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A Single Amino Acid Substitution in a Hydrophobic Domain Causes Temperature-Sensitive Cell-Surface Transport of a Mutant Viral Glycoprotein

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DNA sequences were determined for three cDNA clones encoding vesicular stomatitis virus glycoproteins from the *ts*O45 mutant (which encodes a glycoprotein that exhibits temperature-sensitive cell-surface transport), the wild-type parent strain, and a spontaneous revertant of *ts*O45. The DNA sequence analysis showed that as many as three amino acid changes could be responsible for the transport defect. By recombining the cDNA clones in vitro and expressing the recombinants in COS cells, we were able to trace the critical lesion in *ts*O45 to a single substitution of a polar amino acid (serine) for a hydrophobic amino acid (phenylalanine) in a hydrophobic domain. We suggest that this nonconservative substitution may block protein transport by causing protein denaturation at the nonpermissive temperature. Comparison of the predicted glycoprotein sequences from two vesicular stomatitis virus strains suggests a possible basis for the differential carbohydrate requirement in transport of the two glycoproteins.

One approach to defining aspects of protein structure involved in protein transport to the cell surface is the isolation and sequence analysis of viral mutants encoding proteins which are defective in this process. Vesicular stomatitis virus (VSV) has been ideal for the isolation of such mutants because it grows rapidly and has a relatively small negative-stranded RNA genome encoding only five proteins (33). Numerous temperature-sensitive mutants of VSV have been isolated (23), and those in complementation group V are known to have defects in the transport of a single viral glycoprotein species (14, 17, 35). The *ts*O45 mutant, a spontaneous mutant isolated by Flammand (6), has been used extensively and is probably the best characterized of the mutants in group V. This mutant has a reversible block in transport of the VSV glycoprotein (G protein) from the rough endoplasmic reticulum (rER) to the Golgi apparatus (2), but nothing is known about the molecular basis for the G protein transport defect in *ts*O45 or any other group V mutant.

G protein consists of a single polypeptide chain of 511 amino acids containing two asparagine-linked complex oligosaccharides (24, 29). G protein forms spikes (probably monomeric [5]) that protrude from the membrane envelope of the virus and is required for virus infectivity (3). More than 95% of the protein is exposed on the exterior of the virion, a hydrophobic domain of 20 amino acids spans the viral membrane, and a hydrophilic carboxy-terminal domain of 29 amino acids is inside the viral membrane. A fatty acid chain (palmitate) is linked to the single cysteine residue in this internal domain (19a, 26). The pathway of biosynthesis and transport of G protein to the cell surface is complex. Insertion into the rER, glycosylation, and presumably cleavage of the signal sequence occur while G protein is a nascent chain (19, 30). After synthesis, G protein is transported to the Golgi apparatus (2) and then to the plasma membrane, where it is incorporated into virus particles.

Analysis of the precise defect causing temperature-sensitive transport of G protein from the *ts*O45 mutant was likely to be complicated by the high mutation rate in RNA viruses (reviewed in Holland et al. [12]). We therefore chose to carry out sequence analysis from cDNA clones encoding the G proteins of the mutant, parent, and revertant viruses. These clones could then be recombined in vitro and expressed to distinguish between the mutation(s) responsible for the temperature sensitivity and silent mutations.

We have reported the nucleotide sequence of a cDNA clone encoding the G protein from the San Juan strain of VSV (29). The Orsay strain is of the same serotype (Indiana) as the San Juan strain, but originated from a different VSV outbreak in cattle (4). The deduced Orsay G protein sequence differs extensively from that of the San Juan strain. The unglycosylated form of G protein from the Orsay strain is transported much more efficiently than the G protein from the San Juan strain (9, 10). Comparison of the G protein sequences from the two strains suggests a reason for this difference in carbohydrate requirement during transport.

MATERIALS AND METHODS

Plasmid constructions and virus strains. The cDNA clones were prepared by previously published procedures (29) from VSV mRNA transcribed in vitro by the VSV polymerase. Nearly full-length double-stranded cDNA was purified by gel electrophoresis and tailed with deoxyribosylcytidine (dC) residues before insertion at the *Pst*I site of deoxyribosylguanosine (dG)-tailed pBR322. All sequences were determined by the procedure of Maxam and Gilbert (21). Each cDNA clone contained 19 nucleotides of the 5'-noncoding region from G mRNA preceded by between 13 and 24 dG-dC residues. The first cDNA clone obtained encoding the G protein from the *ts*O45 mutant (pO45-45) contained a frameshift mutation which apparently resulted from the loss of one thymidine residue within a sequence of nine thymine residues (at nucleotide 1352). The sequences from two other cDNA clones (pO45-32 and pO45-51) of the O45 strain were determined in this region, and both contained nine thymidine residues at this position.

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To obtain cDNA clones suitable for expression in simian virus 40 (SV40) vectors, the cDNA inserts from pO45-45 and pO45R (a cDNA clone encoding the G protein from a *ts*O45 revertant) were excised by *Pst*I partial digestion, trimmed with *Bal*31 nuclease to remove dG-dC tails introduced during cloning, and ligated to synthetic DNA linkers containing the *Xho*I recognition site as described previously (27). These DNAs were then cloned at the unique *Xho*I site in the SV40-based expression vector pJC119 (31). Plasmids containing the DNAs in the correct orientation for expression were designated pSVO45-1 and pSVO45R. These plasmids contained the nucleotide sequences encoding the entire G proteins except that pSVO45-1 contained a frameshift mutation which resulted in the expression of a truncated G protein. The coding regions were preceded by 19 untranslated 5′ nucleotides from G mRNA in both clones. All 5′-dG-dC tails were eliminated by *Bal*31 nuclease from pSVO45-1, whereas four 5′-dG-dC tails remained in pSVO45R. We next replaced a *Kpn*I and *Sst*I fragment (nucleotides 1038 to 1417) in pSVO45-1 with the same fragment from a second cDNA clone (pO45-51) which did not contain the frameshift mutation at nucleotide 1352. This recombinant was designated pSVO45-2.

The recombinants (see Fig. 3) were prepared by ligation of the indicated DNA fragments (two or three fragments) into a vector DNA designated pSVGL3 (28) a derivative of pJC119 (31), from which the insert DNA had been excised with *Xho*I and *Bam*HI. For example, the recombinant designated C-E-F (ts-R-ts) was prepared by ligation of the 5′ *Xho*I-*Nco*I fragment from pSVO45-2, the central *Nco*I-*Pst*I fragment from pSVO45R, the 3′ *Nco*I-*Bam*HI fragment from pSVO45-2, and pSVGL3 DNA (29). The reciprocal recombinant was prepared by reversing the starting DNAs. Approximately 2 pmol of each DNA fragment was included in the ligation reactions. These ligation reactions were then used directly to transform *Escherichia coli* C600 to ampicillin resistance. Recombinant DNA methodology was essentially as described by Maniatis et al. (20). The O45 DNA sequence contains a *Hpa*II site at nucleotide 720 (Fig. 1) which is not present in the other sequences. The recombinant plasmids obtained were characterized by restriction enzyme mapping, including verification of the appropriate presence or absence of the *Hpa*II site in each recombinant plasmid.

A VSV strain designated as the Orsay wild type was obtained from Gordon Freeman and Alice Huang, and the *ts*O45 mutant was obtained from John Bergmann. Mutant O45R was a spontaneous revertant of this mutant. Spontaneous revertants were obtained at a frequency of approximately 10^{-5}. All virus stocks were purified from single plaques and passaged no more than twice before use in cDNA cloning. It should be noted that the Orsay wild-type strain may not be the immediate parent of the *ts*O45 virus, although the DNA sequence shows that it is closely related. This uncertainty about the precise origin of *ts*O45 does not affect our conclusions about the mutation responsible for temperature-sensitive transport. This conclusion is based only on analysis of recombinants between cDNAs cloned from the *ts*O45 and *ts*O45R viruses.

Transfection, labeling, immunoprecipitation, and indirect immunofluorescence. The methods for transfection, biosynthetic labeling, immunoprecipitation, and indirect immunofluorescence have been described in detail previously (28). Briefly, COS-1 cells growing on cover slips were transfected with 5 μg of DNA from pSVO45-2 or pSVO45R in the presence of 500 μg of diethylaminoethyl dextran per ml and incubated at either 34 or 39.8°C for 48 h. Cells were fixed in 3% paraformaldehyde, and double-label indirect immunofluorescence was carried out as described previously (28). Cells were labeled with [^{35}S]methionine in medium lacking methionine for the indicated periods of time, and G protein was precipitated from cell lysates with a rabbit anti-VSV serum and analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (16). Double-label indirect immunofluorescence was carried out with rabbit anti-VSV and guinea pig anti-VSV sera as described previously. Conjugated second antibodies were purchased from Cappel Laboratories. Cells were visualized with a Nikon Optiphot microscope equipped with fluorescence epi-illumination and a Nikon 40× oil immersion plan apochromat objective.

RESULTS

Multiple amino acid changes in the mutant G protein. To determine the nature of the mutation(s) responsible for blocking transport of the VSV glycoprotein in the *ts*O45 mutant, we obtained cDNA clones of mRNAs encoding the G proteins from the wild-type Orsay strain, the *ts*O45 mutant, and a spontaneous revertant of the *ts*O45 mutant. Determination of the nucleotide sequences of these cDNA clones revealed nine nucleotide changes and five predicted amino acid changes between the mutant and wild-type proteins (Fig. 1, Table 1). In the revertant, the nucleotide sequence indicated that three of the five amino acids had apparently reverted to the wild-type sequence (Fig. 1, Table 1). These amino acid changes in the revertant were lysine to asparagine (amino acid 50), serine to phenylalanine (amino acid 204), and, surprisingly, a deletion of a phenylalanine residue (amino acid 441). One or more of these changes potentially could have been responsible for reversion.

Conditional transport of the mutant protein. We next decided to map the critical reversion event(s) by recombination of the mutant and revertant genes in vitro, followed by expression of the recombined genes in an SV40-based vector. This approach required that we first demonstrate expression of these cDNA clones, as well as temperature-sensitive transport of the G protein encoded by the cDNA clone from the *ts*O45 mutant. We have previously used an SV40-based vector to express a cDNA clone encoding the VSV G protein from the San Juan strain (27). Expression of cDNAs incorporated at the single *Xho*I site in this vector is directed from the late promoter of SV40 (31). Expression was assayed 2 days after transfection of COS-1 cells (11), at which time the vector DNA had undergone extensive replication.

To determine whether the transport of the G protein encoded by the cDNA cloned from the *ts*O45 strain was temperature sensitive, we first subcloned this DNA and DNA from the revertant strain into the expression vector. Plasmids expected to express the mutant and revertant proteins (designated pSVO45-2 and pSVO45R, respectively) were then transfected onto COS-1 cells. We found that removal of the dG-dC sequences that were introduced during the initial cloning procedure was essential for obtaining protein expression. For unknown reasons, dG-dC tails as short as 15 nucleotides reduced expression of G protein at least 50-fold.

Cell surface and internal expression of G protein were assayed first by double-label indirect immunofluorescence of transfected COS-1 cells. Examples of the fluorescence patterns obtained for mutant and revertant G proteins expressed at either the permissive temperature (34°C) or the nonpermissive temperature (39.8°C) are shown in Fig. 2. Each pair of panels show the surface fluorescence (before permeabilization) or the internal fluorescence (after permea-

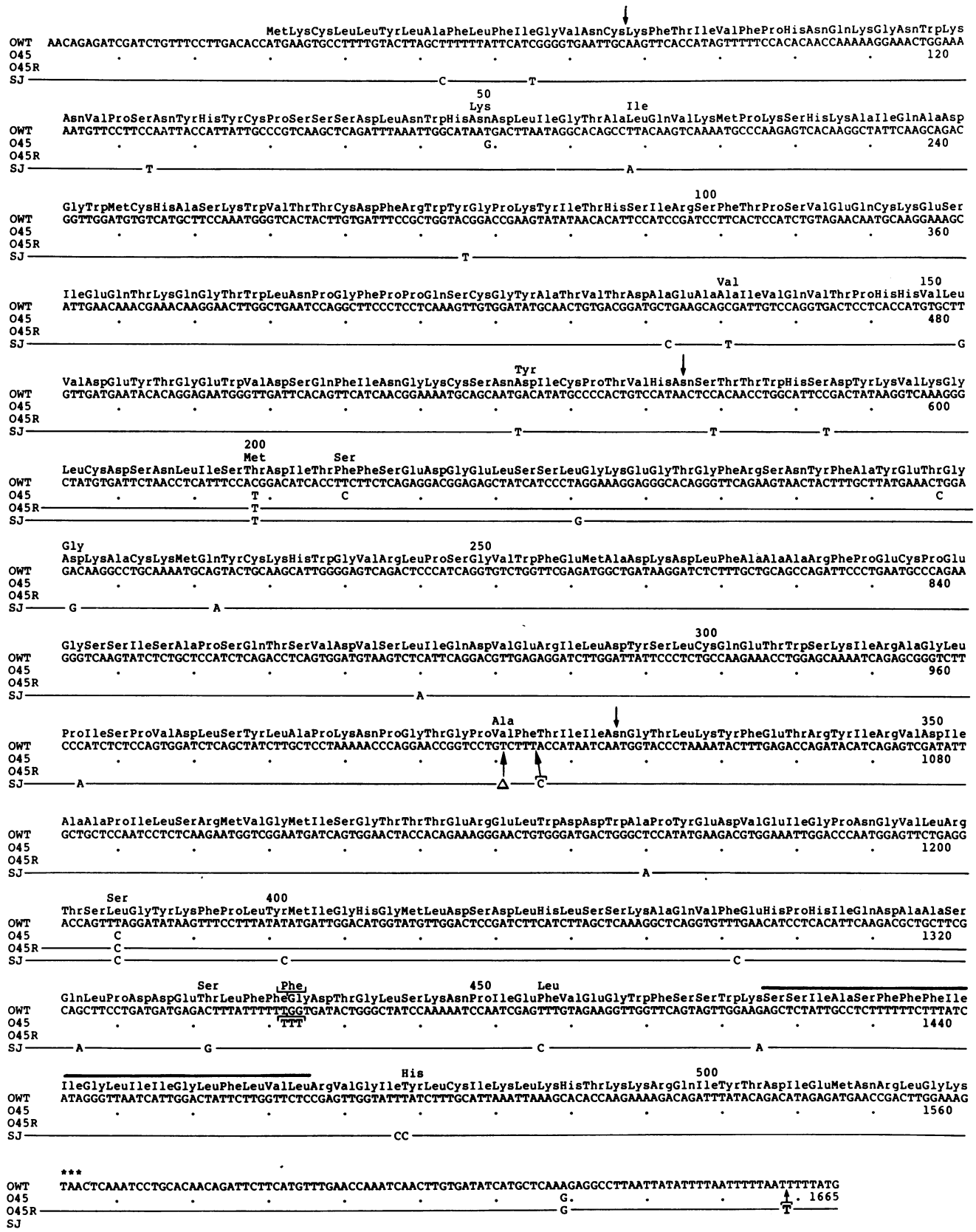

FIG. 1. Comparisons of mRNA and predicted protein sequences derived from cDNA clones encoding the VSV glycoproteins from the Orsay wild-type (OWT) strain, mutant *ts*O45, a revertant of mutant *ts*O45 (O45R), and the San Juan wild-type strain. The nucleotide sequence shown (designated OWT) was determined from a cDNA clone (pOWT9) prepared from the mRNA encoding the VSV glycoprotein

TABLE 1. Predicted amino acid sequence differences among mutant, revertant, and wild-type G proteins

Residue no.	Amino acids in the following proteins[a]:			
	OWT	*ts*O45	*ts*O45R	San Juan
50	Asn	**Lys**	Asn	
200	Thr	Met	Met	
204	Phe	**Ser**	Phe	
394	Leu	Ser	Ser	
441		**+Phe**		
57	Leu			Ile
141	Ala			Val
172	Asp			Tyr
200	Thr			Met
231	Asp			Gly
330	Val			Ala
394	Leu			Ser
438	Thr			Ser
452	Phe			Leu
487	Tyr			His

[a] Amino acid sequence differences among the G proteins from the Orsay wild-type strain (OWT), the *ts*O45 mutant, and the revertant of the *ts*O45 mutant (*ts*O45R) are indicated beside the amino acid residue number. The three amino acids that are changed back to the wild-type sequence in the revertant strain are boldfaced in the *ts*O45 column. Differences between the Orsay strain and the San Juan strain are indicated also. +Phe indicates an insertion.

bilization) of the same cell. Surface expression of the G protein encoded by the cDNA clone from the *ts*O45 mutant occurs at 34°C but is blocked at 39.8°C. In contrast, surface expression of G protein encoded by the cDNA clone from the revertant strain occurred at both temperatures. Expression from the cDNA clone encoding the wild-type Orsay G protein was also temperature insensitive (data not shown).

In many cells, the internal fluorescence pattern for the mutant protein at 39.8°C appeared restricted to a reticular network which extended throughout the cytoplasm. This network is presumably the rER. The protein from the revertant appeared concentrated in a region near the nucleus, presumably the Golgi complex. However, we found that the blockage of the mutant protein in the rER was not absolute at 39.8°C. A low level of apparent labeling in the Golgi region was visible in about 50% of the cells, and trace labeling at the cell surface was seen in about 5% of the cells. As expected, the transport block was reversible (2, 17). Between 10 and 20 min after a shift-down from 39.8 to 34°C, bright staining of the mutant protein appeared in the Golgi region of all cells, and after 30 to 40 min it appeared at the cell surface (data not shown).

Additional evidence that the cDNA clones encoded G proteins with the appropriate transport phenotypes was obtained by analysis of the oligosaccharides present on the proteins expressed at 34 and 39.8°C with endoglycosidase H (endo H). Endo H removes the high-mannose oligosaccharides that are transferred to the nascent protein chain in the rER, but will not remove the oligosaccharides after they have been converted to the endo H-resistant form in the Golgi apparatus (13, 32). We found that the mutant protein expressed from the cDNA clone retained high levels of mannose (endo H-sensitive oligosaccharides) at the restrictive temperature, but showed a wild-type rate of acquisition of endo H resistance at the permissive temperature (data not shown).

Mapping the critical mutation by recombination in vitro and expression. To map the residue(s) critical for temperature-sensitive transport, we made three sets of reciprocal recombinants between the cloned cDNAs encoding the temperature-sensitive and revertant G proteins (Fig. 3). Each cDNA was cloned at an *Xho*I site flanked by *Bam*HI sites in the vector. Therefore, it was possible to make the reciprocal recombinants illustrated by ligating two or three DNA fragments with unique ends into a linear vector which had been prepared with a *Bam*HI site at one end and an *Xho*I site at the other end (28). This approach allowed the efficient cloning of the recombined genes in only the correct orientation for expression. The transport phenotypes of G proteins specified by the recombinant genes were then assayed by indirect immunofluorescence and by the rate of acquisition of endo H resistance. The reciprocal recombinants at the *Kpn*I site (Fig. 3, segments A and B) established that the temperature-sensitive phenotype was due to either the replacement of phenylalanine by serine at position 204 or replacement of asparagine by lysine at amino acid 50. The second set of recombinants at the *Nco*I site (Fig. 3, segments C and D) indicated that the temperature-sensitive phenotype was due to the serine residue at position 204.

To obtain an independent verification of this conclusion, we then made the reciprocal recombinants in which a 160-nucleotide fragment containing the codon for this apparently critical residue was exchanged between the two genes (Fig. 3). The analysis of these recombinants by double-label indirect immunofluorescence is shown in Fig. 4. From this analysis, it is evident that the single change of phenylalanine to serine is sufficient to block cell surface transport of the G protein at 39.8°C but not at 34°C. In support of this conclusion, an analysis of the oligosaccharide processing on the two proteins shows that both acquire endo H-resistant sugars at 34°C, whereas this acquisition is blocked at 39.8°C in the recombinant containing serine at amino acid 204 (Fig. 5).

DISCUSSION

In this study, we identified a single amino acid substitution that is responsible for the temperature-sensitive cell-surface transport of the VSV G protein in the *ts*O45 mutant. The analysis was complicated by the high mutation frequency of VSV which led to multiple sequence differences among the genes encoding the wild-type, mutant, and revertant proteins. However, through the use of an SV40-based expression system and recombination in vitro, it was possible to identify the critical single amino acid change.

One puzzling feature of the differences between the mutant and revertant G proteins was that three amino acids were changed back to the wild-type sequence in the rever-

of the Orsay wild-type strain. The sequence differences between this cDNA clone and the cDNA clones derived from the *ts*O45 mutant (pO45-45) and the revertant O45R (pO45R) are designated O45 and O45R, respectively. Differences from the sequence of a cDNA clone encoding the glycoprotein of the San Juan strain (29) are indicated on the line designated SJ. Predicted amino acid changes are indicated above the predicted amino acid sequence of the Orsay wild-type glycoprotein. Insertions or deletions of nucleotides or amino acids are indicated at nucleotide positions 1021, 1025, 1352, and 1653. The site of signal sequence cleavage and the glycosylation sites are indicated by arrows. The transmembrane sequence is indicated by the heavy line above the protein sequence. U residues are shown as T.

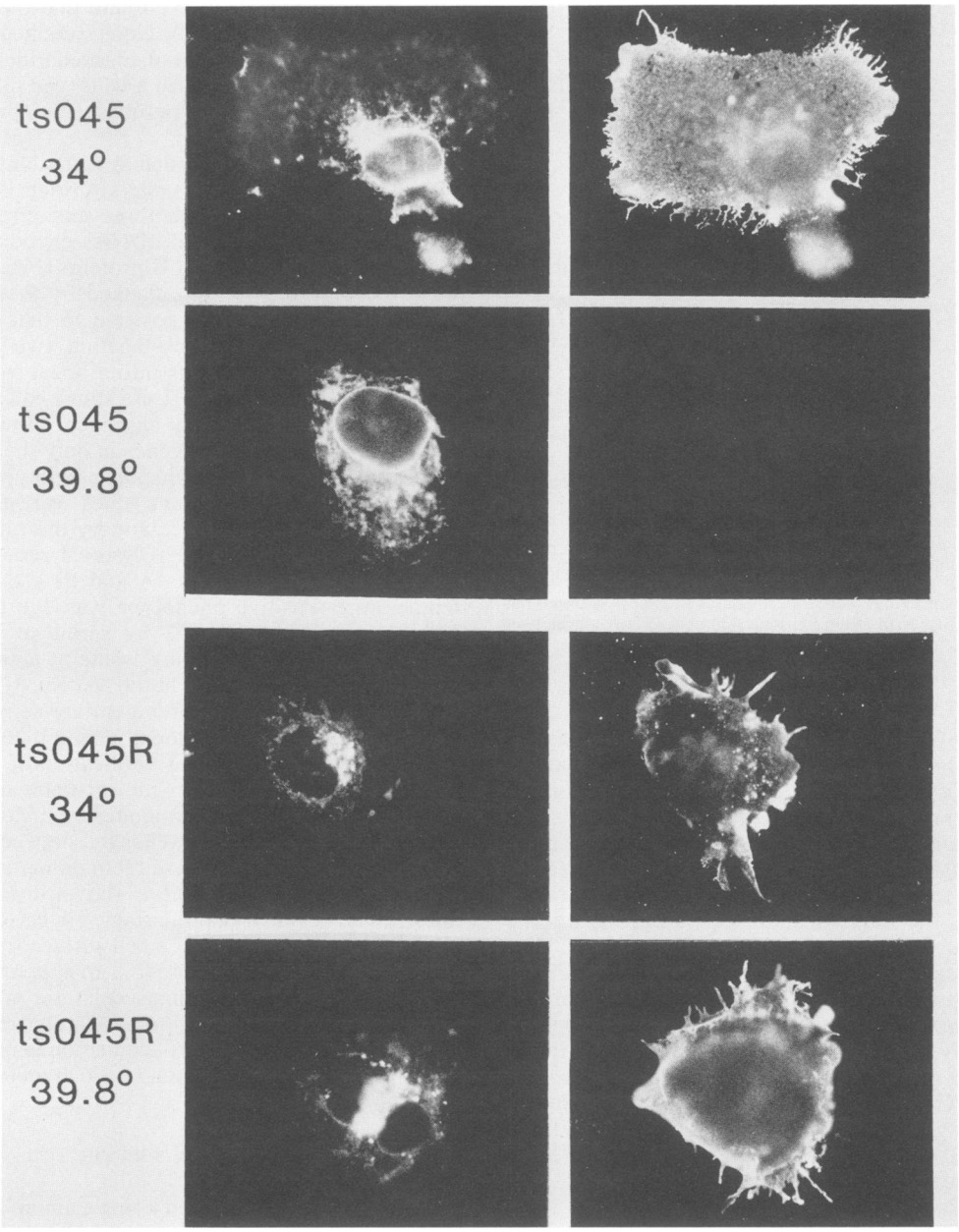

FIG. 2. Indirect immunofluorescence showing normal and temperature-sensitive transport of VSV glycoproteins expressed from cloned cDNA. Cells were transfected with DNA of pSVO45-2 (encoding *ts*O45 mutant G protein), or pSVO45R (encoding the G protein from the *ts*O45 revertant) and incubated at the indicated temperature for 48 h before fixation. Each pair of panels shows the same cell stained either for surface G protein with rabbit anti-VSV serum and rhodamine-conjugated anti-rabbit immunoglobulin G (IgG) before permeabilization with detergent (panels on the right) or for internal G protein with guinea pig anti-VSV serum and fluorescein isothiocyanate-conjugated goat anti-guinea pig IgG after permeabilization with detergent (panels on the left).

tant. Why were there apparently three reversion events when we found that only the substitution of phenylalanine for serine is essential to allow apparently normal transport of the G protein to the cell surface? The explanation we favor is that the *ts*O45 mutant stock from which we derived the cDNA clone contained a mixture of viruses carrying many silent mutations. The particular clone we chose then might have contained two of these silent mutations. The revertant of *ts*O45 used in cloning might have arisen from a *ts*O45 virus which did not contain these silent mutations and thus would not actually have reverted at these sites. One of the apparently silent changes, insertion of phenylalanine, was encoded by the insertion of three uridine residues into a stretch of six uridine residues. The mechanism of polyadenylation by the VSV polymerase is apparently reiterative copying of a stretch of seven uridine residues at intercistronic junctions (22, 25). We propose that during replication of the negative strand complement of the G protein gene, the VSV polymerase may frequently add extra adenosine residues when copying this intragenic sequence of six uridine residues. In support of this model, we obtained one cDNA clone of the *ts*O45 mRNA which contained eight uridine

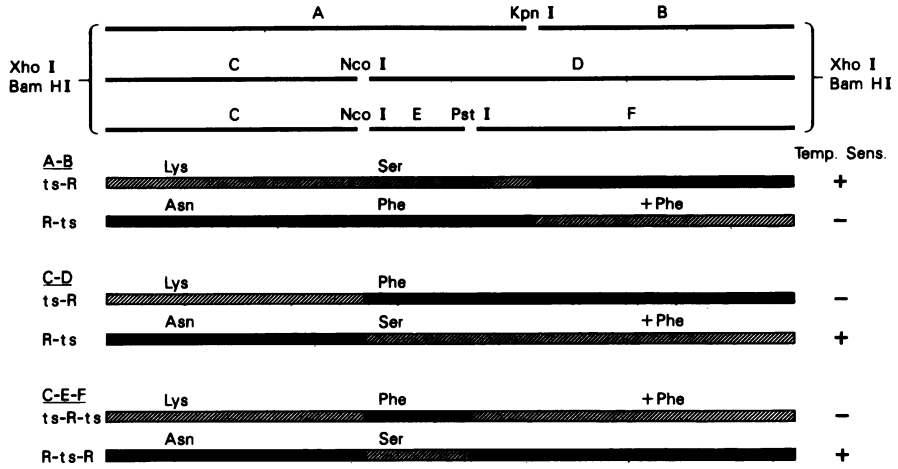

FIG. 3. Mapping of the critical mutation in *ts*O45. Restriction fragments of DNA encoding G proteins from *ts*O45 and the *ts*O45 revertant (derived from pSVO45-2 and pSVO45R) are represented by the lines flanked by *Bam*HI and *Xho*I sites. These fragments (designated A through F) were used to generate the reciprocal recombinants diagrammed below. The predicted amino acid sequence differences between G proteins encoded by each pair of reciprocal recombinants are indicated. Fragments derived from pSVO45R are indicated by solid bars, and fragments derived from pSVO45-2 are indicated by hatched bars. Constructions involved three- and four-part DNA ligations into a modified expression vector as described in the text. Recombinant plasmid DNAs were then transfected onto COS-1 cells. In each case, the temperature sensitivity indicated was assayed both by indirect immunofluorescence and by analysis of the kinetics of oligosaccharide processing.

residues (a frameshift mutation) at this position in the G gene. Also, we found that the revertant strain contained an additional uridine residue in a sequence of five uridine residues within the 3'-noncoding region (nucleotide 1660 [Fig. 1]).

An alternative explanation for the three amino acid changes is that the two apparently silent reversion events affect some important aspect of G protein function other than transport to the cell surface. However, only the critical phenylalanine residue is conserved between the two VSV serotypes, suggesting that this explanation is less likely (8).

Hydrophilic stretches of amino acids have a high probability of being exposed on the surface of native proteins, whereas hydrophobic stretches of amino acids are likely to be buried (for a review, see Kyte and Doolittle, reference 15). An analysis of the local hydrophobicity profile along the G protein sequence by the method of Kyte and Doolittle (15) has been reported previously (7). Interestingly, the mutation responsible for the G protein transport defect occurs in one of the most hydrophobic regions of the protein other than the transmembrane domain or signal sequence. This mutation, a substitution of a polar amino acid (serine) for a hydrophobic amino acid (phenylalanine) presumably alters significantly the protein folding in this region. We suggest that because of this change, the mutant protein may be denatured at the nonpermissive temperature and that this denaturation prevents further transport to the Golgi apparatus and the cell surface. Alternatively, the conformational change in the protein may be more subtle, perhaps preventing recognition by some accessory protein involved in transport.

Although many temperature-sensitive mutants affected in viral glycoprotein transport have been isolated, there is only one published report (1) on the sequence changes responsible for the transport defect, and no direct expression of cDNA clones encoding these mutant proteins has been reported. Sequence analysis of RNA from two mutants of Sindbis virus with defects in glycoprotein transport (1) has shown several amino acid sequence differences between the mutant and parent glycoproteins. Sequence analysis of one revertant has shown that two amino acids were changed back to the wild-type sequence, but it was not clear whether one or both of the changes were required. In the other case, apparently only one amino acid change occurred in the revertant.

Among nonviral proteins, there is one case in which an amino acid change responsible for a transport defect has been identified. A single amino acid near the amino terminus of a lambda$_2$ immunoglobulin chain is apparently sufficient to block its secretion (34), although direct expression of the sequenced gene was not reported.

In vitro mutagenesis and expression of the VSV glycoprotein gene has been used to show that the structure of the 29-amino-acid cytoplasmic domain is critical for a high rate of transfer from the rER to the Golgi apparatus (28). Elimination of the normal cytoplasmic domain can even block transfer completely, suggesting that this domain may be critical for recognition by a transport factor. The analysis of the *ts*O45 mutant presented here shows that the structure of the portion of the protein within the lumen of the rER can also play a critical role in blocking protein transport.

It is well documented that some glycoproteins require carbohydrate for transport to the cell surface, whereas others do not or have a less stringent requirement (13). This situation has even been observed for VSV glycoproteins of different strains. In the presence of tunicamycin, an inhibitor of glycosylation, no detectable G protein is found at the surface of cells infected with the San Juan strain of VSV at 30°C (18). Furthermore, the unglycosylated G protein appears in an aggregated form in the rER (10). However, the requirement for carbohydrate in cell surface transport of the G protein from the Orsay strain of VSV (the sequence reported here) is much less stringent at 30°C (10). At 37°C, however, G proteins from both strains show a stringent requirement for carbohydrate. These results suggest that carbohydrate plays a role in maintaining the conformation of the protein and that sequence changes in the G protein of the Orsay strain might lessen the need for carbohydrate.

Because oligosaccharides are hydrophilic and occur exposed on protein surfaces, one might imagine a model in which the requirement for carbohydrate could be lessened

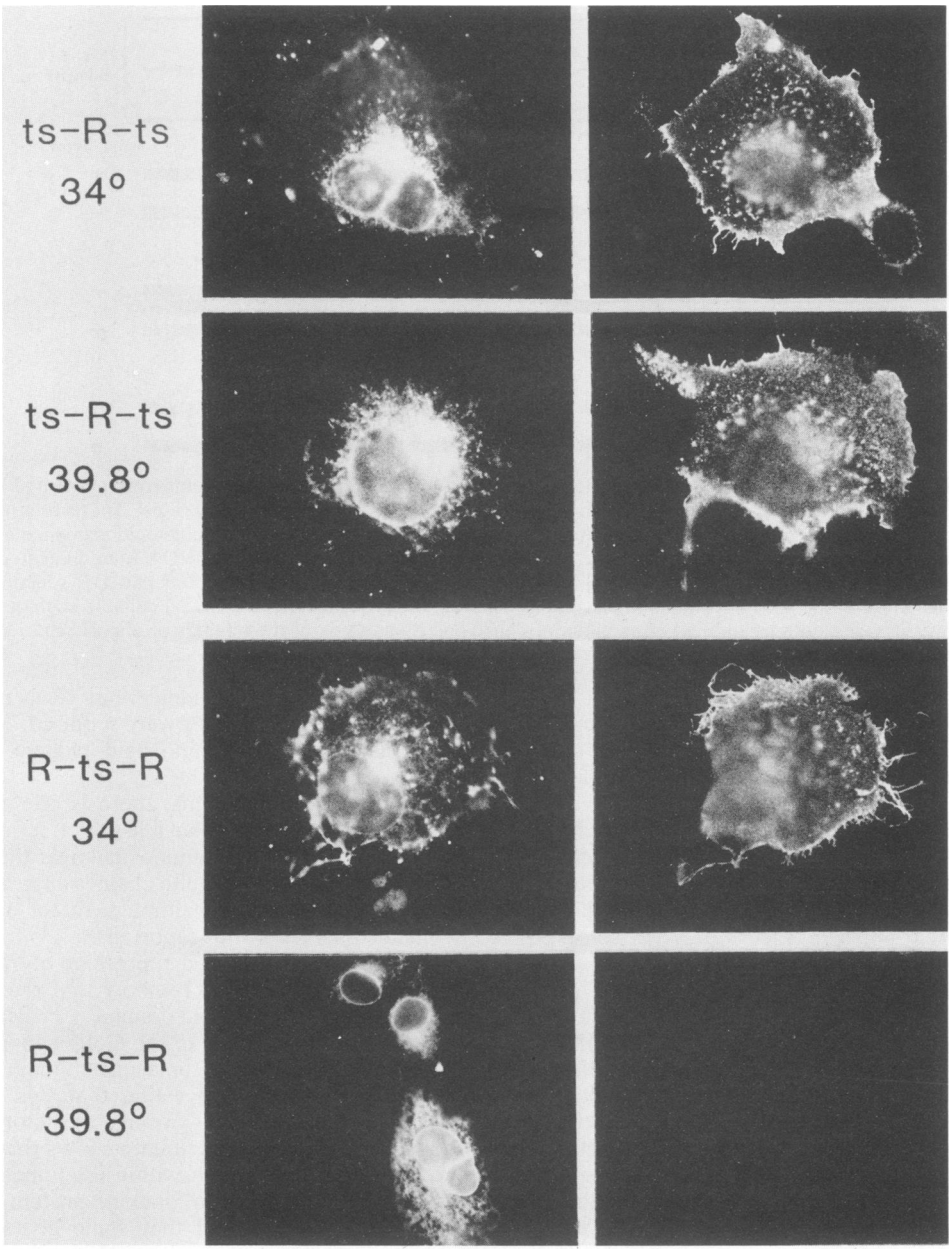

FIG. 4. Indirect immunofluorescence showing temperature-sensitive transport of G proteins encoded by reciprocal recombinants. The plasmid DNAs encoding the recombinant G proteins designated ts-R-ts and R-ts-R (Fig. 3) were transfected onto COS-1 cells as described in the legend to Fig. 2. Cells were maintained at the indicated temperatures for 48 h before fixation and staining. Surface (panels on the right) and internal (panels on the left) staining for the G protein was carried out as described in the legend to Fig. 2.

by amino acid changes which rendered the protein more hydrophilic, especially in the carbohydrate-containing domains. We found nine amino acid sequence differences between the Orsay and San Juan strains in the extracellular domain of G protein (Table 1). The most dramatic change in hydrophilic character was the conversion of tyrosine in the San Juan strain to aspartic acid in the Orsay strain at amino acid 172, which is only six amino acids from the first glycosylation site (asparagine 179). If the first glycosylation site were critical in maintaining protein folding, the addition of a negative charge might be important in lessening the need for carbohydrate. Of the remaining eight changes, three do not change amino acid character, and three render the Orsay glycoprotein more hydrophilic (including the addition of another charged residue at position 231). Only two changes reduce the hydrophobic character of the San Juan glycoprotein. The only change in the primary sequence near the second glycosylation site (Asn 335) is a substitution in the Orsay strain of a more hydrophobic residue (valine for alanine) at amino acid 330. Finally, there is one change in the cytoplasmic domain (amino acid 487) which presumably does not affect the requirement for carbohydrate.

We have reported here the direct expression of a cDNA clone encoding a glycoprotein with a temperature-sensitive transport defect. Our results illustrate that by using cDNA cloning and expression systems it is possible to identify a

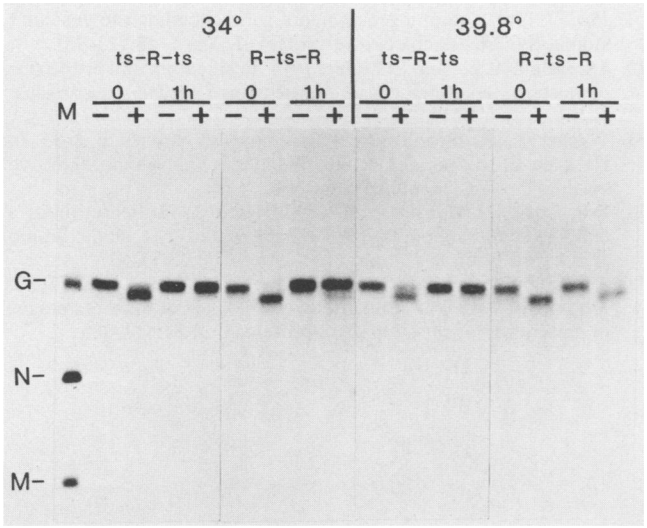

FIG. 5. Kinetics of acquisition of endo H resistance. Plates (5-cm diameter) containing about 10^6 COS-1 cells were transfected with 10 μg of plasmid DNA encoding the indicated recombinant G proteins (Fig. 3). After 40 h at 37°C, the cells were transferred to the indicated temperatures for 30 min and pulse-labeled for 30 min with 50 μCi of [^{35}S]methionine per ml. The cells were either harvested at this time or incubated in chase medium containing 10 mM methionine for an additional 1 h. Cell-associated proteins were then subjected to immunoprecipitation with rabbit anti-VSV serum and fixed *Staphylococcus aureus* as described previously (28). Immunoprecipitates were divided in half, and half was digested with endo H (28, 32). These digested (+) and undigested samples (−) were then electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel (16). An autoradiogram (2-day exposure) of the fluorographed gel is shown. VSV protein markers are shown in lane M.

critical mutation affecting protein transport within a high background of apparently silent mutations. Further analysis of mutants by this approach should tell us whether mutations affecting G protein transport are restricted to specific domains. Use of this system, combined with oligonucleotide-directed mutagenesis, should make it possible to determine whether the relative lack of carbohydrate requirement in the Orsay strain is due to multiple changes or perhaps to just the single amino acid change near the first glycosylation site.

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