Mutations of the Rous Sarcoma Virus *env* Gene That Affect the Transport and Subcellular Location of the Glycoprotein Products

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ABSTRACT The envelope glycoproteins of Rous sarcoma virus (RSV), gp85 and gp37, are anchored in the membrane by a 27-amino acid, hydrophobic domain that lies adjacent to a 22-amino acid, cytoplasmic domain at the carboxy terminus of gp37. We have altered these cytoplasmic and transmembrane domains by introducing deletion mutations into the molecularly cloned sequences of a proviral *env* gene. The effects of the mutations on the transport and subcellular localization of the Rous sarcoma virus glycoproteins were examined in monkey (CV-1) cells using an SV40 expression vector. We found, on the one hand, that replacement of the nonconserved region of the cytoplasmic domain with a longer, unrelated sequence of amino acids (mutant C1) did not alter the rate of transport to the Golgi apparatus nor the appearance of the glycoprotein on the cell surface. Larger deletions, extending into the conserved region of the cytoplasmic domain (mutant C2), resulted in a slower rate of transport to the Golgi apparatus, but did not prevent transport to the cell surface. On the other hand, removal of the entire cytoplasmic and transmembrane domains (mutant C3) did block transport and therefore did not result in secretion of the truncated protein. Our results demonstrate that the C3 polypeptide was not transported to the Golgi apparatus, although it apparently remained in a soluble, nonanchored form in the lumen of the rough endoplasmic reticulum; therefore, it appears that this mutant protein lacks a functional sorting signal. Surprisingly, subcellular localization by internal immunofluorescence revealed that the C3 protein (unlike the wild type) did not accumulate on the nuclear membrane but rather in vesicles distributed throughout the cytoplasm. This observation suggests that the wild-type glycoproteins (and perhaps other membrane-bound or secreted proteins) are specifically transported to the nuclear membrane after their biosynthesis elsewhere in the rough endoplasmic reticulum.

The mechanism(s) by which cells send membrane-bound and secreted proteins to their proper subcellular locations remains an enigma of molecular biology. The mechanism presumably involves the specific interaction of "sorting signals," located within the structure of the newly synthesized proteins, with membrane-bound receptors in the rough endoplasmic reticulum (RER)[1] and Golgi apparatus of the cell (for review, see references 53 and 59). Evidence that protein sorting requires such specific interactions has recently been provided by the observation that cells can transport and secrete a variety of glycosylated and unglycosylated proteins at distinctly different rates (10, 18, 36, 38, 61).

Very little is known about the composition(s) of sorting signals, but it is generally thought that they are composed of protein. In support of this idea, transport defective mutants of secreted proteins have been identified that differ from the wild-type forms by one (44, 71) or two (22, 72) amino acid substitutions. Also, several conditional transport defective mutants of membrane-bound viral glycoproteins have been identified (for example, 33, 37, 47, 73). Furthermore, studies using tunicamycin, an inhibitor of glycosylation, suggest that carbohydrate moieties are not recognized directly by the

[1] *Abbreviations used in this paper*: Endo H, endoglycosidase H; HA, hemagglutinin; RER, rough endoplasmic reticulum; RSV, Rous sarcoma virus; VSV, vesicular stomatitis virus.

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sorting machinery but may be important for maintaining the proper secondary or tertiary structures of (protein-composed) sorting signals (15–17, 23, 52, 61, 63). An indirect role for carbohydrate moieties in the sorting process is perhaps to be expected in view of the fact that many secreted proteins are not glycosylated at all (see, for example, references 61, 67). To be sure, the transport of certain hydrolases to the lysosome (and away from the secretory pathway) does appear to require the addition of a carbohydrate moiety (mannose-6-phosphate [20]), but these additions in turn must require the recognition of signals within the polypeptide chains.

Even less is known about the intramolecular location(s) of sorting signals. In the case of the membrane-spanning glycoproteins, three protein domains exist that together or separately may harbor a sorting signal: (*a*) the internal or cytoplasmic domain, (*b*) the hydrophobic or transmembrane domain, and (*c*) the extracytoplasmic or external domain. Because most secreted proteins (which may be co-transported with membrane-bound glycoproteins [62]) possess only external domains, it would seem reasonable to expect the transmembrane and cytoplasmic domains to be unimportant to the sorting process. This hypothesis has been recently tested for the vesicular stomatitis virus (VSV) G protein (50) and the influenza virus hemagglutinin protein (14, 64). Removal of the transmembrane and cytoplasmic domains of both of these proteins (by means of in vitro mutagenesis of the cloned genes) resulted in secretion of the truncated proteins. Furthermore, removal of portions of the cytoplasmic domain from the Semliki Forest virus E2 glycoprotein (12) and from the H-2L transplantation antigen of the mouse (45, 74) has been shown not to block transport to the cell surface. Subsequently, however, evidence has been obtained that demonstrates that the cytoplasmic domain may indeed be important for the sorting process. Defined alterations in the cytoplasmic domain of the VSV G protein, obtained by in vitro mutagenesis, have shown that deletions within this domain can reduce or eliminate transport to the cell surface (51). Furthermore, spontaneous mutants of the vaccinia virus hemmagglutinin protein that are blocked in the RER, though not well characterized by sequencing, have been shown to contain longer cytoplasmic domains (58). Thus, the structure and locations of sorting signals within membrane-spanning glycoproteins is not as obvious as might initially have been thought.

Unraveling of the complex nature of sorting signals is likely to require the analysis of many well-defined mutant polypeptides. To this end, we have constructed a series of mutations in the envelope gene (*env*) of Rous sarcoma virus (RSV), an avian retrovirus. The RSV *env* gene codes for two glycoproteins, gp85 and gp37, which are found on the outer surface of infectious virions. These proteins are required for the specific binding of the virions to receptors on the surface of susceptible cells and initiate the process of cellular penetration (69). The two glycoproteins are synthesized as a single glycosylated precursor ($Pr95^{env}$), which is subsequently processed to give the mature forms (7, 9, 21, 32, 43). Processing appears to take place in the Golgi apparatus of infected chicken cells (4, 5), and pactamycin-mapping experiments place gp85 at the amino terminus and gp37 at the carboxy terminus of $Pr95^{env}$ (32, 57). The proteins are linked by disulfide bonds to give complexes that contain one molecule of gp85 and one of gp37 (34). Treatment of virions with reducing agents releases only gp85, which suggests that gp37 serves to anchor the glycoproteins to the viral membrane (46). The determinants of host-range specificity appear to map to gp85 (24, 29, 30).

A DNA copy of the entire RSV genome has recently been molecularly cloned (31) and sequenced (56). Amino acid sequencing of the amino termini of gp85 and gp37 has enabled the structural and functional regions of the *env* gene product to be deduced (29). These latter studies have demonstrated the presence of a 27-amino acid-long hydrophobic (and presumably membrane-spanning) domain and a 22-amino acid-long cytoplasmic domain at the carboxy terminus of gp37 (see Fig. 2). Comparison of these domains with those of other exogenous and endogenous strains of RSV has revealed that the sequence within the hydrophobic domain is highly conserved; furthermore, in the cytoplasmic domain, the sequence of the first 18 amino acids (adjacent to the hydrophobic domain) is also highly conserved whereas those at the carboxy termini diverge greatly (26, 29).

In this paper we report the importance of the carboxy-terminal domains of gp37 for transport of the RSV glycoproteins to the cell surface. We show that alteration of the nonconserved region of the cytoplasmic domain does not affect the rate of transport, but a deletion within the conserved region does. Furthermore, deletion of the entire cytoplasmic and transmembrane domains does not result in secretion of the truncated protein, although it apparently remains soluble in the lumen of the rough endoplasmic reticulum. We also present evidence that suggests that the nuclear envelope is a distinct intermediate compartment between the rough endoplasmic reticulum and the Golgi apparatus. A preliminary report of some of this work has recently been published (70).

MATERIALS AND METHODS

DNAs, Viruses, and Cells: The source of the RSV *env* gene was pATV-8, a recombinant pBR322 plasmid containing a complete copy of the genome of the Prague C strain (31). The entire nucleotide sequence of this cloned copy has recently been reported (56). The SV40 late region replacement vector used for the expression of wild-type and mutant RSV *env* genes was constructed from wild-type SV40 DNA. SV40 late functions were provided by a viable, early deletion mutant, d11055 (48, 49). This SV40 helper genome and the SV40 vector genome were molecularly cloned in pBR322 derivatives and generously provided by Dr. Mary-Jane Gething and Dr. Joe Sambrook (Cold Spring Harbor Laboratory). Control experiments were performed using recombinant SV40 viruses carrying cloned influenza hemagglutinin (HA) genes (also obtained from Dr. Gething). The viruses used were SVEHA3 (which expresses wild-type HA [13]) and SVEHA20A$^-$ (which expresses an "anchor-minus" HA [14]).

All recombinant bacterial plasmids were propagated in *Escherichia coli* strain DH-1, which is *recA* and *hsdR* (40). Transformed DH-1 cells were selected and grown in L medium (per liter, 10 g tryptone, 5 g yeast extract, 10 g NaCl) containing ampicillin. The expression and characterization of the recombinant SV40 viruses were studied using CV-1 cells, a continuous line of African green monkey kidney cells that are permissive for SV40 replication (1). These cells were grown in Dulbecco's modified medium containing 10% fetal calf serum.

Manipulation of DNAs: All DNA modification enzymes (restriction endonucleases, T4 DNA ligase, Klenow fragment of DNA polymerase I, Bal31) were purchased from Bethesda Research Laboratories (Gaithersburg, MD). All manipulations and reactions involving DNA (restriction endonuclease digestions, analytical and preparative gel electrophoresis, blunt-end and sticky-end ligations, bacterial transformation, plasmid isolations, Bal31 digestions, end labeling, etc.) were performed according to standard laboratory protocols (40).

Subcloning and In Vitro Mutagenesis of the env Gene: The 1866 base pair (bp) KpnI-XbaI fragment of pATV-8 contains the RSV *env* gene with the sequences coding for the amino terminus of gp85 located near the KpnI site and those for the carboxy terminus of gp37 located near the XbaI site (29, 56). This fragment was subcloned into a derivative of pBR322 after ligating a BamHI linker to the XbaI end with the use of the Klenow fragment of DNA polymerase I and T4 DNA ligase.

To introduce deletions into the subcloned *env* gene, plasmid DNA was cut at the unique BamHI site and digested for various amounts of time with the double-strand-specific exonuclease, Bal31. Reactions giving digestion products of the desired size class were identified by digesting aliquots with restriction

enzymes and analyzing the products on polyacrylamide gels. The DNA from these Bal31 reactions was then ligated in the presence of BamHI linkers, CCGGATCCGG, (Collaborative Research, Lexington, MA) to restore the BamHI site. After transformation of DH-1 cells, clones were screened for the presence of the BamHI site, and then the approximate size of each deletion was determined by restriction enzyme analysis. The precise endpoints of the deletions of interest were determined by the Maxam–Gilbert method of DNA sequencing (41).

Construction of Recombinant SV40 Viruses: Expression of the wild-type and mutant *env* genes was accomplished using an SV40 late region replacement vector. As shown in Fig. 1, KpnI-BamHI fragments containing the *env* genes were inserted in the place of the late region of SV40 DNA between the KpnI and BamHI sites. These substitutions leave a functional SV40 late promoter (6), which allows transcription of the inserted *env* genes. The SV40-*env* recombinant DNAs were cloned in the BamHI site of a pBR322 derivative and propagated in *E. coli* cells. Before transfection of CV-1 cells, the bacterial plasmid sequences (pBR322) were removed by BamHI digestion, and the SV40-*env* sequences were circularized by ligation at low DNA concentration (4 μg/ml).

Virions carrying the recombinant SV40-*env* DNAs were produced in CV-1 cells by complementation with a co-transfected SV40 helper DNA (d11055) using a modification of the method of Gething and Sambrook (13). Briefly, 60-mm plates of CV-1 cells (~80% confluent) were washed once with PBS and once with Tris-buffered saline. 500 μl of Tris-buffered saline containing 200 ng of circularized SV40-*env* DNA, 75 ng of circularized helper DNA, and 250 μg of DEAE-dextran (42) were added to the monolayers, which were then placed at 37°C in a CO_2 incubator. After 60–90 min, the cells were gently washed once with Tris-buffered saline, once with PBS, and then growth medium containing 100 μM chloroquine was added for 3–5 h at 37°C (39). The cells were incubated in medium without chloroquine for 4–6 d until cytopathic effects were observed. High-titer virus stocks were produced by freeze-thawing the transfected cells three times and using the lysates to infect fresh monolayers. The resulting virus stocks were used at concentrations that produced extensive cytopathic effects (rounded or vacuolated but still attached cells) in 3–4 d.

Radiolabeling of Cells: Cells were labeled with radioisotopes 50–72 h after infection. Labeling with D-[2-^{3}H]mannose (10 Ci/mmol, Amersham Corp., Arlington Heights, IL), D-[6-^{3}H(*N*)]glucosamine HCl (26.8 Ci/mmol, Amersham Corp.), or L-[5,6-^{3}H]fucose (84 Ci/mmol, New England Nuclear, Boston, MA) was performed in 0.5 ml of serum-free medium for 6 h using 100 μCi of labeled sugar. Cells to be pulse labeled with L-[4,5-^{3}H(*N*)]leucine (50 Ci/mmol, New England Nuclear) were starved for 1 h in leucine-free, serum-free medium and then incubated with 0.25 ml of the same medium containing 250 μCi of [^{3}H]leucine for 15 min. Chases with unlabeled leucine were performed in 2 ml of growth medium after removing the labeling medium. For those experiments where quantitation between pulse and pulse–chase plates was critical, great care was taken to treat all the plates identically (the same amount of cells, virus inoculum, radioisotope, etc., were used), and each experiment was repeated two to three times.

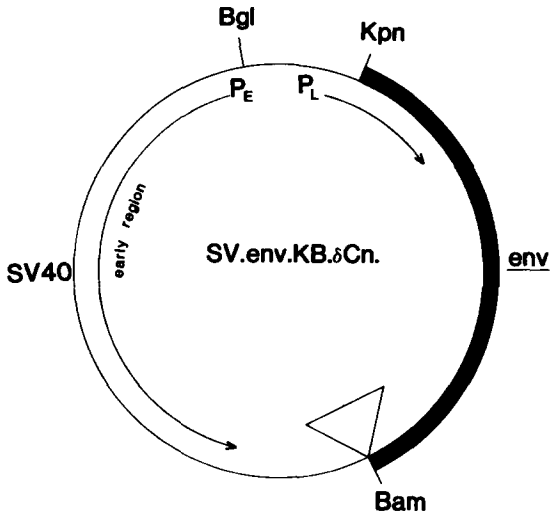


FIGURE 1 Generalized structure of the SV40 late region replacement vector used for the expression of the mutant RSV *env* genes in CV-1 cells. The region of *env* coding for the amino terminus of the glycoprotein product is located near the KpnI site. Three mutations (designated C1, C2, and C3) in the sequences coding for the carboxy terminus were constructed in vitro using the exonuclease, Bal31. The site of the deletions in the *env* gene is indicated by the triangle.

Immunoprecipitation of Viral Polypeptides: Radiolabeled cells were detergent lysed as described before (29). To examine the culture fluids for secreted viral proteins, we centrifuged the medium to remove loose cells and then mixed with detergents (19). Viral proteins were immunoprecipitated using an excess of antiviral antibodies (anti-gp85/anti-gp37 or anti-HA) and the immune complexes were collected with fixed *Staphylococcus aureus* as previously described (13, 29). The anti-gp85 and anti-gp37 antibodies were prepared using detergent-treated, gel-purified RSV glycoproteins and have been shown to react with both glycosylated and nonglycosylated forms of the RSV glycoproteins (29).

SDS Polyacrylamide Gels: Immunoprecipitated viral polypeptides were reduced, denatured, and electrophoresed in 1.5-mm-thick SDS–Tris–glycine slab gels containing 10% acrylamide as described previously (29). To quantitate the amount of radioactivity in bands of viral protein, we carefully cut out the bands of the dried gel and soaked them overnight at 37°C in 0.4 ml of 90% Protosol (New England Nuclear). 4 ml Omnifluor (New England Nuclear)–toluene was then added, and the radioactivity present was measured in a liquid scintillation counter.

Fluorescent Staining of Cells: CV-1 cells were grown on coverslips in 35-mm plates and stained after 50–65 h of infection. To identify the internal locations of the viral proteins, we performed indirect immunofluorescent staining on cells fixed with 95% ethanol, 5% glacial acetic acid (vol/vol). Rabbit antiviral antibodies (anti-gp85 or anti-HA) were added for 30 min at room temperature, and after washing the excess antibodies away, fluorescein-conjugated goat anti-rabbit IgG (Antibodies Inc., Davis, CA) was added for 30 min at room temperature. After thoroughly washing, the coverslips were wet mounted on slides using 50% glycerol, 50% PBS containing 0.1% BSA. To stain the Golgi apparatus (68), we first stained the fixed cells immunofluorescently as described above. After washing away the excess fluorescein labeled, rhodamine-conjugated wheat germ agglutinin (E. Y. Laboratories, San Mateo, CA) diluted 1:10 with PBS was added for 30 min at room temperature. The excess rhodamine label was then washed away, and the coverslips were mounted as described above. To detect viral proteins on the surface of infected cells, we used live cells, and all steps were carried out at 4°C. The cells were thoroughly washed and antiserum from RSV-PRC infected chickens was added for 30 min. This antiserum reacts strongly with the antigenic determinants present on native (non-detergent-treated) RSV glycoproteins. The cells were then washed and fluorescein-conjugated rabbit anti-chicken IgG (Antibodies Inc.) was added for another 30 min. After washing, the cells were fixed with 95% ethanol, 5% glacial acetic acid for 15–30 min. The coverslips were then washed and mounted as described above. All photographs were made on a Nikon Fluophot fluorescence microscope (Nikon Inc., Garden City, NY) using a × 40 objective, and Kodak Tri-X Pan film (400 ASA, Eastman Kodak Co., Rochester, NY) was used for black and white negatives.

Fractionation of Infected Cells: Cells were fractionated into cytosol and membrane portions by the general method of Dickson and Atterwill (8). CV-1 cells were infected and (after 57 h) either pulse labeled or pulse–chase labeled (4-h chase) with [^{3}H]leucine and washed twice with ice cold PBS. The cells were then scraped off the plates in PBS, pelleted at 2,000 *g* for 5 min, and resuspended in hypotonic buffer (1 mM $MgCl_2$, 2 mM $NaHCO_3$). After incubating for 10 min on ice to allow swelling, the cells were ruptured by Dounce homogenization. Nuclei and unbroken cells were pelleted by centrifugation at 2,000 *g* for 10 min. The resulting supernatant was centrifuged at 50,000 rpm in Beckman SW50.1 rotor (250,000 *g*, average, Beckman Instruments, Inc., Fullerton, CA) for 15 min at 4°C. The cytosol fraction (supernatant) and membrane fraction (pellet) were adjusted to equal volumes using detergent-containing buffers, and the viral proteins were immunoprecipitated and electrophoresed as described above.

RESULTS

Construction and Expression of Deletion Mutations Altering the Carboxy Terminus of $Pr95^{env}$

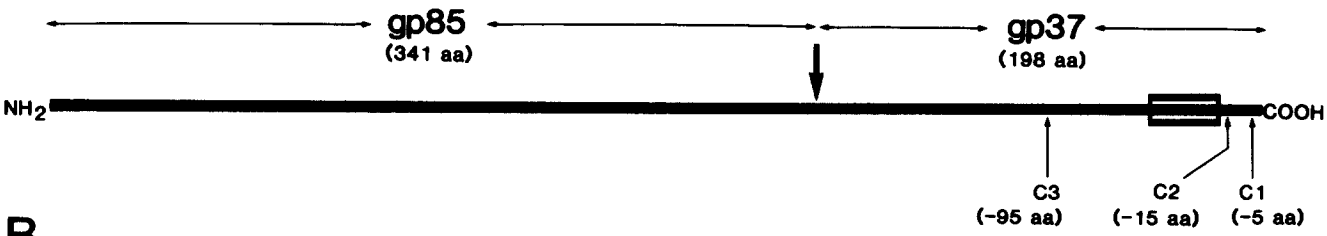
The *env* gene of the Prague C strain of RSV is contained in a KpnI-XbaI fragment of the proviral DNA (56). The sequences coding for the amino terminus of $Pr95^{env}$ are located near the KpnI site, and those coding for the carboxy terminus are located near the XbaI site. Deletions were introduced into the *env* gene by digesting from the XbaI end with the double

strand-specific exonuclease, Bal31 (see Materials and Methods). BamHI linkers were ligated to the digested ends, and the mutant *env* genes were cloned between the KpnI and BamHI sites into the late region of SV40 DNA (Fig. 1). This arrangement puts the *env* genes under the control of the SV40 late promoter. Codons for the termination of protein synthesis were provided by the SV40 sequences flanking the BamHI site.

Before characterizing the phenotypes of the *env* mutations, recombinant SV40 virus stocks were prepared. For this purpose, each recombinant SV40-*env* DNA (vector DNA) was co-transfected into monkey kidney (CV-1) cells along with the DNA of an SV40 early region deletion mutant (helper DNA). Complementation of the early SV40 functions (of the vector DNAs) with the late SV40 functions (of the helper DNA) enabled the production of infectious, recombinant SV40 virions (13). These high-titer virus stocks were then used to infect fresh monolayers of CV-1 cells in order to characterize the wild-type and mutant RSV glycoproteins.

The endpoints of the deletions were determined by DNA sequencing, and the results were used to predict the amino acid sequences of the mutant polypeptides. These predictions are shown in Fig. 2. Mutant C1 polypeptides are predicted to lack the five amino acids at the carboxy terminus of the cytoplasmic domain; these amino acids are replaced by 20 "foreign" amino acids (coded by the BamHI linker and adjacent SV40 sequences). In effect, the C1 polypeptide has lost one nonconserved sequence and gained another (longer) one at the end of gp37. Mutant C2 polypeptides are predicted to lack 15 amino acids of the cytoplasmic domain which are replaced by four foreign amino acids. Thus, a large portion of the conserved region of the gp37 cytoplasmic domain is missing from the C2 polypeptide. Mutant C3 polypeptides are predicted to lack the entire cytoplasmic domain, the entire hydrophobic domain, and a portion (46 amino acids) of the external domain of gp37; these are replaced by the same 20 foreign amino acids that are appended to the C1 polypeptides. In structure, the C3 polypeptide resembles the anchor-minus polypeptides of the VSV G protein (50) and the influenza virus HA protein (14, 64) that have been reported to be secreted. It should be noted that none of the mutant polypeptides described in this report contain the SV40-coded, "transport-poison" sequence that has been found to affect the transport of certain mutant VSV G proteins (51). Further-

A



B

wildtype ...leu ser asp his ser glu ser ile gln lys lys phe gln leu met lys lys his val asn lys ile gly val asp ser asp pro ile gly ser

mutant C1 ...leu ser asp his ser glu ser ile gln lys lys phe gln leu met lys lys his val asn lys ile gly val asp ser asp pro ile gly ser

mutant C2 ...leu ser asp his ser glu ser ile gln lys lys phe gln leu met lys lys his val asn lys ile gly val asp ser asp pro ile gly ser

mutant C3 ...leu ser asp PRO ASP PRO ASP MET ILE ARG TYR ILE ASP GLU PHE GLY GLN THR THR THR ARG MET GLN *** - - - - - - -

trp leu arg gly ile phe gly gly ile gly glu trp ala val his leu leu lys gly leu leu leu gly leu val val ile leu leu leu leu val cys leu pro

trp leu arg gly ile phe gly gly ile gly glu trp ala val his leu leu lys gly leu leu leu gly leu val val ile leu leu leu leu val cys leu pro

trp leu arg gly ile phe gly gly ile gly glu trp ala val his leu leu lys gly leu leu leu gly leu val val ile leu leu leu leu val cys leu pro

- -

cys leu leu gln phe val ser ser ser ile arg lys met ile asn ser ser ile asn tyr his thr glu tyr arg lys met gln gly gly ala val *** - -

cys leu leu gln phe val ser ser ser ile arg lys met ile asn ser ser ile asn tyr his thr glu tyr arg lys met PRO ASP PRO ASP MET ILE ARG TYR

cys leu leu gln phe val ser ser ser ile arg lys met ile asn ser ser ARG ILE GLN THR *** - - - - - - - - - - - - - -

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ILE ASP GLU PHE GLY GLN THR THR THR ARG MET GLN ***

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FIGURE 2 Predicted primary structures of the mutant polypeptides. (*A*) Schematic diagram of the precursor product of *env* ($Pr95^{env}$) showing the relative endpoint and number of amino acids deleted from the carboxy terminus for each of the three mutants (C1, C2, and C3). The box represents the 27 amino acids of the membrane anchor region of gp37. (*B*) Amino acid sequences near the carboxy terminus of each of the mutant polypeptides as predicted by DNA sequencing. The underlined amino acids correspond to those of the transmembrane, hydrophobic anchor region of gp37. Protein synthesis termination codons (***) were provided by the adjacent sequences of the SV40 vector DNA. This foreign amino acids coded by the BamHI linker and SV40 DNA sequences are indicated by uppercase letters.

more, none have lost any of the potential glycosylation sites previously identified (29).

Preliminary Characterization of the Mutant Polypeptides

Experimental evidence in support of the predicted amino acid sequences of the mutant polypeptides was obtained from the electrophoresis of the proteins in SDS polyacrylamide gels. CV-1 cells infected for 62 h with recombinant SV40 viruses were pulse labeled with [^{3}H]leucine for 15 min and either detergent lysed immediately or chased with unlabeled leucine for 2, 4, or 8 h and then lysed. The RSV glycoproteins were collected by immunoprecipitation using a mixture of anti-gp85 and anti-gp37 antibodies. These antibodies were prepared using detergent-treated, gel-purified gp85 and gp37. Anti-gp85 and anti-gp37 recognize both glycosylated and unglycosylated forms of the RSV glycoproteins in the presence of detergents (29). The collected proteins were separated in SDS polyacrylamide gels, and the fluorogram from one such gel is shown in Fig. 3.

After the pulse, the labeled polypeptides of each mutant were contained in a single band. Whereas those of C1 and C2 were similar in size to the wild-type product, the mutant C3 polypeptide was noticeably smaller in molecular weight. The size difference is consistent with the predicted, net deletion of 75 amino acids. During the chase, the electrophoretic mobility of the wild-type, mutant C1, and mutant C2 precursors increased slightly. These increases are probably due to the removal of glucose and mannose residues from the oligosaccharide side chains (4), which occurs in the RER and *cis* compartment of the Golgi apparatus (25). In contrast, the mutant C3 polypeptides maintained the same mobility throughout the chase. This result suggested that the oligosaccharide side chains of this mutant glycoprotein are not trimmed back, perhaps due to a block in transport to the Golgi apparatus. (The existence of such a block will be demonstrated in subsequent sections of this paper.)

During the chase, gp85 and gp37-related proteins were produced in cells expressing the wild-type, mutant C1, or mutant C2 precursors. The amounts of these cleavage products reached a plateau level in all three cases. The failure of gp85 and gp37-related proteins to accumulate to high levels is presumably due to their degradation because none could be detected in the culture medium even under conditions where anti-gp85 and anti-gp37 would be expected to react with such products (in the presence of detergents). Because these proteins appear on the cell surface (see below), we assume that degradation occurs after transport to that location. Mutant C1 has been found to consistently produce lower steady-state levels of gp85 and gp37-related proteins even though the C1 precursor appears to be processed at a rate identical to wild-type (see below). Differences in the steady-state levels of the cleavage products probably reflect differences in their degradation rates; however, we have not tested this hypothesis. In contrast, mutant C3 polypeptides were not cleaved and were degraded very slowly during the chase. This latter result is consistent with the hypothesis that there is a block in transport of this protein to the site of precursor cleavage, as described above (and demonstrated below).

As expected from the method of mutant construction, the electrophoretic mobilities of gp85 produced in cells expressing the wild-type, mutant C1, or mutant C2 polypeptides did not differ. Differences were only observed in the mobilities of the gp37-related proteins. The gp37 of mutant C2 was found to be smaller than that of the wild type, as predicted by the DNA analysis. The gp37 of mutant C1, however, appeared as a doublet band in the gel. The reason for this doublet is not certain, but it may be that the pattern of oligosaccharide addition and modification is altered by the C1 mutation.

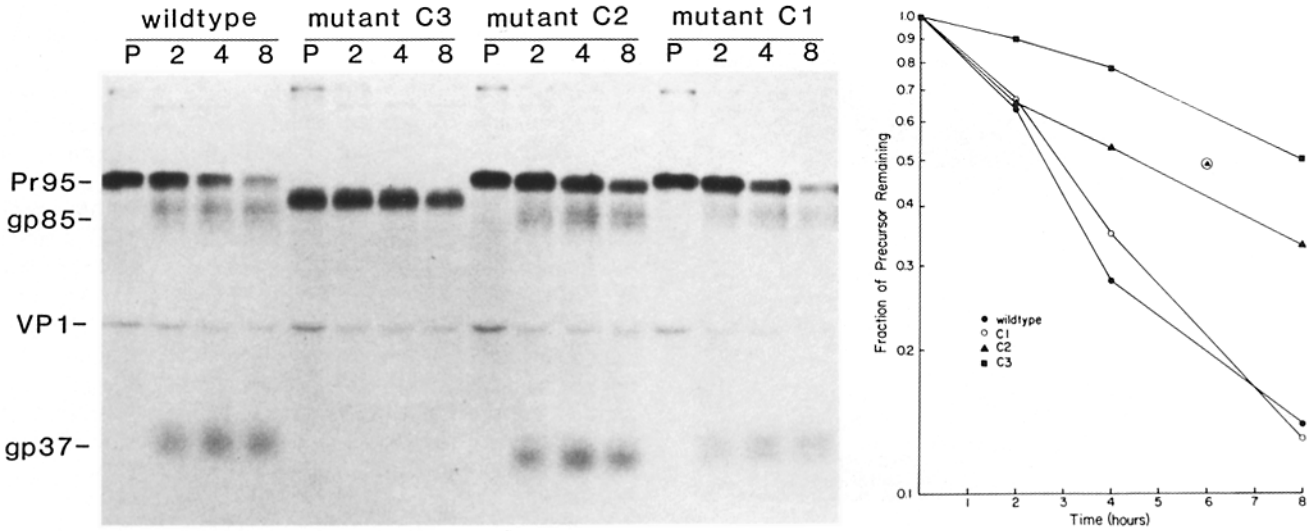


FIGURE 3 Characterization of the mutant polypeptides and their rate of transport from the RER. Plates of CV-1 cells were infected with recombinant SV40 viruses carrying wild-type or mutant (C1–C3) *env* genes. After 62 h of infection, the cells were pulse labeled with [^{3}H]leucine for 15 min and either lysed or chased with unlabeled leucine for 2, 4, or 8 h and then lysed. RSV glycoproteins were immunoprecipitated with a mixture of anti-gp85 and anti-gp37 antibodies, electrophoresed in SDS polyacrylamide gels, and fluorographed. An example of one such fluorogram is shown. (VP1 is an SV40 coat protein that is nonspecifically isolated during immunoprecipitation.) To determine the relative amount of precursor remaining at each time point, the Pr95^{env} bands were cut out of the gels and solubilized, and the radioactivity present was measured in a liquid scintillation counter. Each experiment was repeated three times, and the averages for each time point are shown in the graph. The circled triangle represents the average fraction of precursor remaining when cells infected with mutant C2 were pulse labeled at 50, 62, or 71 h after infection and chased for 6 h. Details of these experiments can be found in Materials and Methods.

Whatever the case, the upper band of the doublet has consistently been found to migrate more slowly than the bulk of the wild-type gp37, and thus the mutant C1 polypeptide appears to be larger than wild type, as predicted.

Effects of the Mutations on the Rate of Precursor Transport from the RER

Processing of $Pr95^{env}$ to produce gp85 and gp37 most likely occurs in the Golgi apparatus of CV-1 cells before the formation of complex oligosaccharides (manuscript in preparation). Evidence consistent with this was obtained by labeling cells expressing *env* with [^{3}H]palmitic acid (a presumptive marker for the *cis* compartment of the Golgi apparatus [54, 55]) or [^{3}H]fucose (a marker for the *trans* Golgi compartment [25]). Briefly, $Pr95^{env}$ was found to be labeled with [^{3}H]-palmitic acid but not with [^{3}H]fucose indicating that precursor cleavage occurs at an intermediate step during transport (data not shown). These results suggested that measurements of the rate of precursor disappearance—concomitant with the appearance of cleavage products—could be used to determine the rates of transport to the Golgi apparatus (see Discussion).

To measure their rates of transport, the wild-type and mutant precursor polypeptides were pulse or pulse–chase labeled with [^{3}H]leucine (as described above), immunoprecipitated, and electrophoresed in an SDS polyacrylamide gel. After determining their positions in the dried gel by fluorography the bands containing the precursors were excised from the gel, solubilized, and the amount of radioactivity in each band was measured. This experiment was repeated three times for the wild type and each mutant. The fraction of the precursors remaining at each of the time points (relative to the pulse) was calculated for each experiment, and the average values from all three experiments are plotted in Fig. 3. From this graph, it is clear that mutant C1 polypeptides disappear (that is, are cleaved) at about the same rate as the wild-type polypeptides with a half-life of 2.5–3.0 h. On the other hand, mutant C2 and mutant C3 polypeptides disappear more slowly with half-lives of 4.5 and 8 h, respectively.

It could be argued that the populations of cells expressing the mutant C2 or C3 polypeptides die at a faster rate and therefore exhibit net rates of transport that are slower than those of the cells expressing the wild-type or C1 polypeptides. That is, the results in Fig. 3 might be hypothesized to reflect differences in the titers of SV40-*env* recombinant viruses used to infect the CV-1 cells. However, this hypothesis does not seem likely inasmuch as the progression of CPE (cytopathic effects) observed with each virus stock appeared to be the same. Nevertheless, we further explored the possibility that the transport rate might decrease as the infection progressed. To this end, identical plates of cells expressing the mutant C2 polypeptides were either pulse labeled or pulse labeled and chased for 6 h at the following times after infection: 50, 62, or 71 h. We found that time (after infection) had no effect on the fraction of precursor remaining after the chase, although the total amount of labeled protein increased. The values we obtained (0.45, 0.51, and 0.50, respectively) were averaged and plotted on the graph in Fig. 3 (circled triangle). In summary, the differences in precursor transport illustrated in Fig. 3 are indeed due to the mutations of the *env* gene and not due to artifacts of the SV40 expression system.

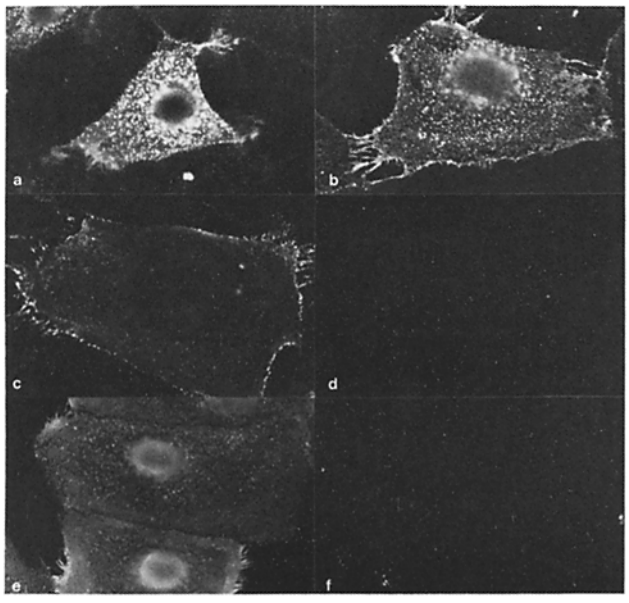


FIGURE 4 Surface immunofluorescence of cells infected with recombinant SV40 viruses. Live CV-1 cells expressing wild-type (panel *a*), mutant C1 (panel *b*), mutant C2 (panel *c*), or mutant C3 (panel *d*) *env* gene products were exposed to chicken anti-RSV antibodies (specific for native RSV glycoproteins) and then to fluorescein-conjugated rabbit anti-chicken IgG as described in Materials and Methods. As controls, cells infected with recombinant SV40 viruses expressing a membrane-bound, wild-type HA polypeptide (panel *e*) or a secreted, anchor-minus HA polypeptide (panel *f*) were also examined using the appropriate antisera. The mutant C3 polypeptides did not appear on the surface of infected cells.

Surface Immunofluorescence of Cells Expressing the Wild-type and Mutant Polypeptides

To determine if any of the mutations block the transport of the RSV glycoproteins to the surface of infected cells, the live infected cells were reacted with chicken anti-RSV antibodies (which recognize the antigenic determinants of the non-detergent-treated glycoproteins and were found to be most effective in surface immunofluorescence assays), stained with fluorescein-conjugated rabbit anti-chicken IgG, fixed, and examined by fluorescence microscopy. Photographs of typical cells are shown in Fig. 4. We found that the wild-type (*a*), mutant C1 (*b*), and mutant C2 (*c*) polypeptides all appear on the surface of infected cells. As a control, we also stained (using anti-HA antibodies) cells expressing the wild-type HA of influenza virus (Fig. 4, *e*), which is known to be expressed on the surface of CV-1 cells infected with SV40-HA recombinant viruses (13). No attempts were made to quantitate differences in the intensities of surface fluorescence exhibited on the cells, but we have observed consistently lower levels of surface fluorescence with mutant C2-infected cells—whether this reflects a slower rate of transport to the surface is not certain.

No fluorescence was detected on the surface of any of the cells expressing the C3 polypeptides (panel *d*). This result is consistent with the evidence that transport of the C3 polypeptide to the cell surface is blocked. On the other hand, this result would also be predicted if the small amount of C3 polypeptide observed to disappear during a chase (see Fig. 3) was secreted, albeit slowly, from the cell, because, as shown in panel *f*, anchor-minus HA polypeptides cannot be detected by surface fluorescence even though they are efficiently secreted from CV-1 cells (14).

Examination of Culture Medium for Secreted Glycoproteins

To determine if the mutant C3 polypeptides are secreted, we examined the culture medium from infected CV-1 cells for the presence of immunoprecipitable glycoproteins. For this experiment, monolayers were infected with a dilution (1:50) of recombinant SV40 virus that gives optimum expression of the mutant C3 *env* gene. To provide an internal control for protein secretion, the monolayers were simultaneously infected with dilutions of another recombinant SV40 virus (SVEHA20A$^-$), which expresses a secreted form of the influenza HA protein (designated HAa$^-$ [14]). At the highest concentration, close to 100% of the cells were infected with SVEHA20A$^-$. After 60 h of infection, the cells were pulse-labeled with [^{3}H]leucine, chased for 5 h with unlabeled leucine, and divided into culture medium and cellular lysate fractions. The electrophoretic results obtained after immunoprecipitation in the presence of detergents with antibodies specific for either RSV glycoproteins (anti-gp85 and anti-gp37) or HA protein are shown in Fig. 5. Virtually all of the labeled C3 protein was found to be cell associated after the chase whether co-infecting (HAa$^-$-producing) virus was present (plates *B–D*) or not (plate *A*). In the same cultures, however, virtually all the labeled HAa$^-$ protein was found in the culture medium after the chase whether co-infecting (C3-producing) virus was present (plates *B–D*) or not (plate *E*).

Trace amounts of RSV-specific protein (barely visible in the autoradiogram) were present in the medium. The amount present is clearly less than would be predicted from the kinetics of precursor disappearance illustrated in Fig. 3 (30% of the total after a 5-h chase) and did not appear to increase during extended periods of chasing (data not shown) or with increasing amounts of coinfecting virus. It is not clear whether this represents protein spilt from disrupted cells or trace amounts of truly secreted protein. It seems unlikely that the C3 protein is secreted and then adsorbed to the surface of the CV-1 cells because the results of the surface immunofluorescence experiments (Fig. 4) and the subcellular localization experiments (described below) argue against this.

Subcellular Localization of the Mutant C3 Polypeptides

Inasmuch as the C3 polypeptide was severely slowed (if not blocked) in transport to the cell surface, we sought to determine the site of the barrier along the transport pathway. The results of the preliminary characterizations suggested that the block occurs before transport to the Golgi apparatus (see above; Fig. 3). To test this hypothesis, we (*a*) labeled wild-type and mutant C3 polypeptides with radiolabeled sugars that are known to be added at specific points along the transport pathway and (*b*) stained fixed, infected cells with a rhodamine-labeled, Golgi-specific lecithin and fluorescein-labeled anti-gp85 antibody to localize the *env*-specific glycoproteins relative to the Golgi apparatus.

For the radiolabeling experiment, CV-1 cells expressing either the wild-type or mutant C3 polypeptides were labeled for 6 h with [^{3}H]mannose (Man), [^{3}H]glucosamine (Gl$_4$), or [^{3}H]fucose (Fuc). The results obtained after electrophoresis and fluorography of the immunoprecipitated glycoproteins are shown in Fig. 6. In the case of fucose labeling, we found that the wild-type gp85 and gp37 (but not Pr95^{env}) were well labeled. As mentioned above, this result demonstrates that Pr95^{env} is cleaved before the addition of fucose in the *trans* Golgi compartment. In contrast, the C3 polypeptides were not labeled with fucose, nor were any labeled cleavage products detected. This result indicates that the transport block

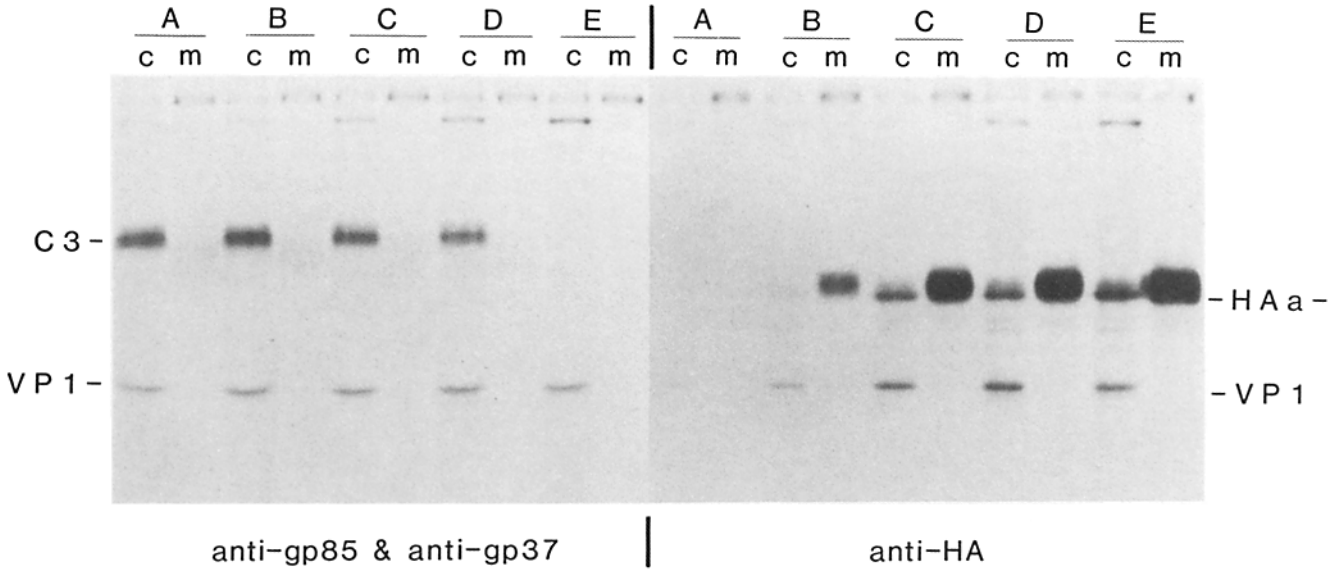


FIGURE 5 Examination of culture fluids for the presence of secreted mutant C3 polypeptides. Four plates of CV-1 cells were infected with 0.25 ml of recombinant SV40 viruses carrying mutation C3 (1:50 dilution). As an internal control, these cells were co-infected with dilutions of SVEHA20A$^-$ (a recombinant SV40 virus that expresses a secreted, anchor-minus HA polypeptide): no co-infecting virus (plate *A*), 0.25 ml of 1:100 diluted SVEHA20A$^-$ (plate *B*), 0.25 ml of 1:10 diluted SVEHA20A$^-$ (plate *C*), or 0.5 ml of 1:10 diluted SVEHA20A$^-$ (plate *D*). A fifth plate was infected only with 0.25 ml of 1:10 diluted SVEHA20A$^-$ (plate *E*). After 60 h of infection, the cells were pulse labeled with [^{3}H]leucine and then chased for 5 h with unlabeled leucine. The cultures were fractionated into cellular (c) and culture medium (*m*) portions having the same volume. Equal volumes of each fraction were immunoprecipitated with either a mixture of anti-gp85 and anti-gp37 antibodies or anti-HA antibodies. The results from SDS PAGE and fluorography are presented. Mutant C3 polypeptides were not found in the culture medium.

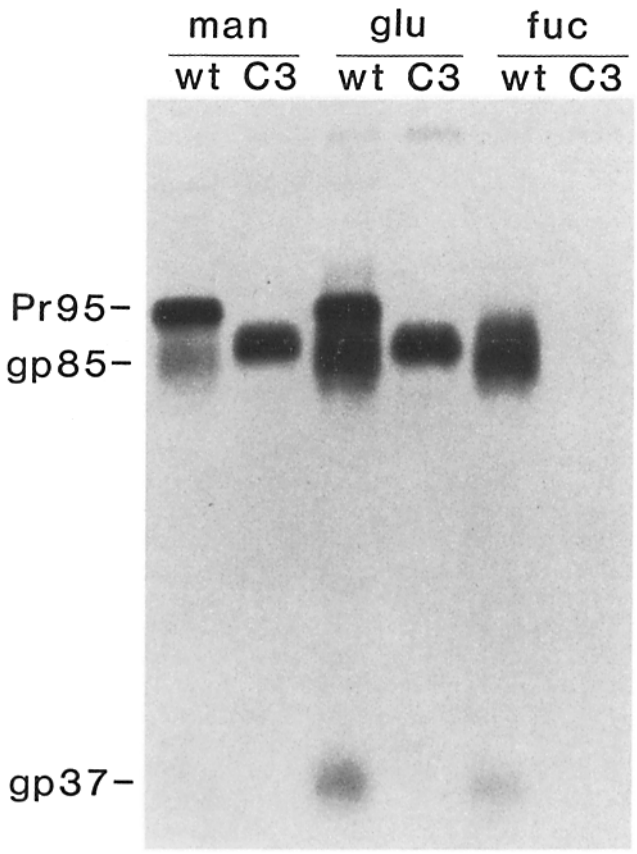


FIGURE 6 Radiolabeling of wild-type and mutant C3 polypeptides with [^{3}H]sugars. Cells infected with 60 h were labeled for 6 h with 100 μCi of [^{3}H]mannose, [^{3}H]glucosamine, or [^{3}H]fucose. The cells were then lysed; the *env*-coded glycoproteins were immunoprecipitated with a mixture of anti-gp85 and anti-gp37 antibodies, and the collected proteins were separated in an SDS polyacrylamide gel. Fucose was not incorporated into mutant C3 polypeptides.

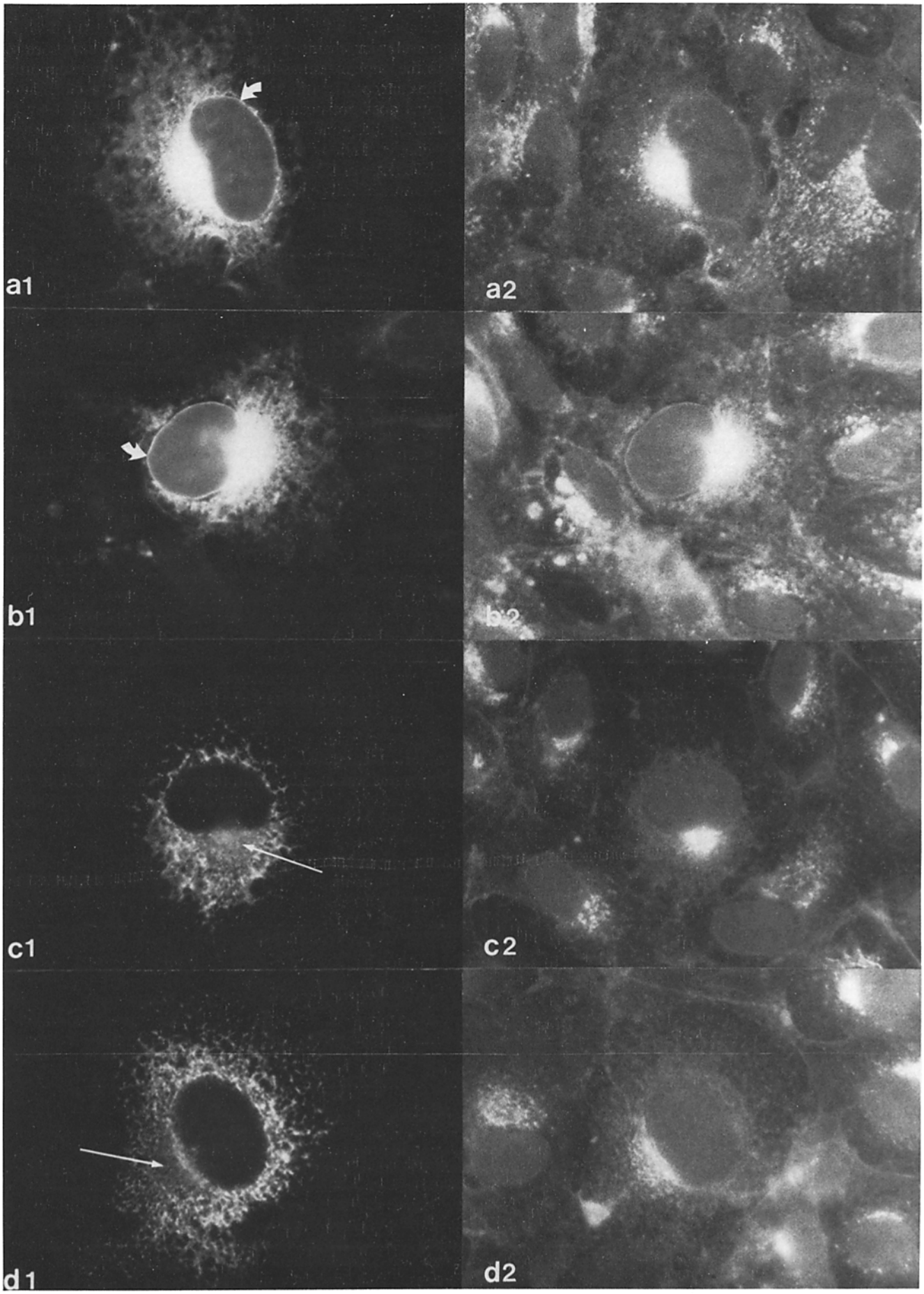
occurs before the site of fucose addition and suggests that transport may be blocked in the RER. In the case of mannose and glucosamine labeling, we found that the wild-type Pr95^{env} was well labeled, as was expected in that both of these sugars are incorporated into the dolichol-linked oligosaccharides that are co-translationally transferred to the polypeptide backbone in the RER (25). In Fig. 6, it can also be seen that wild-type gp85 and gp37 were better labeled with [^{3}H]glucosamine than with [^{3}H]mannose, although the amounts of labeled Pr95^{env} were similar. This observation is consistent with the well-characterized events of oligosaccharide processing during which the number of mannose residues decrease and the number of glucosamine residues (in *N*-acetylglucosamine) increase during transport from the RER to the Golgi apparatus (25). In contrast, the amount of radioactivity incorporated into the C3 polypeptide was about the same for both [^{3}H]-mannose and [^{3}H]glucosamine, indicating that the oligosaccharide side chains of the C3 polypeptide are not processed to the same extent as those of the wild type and supporting the concept that transport from the RER to the Golgi apparatus does not occur. This hypothesis is supported by the finding that none of the C3 protein is resistant to endoglycosidase H (Endo H) digestion (data not shown).

To determine the subcellular location of the wild-type and mutant C3 polypeptides by fluorescence microscopy, we acid-alcohol fixed infected cells 65 h after infection. The *env*-specific proteins were located by immunofluorescence after staining with fluorescein-conjugated antibodies. To determine the position of the Golgi apparatus, we also stained the same cells with rhodamine-conjugated wheat germ agglutinin (68). Photographs of the typical patterns of fluorescence observed are shown in Fig. 7. For cells expressing the wild-type *env* gene, the fluorescein label (Fig. 7, *a1* and *b1*) was found to define three distinct regions: (*a*) a bright area to one side of each nucleus, (*b*) a bright, distinct ring around each nucleus (indicated with short arrows), and (*c*) a diffuse network extending throughout the cytoplasm. It is widely accepted that these regions correspond to (*a*) the Golgi apparatus, (*b*) the nuclear membrane, and (*c*) the endoplasmic reticulum (respectively). Proof that the wild-type protein is indeed transported to the Golgi apparatus was obtained with the rhodamine label (Fig. 7, *a2* and *b2*) whose fluorescence was found to coincide with the bright regions of fluorescein fluorescence to one side of each nucleus. In contrast, for cells expressing the C3 polypeptide, the fluorescein label (Fig. 7, *c1* and *d1*) was only found to reside in one of the three compartments defined by the wild-type protein, namely the endoplasmic reticulum. No rings were ever observed around any of the nuclei, and dark, nonfluorescent areas (indicated with long arrows) were always observed over the Golgi complexes. These observations held true for all times after infection during which expression of the *env* genes could be detected (45–72 h). Proof that the dark areas indicate the position of the Golgi apparatus was obtained with the rhodamine label (Fig. 7, *c2* and *d2*) whose fluorescence was found to coincide with these dark areas rather than the position of the fluorescein label. These results show that transport of the C3 protein is blocked at a very early step in the RER. Furthermore, it suggests that transport of the wild-type *env* product to the nuclear membrane occurs in a specific manner after synthesis elsewhere in the RER.

Fractionation of Cells Expressing Mutant C3 Polypeptides

The failure of a mutant protein to be transported could be due to either (*a*) the loss of a sorting signal or (*b*) the loss of

FIGURE 7 Double fluorescent staining to determine the intracellular locations of the wild-type or mutant C3 polypeptides relative to the Golgi apparatus. Cells infected for 65 h with recombinant SV40 viruses carrying either wild-type (panels *a* and *b*) or mutant C3 (panels *c* and *d*) *env* genes were fixed and treated sequentially with rabbit anti-gp85 antiserum, fluorescein-conjugated goat anti-rabbit IgG, and then rhodamine-conjugated wheat germ agglutinin (as described in Materials and Methods). The panels on the left (*a1–d1*) show the cellular locations of the *env*-coded polypeptides (as indicated by the fluorescein-labeled probe), and the panels on the right (*a2–d2*) show the positions of the Golgi apparatus (as indicated by the rhodamine-labeled probe) in the same cells. The short arrows in panels *a1* and *b1* point to the bright rings of fluorescence around the nuclei, and the long arrows in panels *c1* and *d1* point to the dark areas of nonfluorescence as described in the text. The mutant C3 polypeptides (unlike those of the wild type) were not found in the Golgi and did not form a ring around the nucleus.


a1
a2
b1
b2
c1
c2
d1
d2

solubility and aggregation of the protein. In an attempt to determine which of these situations exists for the C3 protein, infected CV-1 cells were pulse labeled with [^{3}H]leucine or pulse labeled and chased for 4 h. The cells were disrupted by Dounce homogenization in detergent-free, hypotonic buffer and the unbroken cells and nuclei were removed by low-speed centrifugation. The cleared homogenates were then centrifuged at 250,000 *g* for 15 min to separate the membranes (pellet) from the cytosol (supernatant). As controls, the same fractionation was performed using cells expressing the wild-type *env* glycoproteins (*wt*), the influenza virus hemagglutinin (*HA*), or the anchor-minus hemagglutinin (*HAa*$^-$). The results obtained after immunoprecipitation (in the presence of detergents), electrophoresis, and fluorography are presented in Fig. 8.

The pulse-labeled wild-type *env* product (Pr95^{env}) and HA protein were both found to pellet with the membranes during the high-speed centrifugation. This result was expected in that both of these proteins possess membrane-spanning domains. Likewise, after the chase, the wild-type *env* products (Pr95^{env}, gp85, and gp37) and the HA protein were still (as expected) associated with only the membrane fractions. In contrast, the pulse-labeled HAa$^-$ protein was found in about equal amounts in both the membrane and cytosol fractions. This result indicates that the HAa$^-$ proteins remained soluble and were capable of leaking out of the lumen of the RER in the hypotonic conditions employed in the experiment. Because the HAa$^-$ proteins are rapidly transported and secreted from CV-1 cells, no cell-associated HAa$^-$ remained after the 4-h chase.

The behavior of the C3 protein was similar to that of the HAa$^-$ protein, except that the C3 protein (as expected) was not secreted during the chase. Approximately half of the pulse-labeled and pulse–chase-labeled C3 protein was found to leak from the lumen of the RER into the cytosol during the fractionation. These results, though not conclusive (see Discussion), are consistent with the supposition that the C3 polypeptides remain soluble in vivo, and that the formation of aggregates of insoluble protein is not the reason why this mutant glycoprotein is not transported from the RER.

DISCUSSION

We have constructed and characterized carboxy terminal mutants of the RSV glycoprotein and have demonstrated that the amino acids in this region are important for transport to the cell surface. The novel features of these mutant glycoproteins and the limitations of our interpretations are discussed below.

Mutant C3

The mutant C3 glycoprotein lacks the cytoplasmic domain, the transmembrane domain, and a portion of the external domain of the RSV precursor, and thereby resembles the anchor-minus glycoproteins of VSV (50) and influenza virus (14, 64). To be sure, the size of the C3 deletion is proportionately larger than some of these anchor-minus forms (14, 50), but it is also much smaller than others (64). We cannot rule out a very slow rate of transport, but unlike all previously reported anchor-minus proteins, the C3 glycoprotein does not appear to be secreted, and accumulates in the RER.

Perhaps the most interesting feature of mutant C3 polypep-

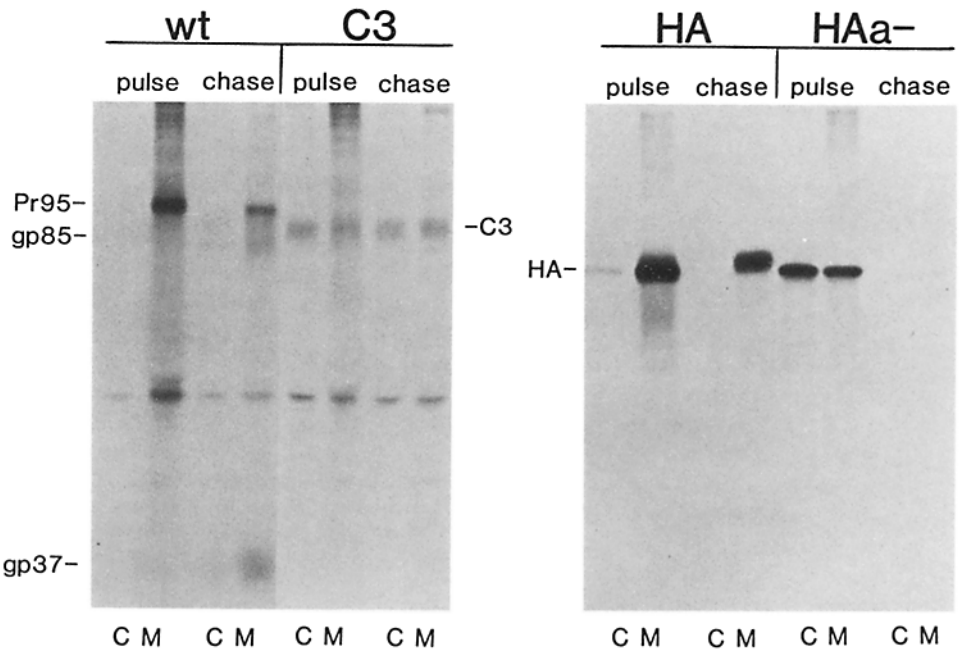


FIGURE 8 Fractionation of cells expressing wild-type and mutant C3 polypeptides. Cells infected for 57 h were either pulse-labeled with [^{3}H]leucine and osmotically lysed or pulse-labeled, chased for 4 h, and then lysed. Cell lysis was accomplished in the absence of detergents by Dounce homogenization in hypotonic buffer. Nuclei and unbroken cells were removed by low-speed centrifugation, and the resulting cleared lysate was fractionated by ultracentrifugation at 50,000 rpm in an SW50.1 rotor (250,000 *g*, average) for 15 min. The viral polypeptides present in the supernatants (soluble or cytosol fraction [*c*]) and the pellets (membrane fractions [*m*]) were immunoprecipitated and electrophoresed in SDS polyacrylamide gels (see Materials and Methods for details). The left panel shows the results obtained using wild-type (*wt*) and mutant C3 (*C3*) RSV glycoproteins, and the right panel shows the results of control experiments using wild-type (*HA*) and anchor-minus (*HAa*$^-$) hemagglutinin polypeptides. Notice that the C3 polypeptides were present in the soluble, cytosol fractions.

tides is their failure to accumulate on the nuclear membrane. The nuclear membrane and the RER have long been known to be continuous, and because of the presence of ribosomes on the outer nuclear membrane, it has generally been thought that glycoproteins could be synthesized randomly either on the RER proper or on the nuclear membrane. Consistent with these ideas are the reports of several mutant glycoproteins whose transport to the Golgi apparatus is blocked; these mutant proteins have been found by immunofluorescence to accumulate in the RER as well as on the nuclear membrane (for example, references 3, 51, 58). Even the VSV anchor-minus glycoprotein, which is secreted at a slow rate but presumably remains soluble, has been found to accumulate around the nucleus of producing cells (11, 50). The wild-type forms of these and other (12, 13) glycoproteins are found predominantly in the Golgi apparatus, and this apparently is due to the rapid rates at which they are transported out of the RER (with half-times of ~10 min). In contrast, the wild-type RSV glycoprotein is transported very slowly to the Golgi apparatus. In RSV-infected avian cells, $Pr95^{env}$ has a half-life of ~2 h (4) and in CV-1 cells the half-life is similar—~2.5 h. Consequently, the wild-type RSV glycoproteins are found by immunofluorescence to be accumulated in the RER and on the nuclear membrane (as well as in the Golgi apparatus). Because of this, we expected the C3 glycoprotein, which is not transported efficiently to the Golgi apparatus, to produce (if anything) even brighter rings of immunofluorescence around the nuclei. To our surprise, however, we found no rings. We propose from these observations that proteins synthesized in the RER move to the nuclear membrane before transport to the Golgi apparatus. Moreover, movement to the nuclear membrane may require a specific transport event even though the RER and nuclear membranes appear to be continuous. These hypotheses are supported by recent evidence that indicates that transitional vesicles (for the transport of glycoproteins to the Golgi apparatus) may be derived from "blebs" in the nuclear membrane (2). To our knowledge, this is the first report of a mutant glycoprotein that accumulates in the RER, but does not accumulate on the nuclear membrane.

The failure of a glycoprotein to be transported is due to either its lack of a sorting signal or its lack of solubility in the environment of the transport pathway. For example, nonglycosylated VSV glucoprotein (synthesized in the presence of tunicamycin) is not transported to the cell surface, but exists intracellularly in a form that is insoluble even in nonionic detergents such as Triton X-100 (35). It appears that the C3 polypeptides are soluble in the lumen of the RER because they were not strictly associated with the pelleted (membrane) material during the fractionation experiment, even though no detergents were included in our experiments. Indeed, they behaved exactly like the pulse-labeled control polypeptides (HAa^-), which are presumed to be soluble owing to their rapid secretion by the cell. Furthermore, the distribution of labeled C3 polypeptides in the membrane and cytosol fractions remained the same after a 4-h chase as it was before the chase. Nevertheless, it could be argued that the C3 polypeptides form only small aggregates that are incapable of being transported but capable of spilling into the cytosol. Alternatively, it could be argued that the C3 polypeptides observed in the cytosol were aggregated in very small vesicles that failed to be pelleted by the centrifugation. We are currently investigating these two possibilities by determining the sedimentation rate and the protease sensitivity of the C3 protein in the cytosol. A shortcoming of these and all other simple methods of evaluating solubility (see, for example, references 58, 60) is that they require disruption of the cell. Thus, regardless of the results, it can always be argued that the polypeptides, though soluble in the cytosol, are aggregated in vivo.

Mutants C1 and C2

Mutants C1 and C2 contain alterations only within the cytoplasmic domain of the RSV glycoproteins. A number of such mutations have been characterized for other membrane-bound glycoproteins. Some of these mutations apparently have no effect on the appearance of the proteins on the cell surface (12, 45, 74), but careful kinetic analyses have not been performed to determine effects on the rates of transport. Other cytoplasmic domain mutations have been found to have striking effects on transport (51, 58).

The rates at which glycoproteins are transported from the RER to the Golgi apparatus are typically measured by determining the rates at which resistance to Endo H are acquired (for example, 38, 51, 61). This enzyme removes the simple, high mannose oligosaccharide (added in the RER) but not the complex, terminal oligosaccharides (generated in the Golgi apparatus) from the polypeptide backbone (25, 65). The use of Endo H to measure the rate of transport of the RSV glycoprotein is complicated by several features intrinsic to the glycoprotein. First of all, determination of the amino acid sequence of $Pr95^{env}$ has shown that a large number (sixteen) of potential glycosylation sites are present, and apparently all of these are occupied in vivo (29). Perhaps because of this, great heterogeneity of oligosaccharide processing is observed (28), such that both cell-associated forms (see Fig. 3) and virion forms (4) of gp85 and gp37 appear as very broad smears in SDS polyacrylamide gels. Some of this heterogeneity is due to the presence of hybrid-type oligosaccharides on the RSV glycoproteins (27). These hybrid-type oligosaccharides are produced in the Golgi apparatus and contain complex oligosaccharide structures but nevertheless remain sensitive to Endo H (27, 66). Thus, when gp85 and gp37 are digested with Endo H, the proteins migrate faster, in even broader bands in SDS polyacrylamide gels than before treatment, even though they have been transported to the Golgi apparatus (data not shown). A second and perhaps more serious limitation to the use of Endo H is the failure of gp85 and gp37 to accumulate in the cell. Because the glycoproteins are transported to the cell surface (and do not appear in the growth medium), we hypothesize that degradation occurs after arrival on the cell surface. It is interesting that the majority of gp85 and gp37 produced in RSV-infected avian cells is also degraded (4).

Because of the limitations prohibiting the use of Endo H, we sought an alternative method for measuring transport rates to the Golgi apparatus. Labeling experiments using [^{3}H]palmitic acid and [^{3}H]fucose (described in Results) suggested that $Pr95^{env}$ is cleaved in the Golgi apparatus to produce gp85 and gp37, so we decided to use precursor disappearance as a measure of transport. However, because the precise subcellular location of palmitate addition has not been resolved for mammalian cells (54, 55), our analyses may reflect transport from one RER compartment to another rather than from RER to the Golgi apparatus. In further support of the latter possibility, we have recently constructed (manuscript in preparation) a mutant RSV glycoprotein with an altered (deleted)

external domain that is clearly transported to the Golgi apparatus (as demonstrated by double fluorescence staining) but not to the cell surface (as shown by surface immunofluorescence). This transport-defective mutant protein is not cleaved to gp85 and gp37, and the precursor disappears at a very slow rate. We believe that precursor disappearance is not due to nonspecific degradation in the RER for two reasons. First, the experiments with mutant C3 polypeptides (which accumulate in the RER) show that protein degradation in the RER occurs slowly. Second, pulse–chase experiments using short chase periods (<2 h) indicate that the rate at which steady-state levels of gp85 and gp37 are established correlate with the rate of precursor disappearance during extended chases (data not shown).

By using precursor disappearance as a measure of transport, we examined the effects of mutations in the conserved and nonconserved regions of the cytoplasmic domain of $Pr95^{env}$ (29). We found that replacement of the nonconserved region with another, longer sequence of amino acids (mutant C1) had no effect on transport; however, deletion of a portion of the conserved region (mutant C2) resulted in slower transport. Thus, it appears that the cytoplasmic domain of the RSV glycoproteins is involved in some manner with transport to the Golgi apparatus, as is the cytoplasmic domain of the VSV G protein (51) and perhaps that of the HA protein of vaccinia virus (58). It will be of great interest to determine what effects the C1 and C2 mutations have upon the assembly of infectious virions in avian cells in that it seems likely that the conserved region of the gp37 cytoplasmic domain might also be involved in interactions with other viral proteins during the assembly process. Because infectious DNA copies of the entire RSV genome are available, it will be straightforward to replace the wild-type *env* gene with the mutant *env* genes for this purpose.

One of the basic unanswered questions of sorting mechanisms is in regard to the number of sorting signals contained in a given protein. In the case of the RSV glycoproteins, we have shown here that the cytoplasmic domain is important for transport. We have also identified mutations of the external domain (of gp85) that alter transport to the cell surface (manuscript in preparation). Do these two domains contain separate and distinct sorting signals or is the conformation of a single sorting signal (perhaps in the external domain) affected by the conformation of amino acids in both the external and cytoplasmic domains? Along these lines of thinking, we are currently investigating the effects of mutations in the transmembrane domain upon the transport of the RSV glycoproteins.

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