A Charged Amino Acid Substitution within the Transmembrane Anchor of the Rous Sarcoma Virus Envelope Glycoprotein Affects Surface Expression but Not Intracellular Transport

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Abstract. Two point mutations were introduced by oligonucleotide-directed mutagenesis into the region of the Rous sarcoma virus envelope gene that encodes the hydrophobic transmembrane anchor of the receptor glycoprotein. Single-nucleotide substitutions ultimately converted a hydrophobic leucine, located centrally within the membrane-spanning domain, to either a similarly hydrophobic methionine or a positively charged arginine. The altered coding region was reinserted into an intact copy of the envelope gene, cloned into simian virus 40 late-replacement vector and expressed in primate cells.

Analysis of envelope gene expression in CV-1 monkey cells revealed normal levels of synthesis of a membrane-spanning precursor for both the mutants; however, the arginine-containing mutant [μ26(arg)] exhibited greatly reduced cell surface expression of mature protein, as determined by indirect immunofluorescence and ²⁵¹I labeling of surface proteins. In experiments in which cells producing the μ26(arg) polypeptide were pulsed with radioactive leucine and then chased for 5 h, no intracellular accumulation or extracellular secretion of mature products (gp85 and gp37) could be detected.

Treatment of μ26(arg)-infected cells with lysosomal enzyme inhibitors (chloroquine and leupeptin) resulted in the accumulation of gp85 and gp37, indicating that they were being degraded rapidly in lysosomes. The fact that terminally glycosylated and proteolytically cleaved env gene products were observed under these conditions showed that modifications associated with passage through the trans compartment of the Golgi apparatus occurred normally on the mutant polypeptide; thus insertion of a highly charged amino acid into the transmembrane hydrophobic region of gp37 results in the postGolgi transport to lysosomes.

It is proposed that the insertion of this mutation into the transmembrane anchor of the envelope glycoprotein does not affect membrane association, orientation with respect to the membrane, or intracellular transport at early stages during maturation. At a step late in the transport pathway, however, the presence of the charged side chain alters the protein in such a manner that the molecules are transported to the lysosomes and degraded. It seems likely that transport of the protein from the trans-Golgi to the cell surface is either directly blocked, or that after expression on the cell surface the mature glycoprotein complex is unstable and rapidly endocytosed.

Membrane glycoproteins of enveloped viruses are synthesized, transported, and processed within the infected cell in a manner identical to those of the host. The bitopic receptor glycoproteins of Rous sarcoma virus (RSV) are no exception (Bosch and Schwartz, 1984; Wills et al., 1984); using the host cell's own transport machinery they transit the generalized exocytotic pathway of rough endoplasmic reticulum (RER) to Golgi to cell surface (Palade, 1975; Rothman and Lenard, 1984). Successful passage through these well regulated compartments has been postulated by Blobel (1980) to require at least three separately recognized sequences of amino acids: signal sequences, stop-translocation sequences, and sorting sequences.

Signal sequences are hydrophobic stretches of amino acids, usually at the amino terminus, that generally are cotranslationally cleaved from the protein after initiation of the process of translocation into the lumen of the RER (Blobel and Dobberstein, 1975; Lingappa et al., 1978; Davis and Tai, 1980; von Heijne, 1984). Comparisons of many leader peptides from both prokaryotes and eukaryotes reveal a weak...
consensus with regard to length, hydrophobicity, and cleavage site (von Heijne, 1984).

Sorting signals that direct protein transport to organelles within the cytoplasm have been well-defined (Kalderon et al., 1984; Hase et al., 1984); on the other hand, signals mediating protein transport along the Palade pathway are for the most part unknown. Lysosomal proteins carry a specific mannos-6-phosphate modification of their oligosaccharides that appears to function as a routing signal (Sly and Fischer, 1982), but the criterion for phosphate addition has not been identified. It seems likely that the signals directing specific transport in the exocytotic pathway may be three-dimensional entities residing in the tertiary structure of the protein.

Stop-translocation sequences have been proposed to be a region of the nascent protein molecule that halts insertion through the membrane by disassembling the translocation apparatus, and thereby creates proteins with three topological domains (Blobel, 1980). These sequences appear to be inseparable from the anchor sequences (Yost et al., 1983; Rettenmier et al., 1985), however, their precise structural and physical properties have not been defined. Wold et al. (1985) have suggested that the cytoplasmic domain of membrane-spanning proteins might act to interrupt translocation; however, this seems unlikely in light of the construction of deletion mutants lacking this domain that are found to be associated with the membrane in a normal manner (Perez et al., 1987; Garoff et al., 1983; Zuniga et al., 1983; Murre et al., 1984).

Although regions described as transmembrane anchors vary widely in length and sequence, they do have characteristics in common. Most often, they are long stretches (19-30 residues) of predominantly nonpolar and hydrophobic amino acids bounded by charged residues at the carboxy terminus of membrane proteins. However, membrane-spanning sequences have also been described at the amino termini of some viral proteins (Bloki et al., 1982; Palmiter et al., 1978; Bos et al., 1985; Markoff et al., 1984; Zerial et al., 1986; Speiss and Lodish, 1986) and in the middle of other proteins (Rettenmier et al., 1985; Kopito and Lodish, 1986; Finer-Moore and Stroud, 1984).

In an attempt to define the functions of membrane-associated protein sequences, several investigators have made large deletions extending into the anchor domains (Gething and Sambrook, 1982; Rose and Bergmann, 1982; Rose and Bergmann, 1983; Florkiewicz et al., 1983; Sveda et al., 1982; Wills et al., 1984; Machida et al., 1985; Perez et al., 1987) or introduced frame-shift mutations that change the composition of the anchor (Sveda et al., 1982). The results from these experiments show that the anchor, while not required for initiating translocation, is necessary for membrane integration.

The transfer of intact transmembrane domains to normally secreted molecules has caused translocation of the constructed hybrid molecules to stop at the added sequences (Yost et al., 1983). In complementary experiments, precise deletions of only the transmembrane domain caused the release of the molecules into the lumen of the RER (Rettenmier et al., 1985; Perez et al., 1987). These data show that transmembrane domains themselves contain the stop-translocation sequences. The RSV env gene product is synthesized as a glycosylated (high mannose) 95-kD precursor in the RER. It is transported to and subsequently cleaved in the Golgi to the disulfide-linked (Pauli et al., 1978) and terminally glycosylated polypeptides, gp85 and gp37 (England et al., 1977; Hayman, 1978; Bosch et al., 1982; Bosch and Schwartz, 1984). While in the Golgi, palmitate is added to gp37 (Gebhardt et al., 1984).

The experiments described in this manuscript focus upon a region at the carboxy-terminal domains of the RSV glycoprotein gp37, which anchors the gp85-gp37 receptor complex to the membrane. The DNA sequence encoding this region (Hunter et al., 1983; Schwartz et al., 1983) predicts that this area of the molecule contains two very different domains: (a) a hydrophilic tail to the cytoplasmic side of the membrane and (b) an anchor domain, rich in hydrophobic and nonpolar amino acids, that would span the lipid bilayer. To investigate the requirements for a functional transmembrane region, and in particular the necessity for a long uninterrupted stretch of nonpolar and hydrophobic amino acids to halt translocation, we have inserted a charged residue (arginine) into the anchor of gp37.

The results of these experiments show that the substitution has no discernible effect on the normal bitopic orientation of the glycoprotein or, by definition, on the stop-translocation function of this region. The mutant polypeptide containing the intramembranal arginine appears to be transported normally through the cell to the trans-Golgi compartment; yet, it never accumulates on the plasma membrane. Instead, the terminally glycosylated glycoprotein complex is transported rapidly to the lysosome and degraded. These data show that small alterations within the transmembrane region of a bitopic polypeptide can drastically alter expression by changing transport routes, and therefore, the ultimate destination within the cell.

Materials and Methods

Cells and Viruses

African green monkey kidney cells (CV-1 cells) used in the analysis of expression were obtained from the American Type Culture Collection (Rockville, MD). Plasmids were propagated in the DH-1 strain of Escherichia coli (Hanahan, 1983), while M13 strains were grown in the E. coli JM101 strain (Messing, 1983). The M13mp80 and M13mp11 strains of the bacteriophage M13 used in the mutagenesis were purchased from Bethesda Research Laboratories (Gaithersburg, MD).

Plasmids and Plasmid Constructions

The source of envelope gene sequences was an infectious molecular clone (pAT6B) of the RSV Prague C strain genome (Katz et al., 1982), kindly given to us by R. V. Gunata (Columbia University, NY). The expression vector pSVenvKX was constructed from the plasmid pHenvX (J. Wills of this institution), which contains the entire env gene (Bam HI [4715] to Xba I [6861]; Schwartz et al., 1983) cloned into the Bam HI and Xba I sites of a pAT53 derivative. Kindly provided to us by M. J. Gething and J. Sambrook (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) were: (a) the plasmid pOD1, containing the 3039-bp Hpa I-Bam HI early gene fragment of SV40 inserted into the Cla I site of pX3, (b) the plasmid pC2, a pAT53 derivative whose unique Eco RI site was converted to an Sst I site, and (c) a molecular clone of the intact simian virus 40 (SV40) deletion mutant d1050 (Pipas et al., 1983).

Expression Vector Construction and Use

To facilitate the transfer of mutated carboxy-terminal envelope gene sequences into the env-SV40 expression vector, a modified SV40 late replacement vector was constructed as shown in Fig. 1. The unique Eco RI and Xba I sites in the vector (pSVenvKX) permitted the insertion of mutagenized

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Oligonucleotide-directed Mutagenesis

Methods describing molecular cloning, recombinant phage isolation, mutagenic template preparation, the mutagenesis itself, and verification of potential mutants have been described in detail by Zoller and Smith (1983). Additional protocols used in the manipulations of the M13 bacteriophage were from Messing (1983).

As a source of single-stranded template for mutagenesis, a 717-bp Eco RI-Xba I subfragment of the RSV env gene isolated from pATV8 was inserted into the M13mp11 replicative form. The recombinants, first located as white plaques using the β-galactosidase system of Messing (1983), were subsequently screened by restriction analysis and then positively verified by DNA sequencing (described below).

Mutagenic primers, designed to create the desired changes in the genome described in Fig. 2a, conformed to the criteria regarding uniqueness and stability as proposed by Gillam and Smith (1979) and Zoller and Smith (1982). The integrity of the primer was verified by sequencing (Maxam and Gilbert, 1980; as modified by Zoller and Smith, 1983). The uniqueness of the primer binding sites, predicted by computer screening of template DNA sequences, was checked by using the primers in dideoxynucleotide sequencing reactions on the template (described below). Oligonucleotide primers were synthesized by C. Naeve (St. Jude Hospital, Memphis, TN).

The conditions for primer annealing, enzymatic extension of the mutagenic primers, purification of covalently closed supercoiled DNA, transfection of host bacteria, and the screening of isolates for recombinant clones were from methods detailed by Zoller and Smith (1983).

DNA Sequence Analyses

The chemical cleavage method of DNA sequencing of Maxam and Gilbert (1980) was used as described. The dideoxynucleotide method (Sanger et al., 1977) was used as modified by W. Herr (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Radioiodination and Surface Protein Kinetics

To study fatty acid modification of viral proteins, infected CV-1 cells on 35-mm plates were labeled with [3H]palmitate by adding 400 μCi of [9,10-3H]palmitic acid (50 Ci/mmol; Amersham Corp., Arlington Heights, IL) in 10 μl ethanol to 200 μl of serum-free medium for 15 min. Then, 200 μl of complete media was added and the incubation continued for 5 h before lysis and immunoprecipitation. SDS-PAGE gels of these immunoprecipitates were fluorographed using the dimethylsulfoxide-2,5-diphenyloxazole (PPO) method of Bonner and Laskey (1974).

Radiodination and Surface Protein Kinetics

Both the relative amounts and the rate of turnover of gp37 and gp85 on the surface of infected cells were measured after labeling the proteins by the lactoperoxidase-catalyzed 125I iodination method of Soule and Butel (1979).

Proteins were immunoprecipitated using the methods described above, ex-
cept that it was necessary to preadsorb the lysates with fixed Staph A to reduce background counts before the addition of antisera. The rate at which membrane-associated env protein turned over was measured by incubating the surface-labeled cells in complete medium for various times before lysis and immunoprecipitation. After fixing and drying, the SDS-PAGE gels were autoradiographed on Kodak XAR-5 film at -70°C using intensifying screens (Cronex Lightning Plus; DuPont Co., Wilmington, DE). Quantitation was performed by excising the labeled protein bands and determining the associated radioactivity in a gamma counter.

**Cell Fractionation and Immunofractionation**

The kinetics of env protein processing and transport to the plasma membrane were determined using an immunofractionation scheme. At 60 h post-infection, 35-mm plates of CV-1 cells were pulse-labeled with [3H]leucine, or pulse-labeled and then chased, as described (Wills et al., 1983). After the pulse or subsequent chase periods, the cells were washed with cold PBS, then incubated with 50 μl of chicken anti-RSV Prague C antisera (1:20 dilution in PBS) at 4°C for 45 min. The plates were washed again in PBS and the cells lysed as described previously. The immunocomplexed cell surface proteins were precipitated by adding 50 μl of rabbit anti-chicken IgG antiserum (Antibodies Inc., Davis, CA) diluted 1:20 in PBS and 100 μl of a 10% suspension of Staph A. After incubation on ice for 30 min and clearing of the Staph A by centrifugation, the supernatant was removed and internal proteins immunoprecipitated with a rabbit antisera directed against gp37 and gp37 as described above. The Staph A pellets of surface proteins and cytoplasmic proteins were processed as usual for SDS-PAGE and fluorography. RSV protein bands, identified in the fluorographs, were excised and quantitated by liquid scintillation (Wills et al., 1984). In some experiments, the second cleared supernatant was immunoprecipitated a third time using a mouse monoclonal antisera directed against the SV40 large-T antigen (kindly provided by E. Harlow; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) as an independent measure of multiplicity of infection.

**Cell Fractionation and Proteinase K Digestion of Microsomes**

The association of the RSV proteins with cellular membrane vesicles was shown by fractionating cells into crude membrane and cytoplasmic fractions. The method used was a modification of the Dickson and Atterwill procedure (1980) as described by Wills et al. (1984).

To determine the presence of a cytoplasmic domain on wild-type (wt) and mutant env proteins, microsomes from Dounce-homogenized infected cells were digested with proteinase K and then analyzed by SDS-PAGE in a modified procedure of the Dickson and Atterwill procedure described by Moscov and Blobel (1982) and Guan and Rose (1984). Briefly, Dounce homogenates of pulse-labeled infected cells were aliquoted equally into three tubes. Proteinase K was then added to two tubes, to a concentration of 5 μg/ml; 10% deoxycholate and 10% Triton X-100 were added to one of these tubes to a final concentration of 1% (proteinase plus detergent control). All three tubes were then incubated at 4°C overnight. Phenylmethylsulfonyl fluoride (PMSF) to 2 mM, ovalbumin to 10 μg/ml, alpha-2-macroglobulin (Calbiochem-Behring Corp., La Jolla, CA) to 0.1 U/ml were added to stop the digestion. The proteinase plus detergent controls were immunoprecipitated directly; the other homogenates were digested with proteinase K and then analyzed by SDS-PAGE in the usual manner. RSV protein bands, identified in the fluorographs, were excised and quantitated by liquid scintillation. In some experiments, the second cleared supernatant was immunoprecipitated three times using mouse monoclonal antisera directed against the SV40 large-T antigen (kindly provided by E. Harlow; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) as an independent measure of multiplicity of infection.

**Lysosomal Enzyme Inhibitors**

To determine if the degradative loss of mutant proteins was occurring in the lysosome, the lysosomal protease inhibitors chloroquine and leupeptin were added to the culture medium at 100 or 20 μM final concentrations, respectively. RSV protein bands, identified in the fluorographs, were excised and quantitated by liquid scintillation. In some experiments, the second cleared supernatant was immunoprecipitated three times using mouse monoclonal antisera directed against the SV40 large-T antigen (kindly provided by E. Harlow; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) as an independent measure of multiplicity of infection.

**Immunofluorescence**

Subcellular localizations of env gene products in the infected CV-1 cells were determined using fluorescent antibody probes. Wills et al. (1984) showed that env gene products in the infected CV-1 cells were localized to the plasma membrane. The method used was a modification of the Dickson and Atterwill procedure (1980) as described by Wills et al. (1984).

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Introduction of point mutations into the transmembrane anchor coding sequences of the env gene. (a) Schematic representation of mutagenic primers annealed to env template sequences. The 717-bp Eco RI-Xba I env gene subfragment, coding for the carboxy terminus of gp37, was cloned into the polylinker of M13mp11. Single-stranded DNA from recombinant phage isolates was the source of the env template. Primer 1 changed a C to an A in the RSV sequence corresponding to nucleotide 6,761; this created a unique Sph I site and caused the substitution of a methionine for a leucine at amino acid 16 (of 27) in the transmembrane anchor. Primer 2 changed a T to a G at nucleotide 6,762, created a unique Nae I site, and converted the same leucine to an arginine. The altered sequences were removed from the replicative forms and recloned into the expression vector for analysis. Dotted lines represent residues composing the newly constructed restriction sites. The direction of the in vitro extension of the primers on the M13mp11 templates was to the left. (b) Schematic diagram of the Rous sarcoma virus gp37 transmembrane region. The hydrophobic amino acid anchor of the receptor glycoprotein gp37 extends bitopically through the lipid bilayer (represented by the shaded area) creating three obvious domains: external, transmembranal, and cytoplasmic. The anchor domain consists of 27 predominantly hydrophobic or nonpolar amino acids and is presumed to assume an alpha helical conformation, except at the kink caused by the proline residue. Strongly basic residues lie external to the domain. At amino acid position 16 (of 27), a leucine (L; indicated by the arrow) was...
methods were followed as described except that the ethanol-acetic acid postfixative was changed to 1% formalin in PBS and antisera were preadsorbed with an acetone powder of CV-1 cells.

Results

Insertion of Point Mutations into RSV env Anchor Sequences

The technique of oligonucleotide-directed mutagenesis was used to insert point mutations into the transmembrane anchor region of gp37. An Eco RI-Xba I DNA fragment, containing the region of the RSV genome encoding the carboxy terminus of gp37, was cloned into the bacteriophage vector M13mp11 to serve as the source of single-stranded template for the mutagenesis.

Substitutions made at nucleotide positions 6761 and 6762 of the RSV sequence (Schwartz et al., 1983; Hunter et al., 1983) changed a leucine (hydrophobic), centrally positioned in the wt anchor, to either a methionine (which conserves the hydrophobic nature of the sequence) or an arginine (which inserts a charged residue into the anchor), as depicted in Fig. 2 b. The substitutions were engineered into the coding sequence using two mutagenic 19mer oligonucleotides (Fig. 2 a), as described in Materials and Methods. In addition to altering the coding potential of the sequence, the mutagenesis created unique Spf I and Nae I restriction enzyme sites.

To identify mutant phage DNAs, the mutagenic primers were 32P-labeled and used in DNA-blot hybridizations. These oligonucleotides, which form more stable hybrids with homologous mutated sequences than with nonmutated (wt) sequences, remained bound to immobilized phage DNA during successively higher stringency washes. Potential mutants identified in this manner were confirmed by demonstrating the presence of the new restriction sites and by DNA sequencing (data not shown).

Expression of Mutated env Genes in an SV40 Slot-in Vector

To assess the effects of the amino acid substitutions within the anchor region of gp37, the mutated Eco RI-Xba I fragments were excised from double-stranded replicative form DNA of the M13 vectors and inserted into the expression vector pSV.env.KX. This SV40 late-replacement vector, a variant of one described previously (Wills et al., 1983, 1984), contains unique Eco RI and Xba I sites, which facilitated replacement of the gp37 carboxy-terminal coding sequences (Fig. 1).

Cotransfection of CV-1 monkey cells with the self-ligated expression vector and circularized dl1055 helper virus results in a productive infection by complementation (Gething and Sambrook, 1981). The particles released from the transfected cells are infectious and contain the packaged genomes of either the SV-KX or the helper dl1055. The high titers of infectious virus produced in this system allow env expression in 80% of cells in the monolayer. The high level of expression in this system facilitates the analysis of wt and mutant glycoprotein biosynthesis, transport, and processing.

The mutant with the methionine substitution, made with primer 1, will be referred to in the text as μ14(met). The mutant containing the arginine substitution, made with primer 2, will be referred to as μ26(arg). Using the convention of Wills et al. (1983), the mutant genomes in the plasmid form of the SV40 expression vector have been given the archival names pSV.env.KX.μC14, and pSV.env.KX.μC26, respectively.

Mutations within the Anchor of gp37 Affect the Cellular Locations of env Proteins

Immunofluorescent staining patterns of env proteins in fixed and permeabilized CV-1 cells were identical for both mutant and wt constructions. Within the infected cells, RSV glycoproteins were observed throughout the cytoplasm, with a marked concentration in a perinuclear spot (Fig. 3, B, C, and D). This region was coincident with the staining produced by rhodamine-conjugated wheat germ agglutinin (Fig. 3, b, c, and d), a probe used to identify Golgi apparatus (Virtanen et al., 1980).

The constructions differed markedly, however, when their expression on the surface of unfixed, infected cells was assessed by fluorescent staining. wt and μ14(met)-infected cells stained brightly, indicating surface accumulation of the RSV glycoproteins (Fig. 4, B and D). The μ26(arg) arginine mutant, on the other hand, had greatly reduced surface fluorescence, which was in most cases indistinguishable from that of the uninfected controls (Fig. 4 C). Rare μ26(arg)-infected cells were observed with a surface-fluorescence intensity approaching wt; while these never accounted for 2% of the monolayer, they are most easily explained as a product of membrane permeabilization occurring during the late stages of the lytic SV40 infection (Norkin, 1977). To quantitate the relative amounts of accumulated env protein on infected cell surfaces, the monolayers were radiolabeled, lysed, and immunoprecipitated. Autoradiographed SDS-PAGE gels of wt and μ14(met)-infected cells showed heavy labeling of gp85 and gp37, but cells infected with the μ26(arg) mutant were only weakly labeled (data not shown). Quantitation of these gels showed that the wt and μ14(met)-infected cells expressed over 10-fold more gp85 and gp37 on their cell surfaces than cells infected with μ26(arg).

Because the fluorescent antibody staining experiments showed clearly that the μ26(arg) mutant polypeptide was being synthesized and transported to the Golgi, but never accumulated on the cell surface, we examined the kinetics of intracellular processing and transport to understand better the nature of the defect.

Intracellular Protein Processing

Infected cells, metabolically pulsed using [3H]leucine, were either lysed immediately or chased with complete medium for 6 h before lysis. To rule out secretion of the mutant protein, immunoprecipitates of both lysates and chase media were analysed by gel electrophoresis and fluorography. The results are shown in Fig. 5.

Immunoprecipitates from pulse-labeled cells infected with the wt construction gave an intense band corresponding to the Pr95 precursor polyprotein on SDS-PAGE gels (Fig. 5). The intensity of this band decreased significantly in a 6-h chase (t2, 90 min), while bands corresponding to the cleavage products, gp37 and gp85, appeared. Culture medium was converted to either a methionine (M) or a charged arginine (R) using oligonucleotide-directed mutagenesis. Amino acids are presented in their single letter codes.
Figure 3. Internal localization of\textit{env} gene products in CV-1 cells. Indirect immunofluorescence identified the intracellular location of\textit{env} gene products using monospecific rabbit antisera to gp85 (A–D). Using rhodamine-conjugated wheat germ agglutinin, regions of the cell associated with the Golgi were identified (a–d). The bright \textit{env}-associated fluorescence scattered throughout the cytoplasm of the wt (B), µ26(arg) (C), and µ114(met)- (D) infected cells was concentrated at a perinuclear spot whose staining was coincident with that of the Golgi (b–d, respectively). The uninfected cells (A and a) showed only weak background fluorescence with the rabbit antibodies but characteristically bright Golgi fluorescence was seen with the rhodamine-conjugated lectin.
except for an occasional bright cell (C). Had levels of fluorescence comparable to the uninfected controls (A) used as a probe for the RSV proteins on unfixed, infected CV-1 cells. Pressed on the surface of the infected cells. A chicken antisera was immunoaffluent staining identified proteins that were being ex-

Figure 4. Surface expression of env gene products. Indirect immunofluorescent staining identified proteins that were being expressed on the surface of the infected cells. A chicken antisera was used as a probe for the RSV proteins on unfixed, infected CV-1 cells. The bright fluorescence patterns found on cells infected with the wt (B) or µ114(met) (D) constructions were identical. The µ26(arg) had levels of fluorescence comparable to the uninfected controls (A) except for an occasional bright cell (C).

from the chase was devoid of env protein. Mutant µ114(met) yielded results identical to the wt. In cells infected with mutant µ26(arg), Pr95 was synthesized and turned over at a rate similar to that of wt, but unlike the latter, no gp85 or gp37 polypeptides were observed after the 6-h chase. No µ26(arg) env protein was detected in the medium, ruling out the possibility that the mutation in this protein had resulted in secretion.

Processing and Transport

By using antisera that separately recognized denatured and native forms of the env proteins, infected cells could be immunofractionated into surface and internal compartments; in this manner, the kinetics of processing and transport of the glycoproteins to the cell surface could be followed. The results of the technique, shown in Fig. 6 a, reveal that the mutant µ26(arg) protein was synthesized and chased to gp85 and gp37. However, the mature forms of the µ26(arg) protein rapidly disappear in comparison to wt. As expected from the results presented above, no significant level of µ26(arg) mature cleavage products was seen on the cell surface, even at the 2-h timepoint when internal levels of gp85 and gp37 are comparable to those of wt. The Pr95 molecules of all three constructions appeared to chase with similar rates and were never detected on the cell surface.

The quantitation of the data from such an experiment is shown in Fig. 6 b where µ26(arg) is compared to the wt; results from the µ114(met) infections are identical to wt and are not presented. Fig. 6 b, top, confirms that both mutant and wild-type Pr95 polypeptides turned over at nearly identical rates. The center panel shows that the appearance of intracellular gp37 followed identical kinetics up to the 2-h timepoint; at this time, however, the µ26(arg)-associated counts declined exponentially while the wt counts remained stable. The level of wt gp37 on the cell surface reflected the levels of protein found inside the cell (bottom panel). In contrast, there were no significant levels of surface-associated

gp37 in the µ26(arg) mutant–infected cells. These data suggest that the arginine mutant precursor proteins are being processed to mature products, but these forms are unstable and are not accumulating on the cell surface.

Surface Stability of env Glycoproteins

Infected cells were surface-labeled with 125I at 50 h postinfection and then incubated up to 24 h to determine the kinetics of RSV env protein turnover on the infected cell surface. The results (Fig. 7) show that labeled RSV protein, predominantly in the form of gp37 and gp85, disappeared from the infected cells with time. Both the intensity of labeling and rate of turnover appeared identical for the wt and µ114(met) polypeptides. In contrast, the µ26(arg)-infected cells showed only minimal labeling of gp85 and gp37 at zero time and this dropped to background levels by 4 h. Since some Pr95 precursor was labeled and immunoprecipitated in these experiments, we cannot exclude the possibility that these low levels of gp85 and gp37 reflect intracellular molecules accessible to iodination.

The rate at which surface protein was lost was quantitated. Since the kinetics of disappearance of iodinated cell surface proteins is the same as that of metabolically labeled surface proteins (Tweto and Doyle, 1976), it is expected that the calculated half-life accurately reflects the rates at which these proteins were removed from the surfaces of cells. For both wt and µ114(met), the rate of decrease in labeled gp85 and gp37 gave a calculated half-life for surface expression of these proteins in the range of 12–18 h. 125I-labeled gp37 on the surface of µ26(arg)-infected cells, on the other hand, ap-
Figure 6. Immunofractionation of mutant-infected cells. (a) Infected cells were fractionated into internal and cell surface-associated protein using two different antisera. Surface proteins were immunoprecipitated using a chicken anti-whole virus antisera that identifies native glycoprotein. Rabbit antisera reactive with denatured env proteins were used to identify solubilized env products. The processing of env proteins in cells infected with either wt or the μ114(met) construction was similar. In both cases, the precursors chased with time inside the cells to the cleavage products gp37 and gp85 (Int). These products could be seen appearing on the surface (Sur) of cells as early as 2 h. After 4 h the amounts of cleavage products appeared stable. The μ26(arg) mutant showed quite different results. The precursor kinetics were identical to the other constructions and cleavage products at 2 h were as intense as those seen with the other constructions, but labeled gp85 and gp37 quickly disappeared, so that at the 8-h chase point they were not apparent. No significant label could be detected in the μ26(arg) surface fractions. (b) To quantitate the amounts of env protein at each time point in the immunofractionation experiment, the bands of protein were excised and counted by liquid scintillation. The radioactivity in each of the env gene products was normalized to that in the coprecipitating SV40 VP1 band. (Top) The results for intracellular Pr95; (center) the kinetics of appearance of the cleavage product gp37 inside the cell; (bottom) the kinetics of appearance of gp37 on the surfaces of infected cells. (Solid symbols) wt construction; (open symbols) μ26(arg) construction. The kinetics of disappearance of Pr95 inside the cells is identical for both mutant or wt. However, the intracellular gp37 of μ26(arg) rapidly degrades after peaking at near wt levels, at the 2-h time point. The μ26(arg) gp37 never appears on the surface of the cells in any significant amount.

Inhibition of Lysosomal Proteases and the Protection from Proteolysis of Mutant env Protein

From both pulse-chase and sugar labeling experiments (not shown) it appeared that the cleavage products, gp85 and gp37, of the μ26(arg) mutant were unstable. To identify the intracellular fate of these mutant proteins, the lysosomal enzyme inhibitors chloroquine and leupeptin (Libby et al., 1980) were added to the culture medium during a pulse-chase experiment. Inhibitor treatment of μ26(arg)-infected cells dramatically affected the turnover of the mutant proteins, as can be seen in Fig. 8a. The disappearance of gp37 and gp85, normally seen when μ26(arg)-infected cells were chased, was completely prevented by blocking lysosomal
proteolytic activity. Chloroquine, leupeptin, and a mix of the two inhibited the proteolysis; however, the effect was weaker when leupeptin was used alone.

To determine if the protected protein was diverted to the lysosome before or after fucose addition, an indicator of terminal glycosylation and a marker for the trans-Golgi (Compan, 1973; Green et al., 1980; Rothman, 1981; Wills et al., 1984), cells were labeled for 6 h with [3H]fucose in medium containing both chloroquine and leupeptin (Fig. 8b).

Under these conditions the gp85 and gp37 proteins, which were only weakly labeled in untreated μ26(arg)-infected cells (Fig. 8b), were as intensely labeled as the untreated wt proteins. The inhibitor treatment caused the appearance of a novel band, in both mutant and wt samples, that migrated slightly above the level of Pr95. This result may reflect an inhibition of precursor cleavage by chloroquine that is not detected in the [3H]leucine labeling experiments (Fig. 8a).

**Mutant Protein is Membrane-associated and Correctly Oriented**

The mutation we have inserted into μ26(arg) might be expected to have an effect on the manner in which this molecule spans the membrane. To determine if the altered carboxy terminus of the env product could still act as a stop translocation signal and allow a stable association with the membrane, infected cells were fractionated into membrane and cytoplasmic components and each was checked for the presence of labeled protein (data not shown). In all cases, the RSV glycoproteins were associated with the membrane fractions, regardless of whether or not they contained mutant transmembrane anchors. This result is consistent with a lack of μ26(arg) polypeptides in pulse-chase cell culture medium (Fig. 5).

The orientation of the mutant glycoprotein with respect to intracellular membranes was determined by making crude microsomal vesicles and digesting the cytoplasmically exposed portion of the proteins with protease. The results (shown in Fig. 9) demonstrate that the cytoplasmic domain of both the wt and μ26(arg) glycoproteins were sensitive to proteinase K digestion. The change in migration of the...
The proteolytic digestion of the cytoplasmic domain of wt and μ26( arg )env products. Microsomal vesicles from homogenized infected cells were digested with proteinase K to detect the cytoplasmic domain of the env protein. Cells labeled with [3H]leucine in a pulse were homogenized, the unbroken cells and debris were removed by centrifugation, and the homogenate digested as described in the text. The gel autoradiograms were scanned by laser densitometry as shown. The precise migrations of molecular mass standards were used in a regression analysis to derive a standard curve. The differences in migration before and after proteinase K digestion were estimated and the molecular mass of the polypeptides calculated. The differences observed reflect a change in mass of ~2.5 kD.

Discussion

We have approached the question of the compositional requirements necessary for membrane anchoring and orientation (stop-translocation) of a membrane-spanning protein by substituting an arginine for a centrally positioned leucine in the hydrophobic anchor region of the RSV env gene product (Fig. 1). The arginine substitution is one of the most drastic compositional point mutations that could be made since it is only rarely found buried in hydrophobic environments (Kyte and Doolittle, 1982) and has a high predicted potential for terminating membrane-buried helices (Rao and Argos, 1986). The substitutions we have made fall within the conserved leucine-rich IC region proposed by Patarca and Haseltine (1984), and near the two cysteine residues where palmitate may be covalently added (Gebhardt et al., 1984; Kaufman et al., 1984).

By changing the anchor's hydrophobic integrity through amino acid substitution in the transmembrane anchor did not affect membrane association or its orientation in the membrane; unexpectedly, however, it affected targeting of the protein at a stage late in the transport pathway.

Regardless of the construction, the early products of processing (the Pr95 precursor molecules) behaved normally: they were synthesized with equal efficiency, had normal bitopic symmetry, and were glycosylated. The kinetics for the turnover of these precursors were nearly identical to those previously reported in infected chicken embryo fibroblasts (Bosch and Schwarz, 1984) and in SV40 expression vectors (Wills et al., 1984). Furthermore, in the Golgi, palmitate was added (Quinn et al., 1983), the precursors were cleaved to gp85–gp37 (Hayman, 1978; Bosch et al., 1982), and they received terminal sugars (Hunt et al., 1979). Only after this last stage did the presence of the charged side chain of the substituted arginine alter expression. At the level of the trans-Golgi, a postGolgi compartment (Saraste and Kuismanen, 1984), or at the cell surface, the gp85–gp37 receptor complex was rapidly shunted to lysosomes and degraded—as shown by the protection afforded the terminally glycosylated env proteins by the lysosomotropic agent (Wibo and Poole, 1974), chloroquine.

The exact pathway that the mutant molecules take to the lysosome is not known. They may be transported directly from the trans-Golgi, or first to the surface where they are rapidly endocytosed. It has been suggested that vesicle-mediated pathways exist from the trans-Golgi directly to the lysosome (Wehland et al., 1982; Griffiths and Simons, 1986) and this may be the route taken by a majority of lysosomal enzymes; alternatively, lysosomal proteins, even enzymes that use the mannose-6-phosphate receptor for targeting, may first be transported to the plasma membrane before internalization through the pathway of receptor-mediated endocytosis (Pastan and Willingham, 1983; Lewis et al., 1985; Von Figura and Hasilik, 1986). Discriminating between the alternate pathways has not been possible from data obtained in CV-1 cells with SV40 vectors, although the lack of labeled proteins on the surface at the 2-h time point in the immunofractionation experiment and the low levels of label detected after surface iodination suggest that if the molecules are reaching the cell surface they are endocytosed very rapidly in these cells. Some of the protein may be transiently expressed on the cell surface, however, since in preliminary experiments where the envelope gene of an avian retrovirus was replaced by the mutant μ26( arg ) gene, low levels of infectious virus were obtained after transfection of susceptible avian cells. Moreover, the protein does not appear to disrupt normal cell metabolism and growth, since avian cell lines that permanently express the mutant protein have been established (Miller, D., G. Davis, and E. Hunter, unpublished data).

We have attempted to accumulate arginine mutant protein on the surface of CV-1 cells by treating them with cytochalasin B, a drug reported to inhibit endocytosis (Axline and Reaven, 1974), at 50 μg/ml. These experiments, however, were unsuccessful (data not presented). Ukkonen et al. (1982) were likewise unable to block the endocytosis of the glycoprotein of ts-1, a temperature-sensitive mutant of Semliki Forest virus. Cells infected with this mutant accumulate viral protein on their surface at the permissive temperature, but if they are shifted to the nonpermissive temperature, the mu-
tant proteins are rapidly removed (half-time, ~30 min) from the cell surface and degraded in the lysosomes. The endocytotic degradative pathway for this mutant protein, like that for μ26(arg), was refractory to cytochalasin B, yet sensitive to chloroquine. By immunofluorescence, the ts-1 viral proteins were found in vesicular structures in chloroquine-treated cells. In our experiments, cells infected with either the arginine mutant or the wt virus also had large immunoreactive vesicles in the cytoplasm after treatment with chloroquine (100 μM for 48 h; data not presented).

Why the insertion of an arginine into the anchor should promote degradation is not obvious. It is possible that the effect of the charged residue is transmitted to the external domain of the protein, altering its tertiary structure such that the cell recognizes the protein as being denatured and removes it. Others have shown that abnormal, denatured proteins can be recognized by the cell and preferentially degraded (Capecci et al., 1974); perhaps more pertinent, the greatly reduced surface expression of the gp55 glycoprotein of the Friend spleen focus-forming virus, compared with that of the related gp70 of murine leukemia virus, has been postulated to be due to spontaneous denaturation (Ruta et al., 1982). While we have demonstrated that the mutant μ26(arg) has a normal cytoplasmic domain, we cannot exclude the possibility that the inserted arginine is extruded to the outside of the membrane, thereby extending the external domain of the protein by 16 amino acids. This seems unlikely, however, since it would leave only 11 uncharged residues to span the membrane. Alternatively, the arginine mutation may affect the interaction of env gene products with one another and with other membrane components. The arginine's charge is generally considered to be incompatible with the hydrophobic environment of the lipid bilayer; to achieve stability, the charged guanidinium group needs to be neutralized; how this is done inside the bilayer is not clear. Parsegian (1969) has postulated that a lone charge sequestered in a membrane must form a pore or tunnel along with localized membrane thining to achieve the lowest energy state. If the charged residue in the gp37 anchor causes the mutant molecules to aggregate and form channels in the membrane in an analogous manner, it would likely kill the cell unless there was a mechanism to remove it rapidly. The antibiotic alamethicin is a 20 amino acid transmembrane polypeptide configured into two cylindrical alpha helices interrupted by a proline residue (Fox and Richards, 1982) and thus has a structure very similar to the one proposed for gp37. This small peptide randomly associates in the membrane to form membrane-spanning ion channels with their polar groups towards the center of the channel. Since the env protein is not an isolated entity in the membrane, it is also conceivable that it aggregates with other components of the membrane to reduce net charge cooperatively, and thereby triggers the endocytotic machinery (Mellman and Plutner, 1984).

Since the experiments described in this manuscript were initiated, several proteins with charged residues within the membrane-spanning domain have been described (Kabcecell and Atkinson, 1985; Saito et al., 1984; Hayday et al., 1985; reviewed by Rao and Argos, 1986). The charged residues in bacteriorhodopsin membrane-spanning alpha helices have been suggested to be neutralized by forming ion pairs (Engelman et al., 1980). However, this is likely to be a special case since the energy required to bury an ion pair in the membrane is not much different from that required to bury the free charged group itself (Parsegian, 1969). Neutralization of strong charges, particularly of lysine and arginine, may occur through the formation of strong hydrogen bonds with tyrosine (Kyte and Doolittle, 1982); however, no tyrosine residues are present in the anchor domain of the env gene product that could participate with the arginine. The T cell alpha, beta, and gamma gene products and the rotavirus VP7 protein have a putative structure similar to the arginine mutant's, with a lysine centered within the transmembrane anchor; however, unlike the molecule we created, they invariably have tyrosine residues adjacent to the lysine (Kabcecell and Atkinson, 1985; Saito et al., 1984; Hayday et al., 1985), which could stabilize the charge through hydrogen bonds (Kyte and Doolittle, 1982).

Further speculations regarding specific alterations caused by the arginine in the bilayer are limited by the dearth of empirical data on protein–lipid physical interactions and protein structure in the hydrophobic environment of the membrane (particularly for proteins that span the membrane a single time). Regardless of the physical perturbations to the lipid bilayer caused by the charge in the membrane, the effect upon the cell biology is clear: forcing the arginine's sidechain into the membrane reduces surface expression by causing the protein to be actively removed only after it has passed through most of the compartments of the endocytic pathway.

Adams and Rose (1985) have described the similar insertion of an arginine (and glutamine) at the center of the transmembrane domain of the vesicular stomatitis virus G protein. Their mutant protein, like the one described here, was bitopic, could be seen localizing in the Goi-gi, and did not accumulate on the cell surface. Since these investigators observed a "lower level of protein expression" it is possible that it also was rapidly degraded in lysosomes after terminal glycosylation. In contrast, Cutler and Garoff (1986) observed no alteration in the biosynthesis and transport of a mutant p62 polyepitope of Semliki Forest virus in which the hydrophobic domain was interrupted by insertion of a glutamic acid residue. In this protein, however, the outer boundary of the hydrophobic domain is not delineated by a charged residue and so it is possible that additional uncharged residues from the external domain were pulled into the membrane. Clearly these phenomena can only be explained by further experimentation.

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