Mutations within the *env* Gene of Mason-Pfizer Monkey Virus: Effects on Protein Transport and SU-TM Association

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By deletion mutagenesis analyses, we have examined the contribution of the immunosuppressive peptide (ISP) region within the transmembrane (TM) protein of Mason-Pfizer monkey virus to viral maturation and infectivity. Deletion of the entire region (mutant D105) results in the production of an Env precursor that is transport defective and therefore unable to be processed to mature glycoproteins. This mutation results in the release of noninfectious virions devoid of surface glycoproteins. A second deletion that removes the most highly conserved 11 amino acids of the ISP (mutant D33) does not affect the production, transport, or processing of the Env precursor yet produces virions that are noninfectious. The mutation was shown to cause the loss of interaction between the surface (SU) and TM proteins and result in the efficient shedding of gp70 into the culture medium. The released gp70 protein was biologically active and could still bind with high specificity to susceptible target cells. Since the ISP domain may represent an area of contact between SU and TM, it could provide an additional explanation for the amino acid sequence homology observed within this region of a variety of retroviruses.

Retroviruses gain entry into their host cells via virally encoded glycoproteins. The glycoproteins are initially translated as a polyprotein precursor from a spliced envelope (*env*) gene-specific mRNA. The glycosylated precursor is cleaved in a late Golgi compartment into the SU and TM proteins (29). The SU, or surface, glycoprotein is responsible for the recognition of and binding to the cellular receptor for the virus, whereas the membrane-spanning, transmembrane (TM) protein anchors the SU protein in the viral membrane. The nature of the SU-TM association is poorly understood. In Rous sarcoma virus, disulfide bonds are responsible for maintaining the interaction between gp85 (SU) and gp37 (TM) and chemical reduction of the virus-associated complex results in the loss of SU (31). In contrast, in most retroviruses, including murine leukemia virus (MuLV) (49), Mason-Pfizer monkey virus (M-PMV) (4), and human immunodeficiency virus type 1 (22), there is no covalent association between SU and TM. This may account, in part, for the fact that the SU protein of human immunodeficiency virus type 1 (gp120) is shed into the medium of infected tissue culture cells and that early attempts to purify the virus by centrifugation resulted in the loss of gp120 from the virions (21).

The glycoprotein precursor of M-PMV, Pr86, is cleaved by a cellular Golgi endopeptidase to yield the mature gp70 (SU) and gp22 (TM) proteins, and then, following virus release, a viral protease-mediated cleavage of the gp22 cytoplasmic domain results in conversion of gp22 to gp20 (6). Within the amino-terminal half of gp22 is a stretch of 35 amino acids that is present in a number of retroviruses to various degrees of homology and which has been referred to as the immunosuppressive peptide (ISP) (45). The prototype of this sequence, exemplified in the *env* gene product of Moloney MuLV, has homologs in human T-cell leukemia virus, feline leukemia virus (FeLV), Rous sarcoma virus, reticuloendotheliosis-associated virus (REV-A), and simian

retroviruses (M-PMV and baboon endogenous virus). Because of its presence in retroviruses capable of inducing an immunosuppressed state in their infected hosts (FeLV, MuLV, and REV-A), this region has been hypothesized to play a role in immunosuppression. In vitro studies have shown that it is indeed possible to decrease the blastogenic potential of T cells to mitogenic stimuli by pretreatment with isolated TM protein from several retroviruses (14, 26, 37). And a synthetic peptide composed of 17 amino acids from the amino end of the ISP of MuLV has been shown to exert an immunosuppressive effect on both T-cell (11) and B-cell (32) mitogenic responses in vitro.

The ability of simian type D retroviruses to suppress host immune function is well documented. In the case of SRV-1 (a serotype of the type D retroviruses that is 85% homologous to M-PMV), approximately 50% of rhesus monkeys infected with the virus succumb to a fatal immunosuppressive disease. Death following natural infection of macaques in primate centers and zoos is usually attributed to opportunistic infections, a direct result of an impairment of immune function. A decrease in both T- and B-cell numbers, as well as the destruction of germinal centers, is normally observed (1). Although M-PMV has only recently been reisolated since its discovery in 1970, a laboratory-derived, infectious proviral genome and virus newly isolated from the original tumor have both shown the capability of inducing an immunosuppressive disease in macaques (7).

We previously reported on the nucleotide sequence of the entire M-PMV genome and pointed out the unexpected degree of homology observed when the amino acid sequences of the TM proteins of M-PMV and REV-A were compared (45). Overall, the TM proteins of these two otherwise quite divergent viruses showed 67% amino acid identity, with a 40-amino-acid region that encompassed the ISP exhibiting 100% identity. Since such a high degree of conservation suggested that this region might play an important common structural or functional role, we have initiated a genetic analysis of this region in an attempt to delineate its role in M-PMV replication and pathogenesis. In this paper

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we show that mutagenesis of the ISP region results in a disruption of the stable association between the M-PMV gp70 and gp22 proteins. An 11-amino-acid deletion in the amino terminus of the ISP, although having no effect on Pr86 expression or processing, results in the release of noninfectious virions. The reason for the loss of infectivity is the lack of gp70 in the released virions. We conclude that the loss of gp70 is due to a structural perturbation in gp22 that interferes with TM-SU associations and results in the efficient secretion of biologically active gp70 from infected cells.

MATERIALS AND METHODS

Cell lines. HeLa, COS-1, and CV-1 cells were obtained from the American Type Culture Collection. BHK cells were a gift from Asit Pattnaik of the University of Alabama at Birmingham. COS-1, CV-1, and BHK cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. HeLa cells were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum.

Oligonucleotide site-directed mutagenesis. Mutagenesis utilizing M13 single-stranded DNA was done according to the method of Zoller and Smith (50). A 2.8-kb fragment containing the entire coding region of the M-PMV *env* gene was cloned into M13mp19 and used as the target for mutagenesis. Oligonucleotides (Genetic Designs, Inc., Houston, Tex.) 26 and 31 nucleotides long (mutants D33 and D105, respectively) were used to create the desired deletions. Resultant plaques from the mutagenesis reaction were initially screened by hybridization with end-labelled oligonucleotides. Mutations were confirmed by the dideoxy sequencing method of Sanger et al. (41).

Mutant proviral genomes were constructed by subcloning a 1.4-kb fragment from M13 replicative forms into pSHRM15, a plasmid containing the entire infectious M-PMV proviral genome as well as a hygromycin resistance expression cassette (39).

Construction of recombinant SV40 expressing the wild-type and mutant *env* genes of M-PMV. The methodology for construction of recombinant simian virus 40 (SV40) has previously been described (48). Briefly, the wild-type *env* gene of M-PMV was inserted into an SV40 late region replacement vector (creating SV40/6A), in which transcription is driven via an intact SV40 late promoter. Mutated *env* genes were subcloned into this construction (creating SV40/D33 or SV40/D105) by using unique sites within the coding region. SV40 stocks were produced by cotransfection of each of these constructions with SV40 helper DNA (*dl1055*) into CV-1 cells by using DEAE-dextran. Lysates containing the recombinants were harvested 4 to 6 days later, when cytopathic effects were quite extensive. A second round of infection using this first lysate was used to produce a higher-titer second lysate, which was used in expression studies. Concentrations of the second lysate were adjusted so that infection resulted in cytopathic effects at 40 to 48 h postinfection, at which time labelling studies were performed.

Pulse-labelling of cells and immunoprecipitation. Infected and transfected cells, as well as stable cell lines, were pulse-labelled with [³H]leucine (Dupont-NEN, Billerica, Mass.; 157 Ci/mmol) at the times indicated. Prior to labelling, the cells were incubated in leucine-free medium (GIBCO) for 1 h. Following removal of this medium, leucine-free medium containing [³H]leucine at 75 μ Ci/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 (28), and viral proteins were immunoprecipitated by the addition of goat antiserum prepared against whole disrupted M-PMV (G α M-PMV; Div. of Cancer Cause and Prevention, National Cancer Institute). Chase lysates were prepared and viral proteins were immunoprecipitated in the same manner from additional sets of cells following incubation in complete medium for 4 to 5 h. Immunoprecipitated proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10 or 12% polyacrylamide) gels and examined by fluorography after impregnation of the gel with En³Hance (NEN).

Transfection of HeLa cells. The transfection of HeLa cells with wild-type and mutant proviral genomes was done essentially as described previously (40). A CaCl₂ precipitate containing 10 μ g of CsCl-purified pSHRM15 (wild-type M-PMV) or mutant derivative pMP-D33 or pMP-D105 plasmid DNA was added to subconfluent monolayers of HeLa cells in 35-mm-diameter tissue culture plates. After incubation at 37°C for 4 h, the medium was removed and the cells were shocked by addition of a 15% glycerol solution at room temperature for 3 min. Culture medium was replaced with complete Dulbecco's modified Eagle's medium, and the cells were incubated overnight prior to being transferred to a 100-mm-diameter tissue culture dish. Reverse transcriptase (RT) assays were performed 3, 5, and 7 days following transfection, day 1 being the day of transfer to 100-mm-diameter dishes.

For selection of stable, genome-expressing cells, clones were selected with 250 U of hygromycin B (Calbiochem, La Jolla, Calif.) per ml, beginning 24 h following transfection. Individual colonies became apparent after approximately 10 to 11 days of treatment, and clones were isolated with the use of cloning cylinders. The cells were grown to confluency and assayed in pulse-chase experiments for their ability to produce M-PMV proteins.

Transfection of COS-1 cells. COS-1 cells were transfected according to the protocol of Chen and Okayama (10). Fresh medium was added to 80% confluent cells in 35-mm-diameter plates 2 h prior to transfection. CsCl-purified plasmid DNA (2.5 μ g) in 50 μ l of H₂O was added to an equal volume of 0.5 M CaCl₂ and mixed thoroughly. Following the addition of 100 μ l of 2 $\times$ BBS buffer (10) and incubation at room temperature for 10 min, the mixture was distributed in the medium over the cells. The cells were incubated at 37°C in 3% CO₂ for 24 h. The medium was then changed, and incubation was continued in 5% CO₂ for an additional 40 to 48 h prior to pulse-labelling.

Cell-free infectivity assay. Culture supernatants collected from COS-1 cell transfections at 40 h posttransfection were cleared of cells and cell debris by centrifugation at 14,000 rpm for 5 min at 5°C in a Brinkman 5415 Microfuge. Two milliliters of supernatant containing equivalent amounts of RT activity was added to 50% confluent monolayers of HeLa cells in the presence of 2 μ g 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. Five days later, supernatants were assayed for infectious virus transmission by RT assay.

RT assay. The RT assay is based on a previously described method (9). A volume of 3 ml of culture medium was clarified of cellular debris by centrifugation at 13,000 rpm for 10 min in a Beckman J-21 rotor. Viral particles were pelleted from the clarified supernatant by centrifugation in a Beckman TLA-100.3 rotor at 80,000 rpm for 10 min at 4°C. The viral

pellet was lysed by the addition of 12 μ l of lysis buffer (50 mM Tris [pH 7.8], 100 mM KCl, 0.05% Triton X-100, and 2 mM dithiothreitol) and incubation on ice for 15 min. From this lysate, 7.5 μ l was added to 30 μ l of RT reaction buffer (final concentrations: 50 mM Tris [pH 7.8], 100 mM KCl, 2 mM dithiothreitol, and 7.5 mM MgCl₂), containing 8 μ Ci of [³H]TTP and 1.25 μ g of poly(A) · poly(dT)₁₀ (Boehringer GmbH, Mannheim, Germany) as a template. The reaction mixture was incubated at 37°C for 1 h and terminated by the addition of 12.5 μ l of 0.2 M sodium PP_i. Forty microliters of this mixture was spotted onto NA45 paper (Schleicher & Schuell, Keene, N.H.), allowed to air dry, and then washed three times in 0.5 M phosphate buffer, pH 6.5. The sample was again air dried, suspended in 3.5 ml of Budget-Solv (Research Products International, Mount Prospect, Ill.), and counted in an LKB 1209 Rackbeta scintillation counter.

Fractionation of culture media. One milliliter of the pulse-chase supernatant from transfected COS-1 cells (see above) was divided into 500- μ l aliquots. One aliquot was lysed directly by the addition of 5 $\times$ lysis buffer (final concentrations: 1% Triton X-100, 25 mM Tris-HCl [pH 8.0], 50 mM NaCl, and 1% sodium deoxycholate) and SDS to 0.1%, and then immunoprecipitation was initiated by the addition of G α M-PMV antiserum. This was referred to as the total supernatant fraction. The second 500- μ l aliquot was fractionated into viral pellet and soluble supernatant by centrifugation in a TLA-100.1 rotor (Beckman) at 100,000 rpm for 10 min at 4°C. The resulting supernatant was carefully removed, in order to avoid disturbing the viral pellet, and then treated in the same manner as the total fraction described above. This fraction was called the supernatant fraction. The pellet was lysed directly in the centrifuge tube by the addition of 1 $\times$ lysis buffer and precipitated by adding G α M-PMV antiserum. This represented the pellet fraction. The immune-precipitated protein from the unfractionated and fractionated medium was analyzed by SDS-PAGE on 10 and 12% polyacrylamide gels.

Binding assay for secreted gp70. A HeLa cell clone (D33/cc3) derived by transfection of the pMP-D33 plasmid was used as a source of efficiently secreted gp70. A 175-cm² flask of subconfluent D33/cc3 cells was labelled overnight with 5 ml of growth medium containing 1 mCi of [³H]leucine. The culture medium, containing both gp70 and viral particles, was harvested and subjected to low-speed centrifugation in order to remove cellular debris. A 500- μ l sample of the resulting supernatant was added to HeLa and BHK cell monolayers in 35-mm-diameter plates. The plates were incubated at 37°C, with occasional rocking, for 1 h. The supernatant was then removed, the cells were washed twice with Dulbecco's modified Eagle's medium, and the washes were added to the supernatant fraction prior to immunoprecipitation. The gp70 in this combined supernatant represented the unbound fraction. The cell monolayers were then lysed and immunoprecipitated. The labelled gp70 immunoprecipitated from the cell lysate represented the bound fraction.

RESULTS

Site-directed mutagenesis of the immunosuppressive peptide region. In order to investigate the role of the ISP region in M-PMV replication, a pair of deletion mutations was constructed by oligonucleotide mutagenesis. The ISP region (within gp22) is located 60 amino acids carboxy terminal to the cleavage site of Pr86 (Fig. 1). The mutagenic oligonucleotides were designed to remove either the entire 35-amino-

FIG. 1. Schematic diagram of M-PMV genomic organization. The *env* gene is translated from a spliced mRNA to yield the precursor, Pr86, which is cleaved after a dibasic sequence to produce the gp70 (SU) and gp22 (TM) glycoproteins. Following cleavage in the Golgi complex, a fusion peptide is exposed at the amino terminus of the TM protein. At the carboxy terminus is a 38-amino-acid cytoplasmic domain (Cyto. Domain), and the gp22 protein is anchored in the membrane by a hydrophobic 28-amino-acid membrane-spanning domain (M.S.D.). The immunosuppressive peptide lies 60 amino acids downstream from the amino terminus. The two mutations, D105, which removes 35 amino acids, and D33, which removes 11 amino acids, are shown below the sequence for the immunosuppressive peptide region.

acid domain (D105) or 11 amino acid residues from within this region (mutant D33). This 11-amino-acid segment represents the most highly conserved sequence within the ISP when the sequence from a number of divergent retroviruses are compared (45). Mutagenesis was carried out by the method of Zoller and Smith (50). Following identification of the mutants by hybridization and confirmation by sequencing, their phenotypes were determined by expression of the mutated genes in the systems described below.

Expression of mutant *env* proteins via a recombinant SV40 system. In order to determine the effects of the mutations on envelope gene expression and processing of the glycoprotein precursor, Pr86, mutated envelope constructions were cloned into an SV40-based expression vector and cotransfected with helper DNA in order to obtain virions with recombinant genomes. When these virions were used to infect CV-1 cells, glycoprotein expression could be monitored by pulse-labelling the cells and immunoprecipitating the *env* gene products (Fig. 2). It can be seen that in cells expressing the wild-type construct (SV40/6A) the precursor to the envelope glycoprotein, Pr86, could be immunoprecipitated following a pulse-label. This protein was subsequently cleaved to the mature glycoproteins, gp70 and gp22, following the 4-h chase period (Fig. 2, lane 4).

Cells infected with the mutant SV40/D33 also express Pr86, which undergoes cleavage to gp70, and a smaller version of gp22 during the chase (Fig. 2, lanes 5 and 6; and lower panel, lanes 5 and 6). This smaller gp22 is consistent

FIG. 2. Synthesis and processing of wild-type and mutant glycoproteins expressed via recombinant SV40 infection. CV-1 cells infected with either wild-type (SV40/6A) or mutant (SV40/D33 and SV40/D105) *env*-expressing virus were pulse-labelled with [3 H]leucine 40 h following infection. Immunoprecipitation with G α M-PMV antiserum showed the glycoprotein precursor, Pr86, to be present in the pulse of all three constructs (lanes 3, 5, and 7), although this protein was noticeably smaller (lane 7) in the case of SV40/D105 because of the introduced deletion. After a 4-h chase, processing to mature forms (gp70 and gp22) occurred in the wild type (lane 4) and SV40/D33 (lane 6), with a smaller gp22 due to the loss of 11 amino acids evident in the latter (indicated by small arrowhead). An overexposure of the same gel (lower panel) shows the SV40/D33 mutant gp22 more distinctly and demonstrates the lack of any corresponding band in either the mock (lane 2) or the SV40/D105 (lane 8) chase lane. In the case of SV40/D105, no processing occurred (lane 8), and this mutant was transport defective (see text).

with the loss of 11 amino acids (approximately 1.2 kDa) predicted from the D33 mutation. In the SV40/D105 mutant-infected cells similar levels of precursor synthesis were observed in the pulse, but this smaller polypeptide (approximately 82 kDa) was not cleaved to SU and TM proteins during the chase (lane 8). Since cleavage of the glycoprotein precursor occurs late in the secretory pathway, this result suggested that the more highly truncated molecule either was not transported to the site of cleavage or was unable to be cleaved because of a deletion-induced change in structure. Endoglycosidase H digestion of the SV40/D105 chase products supports the former hypothesis (data not shown).

Since infection of CV-1 cells with SV40/D33 resulted in apparently wild-type expression and processing of the glycoprotein gene, the mutation was engineered into an infectious genome in order to determine whether it affected the production of infectious virus. The D105 mutated envelope gene was also introduced into the infectious M-PMV genome as a negative control for these experiments. Previous work suggested that the D105 mutant would give rise to noninfectious virions lacking processed glycoprotein (39).

Infectivity of genomes containing the D33 and D105 mutations. The two deletion mutations were subcloned into plasmid pSHRM15, the construction of which has been described previously (39). This plasmid contains the entire infectious genome of M-PMV cloned into pTZ18R. It also contains the hygromycin resistance gene (2) under the transcriptional control of the SV40 early promoter. Since the promoter region contains the SV40 origin of replication, this genomic plasmid can replicate to high copy number in cells

expressing SV40 T antigen and yield high levels of viral proteins in transient assays.

For initial infectivity assays, HeLa cells were transfected with pSHRM15, pMP-D33 (pSHRM15 containing the D33 mutant), or pMP-D105 (the corresponding D105 mutant in pSHRM15) by a modified CaCl₂ technique as described in Materials and Methods. Culture supernatants were collected from the transfected cells on days 3, 5, and 7 posttransfection and assayed for the presence of RT activity. Since the detection of RT activity depends on the spread of infection within the culture, an increase in RT in the medium provides a direct measure of infectivity. As shown in Fig. 3, transfection of HeLa cells with a wild-type genome resulted in a rapid increase in RT activity from day 3 through day 7, consistent with the known infectivity of M-PMV for these target cells. In contrast, cells transfected with either pMP-D33 or pMP-D105 failed to show any RT activity above background levels in the culture medium during the same 7-day period.

To confirm that transfection of pMP-D33 and pMP-D105 genomes resulted in the assembly and release of noninfectious virus particles, we transfected them into COS-1 cells. In these T-antigen-producing cells virus release is independent of infectivity because amplification of plasmid DNA results in a burst of viral protein synthesis 48 to 72 h posttransfection. The results of an RT assay performed on COS-1 cell supernatants 72 h posttransfection are shown in Table 1 and demonstrate that RT-containing virions are released from cells transfected with each of the constructs. Thus, as we would predict from previous experiments (39),

FIG. 3. RT assay of transfected HeLa cells. Plasmid constructs pSHRM15 (containing the wild-type infectious genome of M-PMV) and the two ISP deletion mutants, pMP-D33 and pMP-D105 (both cloned into the wild-type background), were introduced into HeLa cells via a modified CaCl_2 technique (10). On days 3, 5, and 7 posttransfection (see Materials and Methods) the transfected cells were assayed for RT activity in order to monitor the spread of infectious virus in the culture. Values are averages of duplicate counts.

mutations within the *env* gene do not block RT-containing virion assembly and release. Equivalent numbers of virions released from transfected COS-1 cells (normalized by RT activity) were assessed for their ability to infect HeLa cells. In contrast to virions released from wild-type pSHRM15-transfected cells, neither pMP-D33 or pMP-D105 virions were able to establish infection in HeLa cells (data not shown).

Analysis of mutant virus phenotypes. Since pMP-D33 was noninfectious in the HeLa cell transfection and infection assays, COS-1 cells were transfected with this and the control plasmids in order to determine the nature of the defect in this mutant. Seventy-two hours posttransfection, COS-1 cells were metabolically labelled with [^3H]leucine in a 15-min pulse and then either lysed immediately or chased in unlabelled medium for 4 h. The appearance and processing of *gag*, *gag*-related, and *env* gene products in wild-type-transfected cells was determined by immunoprecipitation. Figure 4 shows the results of a typical pulse-chase experiment. In panel A, different antisera were used to distinguish the Gag precursors, Pr95 and Pr78 (lane 1) from the closely migrating Env precursor Pr86 (lane 3). In panel B, following a pulse-label of wild-type M-PMV-expressing cells, four labelled protein bands were immunoprecipitated by using

FIG. 4. Synthesis and processing of Gag, Gag-related, and Env proteins. Transfected COS-1 cells were metabolically labelled for 15 min (72 h following transfection), and proteins immunoprecipitated from both the pulse plates and the 4-h chase plates were examined by SDS-10% PAGE. In the case of the wild type (pSHRM15), all of the precursor polypeptides and the relevant processed products were identified by the use of various antisera. (A) The Gag (Pr78) and Gag-Pro (Pr95) precursors (lane 1, pulse) as well as the processed major capsid protein, p27 (lane 2, chase), are precipitated by a monospecific rabbit antiserum directed against p27. The Env precursor, Pr86, is recognized in the pulse (lane 3) by a goat antiserum against M-PMV glycoproteins, and the mature forms, gp70, gp22, and gp20, are precipitated by the same antiserum in the chase (lane 4). Lanes 5 and 6, wild-type pulse and chase lysates immunoprecipitated with a monoclonal antibody directed against gp20. This antibody precipitates Pr86 and the two forms of the TM glycoprotein (gp22 and gp20) but not gp70. Lanes 7 and 8, pulses of pMP-D33 and pMP-D105, respectively, reacted with the same monoclonal antibody. (B) Transfected COS-1 cells were metabolically labelled for 15 min (72 h following transfection), and proteins immunoprecipitated from both the pulse plates and 4-h chase plates, by the addition of α M-PMV antiserum, were examined by SDS-10% PAGE. The synthesis of Gag (Pr78), Gag-Pro (Pr95), and Gag-Pro-Pol (Pr180) precursors was not affected in either pMP-D33 (lane 3) or pMP-D105 (lane 5) compared with that in pSHRM15 (lane 1). Processing of these precursors was also unaffected, as evidenced by the appearance, during the chase, of p27, the major capsid protein, in all three constructs (lanes 2, 4, and 6). Synthesis of the Env precursor, Pr86, and its processing to gp70 are similar to those observed previously in the SV40 expression system (compare with Fig. 2). Mutant pMP-D33 Env precursor is processed similarly (lane 4) to that of the wild type (lane 2), although the levels of cell-associated mature glycoproteins is lower. In the case of mutant pMP-D105, Pr86 remains unprocessed.

TABLE 1. RT assay of released virions from transfected COS-1 cells^a

Transfected DNA	cpm/0.5ml
pSHRM15	3,626
pMP-D105	2,235
pMP-D33	5,302

^a RT assays were performed as described in Materials and Methods except that 1 ml of medium was used in each case. Values are averages of duplicate counts.

GαM-PMV antiserum (lane 1). These bands correspond to Pr78, the Gag polyprotein precursor; Pr95, the Gag-Pro polyprotein; Pr180, the Gag-Pro-Pol precursor; and Pr86, the Env precursor. Both pMP-D33-transfected (lane 3) and pMP-D105-transfected (lane 5) cells exhibit the same pattern of immunoprecipitated viral protein bands as that of the wild-type in the pulse, indicating that expression of the virus precursor proteins was unaffected by the mutations in the immunosuppressive domain. The migration of the mutant Env precursors was, however, somewhat faster than that observed for the wild type, consistent with the deletions within the coding region for each mutant. The pMP-D105 Env precursor, with a deletion of 35 amino acids, migrates just above the Pr78 precursor (lane 5).

During a chase in unlabelled medium, the processing of Gag precursors can be monitored by the appearance of p27, the major capsid protein. All three chase lanes (Fig. 4B, lanes 2, 4, and 6) showed the p27 band (less intense in the case of pMP-D105, lane 6, because of significantly less precursor in the pulse), with the concomitant loss of intensity of the Gag precursors, indicating that the processing of the Gag polyprotein occurred with wild-type kinetics in the mutant-transfected cells. Glycoprotein precursor processing resembled that observed when the *env* gene was expressed in the absence of other viral proteins by using an SV40 expression system (Fig. 2). In cells expressing mutant pMP-D105, the truncated Pr86 precursor remained uncleaved following the chase (Fig. 4B, lane 6), as we had observed in the *env* expression system (Fig. 2, lane 8), but now migrates with the remaining Pr78, presumably because of trimming of glucose residues in the endoplasmic reticulum. In contrast, in pMP-D33-producing cells (lane 4), Pr86 was processed with kinetics similar to that of the wild type, but compared with wild-type-virus-producing cells (lane 2), significantly less gp70 remained cell associated following the chase period (lane 4). In addition, TM glycoprotein bands are not evident in this mutant, although processing has obviously occurred.

The inability to detect D33 TM products could be the result of degradation or an inability of the antibody to detect the mutated protein. In order to determine whether the deletion in mutant pMP-D33 resulted in an inability of the polyclonal antiserum to recognize the TM glycoprotein, we used a monoclonal antibody directed against gp20. This monoclonal antibody immunoprecipitates the wild-type Env precursor, Pr86 (Fig. 4A, lane 5), as well as both forms of the TM glycoprotein, gp22 and gp20 (lane 6). However, neither the pMP-D105 (lane 8) nor the pMP-D33 (lane 7) Env precursor proteins are precipitated by this antibody. Therefore, it is possible that loss of this epitope contributes to our inability to immunoprecipitate the pMP-D33 TM glycoprotein product (Fig. 4B, lane 4). However, as we will discuss below (next section and Discussion), it is likely that a majority of the pMP-D33 TM glycoprotein is degraded following Pr86 cleavage.

Because lower levels of both gp70 and the TM glycoprotein were detectable in D33-producing cells, we investigated whether the D33 mutant glycoproteins were being degraded or more rapidly released from transfected COS-1 cells. For these studies chase medium supernatants from labelled cells were fractionated into pelleted virus and soluble culture medium fractions to determine whether the released virions showed any abnormalities in glycoprotein content. As can be seen in Fig. 5A, the total culture medium supernatant fraction from wild-type-virus-producing cells contains proteins corresponding to gp70, p27, gp22, gp20, p14, p12, and p10. The high-speed-centrifugation supernatant fraction con-

FIG. 5. Fractionation of culture media from COS-1 cells transfected with either pSHRM15 (wild type) or mutant construct pMP-D33 or pMP-D105. Media containing labelled, released viral particles were collected from transfected cells that had been pulse-labelled for 15 min and chased in unlabelled medium for 6 h. Half of each sample was immunoprecipitated directly by the addition of GαM-PMV antiserum (representing the total [T] fraction). The other half of the sample was subjected to ultracentrifugation in order to pellet and determine the protein content of the released virions. Pelleted particles (P fraction) and the supernatant remaining after ultracentrifugation (S fraction) were then immunoprecipitated in the same manner. (A) The major capsid protein, p27, is seen in the T lanes and in the P lanes, consistent with the pelleting of the virus contained in the supernatants. The T and P lanes for pSHRM15 also contain the two forms of the TM protein (see text), gp22 and gp20, consistent with their presence in the viral membrane. No TM protein is evident for the pMP-D33 mutant (see Discussion) or the unprocessed pMP-D105 mutant. The lack of gp70 in pMP-D105 (lanes 7 to 9) was expected because of the defective transport of the glycoprotein precursor. Other viral components, p14, p12, and p10, are observed on an SDS-12% PAGE gel in all three virus preparations. (B) p27 levels of all three constructs have been equalized in order to show that the level of gp70 released by pMP-D33 is five to seven times greater than that released by pSHRM15.

tained gp70 that was shed from the transfected cells or was stripped from viral particles during centrifugation; very little p27 could be seen in this fraction. In contrast, the pellet contained the gp70 that remained attached to virions as well as the capsid components p27, p14, p12, and p10 and the TM glycoproteins. The corresponding lanes for pMP-D105 (Fig. 5A, lanes 7 to 9) lack any detectable glycoproteins, confirming that the virions released contained no glycoprotein, as would be predicted from the pulse-chase experimental re-

sults. However, the lanes do contain pelletable capsid and associated proteins. The result of the defect in pMP-D33 is apparent from an examination of Fig. 5A, lanes 4 to 6. In contrast to the wild-type gp70, essentially all of the D33 SU glycoprotein remained in the soluble supernatant fraction (lane 5); very little was precipitated from the virion-associated pellet fraction (lane 6). This is consistent with the lower level of gp70 seen associated with the cells in the pulse-chase experiments. It appears likely, therefore, that the effect of the D33 deletion is to interfere with the interaction between gp22 and gp70, such that gp70 is secreted into the culture medium and glycoprotein-deficient, noninfectious virus is produced. Because virtually no detectable TM glycoprotein is incorporated into the released pMP-D33 virions and gp22 does not accumulate within the cell, we believe that following release of gp70, the cell-associated gp22 is rapidly degraded, presumably because of the loss of association with SU. For Fig. 4B, the same protocol was used to fractionate labelled chase medium supernatants as was used for Fig. 4A, except that the amount of material loaded on the gel was normalized to the amount of p27 released into the medium for all three constructs. Quantitation of the excised gp70 gel bands showed that five- to sevenfold more is released into the medium of mutant pMP-D33-transfected cells than from wild-type cells.

To determine whether the glycoprotein was being shed from the transfected cells or was loosely associated with virions and efficiently stripped off during centrifugation, supernatants from CV-1 cells infected with the *env*-recombinant SV40 vectors were examined for the presence of gp70. In SV40/6A-infected cells, only trace amounts of gp70 were observed in the medium, even though substantial amounts of gp70 were present within the chase lysate from infected cells (Fig. 6, lanes 4 and 1, respectively). In contrast, large amounts of gp70 were found in the medium of SV40/D33-infected cells (Fig. 6, lane 5). Thus, it appears that gp70 can be shed directly from D33 *env*-expressing cells and that this, rather than stripping of virions, is responsible for the phenomenon observed with pMP-D33 virus-producing cells.

Binding of secreted gp70 to susceptible cells. In order to determine whether the secreted gp70 retained biological activity and could bind to cells normally capable of supporting a productive M-PMV infection, a cell clone (D33/cc3) derived by transfection of HeLa cells with pMP-D33 and selected by hygromycin resistance was used as a source of gp70. Labelled aliquots of supernatant from HeLa cell clone D33/cc3 were added to monolayers of either susceptible (HeLa) cells or nonsusceptible, nonbinding BHK cells (43). The results of this experiment are shown in Fig. 7. A significant proportion (approximately 20%) of the added gp70 fraction bound to HeLa cells (Fig. 7, HeLa lane B). Binding of the gp70 was also observed in the case of COS-1, CV-1, and Raji cells (all susceptible to M-PMV infection) but not in the case of Raji cells persistently infected with SRV-1, a different serotype of the type D simian retroviruses that utilizes the same receptor (data not shown). As might be expected, capsids (devoid of gp70) did not bind to either type of cell; with both HeLa and BHK cells all of the p27 is recovered in the unbound fraction (Fig. 7, lanes F).

DISCUSSION

Retroviruses have been associated with suppression of host immune function in a variety of species (1, 8, 17, 18, 20, 27, 33), and in some instances the immune dysfunction can

FIG. 6. Analysis of gp70 release from SV40 recombinant-infected CV-1 cells. Forty hours following infection with each of the three recombinants (SV40/6A, SV40/D33, and SV40/D105), 35-mm-diameter plates of CV-1 cells were pulse-labelled for 15 min with [3 H]leucine and then chased for 4 h by the addition of unlabelled medium. Cell lysates from the chase of SV40/6A (lane 1), SV40/D33 (lane 2), and SV40/D105 (lane 3), as well media from the corresponding 4-h chases (lanes 4, 5, and 6), were immunoprecipitated by the addition of G α M-PMV antiserum. Production and processing of the glycoprotein precursor, Pr86, is similar to that observed previously (Fig. 2 and 4). The smaller, less immunologically recognized gp22 of mutant D33 (Fig. 2 and 4 and see Discussion) is not seen. However, only in the case of SV40/D33 (lane 5) is any appreciable gp70 found in the infected-cell medium.

be mimicked in vitro. Preparations of whole, inactivated MuLV virions were shown to inhibit lymphocyte responses to mitogen in vitro (25), and the TM protein (p15E) of FeLV was implicated as the component responsible for decreased responses of human lymphocytes to mitogenic stimuli in an in vitro system (26). Cianciolo et al. (13) demonstrated that the p15E of MuLV could inhibit macrophage accumulation, and other investigators showed that isolated FeLV p15E could suppress the secretion of interleukin-2 by human T cells (14) as well as the proliferative response of murine splenocytes (37).

Because several retroviral TM proteins could induce suppression of immune responsiveness in vitro, Cianciolo et al. proposed that a conserved region in these proteins, the ISP region, mediated this suppression of the immune response (12). In support of this conclusion, these investigators (11) demonstrated that a synthetic peptide (CKS-17) corresponding to the first 17 amino acids of the Moloney MuLV ISP sequence could reproduce the in vitro effects of p15E on lymphoid cell function. These properties included inhibition of both human and murine lymphocyte proliferation in response to alloantigen stimulation (11) and inhibition of interleukin-2 production (34). In addition, CKS-17 has been shown, in vitro, to inhibit natural killer cell activity (24), suppress the polyclonal response of feline B cells (32), and inhibit the production of human gamma interferon (36). And recent in vivo work (35) has demonstrated that the CKS-17 peptide could retard delayed-type hypersensitivity in mice to

FIG. 7. Binding assay for secreted gp70. Labelled aliquots of supernatant from HeLa cell clone D33/cc3 (derived by transfection with pMP-D33 DNA and selection by hygromycin resistance) were added to either HeLa or BHK cells in order to determine the ability of the secreted gp70 to bind. The cells were incubated with the supernatant for 1 h at 37°C and then washed extensively to remove protein bound nonspecifically. HeLa cells (capable of binding M-PMV virions and giving rise to a productive infection) were found to bind a substantial portion of the gp70 (lane B, HeLa) in comparison with the unbound, or free, gp70 (lane F, HeLa). In contrast, BHK cells did not bind any of the gp70 (lane B, BHK), and all of the input protein was recovered in the wash (lane F, BHK). In both cases, all of the p27 protein was found in the wash (lanes F), consistent with the inability of the virions carrying the D33 mutation to bind to cells, due to the loss of gp70.

sheep erythrocytes. This region of the MuLV and FeLV TM proteins thus appears to have a broad range of immunomodulatory activities.

During sequencing of the M-PMV genome, a region corresponding to the ISP was found within the TM domain of the predicted envelope gene product (45). Surprisingly, this 35-amino-acid stretch was demonstrated to be 100% homologous to the ISP of REV-A and showed a high degree of homology to the related regions in TM proteins of FeLV, Moloney MuLV, Rous sarcoma virus and two human retroviruses, human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2. The M-PMV region contains 12 residues that show identity to the CKS-17 peptide. Because the ISP region is found in a wide variety of retroviruses and because we were able to demonstrate such a high level of conservation within this region between two highly divergent viruses (REV-A and M-PMV), we have initiated a genetic analysis to determine the role this region might play in virus replication and pathogenesis. In this paper we have utilized deletion mutations to explore whether this region might contribute to the structural integrity of the glycoprotein complex.

Deletion of the entire 35-amino-acid ISP coding domain (mutant D105) resulted in the synthesis of wild-type levels of a smaller precursor protein, which appeared to be normally

glycosylated. However, when the mutant glycoprotein precursor was expressed either in the absence of other viral structural proteins or in the context of the M-PMV genome, the protein failed to be cleaved to the SU and TM proteins. Since this cleavage event occurs late in the secretory pathway (46, 48), it was possible that this extensive deletion, which includes three cysteine residues potentially involved in disulfide bond formation, resulted in an altered tertiary structure which prevented the mutant precursor from being transported to the *trans*-Golgi compartment of the cell. Indeed, endoglycosidase H digestion of mutant D105 Env protein remaining after a 4-h chase showed that it remained sensitive to the enzyme (data not shown), which is consistent with a lack of transport to the Golgi complex. Following entry into the secretory pathway of the cell, retroviral glycoproteins associate into oligomeric structures that represent the structural complex found on the surface of the virion (16), and this process appears to be critical for transport from the rough endoplasmic reticulum. It seems possible then that one effect of the D105 mutation is to interfere with the normal protein folding required for oligomerization and thus prevent movement of the nascent protein from the endoplasmic reticulum.

In contrast to mutant D105, mutant D33, in which only the 11 most highly conserved residues of the ISP were deleted, encoded a precursor which was transported from the endoplasmic reticulum to the Golgi complex and was proteolytically processed with efficiency similar to that of the wild type. This mutation therefore does not appear to induce structural perturbations that prevent normal intracellular transport. In both the *env* expression system and the context of the viral genome, processing of Pr86 was seen in the chase, although the level of cell-associated gp70 was lower than that in wild-type-Env-producing cells. Nevertheless, this mutation completely abrogated the infectivity of the pMP-D33 proviral DNA clone even though it had no effect on the assembly and release of RT-containing virions. It was determined from an analysis of culture supernatants and released virus that the D33 mutation results in a disruption of the noncovalent association between the SU and TM proteins, causing the dissociation of gp70 from the mutated gp22, secretion of large amounts of gp70 protein into the culture medium, and release of virions lacking the receptor-binding SU domain. This result suggests that deletion of the ISP region results in the loss of a domain within the TM protein that is critical for contact with gp70. It is thus possible that the high level of conservation observed within this region of retroviral TM proteins reflects a need to maintain a highly conserved structural domain that functions in TM-SU interactions. The predicted location of the ISP region near the top of the TM spike (19) is consistent with a role in TM-SU interactions. However, it is also possible that the structural perturbation induced by the loss of 11 amino acids in this region affects other gp22-gp70 interactions distal to the site of the mutation, even though intracellular transport is unaffected and a functional (receptor-binding) gp70 is released. Kowalski and colleagues (30) have shown that insertion mutations within either the human immunodeficiency virus type 1 gp120 (SU) or gp41 (TM) coding regions could result in increases in gp120 shedding into the culture medium, and because the mutations that they have described are in regions distinct from that described here, it is quite possible that other regions that contribute to SU-TM association will be identified. We are currently pursuing additional mutational and reversion analyses to explore the possibility that the ISP region represents a major site of

SU-TM association. Preliminary experiments indicate that point mutations within this region can yield mutant glycoproteins with phenotypes similar to that of the D33 deletion mutant described here (5).

Immunoprecipitation of pMP-D33 virions and the fractionation of pMP-D33 cell supernatants raised the possibility that the mutant gp22 was not incorporated into virions (Fig. 5A). These observations were complicated, however, by our discovery that the mutation ablated reactivity with a monoclonal antibody to the external domain of gp20 (Fig. 4A, lane 7) and thus might also reduce recognition of gp22 and gp20 by the polyclonal anti-M-PMV antibodies used. This is a likely possibility, since the region mutated in these studies correlates with a major immunogenic epitope in the gp41 (TM) of human immunodeficiency virus type 1 (23). Nevertheless, it is likely that in the absence of gp70, the mutant gp22 molecules are both inefficiently incorporated into virions and, because they do not accumulate in cells, targeted for degradation by the cell. It may be that the loss of interaction between SU and TM makes the surface gp22 unstable and results in a majority of the molecules entering the degradation pathway. Attempts to block degradation of the mutant glycoprotein by treating cells with 1 to 100 mM chloroquine (15) were unsuccessful because this drug also blocks transport of Pr86 from the endoplasmic reticulum in COS-1 cells (5).

Several studies (3, 47) have shown that only a small fraction of the envelope glycoprotein synthesized in retrovirus-infected cells is incorporated into virions. The fate of the glycoprotein molecules that are not incorporated into virus has not been clearly defined, but it seems likely that at least some of these are endocytosed from the cell surface and degraded in lysosomes (15). The cytoplasmic domain of M-PMV contains a sequence of amino acids that is highly conserved in the cytoplasmic domains of B-lymphocyte membrane proteins and may be involved in signalling endocytosis (38). The possibility that a significant proportion of M-PMV *env* gene products are turned over in this manner is supported by our observation that the D33 mutation results in a five- to sevenfold increase in extracellular gp70 (Fig. 5B). It is likely that in contrast to wild-type gp70 proteins, which are anchored in the membrane through gp22 associations and can thus be carried into endosomes, the gp70 of the D33 mutant is not associated or is weakly associated with gp22 and is therefore released as a soluble molecule following proteolytic cleavage of the Env precursor; endocytosis and degradation of the mutant gp22 would not therefore affect the level of gp70 seen in the culture medium but would greatly decrease the amount of gp22 that is cell associated.

The D33 mutation appears to result in the release of a normally folded, biologically active gp70 glycoprotein. An assessment of the biological activity of the secreted gp70 was based on its ability to bind specifically to susceptible cells (HeLa and Raji cells) and an inability to bind to cells previously shown to lack the M-PMV receptor (BHK cells [43]). Moreover, preliminary experiments have shown that pretreatment of cells with the soluble gp70 can interfere with attachment and fusion activity of wild-type M-PMV glycoprotein (42). In addition, while the protein was able to bind to susceptible cells, it did not bind to cells that were persistently infected with SRV-1, another serotype of the type D simian retroviruses. This is consistent with the observations of Sommerfelt and Weiss (44), who demonstrated that M-PMV and SRV-1 share a common cellular receptor and exhibit reciprocal virus interference. The finding that the efficiently secreted gp70 binds to susceptible

cells may provide a mechanism for identifying the cellular receptor involved in type D retroviral attachment and entry into cells.

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