Journal of Cellular Biochemistry 23:81–94 (1983)
Protein Transport and Secretion 87–100

Alterations in the Transport and Processing of Rous Sarcoma Virus Envelope Glycoproteins Mutated in the Signal and Anchor Regions

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The *env* gene of Rous sarcoma virus codes for two glycoproteins which are located on the surface of infectious virions. Subcloning of these coding sequences in the place of the late region of SV40 DNA has allowed the expression of a normally glycosylated, functionally active glycoprotein complex on the surface of monkey cells. Through the use of site-directed mutagenesis, the role of specific amino acids in the signal peptide, signal peptidase cleavage site, and membrane anchor region have been investigated. Amino-terminal mutations have shown that deletion of the signal peptidase cleavage site along with one or two amino acids of the hydrophobic signal peptide results in the synthesis of an unglycosylated, uncleaved, and presumably cytoplasmically located precursor. Nevertheless, changing the signal peptidase cleavage site from ala/asp to ala/asn does not block the translocation of the glycoprotein across the membrane or the action of the peptidase. At the other end of the molecule, carboxy-terminal mutations have shown that the deletion of the hydrophobic membrane anchor region is not sufficient for the secretion of the truncated glycoprotein. Interpretations of these results based on recent models for protein transport and secretion are discussed.

Key words: Rous sarcoma virus, *env* gene mutants, membrane anchor region, deletion mutant, signal peptide, cleavage site mutants

The *env* gene of Rous sarcoma virus (RSV) 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 [1]. The two proteins are linked by disulfide bonds to give complexes that contain one molecule of gp85 and one of gp37 [2]. Treatment of virions with reducing agents releases only gp85

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Received June 9, 1983; revised and accepted October 10, 1983.

suggesting that gp37 serves to anchor the glycoproteins to the viral membrane [3]. The determinants of host range specificity appear to map to gp85 [4–6], the molecule to which neutralizing antibodies are primarily directed in animals [7].

It has long been known that the two RSV glycoproteins are synthesized as a single glycosylated precursor (Pr95) which is subsequently processed to give the mature forms [8–12]. The site of the precursor processing event is most likely intracellular [11–14]; however, some conflicting data suggest that processing may occur after the precursor arrives at the cell surface [15,16]. No tryptic peptides are shared by the two mature glycoproteins, and pactamycin mapping experiments place gp85 at the aminoterminus and gp37 at the carboxyterminus of Pr95 [12,17].

Evidence has recently been obtained that the glycoprotein precursor (Pr95) represents a subset of the *env* gene product. Amino acid sequencing of the aminotermini of gp85 and gp37 [6] and nucleotide sequencing of the entire RSV genome [18] have together revealed the existence of 62 additional amino acids, preceding the aminoterminus of gp85, which are rapidly removed during the biosynthesis of the precursor [6]. Evidence in support of this finding was provided earlier by the observation that the unglycosylated product, synthesized in the absence of membranes in vitro (a 64 kD protein) using *env* mRNA [6,19], is larger than the unglycosylated product synthesized in vivo (a 57 kD protein) in the presence of tunicamycin, an inhibitor of glycosylation [20]. Within the 62 amino acid leader peptide is found a sequence of 13 hydrophobic amino acids which are flanked by hydrophilic amino acids [6] in a manner quite similar in structure to the signal peptides of other membrane-bound and secreted proteins [21]. This presumed signal sequence would thus be predicted to be necessary for the translocation of the *env* gene product across the endoplasmic reticulum.

The above mentioned sequence analysis has also revealed a stretch of 27 hydrophobic amino acids near the carboxyterminus of the *env* gene product [6,18]. It has been proposed that these amino acids span the membrane and thereby provide the means by which gp37 is anchored. Such a method of anchorage would be analogous to that of other membrane-bound proteins such as the hemagglutin (HA) protein of influenza virus [22,23], the G protein of VSV [24–26], and membrane-bound IgM [27]. To date, however, no experimental evidence has been reported which directly tests this hypothesis on the RSV *env* gene product.

We have recently succeeded in expressing a molecularly cloned RSV *env* gene in African green monkey kidney cells (CV-1) via SV40 late replacement vectors (manuscript in preparation). Protein synthesis of the cloned gene product is initiated at an AUG codon located at position 5055 in the RSV nucleotide sequence [18]. This AUG is normally (ie, in RSV-infected chicken cells) removed and replaced by the AUG at the 5′ end of the RSV *gag* gene by RNA splicing [6]. As a result, the cloned *env* gene product has a 64 amino acid leader peptide instead of the normal 62 amino acid leader. Nevertheless, this SV40 vector expressed product appears to be normally glycosylated, processed to gp85 and gp37, transported to the cell surface, and biologically active (Hunter et al, in preparation). We have used this expression system to study the effects of various *env* deletions on the translocation of the glycoproteins across the endoplasmic reticulum.

In this paper we report evidence that the leader peptide of the RSV *env* gene product is indeed required for translocation across the endoplasmic reticulum but that removal of the carboxy-terminal, hydrophobic "anchor" region is *not* sufficient for secretion.

MATERIALS AND METHODS

Cells and Viruses

The source of the RSV *env* gene was pATV-8, a recombinant pBR322 plasmid containing the entire genome of the Prague C strain [28]. Wild-type SV40 DNA and DNA from a viable early deletion mutant, d11055 [29,30], were also used. These SV40 DNAs were molecularly cloned in pBR322 derivatives. All recombinant bacterial plasmids were propagated in E coli strain DH-1, which is *recA* and *hsdR* [31]. Transformed DH-1 cells were selected and grown in L broth containing ampicillin. The expression and characterization of wild-type and mutant RSV *env* genes (cloned into SV40 vectors) were studied using CV-1 cells, a continuous line of African green monkey kidney cells that are permissive for SV40 replication [32]. 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. All manipulations and reactions involving DNA (restriction endonuclease digestions, analytical and preparative gel electrophoresis, blunt-end and sticky-end ligations, bacterial transformations, plasmid isolations, BAL31 digestions, end labeling, etc) were performed according to standard laboratory protocols [31].

Subcloning and In Vitro Mutagenesis of the *env* Gene

The 1866 bp KpnI-XbaI fragment of pATV-8 contains the entire RSV *env* gene with the coding sequences for gp85 located closest to the KpnI site and the coding sequences for gp37 located closest to the XbaI site [6,18]. This fragment was cloned into the tetracycline gene of pAT153, a pBR322 derivative [33], between the ClaI and BamHI sites after ligating ClaI linkers and BamHI linkers (Collaborative Research, Inc, Lexington, Massachusetts) to the Klenow reacted KpnI and XbaI ends, respectively. The resulting *env* subclone is called $pENV_{Cla(+)}$.

For the amino-terminal mutations, $pENV_{Cla(+)}$ DNA was opened at the unique XhoI site of *env*, digested with the double-strand-specific exonuclease BAL31, and then religated. For the carboxy-terminal mutations, $pENV_{Cla(+)}$ DNA was opened at the unique BamHI site, digested with BAL31, and religated in the presence of BamHI linkers (CCGGATCCGG; from Collaborative Research) to restore the BamHI site. Individual mutant clones were obtained following transformation of DH-1 cells with the religated, BAL31 products. The end ponts of the deletions were determined by the Maxam-Gilbert method of DNA sequencing [34].

Construction of SV40-*env* Recombinants

Fragments containing wild-type and mutant *env* genes were inserted in the place of the late region of SV40 DNA (Fig. 1). In the case of the mutant *env* genes, ClaI-BamHI fragments from individual mutant clones were inserted between the HpaII and BamHI sites of SV40 DNA, after the ligation of a ClaI linker to the HpaII end. This construction of SV40 and *env* is called SV-CB. In the case of the wild-type *env* gene, a KpnI-BamHI fragment (ie, without a ClaI linker) was inserted between the KpnI (rather than the HpaII) and BamHI sites of SV40 DNA. The resulting recombinant of wild-type *env* is called SV-KB. (A detailed description of the wild-type *env* product produced by SV-KB is in preparation.) Thus, the SV40-mutant *env* constructions

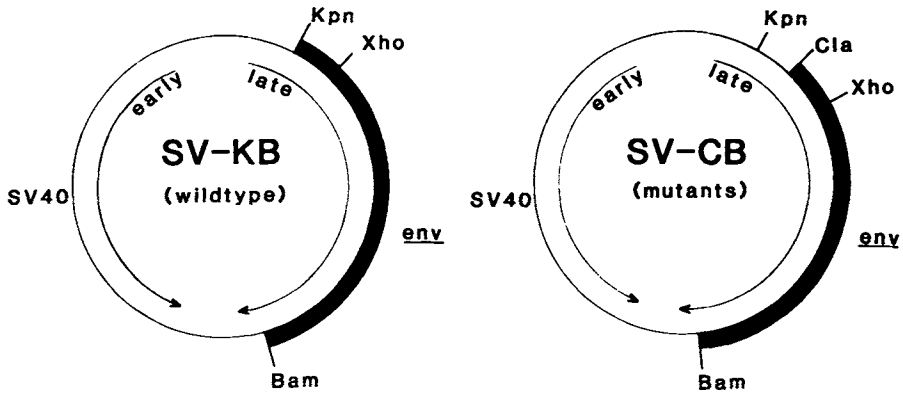


Fig. 1. SV40 late-replacement vectors used for expressing wild-type and mutant *env* genes.

differ from SV-KB in that they contain 52 bp of additional SV40 sequences (those between the KpnI and HpaII sites) and a ClaI linker. In the course of the experiments described here, we found that the presence of these additional sequences reduced the level of *env* expression about tenfold, and we are currently exploring the reasons for this.

CV-1 cells (5×10^5 per 60-mm plate) were transfected with the SV40-*env* recombinants (200 ng) together with the DNA (75 ng) of the helper virus, d11055, using 250 μg of DEAE-dextran (35). After 60–90 min, the DNA suspension was removed and medium containing 100 μM chloroquine was added for 5 hr [36]. The viral stocks resulting from these transfections were used for the experiments described below.

Radiolabeling and Analysis of Polypeptides

CV-1 cells infected for 48–60 hr with SV40-*env* recombinant viruses or uninfected control cells were starved in medium lacking leucine and serum for 1 hr and then incubated with leucine-free medium containing 1 mCi/ml of ^{3}H-leucine (New England Nuclear, Boston) for 15 min. Chases with unlabeled leucine were carried out in Dulbecco's modified medium containing 0.5% fetal calf serum. To inhibit glycosylation, infected cells were treated with 1 μg of tunicamycin per ml of growth medium for 2 hr prior to leucine starvation and for all subsequent labeling periods.

After the labeling of infected CV-1 cells, the medium was removed, the cells were lysed, RSV glycoproteins were immunoprecipitated from the lysates and culture medium fractions, and the immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels as previously described [16,6].

RESULTS

Amino-Terminal Mutants

In order to delete portions of *env* coding for amino acids near the 64 amino acid leader peptide, we digested the cloned gene with the restriction endonuclease XhoI and the double-strand-specific exonuclease BAL31. The amino acid sequence of each

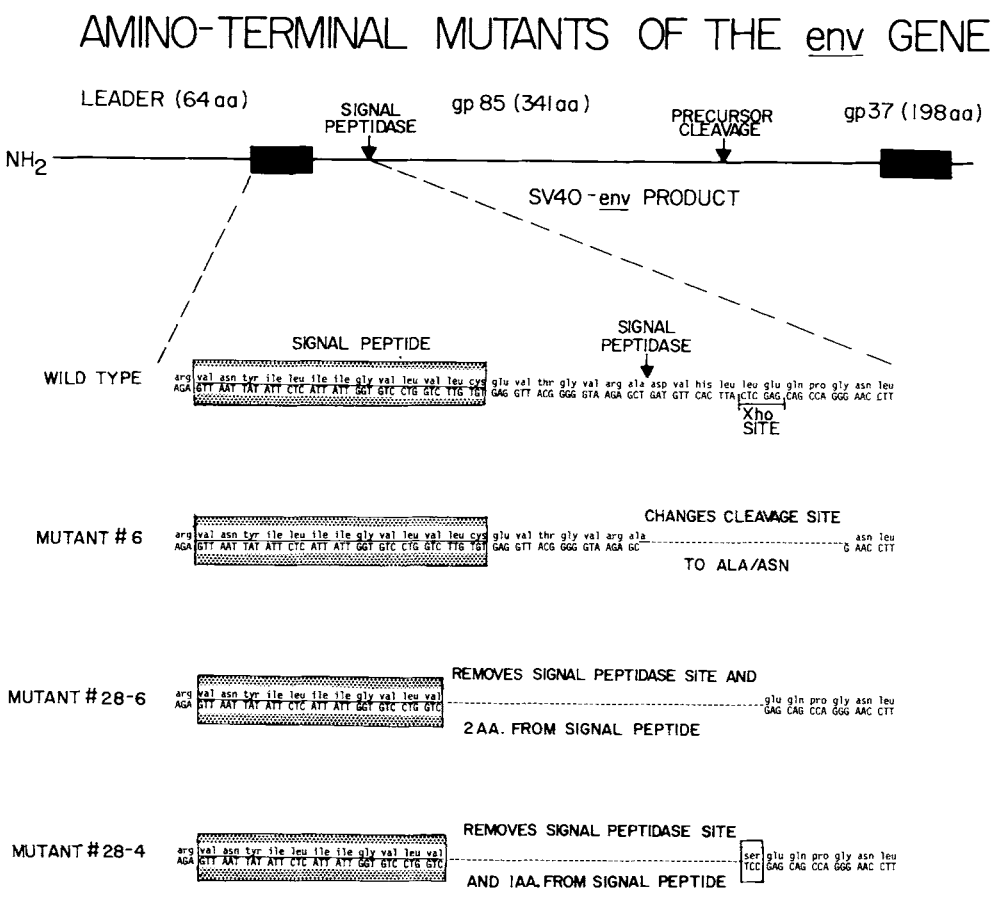


Fig. 2. Schematic representation of the amino-terminal mutants. The upper line represents the primary structure of the wild-type *env* gene product as described in the text. Deletion mutations were obtained by digestion with the exonuclease BAL31 after cutting at the indicated XhoI site. The resulting nucleotide and amino acid sequences of three mutants studied are compared with the wild-type sequences in the lower portion of the figure. The dotted boxes indicate the hydrophobic amino acids of the proposed signal peptide. The open box indicates an inserted uncharged amino acid.

mutant protein was deduced by DNA sequencing. The sequences of the three amino-terminal mutants used in this study are shown in Figure 2.

Mutant 6 was found to lack DNA sequences coding for the first nine amino acids (27 bp) to the right of the signal peptidase cleavage site (ie, at the N terminus of gp85) but to retain all 64 amino acids to the left (ie, the leader peptide). As a result of this deletion, the mutant 6 polypeptide has an altered signal peptidase cleavage site with an uncharged asparagine residue, instead of the wild-type, negatively charged aspartic acid residue, adjacent to the alanine at the C terminus of the leader peptide.

To determine if the leader could be cleaved from the mutant 6 polypeptide, we pulse-labeled the infected cells and ran the immunoprecipitated proteins on an SDS-

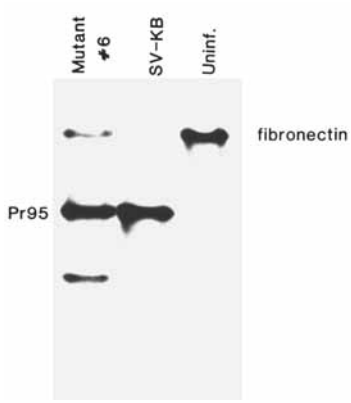


Fig. 3. Pulse-labeling of N-terminal mutant 6 proteins. Infected and uninfected cells were pulse-labeled for 15 min with ^{3}H-leucine and lysed, and immunoprecipitates were obtained using a mixutre of anti-gp85 and anti-gp37 antibodies. The proteins were then electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed.

polyacrylamide gel. The results from the autoradiogram are presented in Figure 3. This figure shows that the wild-type (SV-KB) and mutant 6 precursor polypeptides migrated to approximately the same position in the gel (Pr95). Had the 64 amino acid leader not been removed, a band of protein about 6.5 kD larger in size (ie, greater than 100 kD total) would have been observed. Experiments are in progress to confirm that the site of cleavage in the mutant 6 gene product is the same as that of the wild-type product.

It should be noted that all three lanes in Figure 3 were loaded with about the same amount of radioactivity. As mentioned in Materials and Methods, the wild-type, SV-KB, construction was found to give significantly greater expression than that of the mutants. We have found the relative amount of pulse-labeled SV-KB precursor to be about tenfold greater than that of the mutant constructions. For these reasons, non-specifically precipitated proteins (eg, fibronectin) are not observed in the SV-KB lane.

Mutant 28-6 lacks DNA sequences coding for nine amino acids to the left of the signal peptidase cleavage site as well as five amino acids to the right. The missing 14 amino acids (42 bp deletion) contain not only the cleavage site but also two amino acids of the proposed signal peptide. Mutant 28-4 is similar to mutant 28-6 except that it contains an insert of 3 bp which codes for serine. This additional uncharged amino acid extends the mutant signal peptide from 11 residues (in mutant 28-6) to 12 residues, one short of the original 13 (see Fig. 2).

To determine the effect of these two mutations on the synthesis of *env* gene products, we pulse-labeled the infected cells and ran the *env*-specific proteins on an SDS-polyacrylamide gel. In our initial experiment (data not shown), we found no bands in the position of the wild-type Pr95. Instead, we found a major band of proteins about 63 kD in size. This size corresponds to that predicted for unglycosylated mutant *env* products from which the 64 amino acid leader has not been removed. To determine if the mutant proteins were glycosylated (and perhaps degradation products of about 63 kD), we repeated the labeling experiment in the presence and absence of tunicamycin. As shown in Figure 4, tunicamycin had no effect on the synthesis of the mutant polypeptides although it efficiently prevented the glycosylation

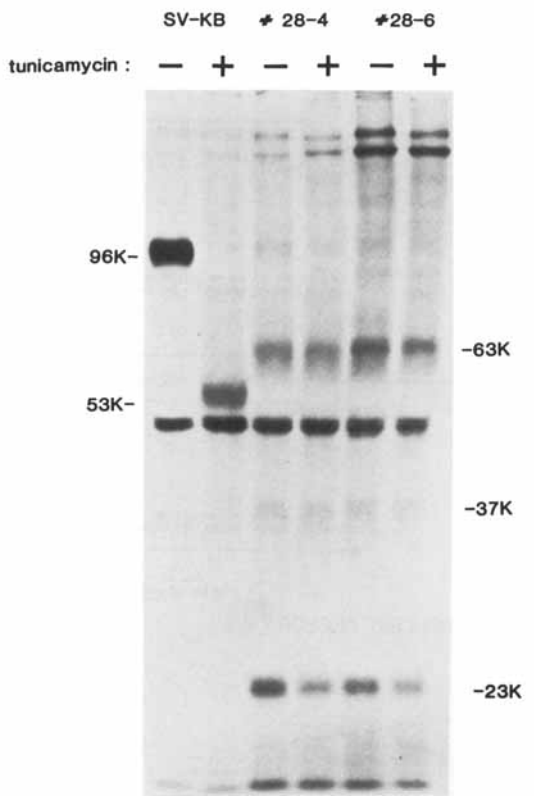
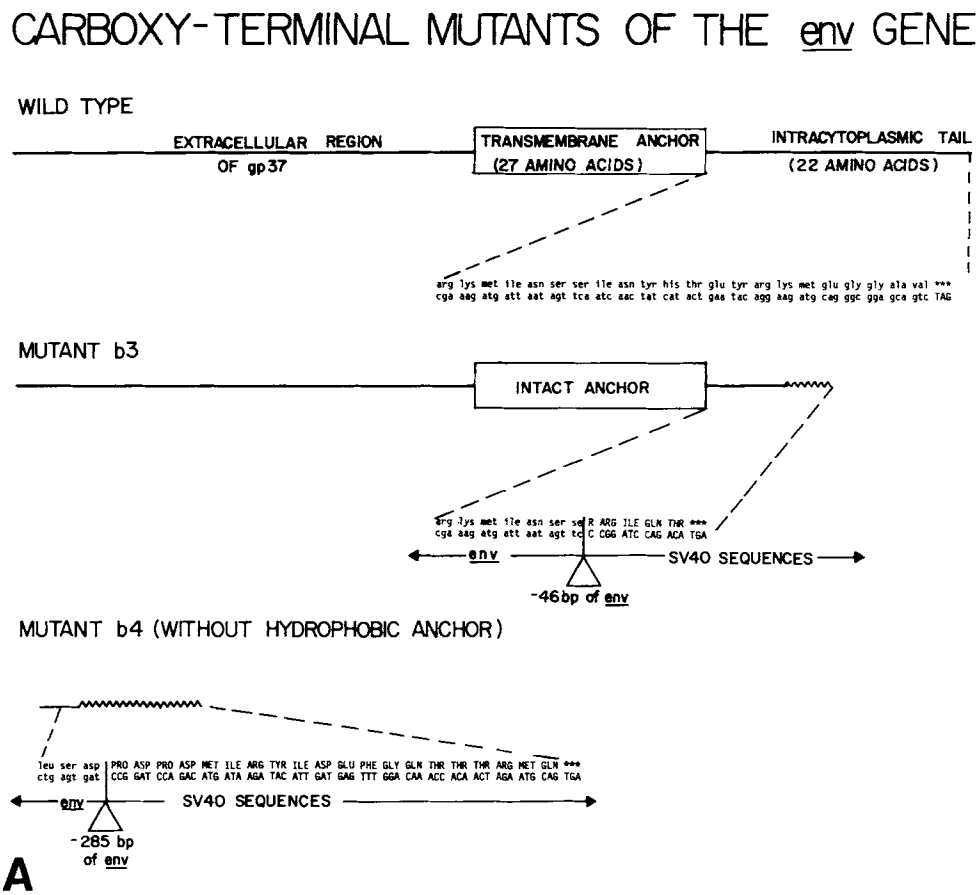


Fig. 4. Effect of tunicamycin on the proteins expressed from N-terminal mutants 28-4 and 28-6. Infected cells were pulse-labeled for 15 min with ^{3}H-leucine in the presence (+) or absence (−) of tunicamycin as described in Materials and Methods. Then the cells were lysed, anti-gp85 and anti-gp37 antibodies were added, and the immunoprecipitated *env* gene products were electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed. The numbers to the right and left of the autoradiogram indicate the measured sizes of the proteins in the corresponding bands obtained using protein standards (not shown).

of the wild-type protein. This suggests that the mutant proteins are not translocated across the membrane. The bands of 37 kD and 23 kD proteins, indicated in the figure, are probably not *env* gene-specific since they are not reproducibly observed and have also been observed in wild-type control experiments. The bands of 45 kD proteins seen in all the lanes contain the SV40 VP1 capsid protein which is nonspecifically precipitated.

Carboxy-Terminal Mutants

In order to delete portion of *env* coding for the intracytoplasmic tail and the presumed anchor region, BAL 31 was also used. After digesting from the 3′ end of the gene, BamHI linkers were attached and the mutant *env* genes were cloned into the SV40 expression vector. The end points of the deletions were determined by DNA sequencing. Codons for the termination of protein synthesis were provided by the flanking SV40 sequences. The structure of the two carboxy-terminal mutants used in this study are presented in Figure 5.


CARBOXY-TERMINAL MUTANTS OF THE env GENE
WILD TYPE
EXTRACELLULAR REGION OF gp37
TRANSMEMBRANE ANCHOR (27 AMINO ACIDS)
INTRACYTOPLASMIC TAIL (22 AMINO ACIDS)
arg lys met ile asn ser ser ile asn tyr his thr glu tyr arg lys met glu gly gly ala val ***
cga aag atg att aat agt tca atc aac tat cat act gaa tac agg aag atg cag ggc gga gca gtc TAG
MUTANT b3
INTACT ANCHOR
arg lys met ile asn ser ser R ARG ILE GLN THR ***
cga aag atg att aat agt tc C CGG ATC CAG ACA TGA
env
SV40 SEQUENCES
-46bp of env
MUTANT b4 (WITHOUT HYDROPHOBIC ANCHOR)
leu ser asp | PRO ASP PRO ASP MET ILE ARG TYR ILE ASP GLU PHE GLY GLN THR THR THR ARG MET GLN ***
ctg agt gat | CCG GAT CCA GAC ATG ATA AGA TAC ATT GAT GAG TTT GGA CAA ACC ACA ACT AGA ATG CAG TGA
env
SV40 SEQUENCES
-285 bp of env
A
CHO
gp37
mutant B4
gp85
Viral Membrane
NH2
COOH
mutant B3
B

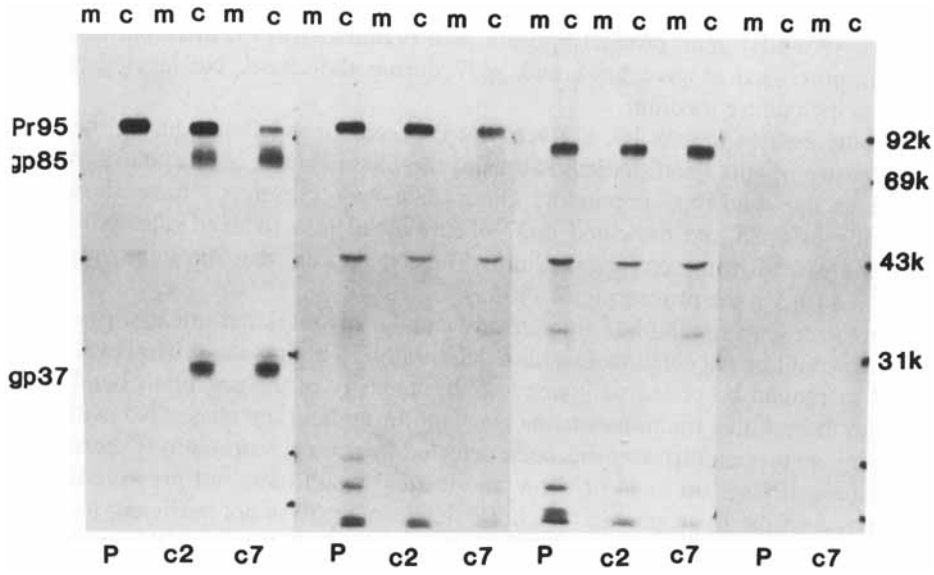


Fig. 6. Pulse-chase labeling of C-terminal mutant proteins. Infected and uninfected cells were pulse-labeled with ^{3}H-leucine for 15 min and chased for the indicated number of hours. Each culture was fractionated into growth medium (m) and cellular lysate (c) portions. A mixture of anti-gp85 and anti-gp37 antibodies was added to each fraction, and the proteins obtained were electrophoresed on 10% SDS-polyacrylamide gels and autoradiographed.

Mutant b3 lacks DNA sequences coding for 15 amino acids at the C terminus of gp37. Termination at the first in-frame stop codon in the SV40 sequence results in a hybrid protein containing four foreign amino acids. This mutant thus maintains an intact hydrophobic anchor but is missing about two-thirds of the intracytoplasmic tail. Mutant b4 lacks DNA sequences coding for the C-terminal 95 amino acids of gp37 which contain the entire cytoplasmic tail and anchor regions. Termination by the SV40 sequences results in a hybrid protein containing 20 foreign amino acids.

In order to determine the effects of these deletions on the synthesis of RSV glycoproteins, we pulse-labeled the infected cells with ^{3}H-leucine and chased with unlabeled leucine for various times. After the specified chase period, the culture medium was removed (and saved) and the cells were lysed. The medium and cellular lysate fractions were mixed with anti-gp85 and anti-gp37 antibodies, and the immu-

Fig. 5. Schematic representation of the carboxy-terminal mutants. A) Deletions (indicated by the triangles) were obtained by digestion with the exonuclease BAL31 and the resulting DNA sequences were determined as described in Materials and Methods. The straight lines represent the amino acids at the carboxy-terminal end of gp37 specified by *env* DNA sequences (lowercase letters). The zigzag lines represent the foreign amino acids coded by the BamHI linker and SV40 DNA sequences (uppercase letters). Stop codons for the mutants are provided by SV40 sequences. B) The end points of the deletions relative to gp37 and gp85 are illustrated. Mutant b3 lacks most of the cytoplasmic tail, and mutant b4 lacks all of the tail and presumed anchor regions of gp37.

noprecipitated proteins were electrophoresed on SDS-polyacrylamide gels. The results of this experiment are shown in Figure 6. In this figure it can be seen that the wild-type (SV-KB) gene product appears as a precursor (Pr95) after pulse-labeling which is processed to give gp85 and gp37 during the chase. No labeled proteins appear in the culture medium.

In the case of mutant b3, a glycosylated precursor is labeled during the pulse. The intensity of this band decreases during the chase, but at a rate that is reduced relative to the wild-type precursor. Other data (not presented) have shown that processing to gp85 and truncated gp37 occurs albeit at a reduced rate. No labeled proteins are seen in the culture medium. Thus, it appears that the intracytoplasmic tail plays a role in the processing of Pr95.

In the case of mutant b4, a noticeably smaller glycosylated precursor is synthesized, as would be expected from a sum deletion of 75 amino acids. However, unlike SV-KB or mutant b3 coded polypeptides, the intensity of the precursor band associated with the cellular fraction remains constant throughout the chase. No evidence of processing or protein turnover has been detected, and no glycoprotein is secreted into the medium. Chases up to 14 hr show an identical result (data not presented). Thus, the removal of the hydrophobic "anchor" region of gp37 is not sufficient to convert the RSV glycoprotein to a secreted form. Moreover, the behavior of this mutant (b4) appears to confirm the observation made with mutant b3 that a signal(s) located near the C-terminus of gp37 is important to the processing of the precursor.

DISCUSSION

The general mechanisms by which membrane-bound and secreted proteins translocate across the membrane of the endoplasmic reticulum are well understood. Briefly, it is known that the synthesis of these proteins can be initiated on cytoplasmic ribosomes [37] and arrested by the binding of the signal recognition particle (SRP), thereby preventing release of the protein into the cytoplasm [38–41]. SRP mediates the specific attachment of the translating ribosome to an integral membrane protein of the endoplasmic reticulum (the "docking" protein), which results in the release of the elongation arrest and translocation of the protein across the membrane [42]. In the course of this translocation process, the hydrophobic signal peptide [21] is cleaved from the protein by another membrane-bound protein, the signal peptidase [43, 44]. Proteins to be secreted are subsequently released into the lumen of the endoplasmic reticulum whereas those to be membrane-bound become anchored (ie, their translocation is stopped) by means of carboxy-terminal hydrophobic regions [22,23,26]. The nature of the mutants described in this paper can be interpreted in view of this general mechanism of protein transport.

Amino-Terminal Mutations

We have found that the removal of as many as nine amino acids to the right of the signal peptidase cleavage site (eg, mutant 6) has no effect on the synthesis of a glycosylated *env* precursor (Pr95). Cleavage occurs in spite of the change of the negatively charged aspartic acid residue to an uncharged asparagine residue at the first position following the leader peptide. This result is consistent with the observations of others that signal peptidases (of both eukaryotes and prokaryotes) are equally active on a wide range of bacterial and eukaryotic protein precursors having different amino acids at the cleavage site [43–46]. The aspects of the substrate important for

peptidase specificity are not clear. Our results suggest that the amino acids of the *env* gene product recognized by the signal peptidase do not extend beyond the leader peptide. This premise is supported by other mutants of the RSV *env* gene that have different alterations in the aminoterminus of gp85 and yet are processed efficiently (unpublished results). Such a hypothesis conflicts, however, with results obtained by Russel and Model [47] using M13 coat protein mutants. They identified a mutant coding for a procoat protein in which leucine replaces glutamic acid as residue 2 of the mature coat protein. Although the mutant precursor was found to be inserted into the membrane, cleavage by the signal peptidase (both in vivo and in vitro) was found to be poor. Their results thus suggest that alterations to the right of the cleavage site do influence the activity of the peptidase. In order to better understand the peptidase specificity, we are currently examining additional *env* mutants that are altered in the region of the cleavage site.

The removal of nine amino acids to the left of the signal peptidase cleavage site, as well as five amino acids to the right, has a drastic effect on the synthesis of the *env* precursor (eg, mutants 28-4 and 28-6) and results in the synthesis of a 63 kD unglycosylated protein. These mutations are complex in that the 14 amino acids deleted are likely to be involved in three transport functions: 1) binding of SRP, 2) insertion into the membrane (ie, hydrophobic signal peptide activity), and 3) cleavage by the signal peptidase.

Three models can be envisioned that may explain the appearance of a 63 kD *env* gene product. First, it may be the case that SRP fails to bind to the translating ribosomes. In this case, translation would not be arrested, and an unglycosylated mutant protein, with leader intact, would be released into the cytoplasm. As mentioned earlier, the predicted size for such an *env* gene product is 63 kD. In the second model, signal peptidase is assumed to catalyze transfer of the *env* gene product across the membrane [44]. In this case, SRP is imagined to bind to the ribosomes, arrest translation, and mediate attachment to the docking protein. Interaction with the docking protein would release the translation arrest and protein synthesis would resume. The absence of cleavage by signal peptidase (due either to the failure of the shortened signal peptide to mediate membrane insertion or to the absence of a cleavage site) would prevent the catalytic translocation of the polypeptide across the membrane, and an unglycosylated mutant product (of 63 kD) would be released into the cytoplasm. In the final model, it is imagined that SRP binds to the signal peptide and that the mutant protein is translocated across the membrane and glycosylated. Because of the lack of a signal peptide cleavage site, the glycosylated mutant protein would be inserted through the membrane at both its amino-terminal and carboxy-terminal ends. This would result in an unstable conformation, and the mutant protein would be degraded to give a glycosylated product of 63 kD and several undetected small polypeptides.

Of the three hypotheses proposed, only the last one can be eliminated. The results of the tunicamycin experiment (Fig. 4) argue that the mutant polypeptides are not glycosylated and thus are not translocated across the membrane. Although we cannot yet distinguish between models 1 and 2, it is clear that we have shown that specific sequences of the proposed signal peptide are indeed required for the normal biosynthesis and posttranslational modification of the RSV glycoproteins.

At this time we cannot explain why the tunicamycin product of SV-KB-infected CV-1 cells (54 kD) is smaller than that of RSV-infected chicken cells (57 kD) [20]. One hypothesis is that cleavage by signal peptidase actually occurs further down the

polypeptide chain in CV-1 cells. This hypothesis is unlikely since all signal peptidases studied to date have been found to accurately cleave both eukaryotic and prokaryotic protein precursors [43,45]. Furthermore, evidence from an additional mutant, No. 1, not discussed in this paper, indicates that amino acid residues 4–18 of gp85 are not required for signal peptidase cleavage. If signal peptidase cleavage were to occur beyond this point in CV-1 cells, the glycosylated precursor would lack at least one glycosylation site and would migrate with a significantly different mobility. However, this is not observed; the glycosylated precursor of SV-KB in CV-1 cells is identical in size to that seen in RSV-infected chicken cells. Experiments are in progress to determine the amino-terminal sequence of the wild-type and mutant *env* precursors from CV-1 cells. A second hypothesis for the smaller size of the CV-1 tunicamycin product is that the intracytoplasmic tail of gp37, which may normally interact with other RSV or chicken cell proteins (eg, *gag* proteins), is lost owing to the absence of these proteins in CV-1 cells. The length of the tail, however, is too short (22 amino acids) to solely account for the 3 kD difference, making this hypothesis also unlikely. A third hypothesis for the size difference is that the tunicamycin product (but not the glycosylated product) is partially degraded in CV-1 cells but is stable in chicken cells. As a fourth explanation, the difference in tunicamycin products may reflect a previously unidentified addition to the RSV glycoprotein that occurs in chicken cells but not CV-1 cells.

Carboxy-Terminal Mutants

The failure of the anchor minus RSV glycoproteins (eg, mutant b4) to be secreted was unexpected. Recent reports have shown that the HA of influenza virus [22,23] and the G protein of VSV [26] are both secreted when their anchors are removed. We are currently performing experiments to determine the cellular location of the truncated RSV glycoproteins.

Several explanations for the lack of secretion can be proposed. For example, it might be the case that another hydrophobic region in the truncated protein could alone act as an anchor. Such a hydrophobic region would be limited to gp37 since gp85 is known to be anchored via disulfide linkages and can be released by treatment of virions with reducing agents [3]. Recently, all the peptides of the *env* gene product longer than nine amino acids lacking charged amino acid residues were identified [6]. Of the peptides found in gp37, none (other than the 27 amino acid "anchor" region) have a hydrophobicity index similar to those of other transmembrane segments and signal peptides. Similarly, none of the foreign (ie, SV40-coded) amino acids at the end of the truncated *env* gene product appear capable of membrane anchorage.

As a second explanation, it might be the case that acylation of gp37 serves to anchor the truncated polypeptide [48]. However, the probable sites of fatty-acid attachment to membrane-bound proteins are thought to occur just outside the lipid bilayer [49,50], and these amino acids have been deleted in mutant b4. Moreover, preliminary data indicate that the RSV anchor minus glycoproteins are not labeled with ^{3}H-palmitic acid, although the wild-type proteins are.

As a third explanation, it may be the case that the deletion of 95 amino acids results in precipitation of the mutant protein owing to incorrect folding. Such precipitates would not be expected to be transported through the cell properly. Experiments are in progress to determine the subcellular location of the mutant polypeptides as well as to determine their solubility.

As a final explanation for the lack of secretion, it may be that the anchor minus protein lacks a cell sorting signal. The loss of such a sorting signal would prevent the transport of the proteins to the cell surface where they could be released to the outside. Alternatively it is possible that a latent translocation signal has been uncovered in mutant b4 polypeptides. Results obtained by immunofluorescence of fixed, infected cells suggest that the truncated mutant b4 proteins are rapidly transported out of the endoplasmic reticulum (ie, do not accumulate) and become located in cytoplasmic vesicles (data not shown). Furthermore, the failure of mutant b4 glycoproteins to be processed to gp85 and truncated gp37 (Fig. 6) suggests that the mutant precursors fail to reach the cellular site of processing.

The experiments described in this manuscript provide the basis for a genetic analysis of those regions of a membrane-spanning polypeptide that signal the cellular transport machinery to export the polypeptide to the cell surface. The application of site-directed mutagenesis together with an SV40 expression system provides a very powerful approach to a more detailed study of this problem.

ACKNOWLEDGMENTS

These studies were supported by grant CA 29884 from the National Cancer Institute. John W. Wills was supported by NIH training grant CA 09202, and J. Marie Hardwick by NIH postdoctoral fellowship CA 06603. Eric Hunter is the recipient of a research career development award CA 00685 from the National Cancer Institute. We thank Melanie Pollard for her expert assistance in the preparation of this manuscript.

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