JOURNAL OF VIROLOGY, Oct. 1987, p. 2981–2988
0022-538X/87/102981-08$02.00/0

Vol. 61, No. 10

Mutants of the Rous Sarcoma Virus Envelope Glycoprotein That Lack the Transmembrane Anchor and Cytoplasmic Domains: Analysis of Intracellular Transport and Assembly into Virions

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Received 16 December 1986/Accepted 15 June 1987

The envelope glycoprotein complex of Rous sarcoma virus consists of a knoblike, receptor-binding gp85 polypeptide that is linked through disulfide bonds to a membrane-spanning gp37 spike. We used oligonucleotide-directed mutagenesis to assess the role of the hydrophobic transmembrane region and hydrophilic cytoplasmic domain of gp37 in intracellular transport and assembly into virions. Early termination codons were introduced on either side of the hydrophobic transmembrane region, and the mutated *env* genes were expressed from the late promoter of simian virus 40. This resulted in the synthesis of glycoprotein complexes composed of a normal gp85 and a truncated gp37 molecule that lacked the cytoplasmic domain alone or both the cytoplasmic and transmembrane domains. The biosynthesis and intracellular transport of the truncated proteins were not significantly different from those of the wild-type glycoproteins, suggesting that any protein signals for biosynthesis and intracellular transport of this viral glycoprotein complex must reside in its extracellular domain. The glycoprotein complex lacking the cytoplasmic domain of gp37 is stably expressed on the cell surface in a manner similar to that of the wild type. In contrast, the complex lacking both the transmembrane and cytoplasmic domains is secreted as a soluble molecule into the media. It can be concluded, therefore, that the transmembrane domain alone is essential for anchoring the RSV *env* complex in the cell membrane and that the cytoplasmic domain is not required for anchor function. Insertion of the mutated genes into an infectious proviral genome allowed us to assess the ability of the truncated gene products to be assembled into virions and to determine whether such virions were infectious. Viral genomes encoding the secreted glycoprotein were noninfectious, whereas those encoding a glycoprotein complex lacking only the cytoplasmic domain of gp37 were infectious. Virions produced from these mutant-infected cells contained normal levels of glycoprotein. The cytoplasmic tail of gp37 is thus not required for the assembly of envelope glycoproteins into virions. It is unlikely, therefore, that this region of gp37 interacts with viral core proteins during the selective incorporation of viral glycoproteins into the viral envelope.

The Rous sarcoma virus (RSV) envelope glycoproteins are representative of a group of membrane-spanning proteins that cross the membrane a single time. They are encoded by the envelope gene (*env*) of RSV and are translated from a spliced, subgenomic mRNA, as a precursor protein of 95 kilodaltons ($Pr95^{env}$). This *env* precursor is cotranslationally translocated into the rough endoplasmic reticulum (RER), where up to 16 carbohydrate side chains are added to the polypeptide chain (17). In a late Golgi compartment, $Pr95^{env}$ is cleaved proteolytically into the two mature viral products, gp85 and gp37 (18, 35). These two glycoproteins are found as a complex, associated by disulfide bonds, on the viral membrane (2, 18). The bitopic organization of the RSV glycoproteins in the viral envelope defines three functional domains: (i) a cytoplasmic domain of 22 amino acids, (ii) a transmembrane domain of 27 amino acids (both located at the carboxy terminus of the small glycoprotein gp37), and (iii) an extracellular domain composed of the amino terminus of gp37 and the entire large glycoprotein gp85, that contains receptor-binding determinants (8).

During budding of type C retroviruses, assembly of the viral core occurs underneath the cell membrane in close association with the viral glycoproteins, and chemical cross-linking experiments have pointed to an interaction between the *gag*-encoded viral structural protein p19 and gp37 (12). It appears likely that such an interaction might be required for normal virus assembly, but the region of gp37 involved is not known. Whether the cytoplasmic domain of this protein plays a role in this process has not been established. However, a comparison of the gp37 carboxy-terminal amino acid sequence of several strains of RSV showed a region of 18 amino acids, adjacent to the inner surface of the viral membrane, that was highly conserved followed by a region of complete amino acid diversity. It seemed possible, therefore, that the conserved region might play a role in virus assembly (17). The function of the transmembrane domain is clearer, since it is typical of other hydrophobic regions that anchor membrane-spanning proteins in the lipid bilayer (7, 15, 23, 33), consisting of a long, uninterrupted stretch of hydrophobic amino acids flanked by charged hydrophilic amino acids. This region presumably interrupts the process of polypeptide translocation into the lumen of the RER, thereby conferring a bitopic orientation on the molecule (1, 36). Moreover, deletion mutants in this region have suggested that additional information may reside in the transmembrane domain (G. Davis, K. Shaw, and E. Hunter, submitted for publication) and it is conceivable that it may also be required for the normal assembly process.

We have been interested in defining functionally important regions of the RSV *env* glycoproteins complex that may be

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involved in biosynthesis, intracellular transport, and viral assembly (16, 24, 34; G. L. Davis and E. Hunter, J. Cell Biol., in press; Davis et al., submitted). The experiments described here focus on the function of the hydrophobic transmembrane and the hydrophilic cytoplasmic domains of the *env* gene product. The complete DNA sequence of the Prague C strain of RSV is known (30), and we have established an expression system for the RSV *env* gene in primate cells under the control of the late promoter of simian virus 40 (SV40) (34). In previous experiments aimed at understanding the role of the carboxy-terminus of gp37 in intracellular transport, we found that a large (95-amino-acid) carboxy-terminal deletion, which removed the cytoplasmic and transmembrane domains of gp37, blocked the intracellular transport of $Pr95^{env}$ immediately after translocation into the RER. Progressive shortening of the cytoplasmic domain of gp37 correlated with a reduction in the rate of intracellular transport of the truncated proteins (35). These mutations suggested that the cytoplasmic tail might play a role in intracellular transport and raised the possibility that the transmembrane domain might be absolutely required for transport from the RER. However, in those experiments the deletions removed the termination codon for the *env* gene and resulted in the addition of different numbers of foreign (SV40-derived) amino acids to the carboxy terminus of the truncated viral glycoproteins. Furthermore, the deletion that removed both the transmembrane and cytoplasmic domains of gp37 extended into the extracellular domain. Thus to determine more directly the role of the cytoplasmic and transmembrane domains of gp37, we have used oligonucleotide-directed mutagenesis to introduce early termination codons in the coding sequences of gp37. These mutations create truncated viral glycoproteins lacking specifically the cytoplasmic domain or both the transmembrane and cytoplasmic domains of gp37. The biosynthesis and transport of the products of these mutant viral glycoprotein genes were analyzed by expression from an SV40 late-region replacement vector, and their ability to be active in viral assembly was investigated by substitution of the mutated genes for the wild-type gene in an infectious avian retrovirus vector. The data presented here show that deletion of the cytoplasmic domain alone, or deletion of both cytoplasmic and transmembrane domains together, had no effect on biosynthesis or intracellular transport of the glycoproteins; however, deletion of the transmembrane domain resulted in a secreted form of the viral glycoproteins. The cytoplasmic domain does not appear, by itself, to be required for anchoring the complex in the membrane or for the process of infectious virus assembly, because retroviral vectors carrying the truncated glycoprotein complex were infectious in avian cells and contained normal levels of glycoprotein.

MATERIALS AND METHODS

DNAs, viruses, and cell culture. All recombinant bacterial plasmids were propagated in DH-1, a *recA hsdR* strain of *Escherichia coli*. The M13 clones were propagated in *E. coli* JM101 (21). The RSV *env* gene cloning and expression in SV40 has been described previously (35). The subcloning of an *Eco*RI-*Xba*I restriction fragment from the 3′ end of the RSV *env* gene into the single-stranded bacteriophage M13mp10 used in this study allows oligonucleotide-directed mutagenesis followed by the direct transfer of the mutated fragment into a slot-in SV40 expression vector. A detailed protocol describing this procedure has been reported by Davis and Hunter (in press). African green monkey cells (CV-1 cells), were grown in Dulbecco modified medium containing 10% fetal calf serum.

Oligonucleotide-directed mutagenesis. The method for in vitro mutagenesis described by Zoller and Smith (37) was carried out with the following modifications. M13 universal primer was used in a 1:1 ratio with the mutagenic primer, and purification of the full-length double-stranded DNA from the single-stranded template was not made, but instead dilutions of the priming reaction mixture were used to directly transform $CaCl_2$-treated *E. coli* JM101 cells. Plaques were transferred to nitrocellulose filters, and the bound DNAs were hybridized to the mutagenic primer labeled at the 5′ end with T4 polynucleotide kinase. The phage of plaques that remained as strong signals after the filters had been washed at 60°C were grown, and the single-stranded DNAs from them were sequenced by using the dideoxynucleotide chain termination method of Sanger et al. (28, 29). After mutant phages were identified, replicative forms were prepared. The *Eco*RI-*Xba*I restriction fragment containing the 3′ end of the RSV *env* gene was recovered from the replicative form of these mutant phages and substituted for the wild-type fragment in the slot-in SV40 expression vector (Davis and Hunter, in press). After removal of plasmid sequences, CV-1 cells were transfected with the SV40 vectors containing the mutant *env* genes, plus helper (*dl*1055) SV40 DNA (35). Viral stocks obtained after transfection were used to infect CV-1 cells for studies on expression of the RSV *env* glycoproteins.

Fluorescent staining of cells, immunoprecipitation of viral polypeptides, and sodium dodecyl sulfate-polyacrylamide gels. At 48 h after infection with the SV40 *env* recombinants, CV-1 cells were pulsed for 15 min with [^{3}H]leucine (65Ci/mmol; Amersham Corp., Arlington Heights, Ill.) to label the RSV glycoproteins and chased for different periods in complete medium. After various chase periods (0 to 8 h), cells were lysed, proteins were immunoprecipitated with a rabbit antiserum against the RSV Prague C glycoproteins, and the immune complexes were removed with Formalin-fixed *Staphylococcus aureus* (35). The immunoprecipitates were electrophoresed in sodium dodecyl sulfate–10% polyacrylamide gels by the procedure described by Hunter et al. (17). Fluorescent staining of the cells was carried out as described previously (35).

Cloning of the RSV *env* gene into an infectious DNA provirus and transfection into avian cells. The infectious, molecularly cloned DNA provirus 779NCTAQ26, kindly provided by S. Hughes, is a *src*$^-$ variant of the Schmidt-Ruppin A strain of RSV that contains an *env* gene flanked by the unique restriction sites *Kpn*I and *Cla*I. This provirus was used for insertion of the wild-type and mutant Prague C *env* genes to test their ability to be incorporated into budding viral particles. The chimeric provirus DNAs were transfected into turkey primary cells by the DEAE-dextran method (8). At 8, 13, and 16 days posttransfection the cell media were assayed for reverse transcriptase activity. In addition, at day 20 the cells were pulse-labeled with [^{3}H]leucine for 15 min and chased for 3 h in complete medium, to analyze the viral products in the cells by immunoprecipitation with a mixture of antibodies against Prague C glycoproteins and whole virus.

Assay for reverse transcriptase. The reverse transcriptase assay is a modification of the method described by Chatterjee et al. (3). In brief, the supernatant fluids from the cells transfected with the different proviruses were first clarified and then centrifuged at 40,000 rpm for 1 h in a Beckman SW41 rotor. Pellets were suspended in 50 μl of suspension buffer (0.1 M NaCl, 0.05 M Tris hydrochloride [pH 7.5],

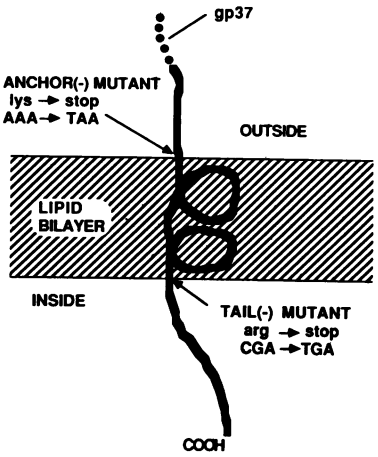

FIG. 1. Schematic representation of the three domains of gp37, the transmembrane glycosylated protein of RSV. The arrows show the positions of the amino acid residues that were changed by oligonucleotide-directed mutagenesis. The codon for the arginine residue (CGA) at the border of the transmembrane and cytoplasmic domains of gp37 was changed to an opal (TGA) codon; this mutant is designated T(−). The codon for the lysine residue (AAA) at the border of the external domain and transmembrane domain in the mutant designated A(−) was changed to an ochre (TAA) codon.

0.001 M EDTA). A 5-μl portion of the virus suspension was mixed with 20 μl of lysis buffer (0.06 M Tris hydrochloride [pH 8.3], 0.125 M NaCl, 0.0125 M dithiothreitol, 0.0075 M $MgCl_2$, 6.25 μCi of [^{3}H]TTP [Amersham Corp.], 0.25 % Nonidet P-40, 50 μg of poly(rA)-oligo(dT)$_{12-18}$ [1:1, Boehringer Mannheim Biochemicals, Indianapolis, Ind.] per ml), and the reaction was carried out as described by Chatterjee et al. (3).

RESULTS

Construction of the truncated *env* glycoproteins. An *Eco*RI-*Xba*I restriction fragment of 717 base pairs, containing the carboxy terminus of gp85 and the entire coding sequence of gp37, was cloned into M13mp10. Single-stranded phage DNA was used as a template to introduce single-base changes into the gp37 coding region by means of two synthetic oligonucleotides. The first was an 18mer oligonucleotide, which changed the first A (nucleotide 6714) of the lysine (AAA) codon, which precedes the hydrophobic domain of gp37 (Fig. 1), to an ochre nonsense codon (TAA); this mutant was designated anchor(−) or A(−). In a similar way, a 17mer oligonucleotide was used to change the C (nucleotide 6797) of the CGA codon corresponding to arginine, the first amino acid of the cytoplasmic domain of gp37 (Fig. 1), to an opal codon (TGA); this mutant was designated tail(−) or T(−). The mutagenized *Eco*RI-*Xba*I restriction fragments were removed from the replicative form of the mutant phage and substituted for the wild-type fragment in the slot-in SV40 *env* expression vector.

Expression of the truncated RSV glycoproteins in CV-1 cells. The SV40 expression vectors, containing RSV *env* genes carrying the early termination codons, were transfected together with *dl*1055 helper DNA into CV-1 cells to obtain infectious virus stocks. These high-titer viral lysates were used at a 1:100 dilution to infect CV-1 cells for expression of the RSV *env* polypeptides. Figure 2 shows the results of immunofluorescent staining of infected CV-1 cells with mutant and wild-type constructions. The immunofluorescent staining of fixed cells (left-hand panels) resulted in identical staining patterns for both mutant- and wild-type-infected cells. The characteristic bright Golgi staining (35) observed in all the cells suggested that intracellular transport to this organelle was unaffected by either mutation. The immunofluorescent staining of the surfaces of unfixed cells is shown in the right-hand panels. Cells infected with the tail(−) mutant show a surface fluorescent staining equivalent to that shown by cells infected with the wild type, indicating that this truncated viral protein was associated normally with the cell surface. Cells infected with the anchor(−) mutant, on the other hand, did not show any surface fluorescent staining above background, indicating that proteins lacking the hydrophobic anchor do not associate in a stable way with the plasma membrane.

Immunoprecipitation of the truncated *env* proteins from infected CV-1 cells. To examine the final location of the truncated viral glycoproteins in CV-1 cells, monolayers were pulsed for 15 min with [^{3}H]leucine. The cells were either lysed immediately after the pulse period or chased for 3 h in complete medium. The cell lysates and chase media were immunoprecipitated, and the immunoprecipitated proteins were separated in sodium dodecyl sulfate–10% polyacrylamide gels. The results of such an experiment are shown in Fig. 3. It can be seen that in wild-type-vector-infected cells the major polypeptide immunoprecipitated from pulse-labeled cells is Pr95^{env}. Similar but somewhat smaller pre-

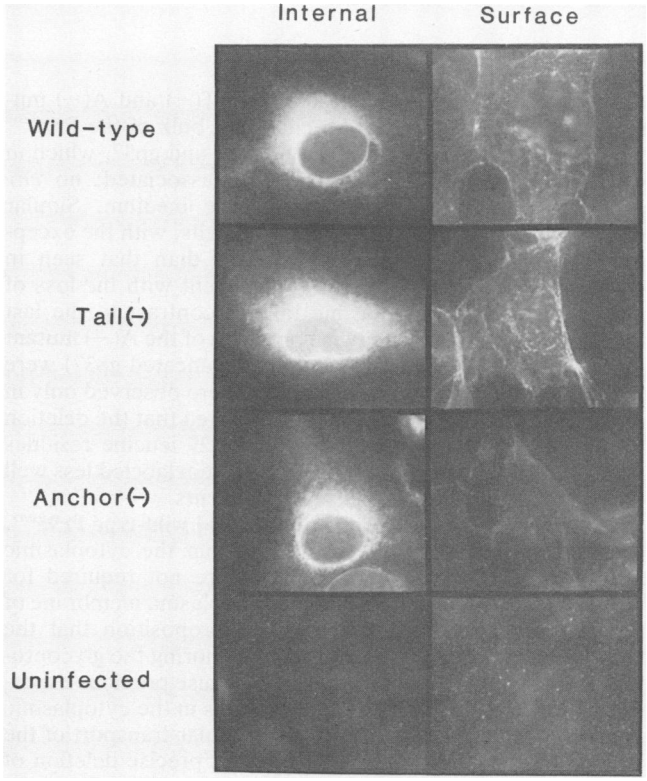

FIG. 2. Indirect immunofluorescent staining of CV-1 cells infected with the SV40 late-region replacement vector carrying the T(−), A(−), and wild-type *env* genes. Fluorescent-antibody staining of fixed cells (internal) is shown in the left panels, and staining of unfixed cells (surface) (right panels) was carried out as described in Materials and Methods. Uninfected cells were included as negative controls.

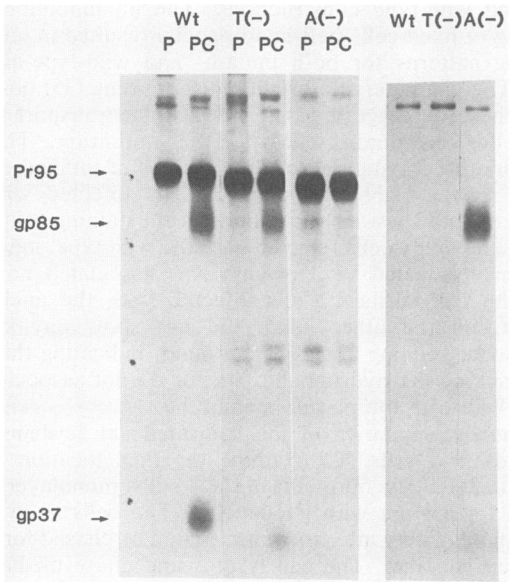

FIG. 3. Pulse-chase labeling of the truncated and wild-type *env* products expressed from the SV40 late replacement vectors in CV-1 cells. At 48 h postinfection, cells were pulse-labeled with [^{3}H]leucine for 15 min and lysed immediately (lane P) or pulse-labeled and then chased for 3 h in complete medium before lysis (lane PC). The immunoprecipitates from the cell lysates (left panel) and the pulse-chase media (right panel) were electrophoresed in a sodium dodecyl sulfate–10% polyacrylamide gel.

cursor proteins were precipitated from T(−) and A(−) mutant-infected cells. After a 3-h chase the bulk of the Pr95^{env} labeled in the pulse was cleaved to gp85 and gp37, which in wild-type-infected cells remained cell associated; no *env* products were released into the chase medium. Similar results were observed in T(−)-infected cells, with the exception that gp37 was detectably smaller than that seen in wild-type-infected cells (Fig. 3), consistent with the loss of the 22-amino-acid cytoplasmic tail. In contrast to the last two constructions, the cleavage products of the A(−) mutant Pr95^{env} (gp85 and the more obviously truncated gp37) were not found associated with the cell but were observed only in the cell medium (Fig. 3). It should be noted that the deletion in the A(−) mutant removes 11 of the 29 leucine residues present in gp37; hence the truncated protein labeled less well than the wild type did in these experiments.

Kinetics of processing of the mutant and wild-type Pr95^{env}. The results presented above indicate that the cytoplasmic and transmembrane anchor domains are not required for transport of the *env* gene product to the plasma membrane of the cell and confirmed our previous proposition that the hydrophobic domain is essential for anchoring the glycoprotein complex in the cell membrane. Because previous experiments (35) had suggested that alterations in the cytoplasmic domain could affect the rate of intracellular transport of the RSV glycoproteins, we studied whether precise deletion of the cytoplasmic and transmembrane domains influenced the rate of intracellular transport. CV-1 cells infected with the three constructions were pulsed with [^{3}H]leucine and chased in complete medium for 0 to 8 h prior to lysis. The *env*-related products were then immunoprecipitated from both cell lysates and culture medium. Figure 4 shows the results of such an experiment. Figure 4A corresponds to the cell-associated glycoproteins that were immunoprecipitated during chase periods of 0 to 6 h following a 15-min pulse-label. For the wild-type *env* and tail(−) constructions, Pr95^{env} was processed to gp85 and gp37 during the chase period and all of the final products remained associated with the cells. The anchor(−) gp85 and truncated-gp37 glycoproteins, on the other hand, were not seen associated with cells at any time during the same period, indicating that as early as 2 h after the pulse the glycoproteins were secreted into the media (Fig. 4B). We have shown previously that cleavage of the precursor protein Pr95^{env} occurs simultaneously with the conversion of the oligosaccharide chains to an endoglycosidase H-resistant form (35). The rate at which Pr95 is cleaved to gp85 and gp37 can thus be used as a measure of the rate at which the precursor is transported to the Golgi complex (35). The bands were excised from the gels and counted in a scintillation counter. The results are quantitated in Fig. 4C, which shows that after an initial lag, the truncated Pr95^{env} products were processed with similar kinetics to those of the wild-type *env* precursor. The basis for the apparent lag in precursor processing is not clear at present. Since both the extracellular secreted form of the anchor(−) mutant protein and the terminally glycosylated, cleaved form of the tail(−) mutant protein were produced with similar kinetics to those of the wild type (Fig. 4A, 2-h chase), it is possible that the antibodies used for immunoprecipitation reacted less efficiently with the nascent translation (pulse-label) product of the two mutants. Nevertheless, we conclude from these studies that the cytoplasmic and anchor domains of gp37 are not required for the efficient biosynthesis and transport of the RSV *env* gene products expressed from the late promoter of SV40 in primate cells.

Role of the cytoplasmic domain of gp37 in virus assembly. Because the above studies indicated that during biosynthesis the truncated RSV glycoprotein complex, lacking the cytoplasmic domain, behaved as a wild-type product in CV-1 cells, we have investigated whether the truncated glycoprotein could be incorporated into virus particles budding from avian cells. A molecularly cloned copy of the Schmidt-Ruppin A strain of Rous sarcoma virus lacking the *src* gene was used for this purpose. The RSV Prague C wild-type, tail(−), and anchor(−) *env* genes were substituted for the Schmidt-Ruppin A *env* gene, as described in Materials and Methods. The recombinant proviruses carrying the wild-type and the mutated *env* genes of the RSV Prague C strain were transfected into turkey cells, and the reverse transcriptase activity present in the media of the transfected cells was measured at 8, 13, and 16 days postinfection. Since only a small fraction of cells are stably transfected, reverse transcriptase activity can be detected only if virus infection can spread through the culture. This method thus provides a sensitive assay for infectious virus production (8). The proviruses containing the wild-type and tail(−) versions of the RSV Prague C glycoproteins yielded similar levels of infectious virus (Fig. 5), as evidenced by a similar rate of increase in reverse transcriptase activity following transfection. Cells transfected with the anchor(−) *env*-containing provirus, on the other hand, showed no detectable reverse transcriptase activity, indicating that this provirus is completely noninfectious.

To confirm that the infectious virions released from avian cells transfected with the tail(−) mutant retained the truncated version of the gp37 glycoprotein, the infected monolayers were pulse-labeled with [^{3}H]leucine for 15 min and chased for 3 h to allow proteolytic cleavage to the viral structural proteins. Lysates of pulse-labeled and pulse-

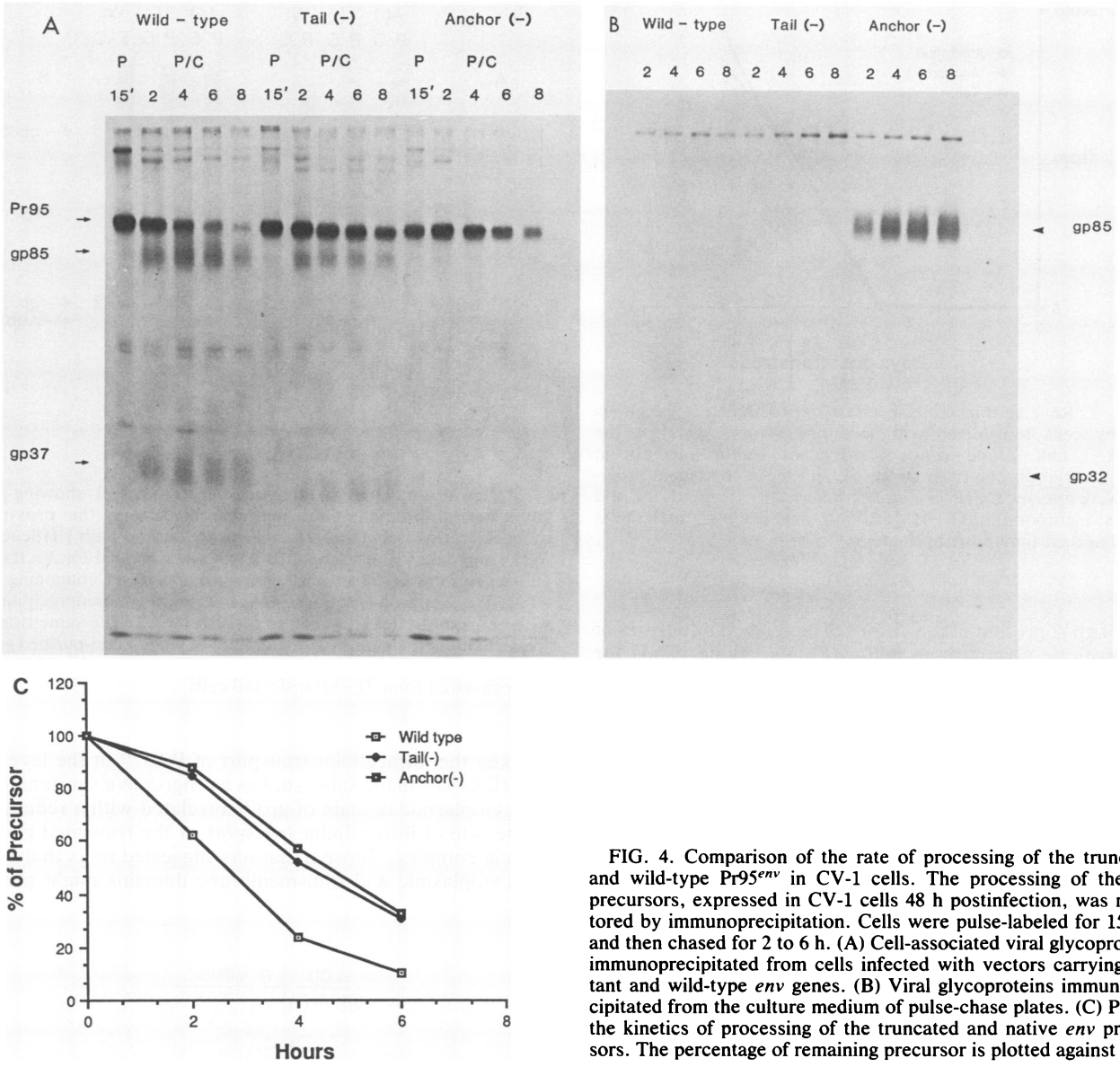

FIG. 4. Comparison of the rate of processing of the truncated and wild-type $Pr95^{env}$ in CV-1 cells. The processing of the *env* precursors, expressed in CV-1 cells 48 h postinfection, was monitored by immunoprecipitation. Cells were pulse-labeled for 15 min and then chased for 2 to 6 h. (A) Cell-associated viral glycoproteins immunoprecipitated from cells infected with vectors carrying mutant and wild-type *env* genes. (B) Viral glycoproteins immunoprecipitated from the culture medium of pulse-chase plates. (C) Plot of the kinetics of processing of the truncated and native *env* precursors. The percentage of remaining precursor is plotted against time.

chased cells were immunoprecipitated with antibody to complete virions, and the polypeptides were separated by polyacrylamide gel electrophoresis. The precursor proteins $Pr76^{gag}$ and $Pr95^{env}$ were immunoprecipitated from the pulse-labeled cells transfected with either the wild-type or tail(−) provirus (Fig. 6). These proteins were absent from both uninfected and anchor(−)-transfected turkey cells, an expected result, since detectable levels of viral protein synthesis require the spread of infectious virus following transfection. During a 3-h chase the precursor polypeptides of the wild-type and tail(−)-infected cells were processed to the mature viral products (Fig. 6A). While the *gag* gene products (p27, pp19, and p12/15) and gp85 from both viruses comigrated on this gel, the gp37 polypeptides showed a clear size difference, confirming the truncated nature of the tail(−) gp37. This was more obvious when the *env* polypeptides were immunoprecipitated with an antibody to gp85/gp37 (Figure 6B); the smaller gp37 band was clearly the only gp37 polypeptide produced during the chase in tail(−) infected cells.

It was possible that the truncated gp37 molecule inefficiently directed the glycoprotein complex into virions, but that such *env*-deficient particles were still infectious. Therefore to determine whether normal or reduced amounts of envelope glycoprotein were present in the virions released from tail(−)-infected turkey cells, virus polypeptides were immunoprecipitated (Fig. 7), with both anti-gp85/gp37 (α gp) antibody and anti-Prague C-virus (α Virus) antibody, from the medium of an overnight chase and separated on a 12% polyacrylamide gel. Virions released from both tail(−)- and wild-type-infected cells had similar protein profiles with equivalent levels of gp85 (Fig. 7). The truncated gp37 is seen less clearly than the wild-type polypeptide with this anti-virus antibody, since its migration is similar to that of p27 (Fig. 6A); however, when anti-gp85/gp37 antibody is used for immunoprecipitation, it is clear that Δgp37 is the only

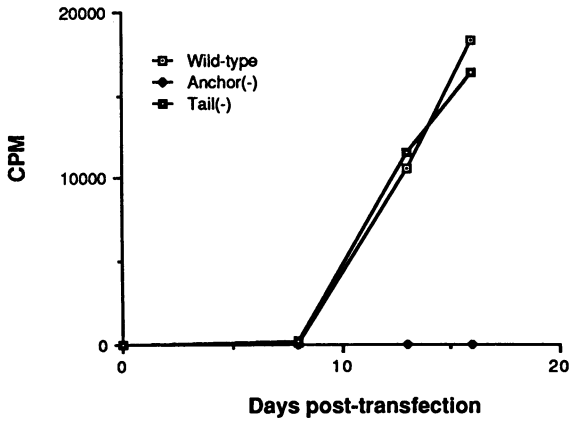

FIG. 5. Reverse transcriptase assay for production of infectious virus by cells transfected with avian retroviruses carrying the anchor(−), tail(−), and wild-type *env* genes. Culture media from turkey embryo fibroblasts transfected with the recombinant proviruses were assayed for reverse transcriptase activity after 8, 13, and 16 days; incorporation of [^{3}H]dTTP into acid-insoluble material is plotted against time posttransfection.

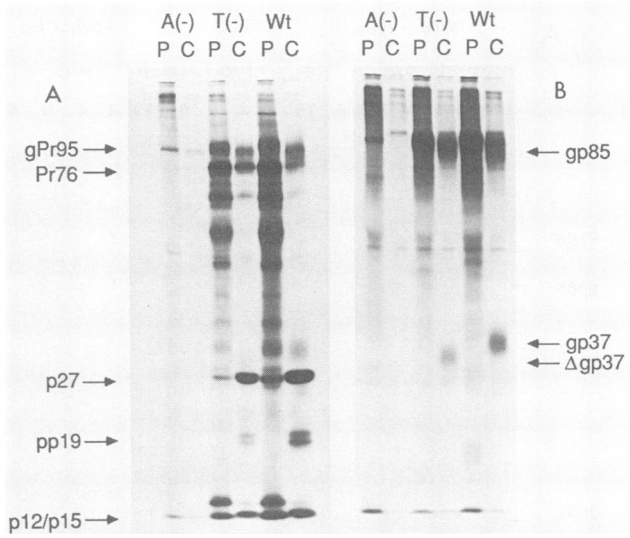

FIG. 6. Autoradiogram of a polyacrylamide gel showing the labeled viral products immunoprecipitated from the provirus-transfected turkey cells. Cells were pulse-labeled with [^{3}H]leucine for 15 min (lanes P) or pulse-labeled and then chased for 3 h (lanes C). (A) Lysates of cells transfected with proviruses containing the A(−), T(−), or wild-type (Wt) *env* genes were immunoprecipitated with antibody to the Prague C strain of RSV. (B) The same lysates were immunoprecipitated with antibody to lentil lectin-purified gp85 and gp37. Δgp37 denotes the location of the truncated gp37 immunoprecipitated from T(−)-transfected cells.

form of gp37 present in the virions. The relative intensities of the envelope glycoprotein and *gag* polypeptide bands for both the tail(−) and wild-type virus are the same (approximately twice the number of counts of wild-type virus were loaded on the gel), indicating that similar numbers of glycoprotein molecules are present in both mutant and wild-type virions. These data show conclusively that the cytoplasmic domain of the RSV *env* glycoprotein complex is not required for its efficient incorporation into avian retrovirus particles or for infectivity of the virus.

DISCUSSION

The maturation of RSV, a member of the simple enveloped viruses (which also includes myxoviruses, paramyxoviruses, rhabdoviruses, and alphaviruses), involves a series of assembly events through which the viral nucleic acid is packaged into a protective coat of protein and lipid (31). In the cytoplasm of the infected cell, the viral nucleic acid is complexed with the five *gag*-encoded proteins p19, p10, p27, p12, and p15 to form a nucleocapsid. Concomitantly, at the cell surface, this complex interacts in a specific manner with the viral glycoproteins and is released into the extracellular fluid enveloped in a piece of host cell plasma membrane that has been modified to contain only the viral membrane proteins. Studies on spike-nucleocapsid interaction, as well as molecular mapping experiments carried out on the viral membrane proteins, should provide information on the domain of these molecules involved in their directed transport and restricted distribution.

It seems reasonable that structural features present in transmembrane proteins destined for the plasma membrane may be involved in the sorting process. For membrane-spanning glycoproteins three domains exist that could harbor a sorting signal; these are the cytoplasmic, the transmembrane, and the external domains. An analysis of the role of each of these domains is required. In previous studies designed to characterize the role of the cytoplasmic and transmembrane domains of the RSV glycoprotein complex, we found that a deletion that removed both of these domains blocked the intracellular transport of Pr95^{env} at the level of the RER (35). In the same studies a progressive shortening of the cytoplasmic domain of gp37 correlated with a reduction in the rate of intracellular transport of the truncated glycoprotein complex. Those mutations suggested to us that both the cytoplasmic and transmembrane domains might play a

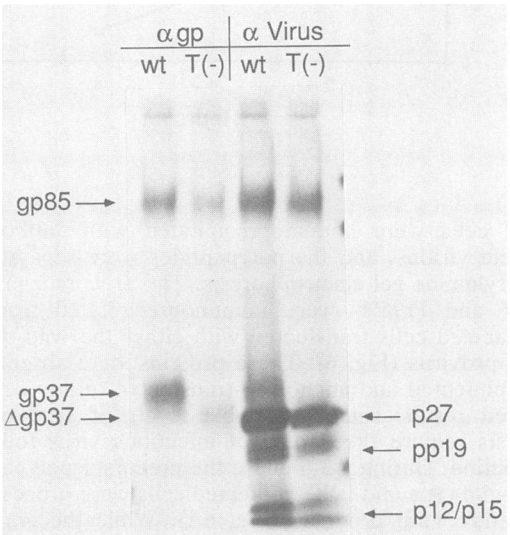

FIG. 7. Autoradiogram of virion proteins released from cells infected with proviruses carrying the tail(−) mutant and wild-type *env* genes. Culture medium from an overnight chase of an experiment similar to that described in Fig. 6 was immunoprecipitated with either anti-gp85/gp37 (α gp lanes) or anti-virus antibody (α Virus lanes) and separated on a 12% polyacrylamide minigel.

role in intracellular transport. Because the deletions that were created in those initial studies introduced a different number of foreign (SV40-derived) amino acids into the truncated protein domains, and because the large deletion that removed both the transmembrane and cytoplasmic domains of gp37 also extended into the extracellular domain, we have used a different approach to define more precisely the role of these two domains in the biosynthesis and intracellular transport of the RSV glycoproteins. The introduction of early termination codons, by using oligonucleotide-directed mutagenesis to modify the lysine and arginine residues flanking the transmembrane domain of the RSV glycoprotein complex, resulted in truncated versions of it that lack only the cytoplasmic or both the transmembrane and cytoplasmic domains with no additional modifications. The expression of these truncated *env* genes, in CV-1 cells, by using the late promoter of SV40, indicates that their biosynthesis and intracellular transport do not differ significantly from those of the wild-type complex. The basis for the lag in $Pr95^{env}$ processing observed with both mutants is not clear. One possibility is that the antibody used in these experiments has a reduced affinity for the nascent polypeptide chain and that the mutant proteins take longer to attain a reactive configuration. The absence of a lag in the appearance of mature products (Fig. 4A and B) would support this interpretation. Thus while the cytoplasmic and transmembrane domains may facilitate protein folding, the signals for processing and intracellular transport of the RSV *env* gene products must reside in the extracellular domain of this viral glycoprotein complex.

The hypothesis that the transmembrane and cytoplasmic domains of a glycoprotein could contain sorting signals has been tested in other viral glycoprotein and membrane receptor systems. The removal of part of the cytoplasmic domain of the Semliki Forest virus E2 glycoprotein or the H-2 transplantation antigen or deletion of both anchor-plus cytoplasmic domains from the vesicular stomatitis virus G protein and the influenza hemagglutinin protein did not block their transport to the cell surface (11, 14, 22, 25, 32, 38). However, defined alterations in the cytoplasmic domain of the vesicular stomatitis virus G protein (analogous to our previous mutations in the carboxy terminus of the RSV *env* gene) reduced or eliminated its transport to the cell surface (26). It is likely that certain mutations in the cytoplasmic domain can affect the conformation of the extracellular domain, thereby resulting in a failure of those mutants to be efficiently transported (13), while other types of mutations may not affect it and consequently have no effect on transport. The inconsistency of our previous results with the ones reported here could be explained in a similar way. We cannot rule out the possibility that in our earlier experiments the extra amino acids added as a consequence of the loss of the *env* termination codon created a conformational change in the extracellular domain of $Pr95^{env}$ that blocked its transport from the RER. Our present results indicate that the cytoplasmic domain of gp37 is neither a recognition signal for transport to the plasma membrane nor a requirement for anchoring the molecule to it. These findings support the idea that the charged amino acids present in most of the cytoplasmic domains of many transmembrane proteins (10, 27) are dispensable for anchor function (6, 9). This question has also been addressed by Cutler et al. (4, 5), who mutated the cytoplasmic domain of the p62 polypeptide of Semliki Forest virus. This region, which normally contains a charge cluster (Arg-Ser-Lys) flanking the hydrophobic domain, was changed into a neutral (Met-Ser-Gly) or an acidic (Met-Ser-Glu) one by using oligonucleotide-directed mutagenesis. Expression analyses of these mutant proteins confirmed that the basic amino acids were not required for cell surface transport, since they reached the surface in a biologically active form. Nevertheless, both mutant polypeptides showed reduced stability when membranes containing them were extracted with high-pH buffer (5). Charged residues within the cytoplasmic domain may thus provide an additional measure of stability to the membrane bound complex.

The secreted form of *env* constitutes direct evidence for the prediction from DNA and protein sequence studies (17) that the stretch of 27 hydrophobic amino acids near the carboxy terminus of gp37 is the membrane anchor of the glycoprotein complex and furthermore establishes that this region alone is sufficient to keep it membrane associated. The wild-type behavior of the secreted form of the RSV glycoproteins is consistent with the study of Davis and Model (7) that indicated that hydrophobic sequences present in proteins can specify transmembrane topology and that this topology is predictably altered when the hydrophobic repeats are relocated or deleted from the protein sequence. Machida et al. (20) have described a naturally occurring mutant of the Rauscher spleen focus-forming virus that is similar to the soluble form of the RSV glycoproteins that we have created in vitro in that it lacks the carboxy-terminal membrane anchor of its envelope glycoprotein. The Rauscher spleen focus-forming virus-encoded glycoprotein terminates prematurely because of a 44-base-pair deletion in the 3′ region of *env* that produces a frame shift and an early termination codon. This mutation makes the virus weakly pathogenic and results in a Rauscher spleen focus-forming virus glycoprotein that has a shortened intracellular life span (19). It will be of interest to determine whether the soluble RSV glycoprotein complex has any biological activity.

The fact that the tail(−) mutant protein was efficiently transported to the cell surface allowed us to analyze the role of the cytoplasmic domain in the process of virus budding. Chemical cross-linking experiments have demonstrated an interaction between gp37 and p19, one of the *gag* gene products that make up the structure of the viral core of RSV (12). Although it is clear that viral assembly can occur in the absence of glycoproteins, it was suggested that the p19-gp37 interaction may be part of the driving force for the process of viral assembly and budding. Furthermore, since host membrane glycoproteins are excluded from the viral membrane, there must be some positive signal for inclusion of the viral *env* gene products in the budding virion. To determine whether the cytoplasmic domain is involved in this interaction and is required for infectious virus assembly, we reconstructed a retrovirus genome carrying the tail(−) *env* gene mutation. The results of this experiment indicate that such mutant viruses are infectious on avian cells and that they spread through the culture with similar efficiency to those containing a native *env* glycoprotein complex. Furthermore, the truncated *env* gene complex appears to be incorporated as efficiently into virus particles as is the wild-type complex. This fact suggests that if an interaction between gp37 and p19 is required to mediate the incorporation of the glycoproteins into the envelope of the budding viral particle, it must occur within the lipid bilayer, presumably within the hydrophobic anchor domain. Indeed, preliminary data indicate that *env* gene products with deletions within the hydrophobic domain but with intact cytoplasmic domains are inefficiently incorporated into virions, despite being expressed on the plasma membrane of the infected cells (D. Miller, G. Davis, and E. Hunter, manuscript in preparation). Thus the data presented

here make it unlikely that interactions between viral capsid proteins and the cytoplasmic domain of the *env* complex constitute a driving force for preferential incorporation of the viral glycoproteins in the viral envelope.

ACKNOWLEDGMENTS

We thank K. Shaw for excellent technical assistance and J. Engler for the synthesis of the oligonucleotides. For critical comments on this manuscript we thank G. Air, S. Chatterjee, R. W. Compans, and F. Suddath. We are grateful to M. Pollard for assistance in preparing this manuscript for publication.

This work was supported by Public Health Service grant CA-29884 from the National Institutes of Health. L. Perez is a predoctoral trainee supported by an ODEPLAN-Chile fellowship. E. Hunter was the recipient of Research Career Development Award CA-00685 from the National Cancer Institute.

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