surface of the lipid bilayer, could confer the specificity necessary for the selective incorporation of viral glycoproteins. In the alphaviruses, immunological studies have suggested that a specific interaction between the cytoplasmic domain of the spike glycoprotein and the protein capsid shell is necessary for incorporation (37) and three-dimensional reconstructions of cryoelectron micrographs indicate that the cytoplasmic domain interacts with a depression in the capsid (12). However, this interactive role for the cytoplasmic domain has not proven to be the case in all of the viral systems examined thus far, since in the case of the avian retrovirus Rous sarcoma virus (RSV), a glycoprotein lacking the entire cytoplasmic domain was effectively incorporated into budding virions (28). Also in studies of influenza virus, mutations which shortened the cytoplasmic domain of the viral hemagglutinin did not block its incorporation into virions (35).

The type D retroviruses present a unique opportunity to study the processes of capsid formation and budding as two separate and distinct steps in the retrovirus group. Unlike the type C retroviruses (such as RSV or human immunodeficiency virus [HIV]), which assemble their protein capsid shells during the process of budding from the plasma membrane, the type D retroviruses preassemble their capsids within the infected cell cytoplasm. These immature capsids then migrate to the plasma membrane, incorporate their complement of glycoproteins as they bud, and are then released. Mason-Pfizer monkey virus (M-PMV), the prototypic type D retrovirus, provides a powerful system in which to examine the contributions made by the cytoplasmic domain of the transmembrane glycoprotein to interactions with the budding capsid that lead to specific incorporation of the virus glycoproteins. In M-PMV, the *env* gene is translated from a spliced mRNA to yield a polyprotein precursor, Pr86. This initial precursor is cleaved by a cellular endopeptidase in a late Golgi compartment (3, 17), producing the mature forms of the surface (SU) glycoprotein, gp70, and the transmembrane (TM) glycoprotein, gp22. The SU glycoprotein mediates binding of the virus to a specific but as yet uncharacterized cell surface receptor. The TM glycoprotein extracellular domain provides an area for association with the SU glycoprotein, consisting of noncovalent interactions that hold the two glycoproteins together (4). This complex is tethered within the plasma membrane by the presence of the TM glycoprotein's 28-amino-acid membrane-spanning domain (Fig. 1A). Immediately inside the infected cell membrane is the 38-amino-acid cytoplasmic domain of the TM glycoprotein.

After release of the immature virus, a viral protease-mediated cleavage occurs within the cytoplasmic domain (6), resulting in the loss of approximately 16 amino acids from the carboxy terminus. This converts the gp22 glycoprotein incorporated at the cellular membrane into a gp20 glycoprotein found within the virion. A similar viral protease-mediated cytoplasmic domain cleavage has been reported for murine leukemia virus, but to date, the functional requirement for this cleavage has not been described (14, 19).

The current study was undertaken to determine the function of the cleavage of gp22 to gp20 within the released virus. The problem was addressed by progressively truncating the cytoplasmic domain through the introduction of stop codons into the carboxy terminus of the TM glycoprotein coding region. We have determined that there is an obligatory requirement for the carboxy-terminal 12 amino acids of the cytoplasmic domain for efficient incorporation of the M-PMV glycoproteins in order to produce a productive infection. Moreover, we have found that the progressive shortening of the cytoplasmic domain leads to increased fusogenic activity of the TM glycoprotein. Our results therefore indicate that the normal, post-

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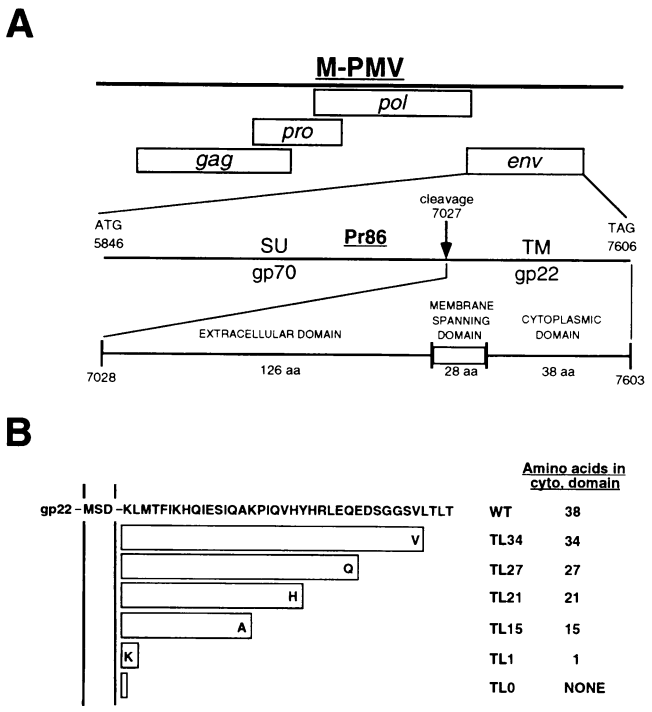

FIG. 1. (A) Genomic organization of M-PMV. M-PMV contains the four essential coding domains common to other retroviruses (*gag*, *pro*, *pol*, and *env*) that are translated to yield four precursor proteins: Pr78, the Gag precursor which yields the capsid components of the virus; Pr95, the Gag-Pro precursor which yields the viral protease; Pr180, the Gag-Pro-Pol precursor which yields RT; and Pr86, which yields the SU and TM glycoproteins. An expanded view of the TM glycoprotein (gp22) shows its division into three distinct domains, extracellular, membrane spanning, and cytoplasmic. The numbers shown below correspond to the nucleotide positions within the published M-PMV sequence (36). (B) The length and amino acid sequence of the wild-type (WT) cytoplasmic domain is compared with the predicted sizes of the mutant proteins (designated by the shaded boxes). The C-terminal amino acid of each truncated mutant is shown within each box. The mutant designation and the number of amino acids contained within its cytoplasmic (cyto.) domain are indicated.

release cleavage of gp22 to gp20 is necessary to activate the fusogenic potential of the TM glycoprotein, allowing released virus to initiate infection upon binding to a new receptor-expressing target cell.

MATERIALS AND METHODS

Cell lines. HeLa, COS-1, and CV-1 cells were obtained from the American Type Culture Collection. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. COS-1 were grown in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum.

Oligonucleotide mutagenesis. Mutagenesis utilizing M13 single-stranded DNA synthesized in a Dut^- Ung^- host (*Escherichia coli* CJ236) was done as previously described (21, 22). CJ236 cells were infected by an M13 phage construct, M13.MP.*env*724, containing the entire coding region of the M-PMV *env* gene. Mutations were identified directly by dideoxy sequencing of resultant plaques from the mutagenesis reaction.

Mutated *env* genes were subcloned into the expression plasmid pTMO (5), which contains the M-PMV *env* gene under the transcriptional control of the myeloproliferative sarcoma virus promoter element (1). Constructs were transiently expressed in COS-1 cells after introduction by a modified $CaCl_2$ precipitate method (7).

Mutant proviral genomes were constructed by subcloning a 1.4-kb fragment from the corresponding pTMO constructs into pSHRM15, a plasmid containing the entire infectious M-PMV proviral genome (31). Each mutation was confirmed by double-stranded DNA sequencing, using the Sequenase system (United States Biochemical Corporation, Cleveland Ohio) after each subcloning step.

Transfection of COS-1 cells. COS-1 cells were transfected by the protocol of Chen and Okayama (7) as previously described (4).

Pulse-labeling of cells and immunoprecipitation. Transfected cells were pulse-labeled with [^{3}H]leucine (DuPont-NEN, Billarica, Mass.; 157 Ci/mmol) 72 h after the addition of DNA. Before being labeled, the cells were incubated in leucine-free medium (GIBCO) for 1 h. After removal of this medium, leucine-free medium containing [^{3}H]leucine at 75 μCi/35-mm-diameter plate was added and incubation was continued for 15 min, with occasional rocking. At the end of the pulse period, the radioactive medium was removed. One set of cells was lysed immediately by the addition of 1 ml of lysis buffer A (16), and viral proteins were immunoprecipitated by the addition of goat antiserum prepared against whole disrupted M-PMV (National Cancer Institute, Division of Cancer Cause and Prevention, Bethesda, Md.). Chase lysates were prepared, and viral proteins were immunoprecipitated in the same manner from additional sets of cells after incubation in complete medium for 4 h. Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 or 12% polyacrylamide) and examined by fluorography after impregnation of the gel with EnHance (NEN).

Determination of the amount of Pr86 in pulse-chase experiments. COS-1 cells, in 60-mm-diameter plates, were transfected with either pTMO, pTMO.TL27, or pTMO.TL1. After 24 h at 5% CO_2, the transfected cells from each construct were split equally into four 35-mm-diameter plates and grown for an additional 24 h. Each 35-mm-diameter plate was pulse-labeled with 60 μCi of [^{35}S]Trans-Label for 20 min. One plate was lysed and immunoprecipitated (representing the pulse), using a monoclonal antibody raised against the simian retrovirus type 1 TM glycoprotein that recognizes the M-PMV Pr86 (23). The other three plates were chased for 2, 4, or 8 h and treated as outlined above. The dried gel was examined on a Molecular Dynamics (Sunnyvale, Calif.) Imagequant Phosphorimaging system, and the counts in each Pr86 band were determined.

Examination of protein content of released virions. Chase media were collected from COS-1 cells and transfected with the various genomic constructs at the times indicated in the text. Virus pellets were prepared by centrifugation of the media in a Beckman TLA-100.2 rotor for 10 min at 80,000 rpm at 4°C in a TL-100 ultracentrifuge. Pellets were disrupted by the addition of 0.5 ml of lysis buffer A, with the addition of SDS to 0.1%, and proteins were immunoprecipitated and examined as discussed above.

Fusion assay. COS-1 cells that had been transfected with the pTMO constructs were harvested 72 h after the addition of DNA. These cells were then mixed, at a 1/20 effector cell to target cell ratio, with human osteosarcoma (HOS) cells and grown in medium containing 2 μg of Fungizone (GIBCO) per ml. After 20 h of growth, cell monolayers were treated with May-Grundwald stain followed by Giemsa staining and syncytia were observed under light microscopy. Well-separated

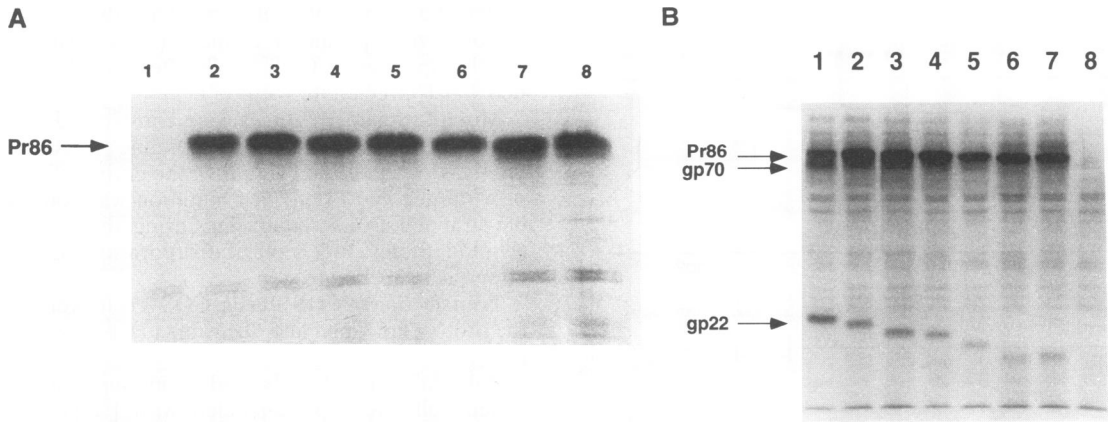

FIG. 2. Synthesis and processing of wild-type and cytoplasmic domain truncation mutations in an *env* expression system. (A) Synthesis of the pTMO (wild-type) Pr86 (lane 2) is compared with that of the precursors of pTMO.TL34 (lane 3), pTMO.TL27 (lane 4), pTMO.TL21 (lane 5), pTMO.TL15 (lane 6), pTMO.TL1 (lane 7), and pTMO.TL0 (lane 8). Lane 1 represents the mock-transfected control. (B) Intracellular cleavage products gp70 and gp22 observed after a 4-hour chase. The order of constructs is pTMO (lane 1), pTMO.TL34 (lane 2), pTMO.TL27 (lane 3), pTMO.TL21 (lane 4), pTMO.TL15 (lane 5), pTMO.TL1 (lane 6), pTMO.TL0 (lane 7), and the mock control (lane 8).

syncytia were used to count the number of nuclei contained within each, and values are reported as averages of at least 10 distinct fusion events.

RT and cell-free infectivity assays. Reverse transcriptase (RT) assays were performed as described previously (4). Culture supernatants exhibiting equivalent amounts of RT activity, collected from transfected COS-1 cells, were added to 50% confluent monolayers of HeLa cells in the presence of 2 μg of Polybrene (hexadimethrine bromide; Sigma, St. Louis, Mo.) per ml. The virus was allowed to adsorb for 1 h at 37°C, and then fresh culture medium was added. Supernatants were assayed for infectious virus transmission by RT assay at the times indicated in the text.

RESULTS

Mutagenesis of the cytoplasmic domain. Figure 1A shows the genomic organization of M-PMV, the initial translation product of the *env* gene, Pr86, and a simplified representation of the gp22 transmembrane glycoprotein. The extracellular domain of gp22 consists of 126 amino acids (with the putative fusion peptide at the extreme amino terminus), followed by a membrane-spanning domain of 28 amino acids and a cytoplasmic domain (or tail) of 38 amino acids. Figure 1B shows the wild-type amino acid sequence of the cytoplasmic domain. Below this sequence are the mutations that produce truncated gp22 molecules as the result of the introduction of premature stop codons. Each mutation is preceded by the designation TL (for tail length), followed by the number of amino acids remaining in the shortened cytoplasmic domains. The truncated molecules are shown in the shaded boxes with the C-terminal amino acid indicated. All mutations were obtained with specific mutagenic oligonucleotides designed to introduce a stop codon at the specified position.

Expression of mutant Env proteins. After verification by sequencing the stop codons introduced by mutagenesis (see Materials and Methods) into the cytoplasmic domain, 1.1-kb fragments from M13 replicative forms were subcloned into the wild-type Env expression vector pTMO (5). Constructs were designated pTMO followed by the TL number as outlined above. For example, pTMO.TL34 is the mutated TM glycoprotein containing 34 amino acids in its cytoplasmic domain, expressed in the pTMO background.

The results of a pulse-chase experiment on COS-1 cells transfected with pTMO, as well as with the six constructs containing cytoplasmic domain truncations, are shown in Fig. 2. Truncated forms of the Env precursor polyprotein Pr86 were produced during a 15-min pulse of each mutant-expressing cell culture (Fig. 2A, lanes 3 to 8) at levels similar to that observed with the wild-type gene (Fig. 2A, lane 2). The extent of the truncation introduced into each construct was reflected in the altered mobilities of the mutated precursor proteins in comparison to the wild-type Pr86. During the chase (Fig. 2B), the wild-type and mutated *env* gene precursor proteins were processed to the mature products, gp70 and gp22 (and truncated forms of gp22). The size differences among the gp22 glycoproteins were consistent with the differences predicted by the truncations introduced into the cytoplasmic domain, with the exception of the TM of TL21 (lane 4) which migrated in a similar manner as that of TL27 (lane 5).

Cytoplasmic domain truncations have been reported to affect the intracellular transport of glycoproteins in other systems (27, 30). To determine if the truncations adversely affected the transport (and subsequent processing) of the M-PMV *env* precursor Pr86, a time course study was undertaken. Figure 3 shows the amount of Pr86 remaining after 2, 4, and 8 h of chase for the wild-type pTMO and for pTMO.TL15 and pTMO.TL1. No significant differences were observed in the rate of processing of Pr86 among these three constructs, the smallest of which retains at least one cytoplasmic amino acid. Similarly, equivalent amounts of the cleavage products, gp70 and truncated gp22, were produced after a chase (Fig. 2B).

Virus incorporation of truncated TM glycoproteins. To determine if this series of mutant TM glycoproteins containing truncations in their cytoplasmic domains could be efficiently incorporated into budding virions, mutations were cloned into the M-PMV genomic expression vector pSHRM15 (31), which contains the entire infectious genome of M-PMV. These constructs were preceded by the designation pMP, followed by the same TL nomenclature mentioned above. COS-1 cells that had been transfected with each construct were pulse-labeled at 48 h posttransfection and then chased by the addition of growth medium. Lysates of the pulse-labeled and pulse-chased cells were immunoprecipitated and examined by SDS-PAGE.

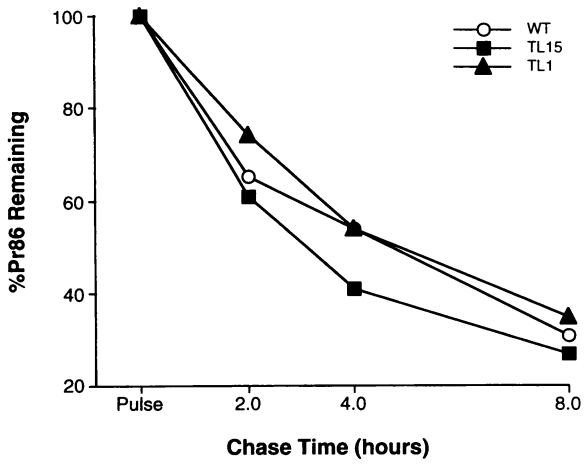

FIG. 3. The rate of loss of the *env* precursor Pr86 monitored in a pulse- and in a 2-, 4-, and 8-h chase. The comparison is between three constructs representing the full-length (WT), partially truncated (TL15), and almost completely truncated (TL1) cytoplasmic domain of the TM glycoprotein. The results represent the data from two separate transfections of each construct.

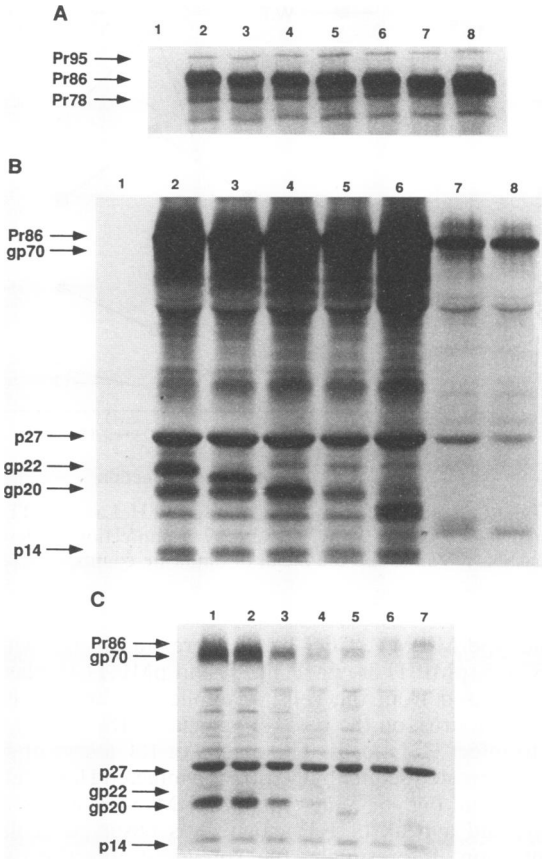

FIG. 4. Analysis of proteins expressed from M-PMV genomic constructs transfected into COS-1 cells from the pulse, chase, and pelleted virions. (A) Protein precursors immunoprecipitated from a 15-min pulse from cells transfected with mock control (lane 1), pSHRM15 (lane 2), pMP.TL34 (lane 3), pMP.TL27 (lane 4), pMP.TL21 (lane 5), pMP.TL15 (lane 6), pMP.TL1 (lane 7), and pMP.TL0 (lane 8). (B) Intracellular *gag* and *env* gene products immunoprecipitated from cell lysates after a 15-min pulse-label and a 4-h chase. Lanes are labeled as described for panel A. (C) Polypeptide analysis of pelleted virions that were released into the culture medium during the 4-h chase. Lanes: 1, pSHRM15; 2, pMP.TL34; 3, pMP.TL27; 4, pMP.TL21; 5, pMP.TL15; 6, pMP.TL1; 7, pMP.TL0.

Figure 4A presents the results of a 15-min pulse-labeling, showing the production of similar levels of the Gag and Gag-Pro precursor polyproteins Pr78 and Pr95. The glycoprotein precursor (Pr86 in the wild type) was produced at similar levels for each of the constructs, as we observed in the *env* expression system (Fig. 4A). The decreasing size of the precursor is highlighted by a gradual increase in migration toward the $Pr78^{Gag}$ band as the length of the truncation increased (lanes 3 to 8). The Env precursors produced by the pMP constructs containing the TL1 (lane 7) and TL0 (lane 8) mutations migrated just above the $Pr78^{Gag}$ band, consistent with the loss of approximately 4-kDa of protein.

After a 6-h chase (Fig. 4B), the Gag protein products, p27(CA) and p14(NC), that result from the activity of the retroviral protease on the Gag precursors were observed. As in the *env* expression system, mature forms of both SU and TM glycoproteins were apparent in each mutant, with TM glycoprotein size decreasing with the extent of the cytoplasmic domain truncations. In contrast to the *env* expression system, both cell-associated gp22 and the processed gp20 can be seen in these virus (protease-containing)-expressing cultures. Interestingly, although the size of the TL34 TM protein is reduced compared with that of the wild-type gp22 (Fig. 4B, lanes 3 and 2, respectively), the gp20 protein is the same size, confirming that cleavage is N terminal to the site of the TL34 mutation. The product of TL27 migrates with the mature gp20 of both the wild type and TL34 (Fig. 4B, lane 4), and so it is not possible to determine whether cleavage of a small number of amino acids occurs in this case. Nevertheless, the fact that the products of TL27 and TL21 migrate with the processed gp20 indicates that cleavage must occur within this region of the protein. Variations in the amount of each protein were due to variations in transfection efficiency, but the relative ratios of capsid proteins and glycoproteins for each construct were maintained.

An examination of the protein content of the pelleted virions released by each of the genomic constructs is shown in Fig. 4C. The amounts of p27(CA) and p14(NC) contained in the mutant virions closely resembled the amounts found in wild-type virus, indicating that processing and assembly of the *gag*-encoded components of the virions were indistinguishable from that of wild type. However, the levels of mature glycoproteins incorporated into each of the pelleted virus preparations varied significantly. The pMP.TL34 virions contained approximately the same amount of (mature gp20) glycoprotein as those of wild-type pSHRM15 virions, whereas the pMP.TL27 virions contained only a small fraction of the wild-type glycoprotein complement, and virions released from cells transfected with pMP.TL21, pMP.TL15, pMP.TL1, and pMP.TL0 contained only trace amounts of the mutated glycoproteins.

Infectivity of mutated genomes. The ability of M-PMV, as well as other retroviruses, to productively infect cells is initially dependent on the capacity of the SU glycoprotein on the particle to recognize and bind to specific cell surface receptors. Since the virions released by the cytoplasmic domain truncation mutant genomes displayed a range of glycoprotein content, we initiated a study of the ability of these mutant viruses to establish an infection in a cell line that could support growth

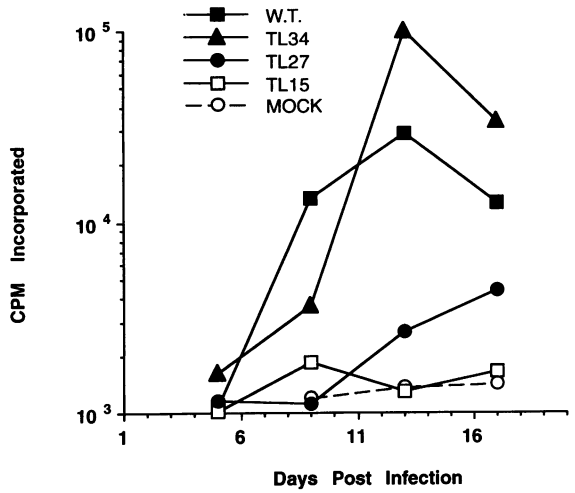

FIG. 5. Cell-free infectivity assay of infected HeLa cells. RT levels were monitored on days 5, 9, 13, and 17 postinfection as described previously (4). Values are the mean of duplicate counts.

of wild-type M-PMV. COS-1 cells were transfected with the pSHRM15, pMP.TL34, pMP.TL27, and pMP.TL15 plasmids. Virus released from the cells was collected, and equivalent amounts of virus, on the basis of associated RT activity, were used to infect HeLa cells. The results of RT assays of supernatants harvested sequentially from the infected HeLa cells are presented in Fig. 5. Wild-type pSHRM15 virus replicated rapidly, and a peak of RT activity was observed at day 13 postinfection. Interestingly, the spread of infection of the mutant virions paralleled the glycoprotein content of the released virus, so that pMP.TL34 replicated and spread through the culture with kinetics only slightly delayed when compared with those of the wild-type virus. In contrast the pMP.TL27 virions, which contain a reduced amount of glycoprotein, showed a slow increase in RT activity that remained almost 10-fold lower than the wild-type peak and appeared only after an extended infection period. The pMP.TL15 virus showed no significant RT activity over that of the mock-infected cells, consistent with the lack of incorporation of glycoprotein into these virions.

Effects of cytoplasmic domain truncations on fusogenic activity. Because the introduction of the stop codons into the cytoplasmic domain of the M-PMV TM protein resulted in the production of glycoprotein products that were incorporated with an altered efficiency into virions, we sought to determine if the glycoproteins were expressed in biologically active form on the plasma membrane of expressing cells. A fusion assay in which COS-1 cells transfected with various mutant pTMO constructs were mixed with HOS target cells was employed. The results of a typical experiment are shown in Fig. 6. The wild-type M-PMV glycoprotein shows only limited fusogenic capacity in this assay, with a majority of the multinucleated cells containing three to five nuclei. Similar results were obtained with the TL34 and TL27 mutant proteins, although a small increase in the number of nuclei per syncytium was seen. In contrast, COS cells expressing TL15 showed a significant increase in the number of nuclei per syncytium, with large multinucleate cells predominating on the plate. A quantitative comparison of syncytium formation by each of the pTMO constructs is shown in Fig. 7. A dramatic increase in fusogenic potential was observed once the protein was truncated to a site near that predicted for viral proteolysis during maturation

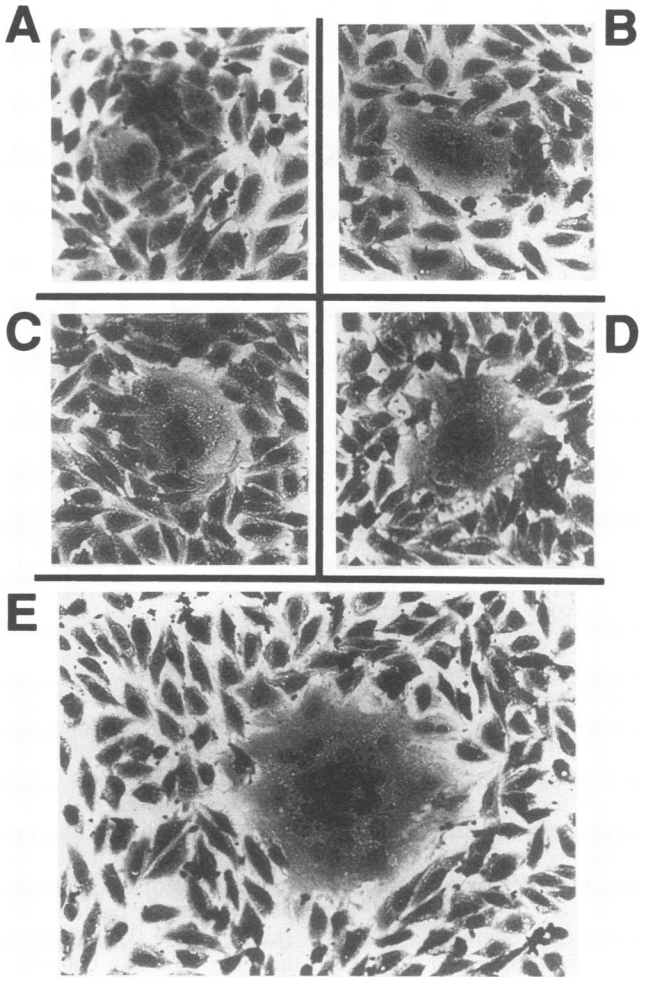

FIG. 6. Photomicrographs of the results of a typical fusion assay. COS-1 cells transfected with pTMO constructs expressing wild-type (panel B), mutant TL34 (panel C), mutant TL27 (panel D), or mutant TL15 (panel E) *env* genes, were mixed with HOS indicator cells and examined after 20 h of incubation and staining. Mock-transfected COS-1 cells mixed with HOS cells are shown in panel A.

(TL21). A further truncation to yield TL15 resulted in even greater fusion (an average of 51 nuclei per syncytium). Thus, in contrast to the case for the incorporation of glycoprotein into virions, in which progressive truncations had resulted in a reduced capacity to be incorporated, in this assay of function, the fusogenic activity increased dramatically as the length of the cytoplasmic domain was decreased.

DISCUSSION

The process by which viral glycoproteins are selected for incorporation into retroviral particles has not been defined. The demonstration by Perez et al. (28) that the cytoplasmic domain of RSV was essentially dispensable for both incorporation into budding virus and subsequent infectivity was surprising. The existence of a cytoplasmic domain, which is present in all retroviral transmembrane glycoproteins and is significantly conserved at the amino acid level within different retroviral subgroups (17), suggested that it should be a functional domain. Indeed, results obtained with the alphaviruses

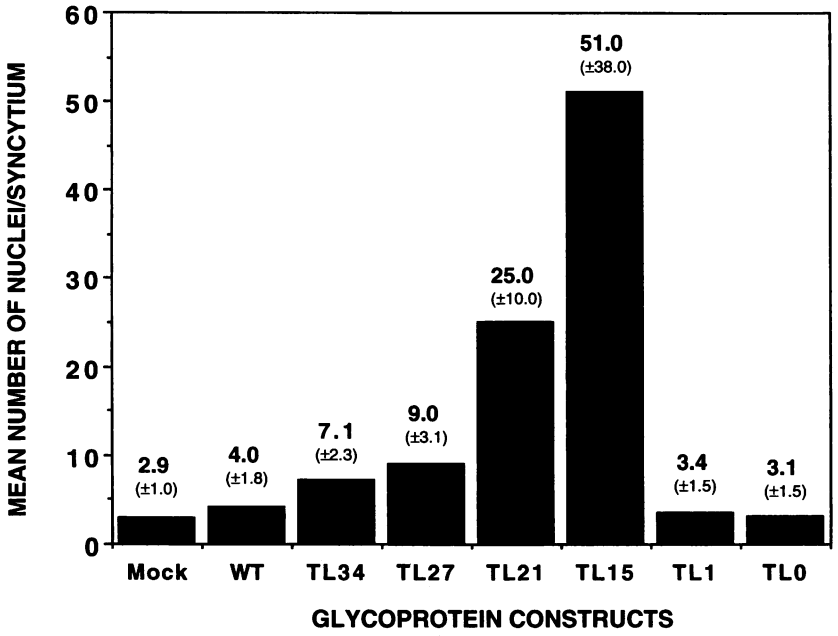

FIG. 7. Graphic presentation of the mean number of nuclei observed within individual syncytia in the fusion assay, following expression of each of the mutant and wild-type glycoproteins. Two plates from each fusion assay were stained, and 10 to 20 separate syncytia were randomly chosen to determine the number of cells included in each fusion event. Numbers above the bars indicate the mean number of nuclei per syncytium for each mutant glycoprotein. Numbers in parentheses indicate the standard deviation from the mean for the number of nuclei in each group of syncytia.

provided compelling immunological and structural evidence that the cytoplasmic domain of the spike glycoprotein interacts with complementary depressions in the capsid shell, thereby enabling the incorporation of the spike glycoprotein and initiating viral budding (37). Nevertheless, the ability of an RSV glycoprotein lacking a cytoplasmic domain to be efficiently incorporated into virus, coupled with our recent observation that the presence of the membrane-spanning and cytoplasmic domains of the RSV Env did not facilitate the functional incorporation of the influenza virus hemagglutinin into RSV virions (9), argues against a role for these regions in specifying incorporation. Moreover, mutations which truncate the cytoplasmic domain of simian immunodeficiency virus have recently been shown to result in enhanced incorporation of glycoproteins into virions (18), suggesting that this long (161-amino-acid) domain is not required for efficient incorporation of the glycoprotein complex in this particular virus.

Nevertheless, other studies have suggested that the TM protein cytoplasmic domain can make important contributions to viral morphogenesis and infectivity. In the case of HIV-1, the cytoplasmic domain has been implicated in the pathogenic effects of the virus in tissue culture (24) and has been shown to play a role in facilitating the incorporation of glycoprotein into virions (10, 39). The potential role of Env-capsid interactions in this system is supported by the observation that deletions within the HIV matrix protein that appear to have no effect on capsid assembly and release block incorporation of the HIV Env complex (38). In addition, a hybrid envelope glycoprotein containing the extracellular and transmembrane domains of HIV-1, fused to the cytoplasmic domain of the vesicular stomatitis virus (VSV) G protein, was shown to rescue a temperature-sensitive VSV mutant lacking the G protein, whereas the wild-type HIV-1 envelope protein did not (26). This would indicate that the cytoplasmic domain of the VSV G protein contains a signal that is sufficient to direct the incorporation of foreign glycoproteins into VSV particles.

The type D retroviruses, exemplified in this paper by M-PMV, employ a morphogenic process that is distinct from that of retroviruses that assemble at the plasma membrane, such as RSV and HIV-1. Immature, preassembled type D capsids migrate from their sites of assembly within the cytoplasm (31, 32) to the plasma membrane. It is at this point that the interaction between the cytoplasmic domain of the TM glycoprotein and the capsids would be postulated to occur. The budding process is not dependent on the presence of viral glycoproteins, since it has previously been demonstrated (4, 31) that in the absence of viral glycoproteins virus particles are still released. However, the nature of the specific process of viral glycoprotein incorporation remained unclear. Our previously published results (6) on the occurrence of a postassembly cleavage within the cytoplasmic domain of the M-PMV TM glycoprotein (converting gp22 into gp20), which could be modulated by mutations in the capsid matrix (MA) protein, raised the distinct possibility that an intimate interaction between the capsid and the gp22 cytoplasmic domain mediated glycoprotein incorporation. In addition, the removal of a portion of the cytoplasmic domain after virus release implied a functionality both in the position and the timing of the cleavage event. We have sought in this study, therefore, to explore the possibility that the cytoplasmic domain of gp22 was involved in the incorporation process and also to investigate the role of the cleavage in this domain, by truncating the cytoplasmic domain through the introduction of stop codons along its length. These stop codons were positioned in order to flank the area hypothesized to be the cleavage site so that the cleavage event itself, as well as the effect of the length of the cytoplasmic domain on incorporation and virus infectivity, could be studied.

In order to investigate the ability of the truncated gp22 glycoproteins to be incorporated into virus, the mutated *env* genes were cloned into the wild-type M-PMV genomic background, pSHRM15. The results of these experiments showed that pMP.TL34 virions incorporated and processed gp22 (to gp20) in a manner similar to that of the wild type. Virus from pMP.TL27, on the other hand, incorporated significantly less glycoprotein in comparison to that from the wild type. The other mutations, TL21, TL15, TL1, and TL0, resulted in truncated proteins that were not incorporated into virions, and particles lacking significant amounts of glycoprotein were released from the cells. Thus, it appears that a cytoplasmic domain longer than 21 amino acids is necessary to direct the M-PMV glycoprotein complex into virions and that residues 22 to 34 encode an incorporation signal that is required for this process. It is likely that these longer truncations remove a sequence or structure necessary for Env incorporation, since in preliminary mutagenesis experiments in which the tyrosine residue at the proposed cleavage site was replaced by histidine, alanine, or glutamine we observed decreased Env incorporation (3a). The results of the mutations reported here are quite different from those we have obtained with the RSV glycoprotein (9, 28) and from those observed with the influenza virus hemagglutinin (35) and support our previous conclusion that the capsid and glycoproteins of M-PMV interact in a specific manner (6). It is of interest to note that the truncations did not appear to affect the intracellular transport and processing of the M-PMV glycoprotein complex, as has been reported for the VSV G protein (8, 34), since the kinetics with which terminally glycosylated cleavage products (gp70 and gp22) could be detected were similar for each of the mutants.

It was not clear whether the incorporated pMP.TL27 TM glycoprotein underwent the maturational processing observed with the wild-type protein, since the mutant TM glycoprotein migrated at the same position as the gp20 glycoprotein. Therefore, the slower spread of infection by this mutant virus (as evidenced by the delay in the increase of RT activity in the infectivity assay) could be due either to the lower amount of glycoprotein on the virus or to the TM glycoprotein not undergoing cleavage (or a combination of both). The four constructs containing larger truncations would not be expected, on the basis of our estimate of the point of cleavage (6), to be able to be cleaved, since they are truncated N terminal of this point. These mutants shed little light on the role of TM cleavage per se, since the lack of infectivity of these mutants was most likely due to the reduced amount of glycoprotein incorporated into virions.

In contrast to the results from the infectivity assays, the assay of cell fusion activity provided new insights into the role of the maturational processing of the cytoplasmic domain. These studies showed that while the wild-type M-PMV glycoprotein was not highly fusogenic, truncation of the TM protein near to the predicted cleavage site greatly enhanced cell fusion activity. The viral protease-mediated cleavage is hypothesized to remove 16 amino acids, leaving 22 cytoplasmic amino acids at the C terminus of the TM glycoprotein of mature virus. The TL21 mutation (retaining 21 amino acids of the cytoplasmic domain) demonstrated an almost fivefold increase in fusion activity in comparison to that of the wild type, and a further increase in fusion (greater than 12-fold) was observed with the *env* construct (pTMO.TL15) encoding an even shorter cytoplasmic domain. Interestingly, mutants completely lacking a cytoplasmic domain (TL1 and TL0) were nonfusogenic.

Thus, changes at the C terminus of the glycoprotein can have dramatic effects (both positive and negative) on the process of fusion that is dependent on the conformation of the ectodomain of the complex. There are other examples of modifications within the cytoplasmic domain of a glycoprotein that affect its biological activity. A naturally occurring mutation that resulted in the truncation of the TM glycoprotein of a noncytopathic HIV-2 resulted in a more fusogenic glycoprotein and a cytopathic virus (15, 25). Similarly, truncations engineered into the TM glycoprotein of human T-cell leukemia virus type I (29) and glycoprotein B of herpes simplex virus type 1 (2, 13) have been shown to result in glycoproteins with increased fusion activity. The mechanism by which C-terminal truncations affect fusogenicity is not known. It is possible that the truncations affect the surface stability of the mutated glycoproteins such that higher levels accumulate on the cell surface. However, preliminary experiments employing immunofluorescent staining of unfixed cells with an anti-glycoprotein antibody, as well as surface biotinylation approaches, showed that the levels of gp70 on the surfaces of mutant and wild-type *env*-expressing cells were indistinguishable (data not shown). We therefore hypothesize that truncation of the M-PMV TM protein induces a conformational change in the trimeric glycoprotein array such that the fusion peptide can interact with the target cell membrane in a more efficient manner.

Cleavage of the cytoplasmic domain, which occurs as part of the maturation process of M-PMV, appears to be necessary to fully activate the fusogenic capability of the glycoprotein complex of released virus. Although this type of cleavage has been observed late in the maturation process of murine leukemia virus (19) and some of the parameters concerning this cleavage event have been reported (11, 14, 20), no functional significance had been assigned to the cleavage itself. A cleavage of the cytoplasmic domain of the equine infectious anemia virus glycoprotein, which takes place in the cytoplasm of infected cells, has also been detected (33), but again, the basis for this has not been elucidated.

The work presented here, together with our previous observations (6) that mutations within the M-PMV MA protein can modulate the protease-mediated cleavage within the cytoplasmic domain of TM, leads us to postulate a multifunctional role for the cytoplasmic domain of the M-PMV TM glycoprotein. We propose that as the cytoplasmically assembled, immature M-PMV capsid reaches the cell membrane the cytoplasmic domain of the TM glycoprotein interacts specifically with the MA protein and that this interaction of the cytoplasmic domain is necessary for efficient incorporation of the Env complex into virions. After virus budding, the maturational cleavage of TM occurs, thereby removing the incorporation signal and activating the fusogenic potential of the glycoprotein complex necessary for further rounds of infection.

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ADDENDUM IN PROOF

Rein et al. (A. Rein, J. Mirro, J. G. Haynes, S. M. Ernst, and K. Nagashima, J. Virol. **68**:1773–1781, 1994) recently demonstrated increased fusogenicity of a similarly truncated murine leukemia virus TM protein.

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