Amino-terminal Deletion Mutants of the Rous Sarcoma Virus Glycoprotein Do Not Block Signal Peptide Cleavage but Can Block Intracellular Transport

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Abstract. Protein sequence requirements for cleavage of the signal peptide from the Rous sarcoma virus glycoprotein have been investigated through the use of deletion mutagenesis. The phenotypes of these mutants have been characterized by expression of the cloned, mutated *env* genes in CV-1 cells using a late replacement SV40 vector. The deletion mutations were generated by Bal31 digestion at the XhoI site located near the 5′ end of the coding sequence for the structural protein gp85, which is found at the amino terminus of the precursor glycoprotein, Pr95. The results of experiments with three mutants (X1, X2, and X3) are presented. Mutant X1 has a 14 amino acid deletion encompassing amino acids 4–17 of gp85, which results in the loss of one potential glycosylation site. In mutants X2 and X3 the amino terminal nine and six amino acids, respectively, of gp85 are deleted. During the biosynthesis of all three mutant polypeptides, the signal peptide is efficiently and accurately cleaved from the nascent protein, even though in mutants X2 and X3 the cleavage site itself has been altered. In these mutants the alanine/aspartic acid cleavage site has been mutated to alanine/asparagine and alanine/glutamine, respectively. These results are consistent with the concept that sequences C-terminal to the signal peptidase site are unimportant in defining the site of cleavage in eucaryotes.

Mutants X2 and X3 behave like wild-type with respect to protein glycosylation, palmitic acid addition, cleavage to gp85 and gp37, and expression on the cell surface. Mutant X1, on the other hand, is defective in intracellular transport. Although it is translocated across the rough endoplasmic reticulum and core-glycosylated, its transport appears to be blocked at an early Golgi compartment. No terminal glycosylation of the protein, cleavage of the precursor protein to the mature products, or expression on the cell surface is observed. The deletion in X1 thus appears to destroy signals required for export to the cell surface.

MANY cellular and viral gene products are transported, from their site of synthesis on the rough endoplasmic reticulum, through the secretory pathway to the cell surface, where they are released as soluble products or inserted into the plasma membrane of the cell. Whereas the general principles of this transport pathway are understood, many of the finer details remain to be elucidated and attempts are being made to use both biochemical and genetic approaches to this end (reviewed by Sabatini et al., 1982; Silhavy et al., 1983; Gething, 1985). We have chosen the envelope glycoprotein gene of Rous sarcoma virus (RSV)[1] for genetic analyses, with the goal of identifying and characterizing protein domains that might play important roles in glycoprotein transport (Wills et al., 1983; 1984). We describe in this paper mutants in the amino-terminus of the *env* gene product that address both the topic of protein sorting and that of the cotranslational proteolytic events necessary to remove amino-terminal signal sequences.

Secretory and membrane-spanning proteins appear to contain specific amino acid structures that serve as "sorting signals", in addition to those sequences that confer specialized functions. These signals are recognized by cellular components that guide these proteins through the appropriate assembly and sorting processes and distribute them to their respective destinations. Insights into these mechanisms has largely come from the study of cell surface and viral envelope glycoproteins (reviewed by Michaelis and Beckwith, 1982; Sabatini et al., 1982; Silhavy et al., 1983; Gething, 1985). Typical membrane glycoproteins appear to have several functional domains that may be involved in transport. The most frequently encountered organization includes: (*a*) an amino-terminal signal peptide that is responsible for initiating translocation of the protein across the endoplasmic reticulum and that is usually cleaved off by a specific pepti-

1. *Abbreviations used in this paper*: HA, hemagglutinin; RSV, Rous sarcoma virus.

dase before synthesis is completed; (*b*) an extracellular domain, which may confer a specific function on the protein; (*c*) a hydrophobic anchor that spans the lipid bilayer; and (*d*) a cytoplasmic tail. There are probably additional signals yet to be delineated, which are responsible for directing such glycoproteins through different subcellular compartments such as the *cis*- and *trans*-Golgi cisternae and transport vesicles. For example, while influenza glycoproteins are sent to the apical membrane, the vesicular stomatitis virus glycoprotein is transported to the basolateral membranes of polarized cells (Roth et al., 1983; Rodriguez-Boulan and Sabatini, 1978). Also, in certain cell types some cellular proteins are stored in secretory granules and are secreted only when induced, while other proteins are targeted to the constitutive pathway and are released from the cell immediately (Kelly, 1985). These less well defined sorting signals are assumed to be inherent structural features of each protein, but have yet to be identified.

After translocation of a nascent chain across the endoplasmic reticulum has been initiated, the signal peptide is removed. This cleavage is carried out by signal peptidase, a cellular gene product. Two classes of signal peptidases have been described. A signal peptidase of *Escherichia coli* (SPase I) has been cloned into pBR322 (Date and Wickner, 1981), and has been shown to accurately cleave eucaryotic precursor proteins as well as bacterial protein precursors (Talmadge et al., 1980). Conversely, the eucaryotic signal peptidase will accurately cleave procaryotic proteins (Watts et al., 1983). The latter enzyme has been studied using detergent-solubilized dog pancreas signal peptidase (Jackson and White, 1981) and hen oviduct signal peptidase (Lively and Walsh, 1983), demonstrating that it is an integral membrane protein that can be solubilized only when the lipid bilayer is dissolved. A second procaryotic signal peptidase (*E. coli* SPase II) has been described that is specific for prolipoproteins (Hussain et al., 1982; Tokunaga et al., 1982) and membrane-bound penicillinases (Nielsen and Lampen, 1982). This enzyme maps to a different locus on the *E. coli* genome and requires a glyceride-modified cysteine for cleavage.

Perlman and Halvorson (1983) and von Heijne (1984) have examined the sequences of a number of membrane proteins and have described amino acid sequence patterns that allow prediction of signal peptidase cleavage sites with >90% accuracy. The most striking feature of signal peptidase cleavage sites is the presence of an amino acid with a small, uncharged side chain at the carboxy-terminus of the signal peptide. The most common amino acids found at this position are alanine and glycine. From their statistical analyses, the peptidase cleavage site appears to be determined by sequences within the signal peptide and not by sequences beyond the cleavage site. This is in contrast to the observations that mutations within the structural protein itself appear to prevent signal peptidase cleavage of the *lam*B gene product and the M13 coat protein (Emr and Bassford, 1982; Benson and Silhavy, 1983; Russel and Model, 1981).

We report here on studies on the signal peptidase processing and intracellular transport of the RSV envelope glycoprotein through the construction, in vitro, of mutated envelope genes, and by expression of such genes from an SV40 vector in primate cells. The RSV envelope glycoprotein is representative of highly glycosylated membrane proteins that undergo proteolytic processing during transport to the plasma membrane. The *env* gene encodes a precursor protein (Pr95) that is cleaved late in the Golgi (Hunter et al., manuscript in preparation) to the two viral structural proteins, gp85 and gp37. The larger component, gp85, is the receptor-binding domain of the glycoprotein complex, whereas gp37, a membrane spanning protein, serves to anchor the complex into the lipid bilayer. These two glycoproteins mediate viral attachment to, and penetration of susceptible cells thus initiating the process of infection. A hydrophobic signal sequence that mediates translocation across the rough endoplasmic reticulum is located within a long amino-terminal signal peptide; the latter being cleaved cotranslationally by signal peptidase from Pr95.

We previously reported preliminary results on a mutant of the RSV *env* gene that is cleaved normally despite a mutation at the signal peptidase site (Wills et al., 1983). We describe here three mutants of the RSV glycoprotein that have wild-type signal peptides but that have deletions in the amino-terminal portion of the mature protein adjacent to the signal peptidase cleavage site. That these deletions have no effect on protein processing provides experimental support for the conclusions drawn from the study of compiled amino acid sequence data (Perlman and Halvorson, 1983; von Heijne, 1984). Indeed, signal peptide cleavage appears to be less sensitive to the deletion of sequences near the cleavage site than are signals required for transport and sorting, because one of these mutants (X1), which has a 14 amino acid deletion near the amino terminus of the mature protein, is not transported past an early compartment of the Golgi apparatus.

Materials and Methods

Cells, Viruses, and DNAs

The coding sequences for the *env* gene of the Prague C strain of RSV were moleculary cloned into the ClaI and BamHI sites of pAT153 after excision from pATV-8 that contains the entire RSV genome (Katz et al., 1982; Schwartz et al., 1983). This recombinant plasmid containing the *env* gene

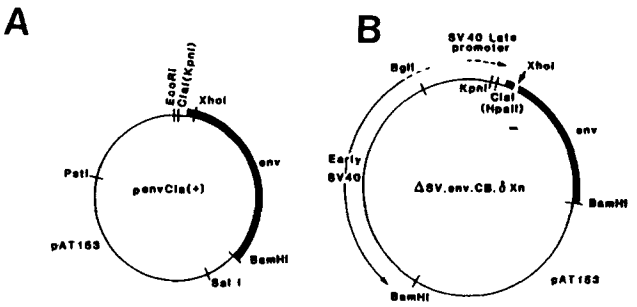


Figure 1. Construction of amino-terminal mutants. (*A*) The KpnI-XbaI fragment of the RSV genome contains the entire *env* gene. A ClaI linker was added at the KpnI end and a BamHI linker was added to the XbaI end of the RSV KpnI-XbaI fragment (solid bar). This fragment was ligated into the plasmid pAT153. Mutations were generated by Bal31 digestion from the unique XhoI site. (*B*) The HpaII site of SV40 was converted to a ClaI site by linker addition and the mutated ClaI-BamHI *env* fragment was ligated into this late replacement SV40 vector. The details of the construction are described in Materials and Methods. The direction of transcription is indicated by the arrows and the promoters are indicated by dashed lines.

is referred to as *penv*Cla(+) and was used for *env* gene mutagenesis (Fig. 1 *A*).

All recombinant plasmids were propagated by transforming *E. coli* strain DH-1 (*recA hsdR*) (Maniatis et al., 1982). Transformed DH-1 cells were grown in L broth containing ampicillin. A derivative of SV40 with the late region replaced by the RSV *env* gene was used as an expression vector (Gething and Sambrook, 1981; Wills et al., 1983). For propagation of recombinant virus, the late SV40 gene functions were supplied by mutant helper virus, dl-1055, that has a small deletion in the early region (Pipas et al., 1980, 1983). The SV40 vector containing the *env* gene in place of late SV40 sequences was expressed in CV-1 cells, a continuous line of African green monkey kidney cells (Acheson, 1981). CV-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

In a natural infection with RSV, the 5' leader sequences for the *env* gene mRNA are derived from sequences at the 5' end of the RSV genome by a splicing event. The result is a signal peptide with six amino-terminal amino acids identical to the amino-terminus of the *gag* gene product (Schwartz et al., 1983; Hunter et al., 1983; Ficht et al., 1984). In the constructs used here, where no *gag* sequences exist, translation is initiated at a naturally occurring, in frame, initiator codon (ATG) at the 5' end of the open reading frame that encompasses the *env* gene (Hunter et al., 1983). This results in the replacement of the six *gag*-related amino acids with eight amino acids encoded upstream of the splice acceptor site. The protein product nevertheless appears to be translated efficiently and transported normally through intracellular pathways to the cell surface (Hunter et al., manuscript in preparation; Wills et al., 1984).

Enzymes

All DNA modification enzymes including restriction endonucleases, T4 DNA ligase, Klenow fragment of *E. coli* DNA polymerase I, and Bal 31 were purchased from Bethesda Research Laboratories (Gaithersburg, MD). All manipulations, reactions, and analysis of DNAs were performed according to the standard procedures described in the Cold Spring Harbor cloning manual (Maniatis et al., 1982).

In Vitro Mutagenesis of the env Gene and Construction of Recombinant Viruses

The KpnI-XbaI fragment of pATV-8 that contains the *env* gene was inserted into a plasmid vector by blunt-ending with the Klenow fragment of DNA PolI and adding a ClaI linker to the KpnI end and a BamHI linker to the XbaI end (Fig. 1 *A*). This plasmid, designated *penv*Cla(+), was digested with XhoI at the unique site 13 base pairs (bp) 3' of the first codon of gp85, between the signal peptide leader sequence and the major portion of the mature glycoprotein coding sequence (Fig. 2). The restricted ends were trimmed by digestion with a double-strand exonuclease, Bal 31, to generate small deletions. Without further treatment the new ends were religated. The mutagenized *env* gene was excised from *penv*Cla(+) by digestion with ClaI and BamHI and ligated into the ClaI and BamHI sites of the SV40 expression vector. The ClaI site in SV40 was generated by addition of ClaI linker at the HpaII site. This construction placed the mutagenized *env* gene under control of the SV40 late promoter (Fig. 1 *B*). The DNA sequence for all mutants was determined by the Maxam-Gilbert procedure (Maxam and Gilbert, 1980).

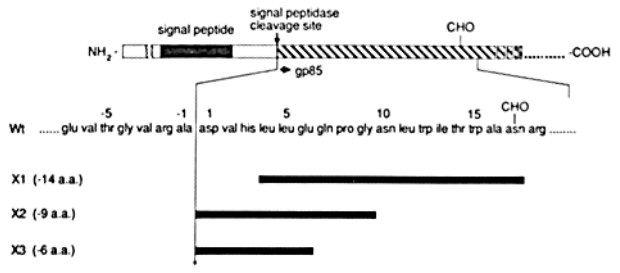


Figure 2. Amino acid sequence deduced from DNA sequence of mutants X1, X2, and X3. The RSV glycoprotein is schematically represented: the location of the hydrophobic signal sequence within the long (64 amino acid) leader peptide is denoted by a stippled bar, and the mature gp85 glycoprotein by a slashed bar. The signal peptidase cleavage site in both the cartoon and the numbered amino acid sequence is denoted by a long arrow. The potential glycosylation site in the amino-terminus of gp85 is shown as a CHO. The amino acid sequence of the last seven amino acids of the leader peptide and the amino-terminal 18 amino acids of gp85 are shown for the wild-type gene product. The solid black bars show the length and position of the deletions in mutants X1, X2, and X3.

The initial mutant constructions in which the *env* gene was placed at the altered SV40 HpaII site, did not give suitable levels of *env* gene expression (Wills et al., 1983). This appears to be due to initiation of translation at the SV40 *agno* gene ATG, located upstream from the *env* sequences, which prevents initiation from the *env* ATG (Perez et al., manuscript in preparation). To increase expression, the mutant genes were cloned into an SV40 vector in which the *agno* ATG was deleted (Perez et al., manuscript in preparation). This vector is referred to as ΔSV.CB.

For expression of the wild type *env* gene products, a similar construction was made except that viral sequences, with only the XbaI site converted to a BamHI site, were ligated directly between the SV40 KpnI and BamHI sites (Hunter et al., manuscript in preparation). This construction is referred to as SV.KB. Thus, SV.KB differs from the mutant constructs in that it lacks the 50 bp KpnI-HpaII fragment that contains the SV40 *agno* gene ATG. The levels of mutant and wild-type *env* gene expression from ΔSV.CB were similar to SV.KB, and their protein products were indistinguishable.

Nomenclature

The mutant constructions described here have been designated Δ*SV.env*.CBδXn, where ΔSV signifies the SV40 vector with the *agno* gene ATG deleted; *env* indicates that the RSV gene for the envelope glycoprotein was inserted into the vector; CB indicates that the construction of the *env* and SV40 sequences was made at the ClaI and BamHI sites; δX signifies that a deletion has been made at the XhoI site; and n indicates the number of a specific mutant. Here we describe the characteristics of three deletion mutants: X1, X2, and X3.

Growth of Virus

After amplification, the vector shown in Fig. 1 *B* was digested with BamHI and recircularized to remove the plasmid sequences and to reduce its size to allow packaging into SV40 virions. This recombinant virus and a late function helper (dl-1055) were used to cotransfect CV-1 cells by a modification of the procedure of Gething and Sambrook (1981). Approximately 300 ng of SV40-*env* DNA and 75 ng of helper DNA was added to cells in 500 ul of Tris-buffered saline containing 250 ug of DEAE-dextran (McCutchan and Pagano, 1968). After 60–90 min cells were washed with Tris-buffered saline and PBS, and incubated with medium containing 100 uM chloroquine (Luthman and Magnusson, 1983) for 3–4 h. After 5–7 d when cytopathic effects were observed, cells were frozen and thawed three times, sonicated, and stored at −70°C as virus stocks.

Radiolabeling Gene Products

Semiconfluent CV-1 cells were infected with a dilution of virus stock that produced extensive cytopathic effects in 72–96 h. For biosynthetic labeling of *env* gene products, infected cells in 35-mm dishes were radiolabeled in 1 ml of medium for 6 h with 100 μCi [^{3}H]fucose (84 Ci/mmol, Amersham Corp., Arlington Heights, IL) or with 400–500 μCi [^{3}H]palmitate (40–60 Ci/mmol, New England Nuclear, Boston, MA) 60–72 h postinfection. For pulse–chase experiments, cells were starved for leucine and serum for 1 h before labeling with 250 μCi (1 mCi/ml) of [^{3}H]leucine (60 Ci/mmol, Amersham Corp.) for 15 min. Duplicate dishes were chased with medium containing an excess of unlabeled amino acids for 3 h. To block glycosylation, cells were treated with 1 μg/ml tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) 3 h before labeling with [^{3}H]leucine and continuously during labeling.

Immunoprecipitation and Gel Electrophoresis

Cells were disrupted with 1 ml of lysis buffer A (1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, and 25 mM Tris-HCl [pH 8.0]). Nuclei and cell debris were pelleted in a microcentrifuge. The supernatants were adjusted to a final concentration of 0.1% SDS. RSV-specific proteins were immunoprecipitated as previously described (Hardwick and Hunter, 1981; Hunter et al., 1983) using antiglycoprotein antiserum and formalin-fixed *Staphylococus aureus* (*Staph A*). The antigen–antibody complex was dissociated from the *Staph A* by boiling in loading buffer (2% SDS, 0.2 M 2-mercaptoethanol, 10% glycerol, and 50 mM Tris-HCl [pH 6.8]), and pelleted in a microcentrifuge. Samples were loaded onto 1.5-mm-thick Tris-

glycine-buffered 10% polyacrylamide gels as previously described (Hunter et al., 1983). Radiolabeled polypeptides were detected by fluorography using Enhance (New England Nuclear, Boston, MA).

Fluorescent Staining of Cells

Virus-specific glycoproteins were detected by immunofluorescent staining of fixed cells to detect intracellular material, and of live, unfixed cells for detection of glycoproteins on the cell surface, as described previously (Wills et al., 1984). The Golgi apparatus was specifically stained in fluorescein-labeled fixed cells by treating them with rhodamine-conjugated wheat germ agglutinin (E. Y. Laboratories, Inc., San Mateo, CA) that was diluted 1:10 with PBS. Cells were treated for 30 min at room temperature (Wills et al., 1984).

Amino Acid Sequencing

Microsequencing (Bhown et al., 1980) of [^{3}H]tryptophan or [^{3}H]leucine-labeled protein was accomplished by sequential Edman degradation in a Beckman 890M, automated sequencer (Beckman Instruments Inc., Fullerton, CA) as previously described (Hunter et al., 1983). [^{3}H]tryptophan (50 Ci/mmol, Amersham Corp.) or [^{3}H]leucine-labeled polypeptides were isolated from 35-mm dishes of infected cells, pulse-labeled with 250 μCi of the appropriate radiolabeled amino acid, after a 1-h incubation in medium lacking either tryptophan or leucine. Wild-type or mutant *env* gene products were immunoprecipitated as described above. To obtain the immunoprecipitated glycoproteins in a buffer suitable for injection into the protein sequencer, the supernatants were subjected to four cycles of centrifugation and dilution in 1 mM dithiothreitol and 0.01% SDS using a Centricon-30 microconcentrator (Amicon Corp., Danvers, MA), before sequencing.

Results

Construction and Expression of Deletion Mutants

Deletion mutations were introduced into the coding region for the envelope glycoprotein of RSV by digestion of a plasmid containing the *env* gene (Fig. 1 *A*) at a unique XhoI site located 13 bp from the 5′ end of the coding sequence for gp85, followed by digestion with the double-strand exonuclease, Bal. 31. Linear DNA was recircularized by ligation and then used to transform bacteria. Potential mutants were identified by restriction enzyme analysis and DNA sequencing. From the DNA sequence, it was inferred that the polypeptide product of mutant X1 had a 14 amino acid deletion that resulted in the loss of one potential glycosylation site; the signal peptidase cleavage site of this mutant protein remained unaltered (Fig. 2). The predicted products of mutants X2 and X3 both had deletions that began at the signal peptidase cleavage site and extended into gp85, nine amino acids and six amino acids, respectively.

The mutant genes were cloned into the late region of SV40 such that the *env* gene replaced the SV40 late genes and was under control of the late SV40 promoter (Fig. 1 *B*). This recombinant virus was cotransfected into CV-1 cells with a helper virus (dl1055) that provided late gene functions and allowed virus assembly. Recombinant virus (and helper) progeny were harvested and used to infect CV-1 cells at high multiplicities to study the biosynthesis and maturation of the mutated *env* gene products. Mutant and wild-type constructions are detailed in Materials and Methods.

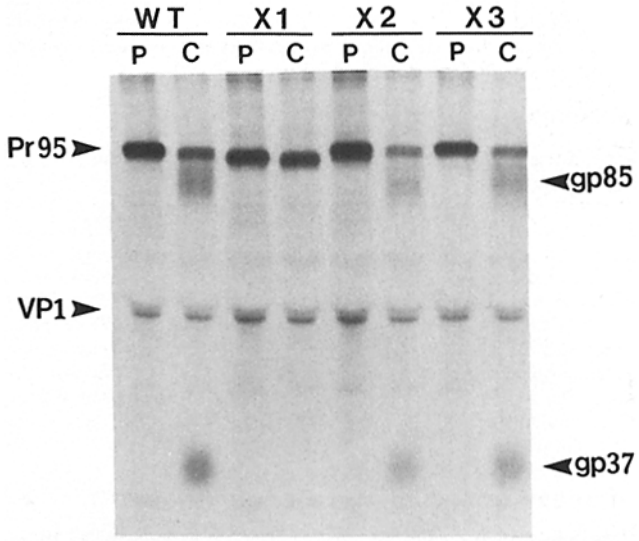


Figure 3. Immunoprecipitation of intracellular glycoproteins. At 60–72 h post infection, cells were incubated in medium without leucine or serum for 1 h and then pulse-labeled with 250 μCi [^{3}H]-leucine per 35 mm dish for 15 min (P lanes). Duplicate cultures were chased with unlabeled medium for 3 h (C lanes). Cell lysates were immunoprecipitated with anti-glycoprotein rabbit antiserum and electrophoresed on an SDS-polyacrylamide gel. Viral precursor glycoprotein Pr95 and mature products gp85 and gp37 were observed as well as SV40 VP1 that was nonspecifically precipitated.

Cleavage of the Precursor Pr95 to Mature Glycoproteins gp85 and gp37

To determine if the mutant *env* genes yielded functional gene products, infected cells were pulse-chased using [^{3}H]leucine, then *env*-related products were immunoprecipitated and electrophoresed on SDS-polyacrylamide gels. In a 15-min pulse, the only *env*-related product synthesized was the Pr95 precursor (Fig. 3, P lanes). The cloned gene products appeared to be very similar to those produced in chicken cells infected with RSV (data not shown), indicating that the precursors were translocated normally into the rough endoplasmic reticulum and glycosylated. The uncleaved precursor that remains after the chase in both wild-type and mutant infected cells has an apparent molecular mass some 3 kD smaller than that first seen in pulse-labeled cells (Fig. 3, *C* lanes). This reduction may be due to the trimming of glucose residues from the carbohydrate side chains, which has been shown to occur early in the transport pathways and before addition of the terminal sugars (Hubbard and Ivatt, 1981). The pronounced size reduction of the glycosylated X1 precursor is presumably because the deletion, in addition to removing 14 amino acids, resulted in the loss of one glycosylation site.

After a 3-h chase, the cleavage products gp85 and gp37 were present for mutants X2 and X3 but not in the case of mutant X1 (Fig. 3, C lanes). Therefore, the deletion mutations in X2 and X3 have no apparent effect on processing of the mutant precursor to mature glycoproteins. The 14 amino acid deletion of X1, however, does appear to prevent processing of the altered precursor, although it does not cause a rapid degradation of the polypeptide. The nature of this defect will be discussed in more detail below.

Cleavage of the Signal Peptide

One of the earliest processing events in the biosynthesis of the *env* gene product is the cleavage of the signal-containing leader sequence from the amino end of the nascent polypeptide (Hunter et al., 1983). To determine if the signal peptide was cleaved efficiently from the deletion mutants, infected

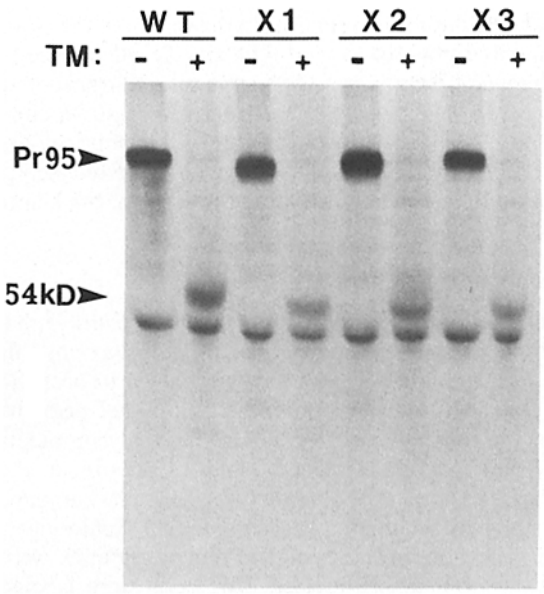


Figure 4. Evidence for cleavage by signal peptidase. Infected cultures were labeled with [^{3}H]leucine in the absence (−) or presence (+) of 1 μg/ml tunicamycin (TM). Tunicamycin was added to the cells 3 h before labeling. Cell lysates were immunoprecipitated with anti-glycoprotein rabbit antiserum and electrophoresed on an SDS-polyacrylamide gel. The unmarked band present in all lanes is nonspecifically precipitated SV40 VP1.

cell cultures were treated with tunicamycin, which blocks glycosylation of RSV glycoproteins (Stohrer and Hunter, 1979). Treated and untreated cells were then pulsed with [^{3}H]leucine, immunoprecipitated, and electrophoresed on polyacrylamide gels (Fig. 4).

In the presence of tunicamycin, the wild-type *env* gene polypeptide precursor migrated as a protein of 54,000 D– the unglycosylated form of this molecule. The unglycosylated mutant precursors were detectably smaller than the wild-type, consistent with the deletions having no effect on signal peptide cleavage. If the signal peptidase had failed to cleave these mutant precursors, the additional 64 amino acids of the leader peptide would have resulted in an increase in size of the unglycosylated product to 60–63 kD, which would have been easily discernible in the polyacrylamide gel system used (Hunter et al., 1983). From these experiments, we conclude that all three mutants are translocated across the endoplasmic reticulum, are cleaved by the signal peptidase, and are glycosylated in a normal manner. The sizes of the unglycosylated forms of X1 and X2 are indistinguishable from each other (Fig. 4, TM [+] lanes), whereas the sizes of their glycosylated forms show a small but reproducible difference (Fig. 4, TM [−] lanes and Fig. 3, p lanes). This provides further evidence that position 17 in gp85 is normally glycosylated in the wild-type protein.

Mutant X1 has a deletion beginning with the fourth amino acid of gp85 and thus retains an unaltered signal peptidase cleavage site. Mutants X2 and X3 on the other hand, have deletions of nine and six amino acids, respectively, from the amino terminus of gp85, and thus alter the C-terminal side of the signal peptidase cleavage site. Amino acid sequencing was performed to determine directly whether the signal was removed and whether proteolysis occurred at the modified cleavage site in these two mutants. Infected cells were pulse-labeled with [^{3}H]tryptophan, the glycoproteins were immunoprecipitated, subjected to Edman degradation on an automated sequencer, and the amount of radioactivity released at each cycle determined (Fig. 5). Radiolabeled tryptophan was recovered at positions 12 and 15 for the wild-type polypeptide at positions 3 and 6 for mutant X2 product and at positions 6 and 9 for mutant X3 protein (the counts in cycle 1 for the latter appear to represent loss of sample from the spinning cup). Sequence analyses of [^{3}H]leucine-labeled proteins yielded results consistent with those described above (data not shown). These results confirm that the signal peptidase cleavage site is ala/asp for wild-type, ala/asn for X2, and ala/gln for X3. Thus, the signal peptidase cleaves at the same position relative to the signal peptide, even though the cleavage site sequence itself is altered.

Localization of env Gene Products by Immunofluorescence

CV-1 cells expressing the *env* gene were reacted with specific antiserum and tagged with fluorescein-conjugated second antibody to determine if the mutant proteins could be found on the cell surface and distributed normally in intracellular compartments (Figs. 6 and 7). Mutants X2 and X3 were expressed on the cell surface as efficiently as the wild-type, judging from immunofluorescence (Fig. 6). Therefore, a deletion of six or nine amino acids at the amino terminus of gp85 did not block transit to the cell surface. In contrast, CV-1 cells infected with the X1 mutant exhibited only trace surface fluorescence (Fig. 6). This observation correlates with

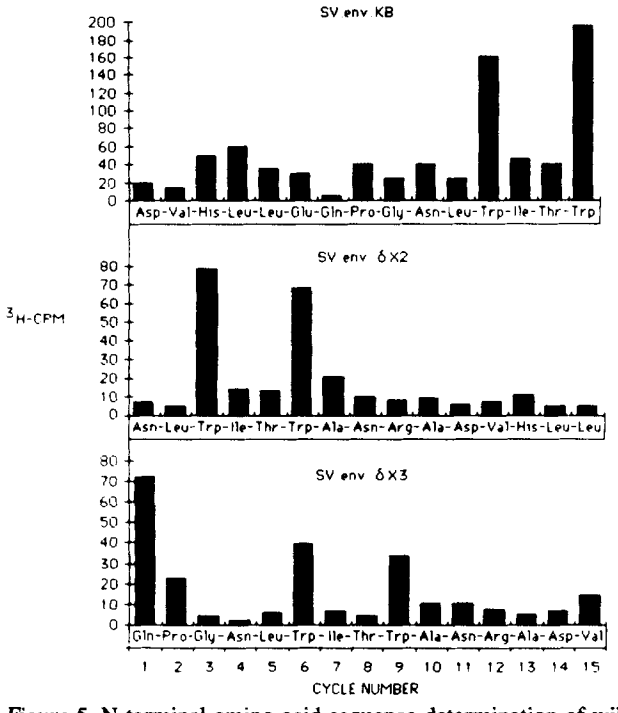


Figure 5. N-terminal amino acid sequence determination of wild-type and mutant Pr95 polypeptides. Infected SV-1 cells were labeled with [^{3}H]tryptophan for 20 min. Proteins immunoprecipitated from cell lysates were subjected to automated Edman degradation and the fractions were counted by liquid scintillation to determine which cycles contained radioactive tryptophan. The peaks correspond to the predicted tryptophan positions in the amino acid sequence shown below.

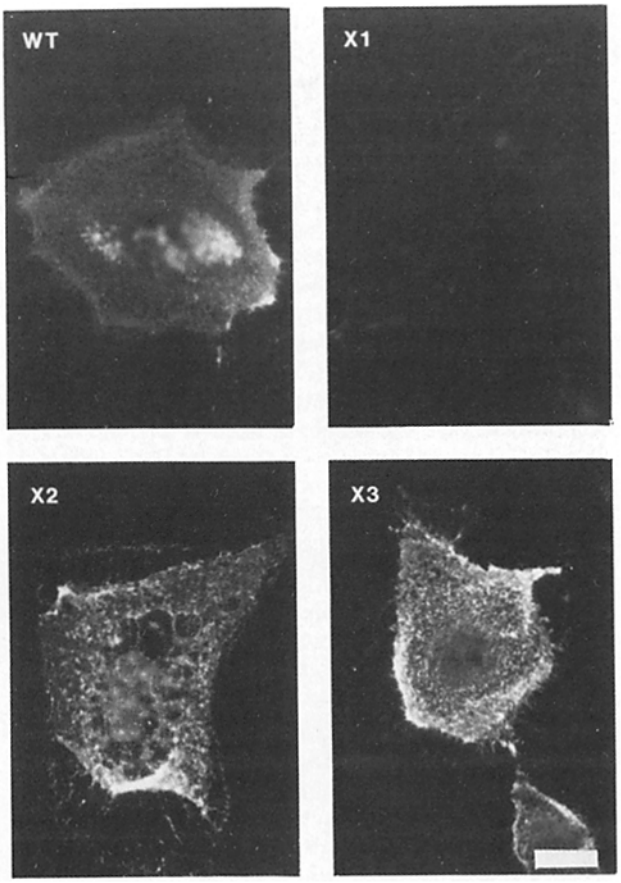


Figure 6. Surface immunofluorescence of cells expressing wild-type and mutant polypeptides. Live infected CV-1 cells were stained to detect surface expression of the wild-type and mutant glycoproteins. Chicken anti-glycoprotein antibodies were tagged with fluorescein-conjugated rabbit anti-chicken IgG for cell surface labeling. Bright surface fluorescence can be observed with cells infected with wild-type and mutant-X2-and X3-containing vectors, whereas those containing the X1 mutant gene stained no brighter than uninfected cells. Bar, 10 μm.

the lack of cleavage of the X1 Pr95 to gp85 and gp37, suggesting that X1 is blocked at a point in maturation before cleavage, which normally occurs before transport to the cell surface.

To determine the intracellular distribution of the mutant X1 polypeptide, fixed cells were stained with anti-glycoprotein antibody and treated with rhodamine-conjugated wheat germ agglutinin which specifically stains the Golgi (Virtanen et al., 1980). The glycoproteins of wild-type and mutant X1, exhibited brilliant intracellular fluorescence that colocalized with that of the rhodamine-conjugated wheat germ agglutinin (Fig. 7). In contrast, mutant C3, an *env* gene mutant with a deletion in the C-terminus of gp37 whose transport is arrested in the endoplasmic reticulum (Wills et al., 1984) shows reduced fluorescein staining in the region of the Golgi rather than a bright area in wild-type and X1 infected cells. Thus the mutant X1 glycoproteins appear to be transported from the rough endoplasmic reticulum to a subcellular compartment that colocalizes with the Golgi. The intracellular transport of mutant X1, therefore, appears to be blocked at a point beyond the rough endoplasmic reticulum, but before the plasma membrane.

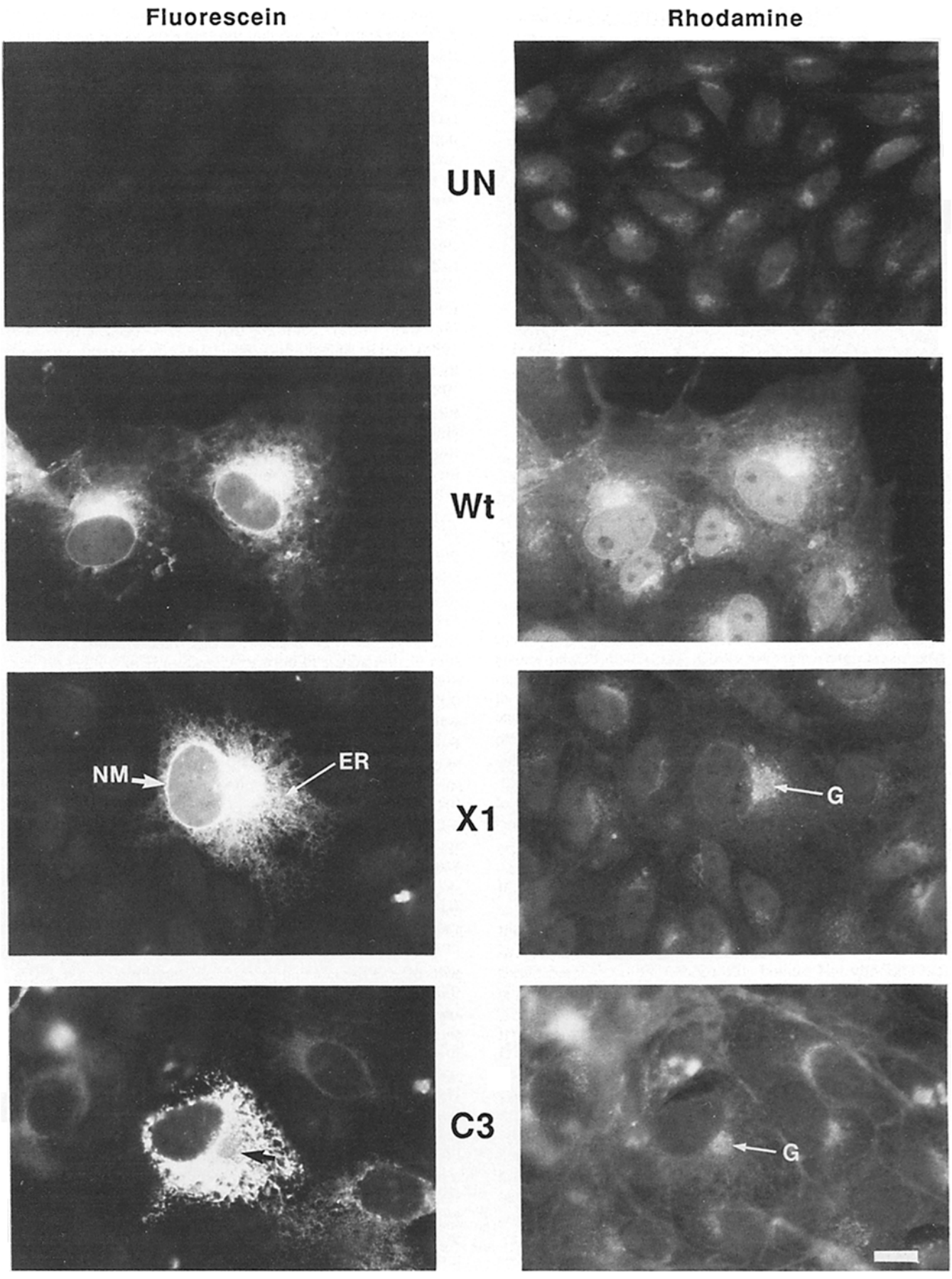
Modifications of Glycoproteins

To further investigate the point in the transport pathway that X1 was blocked, we determined whether the glycoproteins had undergone modifications which are known to occur in the *cis*- and *trans*-Golgi. Palmitic acid is added near the membrane-spanning region of many viral glycoproteins (Schmidt, 1982) including that of RSV (Gerhardt et al., 1984), and its addition is believed to occur in the *cis* compartment of the Golgi apparatus (Schmidt and Schlesinger, 1980). To determine whether all the mutant proteins were modified in this manner, infected CV-1 cells were labeled with [^{3}H]palmitic acid for 6 h and the *env*-specific products were immunoprecipitated for gel analysis. [^{3}H]Palmitic acid predominantly labeled the gp37 polypeptide and, to a lesser extent, Pr95 of the wild-type. The gp85 did not label, as might be expected since it does not traverse the lipid bilayer; gp37 being the bitopic membrane-spanning component of the glycoprotein complex (Fig. 8). Mutants X2 and X3 behaved in an identical manner to wild-type in that the precursors showed trace labeling while the gp37 polypeptide of each was heavily labeled. Because X1 is not cleaved to gp37 and gp85, the bulk of palmitic acid modification (i.e., the sum of that added to both Pr95 and gp37 of wild-type, X2 or X3) should occur on the uncleaved precursor if it enters the correct subcellular compartment. This does not appear to be the case because only trace levels of precursor labeling was observed. To rule out the trivial possibility that the low level of palmitate addition with X1 was due to poor expression or recovery, parallel dishes were pulse-labeled with [^{3}H]leucine and the cell lysates were processed simultaneously with the [^{3}H]palmitate-labeled samples. Similar levels of [^{3}H]leucine-labeled precursor were recovered for all three mutants and the wild-type (Fig. 8). Therefore, we conclude that the low level of palmitic acid labeling for X1 was due to the lack of palmitic acid modification and not due to low levels of expression or recovery.

The labeling of wild-type Pr95 with palmitic acid supports other evidence that palmitic acid addition occurs before cleavage of Pr95 to gp85 and gp37 (Wills et al., 1984). The low level of incorporation of [^{3}H]palmitate into the X1 polypeptide may reflect some leakiness in the transport block or alternatively could indicate low levels of palmitate addition before the *cis*-Golgi compartment.

Fucose is a terminal sugar of N-linked oligosaccharides and is believed to be added to the carbohydrate side chains

Figure 7. Intracellular immunofluorescence of cells expressing wild-type and mutant polypeptides. Fixed infected CV-1 cells were stained to detect the intracellular localization of wild-type and mutant glycoproteins. Rabbit anti-glycoprotein antibodies were tagged with fluorescein-conjugated goat anti-rabbit antibodies which in wild-type and X1 infected cells could be localized on the nuclear membrane (*NM*), endoplasmic reticulum (*ER*), and Golgi apparatus (*G*). The latter was localized by staining the same cells with rhodamine-conjugated wheat germ agglutinin. In mutant C3 infected cells neither the nuclear membrane nor the Golgi apparatus stained with the anti-glycoprotein antiserum. Bar, 10 μm.


Fluorescein
Rhodamine
UN
Wt
X1
C3
NM
ER
G
G

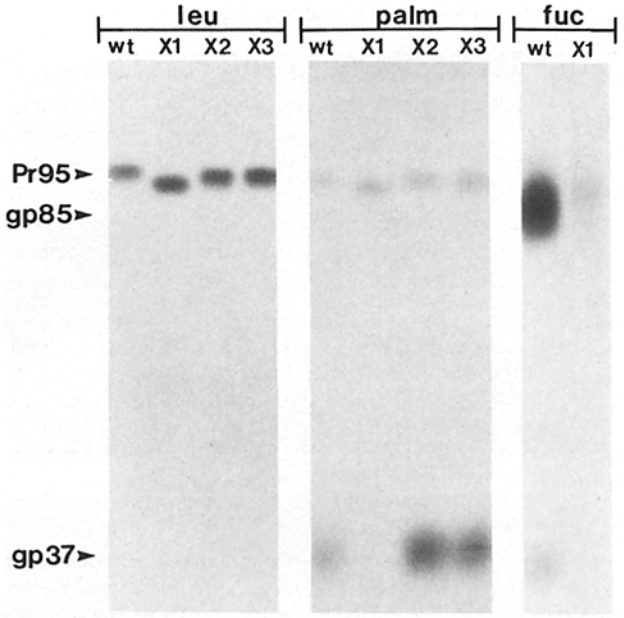


Figure 8. Modification of glycoproteins by palmitic acid addition and fucosylation. Infected CV-1 cells were labeled with [^{3}H]leucine, [^{3}H]palmitic acid, or [^{3}H]fucose, and the glycoproteins were immunoprecipitated and electrophoresed on an SDS-polyacrylamide gel, as described in Materials and Methods. The RSV *env* precursor and mature cleavage products are indicated by arrows.

of glycoproteins in the *trans* compartment of the Golgi apparatus (Hubbard and Ivatt, 1981). The data in Fig. 8 indicate that [^{3}H]fucose was incorporated in only trace amounts into mutant X1 polypeptides. Since the transport of a majority of the X1 polypeptides was blocked before proteolytic processing to gp37 and gp85 and palmitic acid addition, it would also be expected to be blocked before fucose addition. The trace labeling of X1 polypeptides with fucose is primarily in a protein with the mobility of gp85, thus a very small fraction of the mutant gene product does appear to escape the transport block.

Discussion

In this investigation we have studied the role of amino-terminal amino acid sequences of gp85 in signal peptide cleavage and intracellular protein transport. Three mutants with deletions in the amino-terminal end of the RSV gp85 were generated by Bal31 digestion from an XhoI site in the cloned *env* gene. These mutant genes were expressed in CV-1 cells under the control of the SV40 late promoter. Mutant X1 had a 14 amino acid deletion that began with the fourth amino acid of gp85 and extended to amino acid 17; this sequence includes one potential glycosylation site. This deletion resulted in the synthesis of a slightly smaller precursor polypeptide that lacked one carbohydrate side chain, but which otherwise appeared to be glycosylated normally. Based on size estimations in pulse-labeling experiments, in the presence and absence of tunicamycin, the precursor polypeptide lacked the long, signal-containing leader peptide. Thus, although the mutation in X1 significantly alters the sequences near the signal peptidase site, the signal peptidase still recognized and removed the leader peptide. Nevertheless, immunofluorescence experiments and posttranslational modification probes indicate that the transport and maturation of the glycoprotein of X1 is halted shortly after exiting the endoplasmic reticulum, perhaps within pre- or *cis*-Golgi vesicles. A determination of the exact location of the mutant molecules should be amenable to immuno-electron microscopy. It cannot be determined from these experiments if the amino acid deletion, the lack of a carbohydrate side chain, or both, was responsible for the deficiency in transport of X1. The carbohydrate itself is probably not a signaling structure for transport to the Golgi because there are unglycosylated proteins that are transported through the cellular machinery (Strous and Lodish, 1980), and although several of these proteins are normally glycosylated, they continue to be transported in the presence of tunicamycin, an N-linked glycosylation inhibitor. Nevertheless, loss of a carbohydrate side chain may alter the folding and tertiary structure of normally glycosylated proteins. It will be of interest to determine whether addition of a glycosylation site near the amino terminus of X1 restores its ability to be transported to the cell surface, because the addition of a glycosylation site to a membrane anchored form of the rat growth hormone confers on it the ability to be transported to the plasma membrane (Guan et al., 1985). These experiments are in progress.

We have previously reported on a different mutant of the RSV *env* gene that was also blocked in intracellular transport. This mutant, C3, has an engineered deletion at the carboxy-terminus of gp37 that removed the cytoplasmic tail and transmembrane region (Wills et al., 1984). Its transport is clearly blocked at an earlier stage than the X1 mutant since it was localized in the endoplasmic reticulum and never reached the Golgi apparatus. Although these mutants contain alterations at opposite ends of the *env* gene product, they both appear to lack an element that normally signals their transport to and beyond the *cis*-Golgi. The nature of such signals is not understood. While there may be a specific amino acid sequence (analogous to the amino-terminal signal sequence) that is required for recognition and transport from the endoplasmic reticulum to the Golgi apparatus, and from the Golgi to the plasma membrane, it is possible that a correctly aligned tertiary structure is the most critical factor in protein transport.

Like the RSV glycoprotein, the influenza hemagglutinin (HA) glycoprotein is also synthesized as a precursor molecule with an amino-terminal signal peptide that is removed during translation. The precursor is further cleaved into HA1 and HA2 either intracellularly or extracellularly. The three-dimensional structure of HA determined by X-ray crystallography (Wilson et al., 1981) showed that the amino-terminus of HA1 is in close proximity to the HA2, because HA1 is folded back down towards the membrane where HA2 traverses the lipid bilayer. Therefore, the amino-terminus of HA1 may be critical in conferring an appropriate conformation required for stability of a tertiary structure constrained within the lipid bilayer, and thus allow interaction with possible sorting factors. Indeed, small changes in the amino-terminus of HA1 can disrupt assembly and transport of the HA trimer (Wills, J. W., E. Hunter, and M.-J. Gething, manuscript in preparation). The deleted amino acids unique to the X1 mutation may similarly play a critical role in the tertiary structure of the *env*-complex, such that sorting signals required for transport through the Golgi complex are lost.

The other two mutants described here have deletions that begin at the amino-terminus of gp85 and extend nine amino acids (X2) and six amino acids (X3) into this structural protein. Neither of these deletions include a glycosylation site, but they do overlap with the deletion in X1. Despite these changes, the X2 and X3 mutant glycoproteins were transported to the cell surface and were indistinguishable from the wild-type. Mutants X2 and X3 thus indicate that the terminal nine amino acids of Pr95 are not required for normal intracellular transport.

The deletion mutants X2 and X3 encode gp85 polypeptides with novel amino-termini that alter the signal peptidase cleavage site from ala/asp-val-his to ala/asn-leu-trp and ala/gln-pro-gly, respectively. Thus both the charge and secondary structure of the cleavage site would be predicted to be altered by the loss of asp and his (X2), and the relocation of proline near the cutting side (X3). Nevertheless, the signal peptidase efficiently cleaves the leader peptide from the nascent polypeptide and its specificity of cleavage is unaffected by these alterations. Perlman and Halvorson (1983) and von Heijne (1983, 1984) have examined the sequence of almost 100 different procaryotic and eucaryotic signal sequences to determine if there are specific sequences required for recognition by the signal peptidase. Although there is no consensus sequence, there are definite patterns of amino acids that allow an accurate prediction of the cleavage site for 93% of the eucaryotic signal peptides. In general, signal peptides have a positively charged amino acid preceding a hydrophobic core, and although signal peptides vary in length, the distance from the end of the hydrophobic core to the cleavage site is quite highly conserved; the most common length being five amino acids for eucaryotes and six for procaryotes (von Heijne, 1985). The last three amino acids of the signal peptide have a pattern of A-X-B where A is usually a small neutral residue (Ala, Val, Cys, Ser, or Thr) and B is usually ala, gly, or ser, while aromatic and charged residues are forbidden at these positions. No amino acid distribution patterns were observed in the post-cleavage region. Whereas it is clear that predicting the peptidase cleavage site from statistical studies can be reasonably accurate, there are still other unknown contributing factors because there are a few examples where the cleavage site is not at the expected position. Nevertheless, the experiments we report here support the generalized conclusion from statistical analysis, that the sequence to the right of the peptidase cleavage site is not critical for signal peptidase cleavage.

None of the deletions generated in the RSV glycoprotein gene resulted in a loss of recognition and cleavage by the signal peptidase. This result contrasts with previously described procaryotic mutants. A 12 amino acid deletion starting at the fifth residue beyond the signal peptidase site of the *lam*B gene product blocked cleavage of the signal (Emr and Bassford, 1982; Emr et al., 1981) and a deletion of 130 amino acids beginning 70 amino acids downstream from the signal peptidase cleavage site of the same molecule, resulted in the abolition of signal cleavage although the shortened protein was localized correctly (Benson and Silhavy, 1983). In addition, the substitution of a leucine, in place of the glutamic acid, at residue two of the mature M13 coat protein also inhibited signal peptidase cleavage, however, in this latter instance the procoat protein was transported inefficiently across the inner membrane (Boeke et al., 1980; Russel and Model, 1981). Although more mutants will be required to properly define these systems, the procaryotic cleavage site appears to be more sensitive to manipulation than that of eucaryotes. There is accumulating evidence that transported procaryotic proteins, unlike those of eukaryotes, may not be transferred across membranes in a strictly cotranslational manner (Randall and Hardy, 1984). Thus altered regions within the structural protein portion of a molecule would have the opportunity to interact and interfere with signal peptidase cleavage; such an interaction would not be possible in the cotranslational system described for eukaryotes.

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Note Added in Proof. Through using a mutagenic oligonucleotide we have modified the NH_2-terminus of the mutant X1 gp85 from asp-val-his-arg-thr- to asp-val-*asn-arg-thr-*, thereby reinserting the glycosylation site missing from this mutant. Based on the size of the Pr95 produced from this modified gene, we conclude that glycosylation occurs at the new site, however, the transport defect present in the original X1 mutant is retained. Thus the transport block reported for mutant X1 appears to be the result of the amino-acid deletion rather than loss of a carbohydrate side chain.

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