Mutations in the C-Terminal Hydrophobic Domain of Pseudorabies Virus gIII Affect both Membrane Anchoring and Protein Export

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The transmembrane and anchor region of pseudorabies virus gIII is postulated to be in the 35 hydrophobic amino acids (residues 436 to 470) found near the carboxy terminus of the 479-amino-acid envelope protein. In this study, we used a genetic approach to localize the functional gIII membrane anchor between amino acids 443 and 466. Mutant gIII proteins lacking the membrane anchor were not associated with virus particles, indicating that membrane retention is a prerequisite for virion localization. Unexpectedly, the specific hydrophobic gIII sequence defined by these deletions was not required for membrane anchor function since the entire region could be replaced with leucine residues without affecting gIII membrane retention, export, or virion localization. The hydrophobic region appears to encode more than the membrane anchor domain since both efficiency of posttranslational processing and localization to virions are affected by mutations in this region. We speculate that the composition of the hydrophobic domain influences the overall conformation of gIII, which in turn effects the efficiency of gIII export and processing. The virion localization phenotype is probably indirect and reflects the efficiency of protein processing. This conclusion provides insight into the mechanism of glycoprotein incorporation into virions.

Members of the alphaherpesvirus family contain a variety of glycoproteins within the virus envelope. The virally encoded glycoproteins are known to play strategic roles in the virus life cycle including adsorption, fusion, and entry into cells as well as release of progeny virus from cells (4, 12, 20, 24, 44). Many enveloped RNA viruses, such as influenza virus (32, 42) and vesicular stomatitis virus (2, 5), contain only one or two glycoproteins to carry out many of these same functions. The complexity of the herpesvirus envelope and the biological functions of the glycoproteins, therefore, are of particular interest.

In a herpesvirus-infected cell, the newly synthesized viral glycoproteins not only assemble into virus particles but also are localized to most host cell membranes including the endoplasmic reticulum (ER), Golgi apparatus, and the cell surface. Virus particle assembly and virion egress as well as intracellular localization of the viral glycoproteins require exploitation of the cellular export machinery. Currently, a clear definition of the host and virus contributions to efficient trafficking of viral glycoproteins between the virus assembly and export pathways is not available.

We have been studying the gIII envelope glycoprotein of pseudorabies virus (PRV) to gain insight into the signals required for virion and cellular localization of herpesvirus envelope glycoproteins. gIII is not required for viral infection in culture, is homologous to herpes simplex virus type 1 gC, and is conserved among all members of the alphaherpesvirus family isolated and sequenced to date (11, 26, 27, 37). The sequenced gIII homologs contain two hydrophobic domains, one at the amino terminus, predicted to be a signal sequence, and a carboxy-terminal hydrophobic domain predicted to be involved in membrane anchoring. Ryan and colleagues (10, 29) have demonstrated that the 22-amino-acid N-terminal hydrophobic domain of PRV gIII indeed functions as a signal sequence.

The subject of this report is the gIII membrane-anchoring domain predicted to be contained within a 35-amino-acid C-terminal hydrophobic region spanning residues 436 to 470 (Fig. 1A). Transmembrane regions of membrane-bound proteins are typically composed of 20 to 24 hydrophobic amino acids arranged in an alpha-helical structure, which is of sufficient length to span a 3.0-nm lipid bilayer (9). Because of the unusual length of the gIII C-terminal hydrophobic domain (35 amino acids), it was of interest to determine what part of this region, if any, plays a role in gIII membrane retention. Hydrophobicity analysis (19) and consensus tables of amino acids found in known transmembrane segments predicted that the gIII transmembrane domain would span residues 447 to 470 (Fig. 1B). The predicted association of gIII with the membrane would result in a C-terminal hydrophilic extension of 9 amino acids (the putative cytoplasmic tail). We have shown in a recent study that gIII mutants lacking these 9 amino acids were indistinguishable from wild-type gIII in membrane retention, export, and virion localization (34).

Our approach to define the membrane-spanning domain of gIII involved examining the role of the entire 35-amino-acid hydrophobic region in gIII export and virion localization. To do so, we constructed viruses carrying defined mutations within the gIII hydrophobic domain and determined whether the mutant proteins were secreted into the medium. This phenotype characterized mutants with a defect in membrane anchoring. We also determined whether the mutant proteins were found in virus envelopes and calculated the rate of export from the ER to the Golgi apparatus as seen by posttranslational processing (29). Using these methods, we showed that the gIII membrane anchor domain is contained within sequences 443 to 466. Our results also demonstrated that this specific sequence is not required for membrane anchor function since the entire region could be replaced with leucine residues without affecting gIII membrane retention, virion localization, or export.

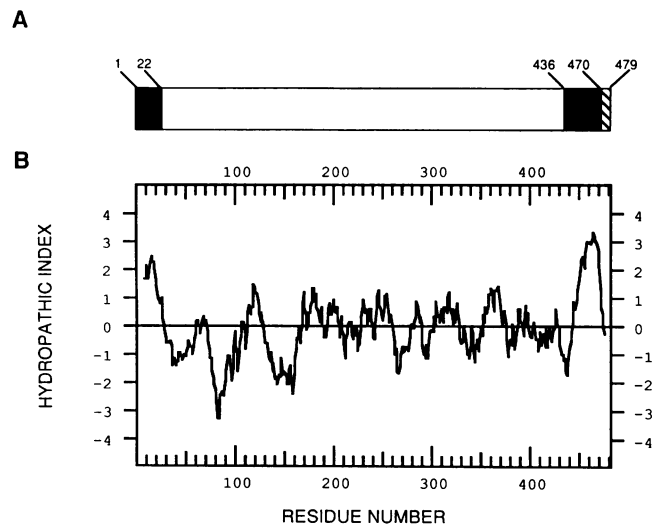* Corresponding author.

A

B

FIG. 1. (A) Domains of the PRV gIII glycoprotein. The regions of gIII containing an uninterrupted stretch of hydrophobic amino acids are depicted with a black box. The putative cytoplasmic domain is shown as a hatched box. The length of these regions as well as the length of the entire gIII protein (in number of amino acids) is indicated above the diagram. (B) Sequence of PRV gIII analyzed by using the Kyte and Doolittle (19) hydropathy scale with a window of 9 amino acids with smoothing. The residue numbers are indicated on the horizontal axis, and the relative hydropathic index is plotted on the vertical axis. Points above 0 are hydrophobic and those below 0 are hydrophilic. The C-terminal hydrophobic peak of the profile corresponds to amino acids 447 to 470, which is therefore predicted to be the gIII transmembrane domain.

MATERIALS AND METHODS

Cell, viruses, and DNA. PK15 swine kidney cells, the Becker strain of PRV (PRV-Be) and PRV2, and *Escherichia coli* PR382 and NF1829 have been described previously (26, 27, 34). The starting plasmid, pIG1, contained the wild-type gIII gene plus approximately 1,000 bp of downstream flanking PRV sequence and the M13 intergenic region for production of single-stranded DNA directly from the pIG1 plasmid without requiring subcloning into the M13 bacteriophage.

Oligonucleotide-directed mutagenesis. The method of Kunkel (18) was followed, using the Bio-Rad Muta-gene in vitro mutagenesis kit as described previously. Uracil-containing single-stranded DNA was obtained by transforming the *dut ung* double-mutant strain PR382 with the double-stranded plasmid pIG1 and infecting with a suitable M13 helper phage (R408). The single-stranded DNA was used as a template to convert the TAC tyrosine codon at residue 436 to a TAG amber codon. The mutagenic primer used to generate this mutation contained the sequence 5'-GGAGG CGTCCTAGCGCTGCGT-3'. The presence of the mutation was confirmed by sequencing the mutagenized DNA from the gIII poly(A) site up to the *Xho*I site (nucleotide 1258). The mutated region was subcloned into parent plasmid as previously described (34) to eliminate possible second-site mutations that may have been generated as a result of the mutagenesis. The resultant plasmid was designated pKS1007.

Two deletion mutations were made by creating unique restriction sites on either side of the region to be deleted. One deletion was made by generating two *Bcl*I sites between gIII nucleotides 1328 to 1333 and 1397 to 1402, using the

mutagenic primers 5'-CACGACGGGCCTGATCACGGA CGC-3' and 5'-CGCCATGCTCACCAGCACG-3', respectively. The resultant plasmid, pKS1004, was digested with *Bcl*I and religated to produce a mutant gIII gene deleted of nucleotides 1328 to 1402. The mutated region was subcloned into parent pIG1 plasmid and designated pKS1005. A second deletion mutant was generated by preparing uracil-containing single-stranded DNA of pKS1005 and using the DNA as a template to generate a *Bam*HI site between nucleotides 1304 and 1309. The following mutagenic primer was used to generate this mutation: 5'-CGCGGGGGAGGCGTGGATC-CGCTGCGTGTCCTC-3'. Subsequently, the resultant plasmid (pKS1008) was digested with *Bam*HI and *Bcl*I and religated to produce a mutant gIII gene deleted of nucleotides 1304 to 1402. The mutated region was subcloned into the parent pIG1 plasmid and was designated pKS1009.

A third deletion mutant was constructed by Calvin Keeler. He digested the pALM3 plasmid (27) with *Hind*III and *Sau*3A or *Hind*III and *Bam*HI. Upon religation of the appropriate purified fragments, a plasmid was isolated containing a gIII gene with a deletion of nucleotides 1357 to 1380. The deletion was verified by sequencing the plasmid. This deletion removed 8 amino acids (residues 453 to 460) from the gIII protein. This plasmid was designated pALM54.

Two deletion-substitution mutations were made in gIII in which the wild-type gIII hydrophobic sequences were removed and replaced with a polyleucine tract. The first contained a deletion of 23 amino acids (residues 443 to 467), and the second contained a deletion of 31 amino acids (residues 436 to 467). The deletion and substitution of the polyleucine tract were accomplished as follows. Two linkers were synthesized so that after annealing with each other they formed a 63-bp oligomer containing *Bam*HI ends and encoding 1 isoleucine residue and 20 leucine residues. This linker was ligated into *Bcl*I-cut pKS1004 and *Bcl*I-*Bam*HI-digested pKS1008. The resultant plasmids were designated pKS1006 and pKS1010, respectively.

All restriction digests, DNA ligations, agarose gel electrophoresis, and gel purification of DNA fragments were done essentially as described by Maniatis et al. (23). The nucleotide sequence of the mutated regions was determined by the dideoxynucleotide-chain termination method of Sanger et al. (30), using the Sequenase kit (U.S. Biochemical Corp.). Restriction enzyme and DNA-modifying enzymes were purchased from Bethesda Research Laboratories and New England Biolabs.

Isolation of recombinant viruses. Recombinant viruses were obtained by cotransfection of PK15 cells with 1 μg of plasmid DNA (*Nco*I-digested pALM54, pKS1005, pKS1006, pKS1009, or pKS1010) and 2 μg of PRV2 genomic DNA essentially as described by Robbins and colleagues (27). PRV2 lacks the internal 402-bp *Sac*I fragment of the gIII gene and expresses a mutant gIII protein that does not react with the gIII-specific monoclonal antibody, M1. Recombinant viruses with the PRV2 gIII gene replaced with the gIII gene carried by the plasmid could be identified by the black plaque assay and the M1 monoclonal antibody (15, 33). Viral plaques that reacted with the M1 monospecific antibody (black plaques) were plaque purified twice and analyzed further.

Purification and analysis of viral DNA. Viral DNA was isolated from cells infected with the recombinant virus by standard techniques (1). After digestion with appropriate restriction enzymes, the gIII-specific DNA fragments were analyzed by the Southern blot method (35) with a nick-translated, gIII-specific probe labeled with [α-^{32}P]deoxyri-

bonucleotide triphosphates. Recombinant viruses containing hydrophobic-domain mutations were named according to the pKS and pALM plasmid from which they were derived, i.e., PRV1007 contains the nonsense mutation at residue 436 in gIII that was carried on plasmid pKS1007, etc. (see Table 1).

Steady-state labeling experiments. PK15 cells were infected at a multiplicity of infection of 5 with PRV-Be or each of the mutant viral strains and labeled continuously for 16 h in the presence of 55 μCi of [³H]glucosamine per ml. Cultures were separated into three fractions: (i) infected cells washed free of extracellular virions, (ii) extracellular virions, and (iii) virion-free media essentially as previously described (1, 29), except that virions were subjected to a second 30% sucrose cushion purification step before further analysis. The gIII envelope glycoproteins were immunoprecipitated from each fraction with the 282 goat polyclonal serum that recognizes both the precursor and mature forms of the gIII glycoprotein (26). All infected-cell fractions were boiled with sodium dodecyl sulfate (SDS) and dithiothreitol before immunoprecipitation with 282 serum. The relative efficiency of infection and the integrity of fractions were determined by using the PRV gII-specific goat polyvalent serum, 284, to immunoprecipitate the unrelated envelope glycoprotein gII from each fraction (41).

Pulse-chase labeling experiments. The protocol for pulse-chase analysis has been described previously (29). Briefly, 60-mm plastic dishes containing PK15 cells (4 × 10⁶) were infected at a multiplicity of infection of 10 with the designated virus. At 5.5 h postinfection, the cells were starved for cysteine and methionine. At 6 h postinfection, 100 μCi of [³⁵S]cysteine in 0.5 ml was added to the cells for 2 min, at which time the radiolabel was removed and the cells were incubated with excess nonradioactive cysteine for various times. Infected-cell monolayers and medium fractions were harvested at the appropriate times and the gIII-specific proteins were immunoprecipitated with the gIII-specific antiserum, 282.

Polyacrylamide gel electrophoresis. All immunoprecipitations were fractionated by electrophoresis with SDS–10% polyacrylamide slab gels. Fluorography was conducted with sodium salicylate and then by autoradiography (6). Densitometric analysis of autoradiographs was done with an LKB 2222-020 Ultroscan XL laser densitometer.

RESULTS

Membrane retention and virion localization require the gIII C-terminal hydrophobic domain. To test the prediction that the C-terminal 35-amino-acid hydrophobic domain of gIII contains the transmembrane or membrane anchor signal, we changed the tyrosine at residue 436 (the first amino acid of the hydrophobic domain) to a termination codon (TAG). The virus carrying this gIII mutation is PRV1007. Our expectation was that if the hydrophobic domain contained the membrane anchor function, the mutant protein should be secreted from cells into the media. As shown in Fig. 2B, the media of infected cell cultures contained a significant amount of the truncated gIII protein, indicating that the mutation had indeed affected the ability of gIII to be retained in cellular membranes. Analysis of purified virions (Fig. 2C) indicated that the mutant gIII glycoprotein was not found in virus particles. Comparison of the amount of gII immunoprecipitated from purified PRV-Be and PRV1007 virions indicated that this effect was not due to low recovery of PRV1007 virus particles (Fig. 2C, lanes 284).
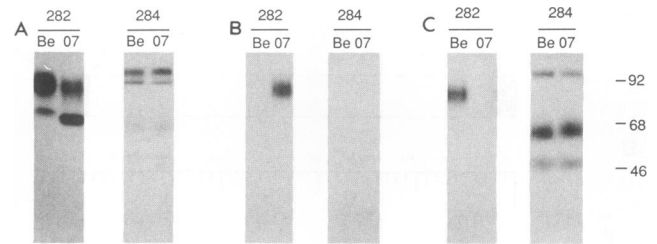
As seen in Fig. 2A, both the precursor and mature forms

FIG. 2. Localization of PRV-Be and PRV1007 gIII proteins during steady-state radiolabeling conditions. PK15 cells were infected at an multiplicity of infection of 5 with parental and mutant virus and labeled with [³H]glucosamine for 16 h. The cultures were divided into cell (A), medium (B), and virion (C) fractions as described in Materials and Methods, and aliquots of each fraction were subjected to immunoprecipitation with either the gIII-specific polyclonal antiserum (282) or the gII-specific polyclonal antiserum (284). Immunoprecipitates were resolved on an SDS–10% polyacrylamide gel, and the ³H-labeled proteins were visualized by fluorography. The infecting virus strains are indicated above each lane: Be, PRV-Be; and 07, PRV1007. The positions of molecular mass standards (in kilodaltons) are shown at right.

of the wild-type and mutant gIII proteins were observed. The level of mature PRV1007 gIII protein in infected cells, however, was lower than that of wild-type gIII. This observation most likely reflects the release of mature PRV1007 gIII protein into the medium. As predicted, the precursor and mature forms of the PRV1007 gIII protein exhibited an increase in electrophoretic mobility owing to the truncation of the gIII protein at amino acid 436.

Compared with export of wild-type gIII protein, export is slower for a mutant gIII protein lacking the entire hydrophobic domain and cytoplasmic tail. Conversion of the gIII precursor (containing high-mannose glycosylation) to the mature form (containing complex glycosylation) is accompanied by a distinct shift in electrophoretic mobility. Furthermore, since this conversion requires transport from the ER to the correct compartment within the Golgi apparatus, kinetics of glycoprotein processing serves as a measure of the efficiency of gIII export. Figure 3A demonstrates that the processing of the gIII protein lacking the C-terminal hydrophobic domain and cytoplasmic tail was slower than that of wild-type gIII. Conversion of the precursor form to the mature form of the protein did not begin to occur until 45 min into the chase period compared with 30 min for wild-type gIII. Furthermore, the rate of processing of this mutant gIII protein, expressed as the time at which 50% of the precursor form was converted to mature form ($T_{1/2}$), was 65 min, about 25 min slower than wild-type gIII (Fig. 3B). The maximum precipitable amount of the intracellular mature form of the PRV1007 gIII protein was observed at the 45-min chase point. This signal subsequently decreased at later chase points which correlated directly with the appearance of the mature protein in the medium. Analysis of the medium fractions indicated that the majority of the mature PRV1007 gIII protein was secreted into the medium by 60 to 90 min after the labeling period. Despite a reduced rate of export following removal of the hydrophobic domain and cytoplasmic tail, the extent of processing, as determined by the amount of precursor left at the 120-min chase point, was relatively unaffected (Fig. 3B).

Hydrophobicity, but not specific amino acid sequence, is sufficient for membrane retention. Computer analysis (19) and consensus tables of known transmembrane domains
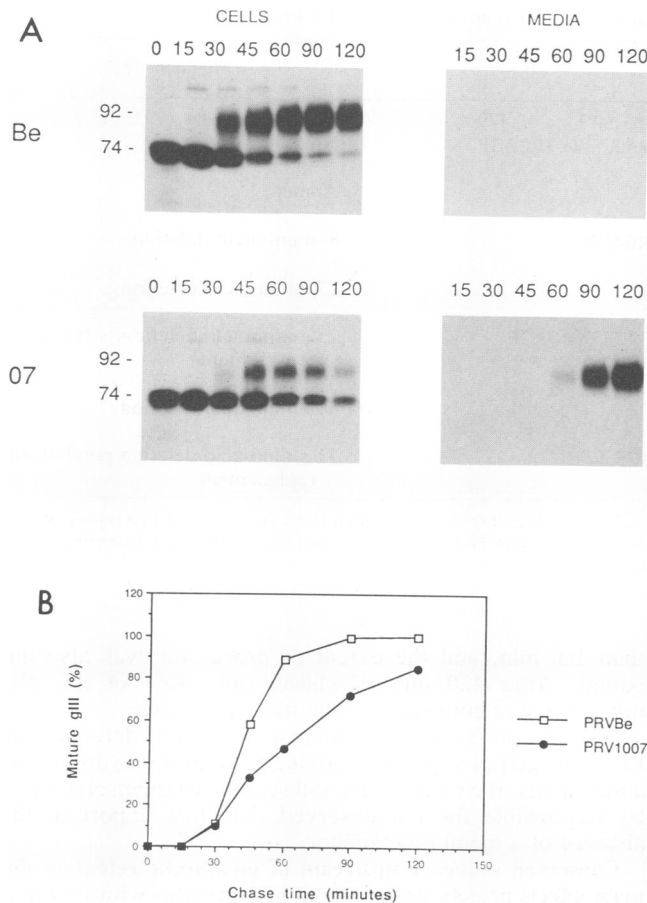
FIG. 3. (A) Pulse-chase analysis of gIII expressed from PRV-Be or PRV1007. PK15 cells (infected at a multiplicity of infection of 10) were pulse-labeled at 6 h postinfection with [^{35}S]cysteine for 2 min and then incubated in the presence of excess nonradioactive cysteine and methionine. At the chase times indicated (in minutes) above each lane, the infected-cell and medium fractions were harvested and the gIII species were immunoprecipitated with 282 antiserum. Immunoprecipitations were then resolved on an SDS–10% polyacrylamide gel and visualized by fluorography. The infecting viral strains (Be, 07) are indicated at the left of each panel. The 74-kDa (precursor) and 92-kDa (mature) forms of gIII are indicated. (B) To quantitate the amounts of precursor and mature forms of gIII at each time point in the pulse-chase experiment, we scanned the autoradiographs by densitometry. The amount of mature gIII (percent) present at each time point (including the amount present in the medium fractions) was calculated by the following equation: [total 92 kDa/(total 74 kDa + 92 kDa)] × 100. From the graph, the rate of gIII precursor to mature processing is measured as the time at which 50% of the precursor form is converted to the mature form ($T_{1/2}$). The extent of processing is measured by the amount of precursor left at the 120-min chase point. The curve corresponding to each of the virus strains is indicated.

predicted the transmembrane domain of gIII to be contained within the stretch of 24 amino acids spanning residues 447 to 470 (Fig. 1B). We constructed a series of gIII mutations in this region to ask two questions. First, is the computer prediction of the putative transmembrane domain correct, and second, does gIII membrane retention and virion localization depend on specific amino acid sequences within the transmembrane domain?

We addressed the first question by constructing two deletions within the proposed transmembrane domain of gIII (Table 1). The mutant gIII protein produced from viral strain PRV54 contained a deletion of 8 amino acids (453 to 460), and the PRV1005 mutant gIII protein contained a deletion of 23 hydrophobic amino acids (443 to 465), 19 of which came from the putative transmembrane domain (Table 1).

We addressed the second question by deleting a segment of the hydrophobic domain and replacing it with a polyleucine tract. PRV1006 expresses a gIII protein containing a replacement of 23 hydrophobic amino acids (443 to 465) with a stretch of 20 leucine residues and 1 isoleucine residue (Table 1).

The results of steady-state labeling and fractionation experiments are shown in Fig. 4. The left panels depict proteins precipitated with the gIII-specific 282 serum, and the right panels depict the gII family of proteins precipitated with 284 serum. The gII protein controls for variation in infection and sample loading.

All three viral strains carrying mutations in the transmembrane domain produced normal quantities of precursor and mature gIII in infected cells (Fig. 4A). However, the relative amount of mature intracellular gIII expressed by PRV1005 (23-amino-acid deletion) was lower than the amount expressed by PRV-Be, PRV54, and PRV1006. As can be seen in Fig. 4B, the mature PRV1005 gIII protein is released into the medium, which most likely accounts for the reduced amount of mature protein seen in infected cells. Both the mature and precursor forms of gIII expressed by PRV1005 exhibited an expected increase in electrophoretic mobility owing to the deletion of 23 hydrophobic amino acids.

Deletion of 8 amino acids from the putative transmembrane domain (PRV54) had no effect on the membrane anchor function since no protein is detected in the medium (Fig. 4B, lane 54). The efficiency of localization of the mutant protein to virions, however, was significantly reduced (Fig. 4C, lane 54). Deletion of 23 C-terminal hydrophobic amino acids (PRV1005) produced a strong defect in membrane retention because significant amounts of protein were released into the medium (Fig. 4B, lane 05). In addition, PRV1005 virus particles did not contain the mutant gIII protein (Fig. 4C, lane 05). Significantly, replacing 23 hydrophobic amino acids with 1 isoleucine residue and 20 leucine residues resulted in a mutant gIII protein indistinguishable from the wild-type gIII protein in both membrane retention and virion localization (Fig. 4B and C, lanes Be and 06).

These data strongly support the computer prediction that residues 447 to 470 contain the gIII membrane retention domain. However, this function does not require specific gIII amino acid sequences since a stretch of leucine residues was sufficient to confer membrane retention and virion localization properties to gIII.

Number of hydrophobic residues in membrane anchor domain affects membrane retention and export efficiency. The rate and extent of gIII protein export were determined by the pulse-chase protocol described in Materials and Methods. Viral strains PRV54, PRV1005, and PRV1006 were analyzed, and the data are given in Fig. 5. Deletion of either 8 or 23 amino acids from the hydrophobic domain of gIII caused a reduction in both the rate and extent of gIII processing (Fig. 5). The mature form of the gIII protein expressed by PRV54 and PRV1005 was not obvious until 45 and 60 min into the chase, respectively. Quantitation of the pulse-chase data indicated that the $T_{1/2}$ for processing of the precursor to the mature form of the PRV54 gIII protein was 85 min, 45 min slower than wild type (Fig. 5B). The $T_{1/2}$ of processing of the secreted gIII protein produced from PRV1005 was

TABLE 1. PRV viral strains and C-terminal sequence of wild-type and mutant gIII proteins

Viral strain	gIII C-terminal sequence[a]	Mutation type
PRV-Be	DTQṘ ⁺436 YDASPASVSẆP[VVSSMIVVIAGIGILAIVLVIMAṪ] 471 ⁺ 479 CVYẎRQAGP	None
PRV1077	DTZṘ	Amber
PRV54	DTQṘ YDASPASVSẆP[VVSSMILAIVLVIMAṪ] ⁺ CVYẎRQAGP	8-amino-acid deletion
PRV1005	DTQṘ YDASPAṠ[VIMAṪ] ⁺ CVYẎRQAGP	23-amino-acid deletion
PRV1006	DTQṘ YDASPASVILLLLLLLLLLLLLLLLLLLLLIMAT ⁺ CVYẎRQAGP	23-amino-acid deletion/polyleucine replacement
PRV1009	DTQṘ [IMAṪ] ⁺ CVYẎRQAGP	31-amino-acid deletion
PRV1010	DTQṘ ILLLLLLLLLLLLLLLLLLLLIMAT ⁺ CVYẎRQAGP	31-amino-acid deletion/polyleucine replacement

[a] C-terminal sequence of mutant and wild-type gIII proteins spanning residues 432 to 479. The boxed region within the wild-type sequence represents the predicted membrane-spanning domain of gIII. The boxed regions within the PRV54, PRV1005, and PRV1009 gIII proteins signify nondeleted transmembrane amino acids. Insertion sequences within PRV1006 and PRV1010 gIII proteins are underlined.

greater than 120 min (Fig. 5B), 55 min slower than the secreted gIII protein produced from PRV1007. Furthermore, the extent of processing, as determined by the amount of gIII precursor left at the 120-min chase point, was reduced as a result of the deletion mutations (Fig. 5B). Only 60% of the gIII protein lacking 8 hydrophobic amino acids (PRV54) and 40% of the gIII protein lacking 23 hydrophobic amino acids (PRV1005) was processed to the mature form by the 120-min chase point. The gIII protein produced from PRV54, but not from PRV1005, retained membrane anchor function since the latter but not the former was secreted into the medium (Fig. 5A). Thus, two classes of gIII proteins were made by decreasing the length of the hydrophobic domain; one secreted and another membrane bound. Both classes have significant defects in processing the gIII precursor to the mature species.

The results from the pulse-chase analysis of PRV1006 are also shown in Fig. 5. This mutant gIII protein, which contains a replacement of 23 hydrophobic amino acids with 1 isoleucine residue and 20 leucine residues, exhibited a rate and extent of processing essentially like that of wild-type gIII. These results indicate that the specific sequence encompassing this stretch of 23 hydrophobic amino acids in wild-type gIII is not required for efficient gIII export.

Cytoplasmic tail adversely affects export in mutants lacking membrane retention signals. The export rate of the secreted PRV1005 gIII protein was 55 min slower than that of the secreted PRV1007 gIII protein. Comparison of the sequence of these gIII mutants (Table 1) implied that the export defect exhibited by the PRV1005 gIII protein must be due to all or part of the amino acids remaining in the hydrophobic domain and cytoplasmic tail. We tested this by constructing PRV1009, a virus whose gIII protein lacks 31 amino acids of the hydrophobic region but still retains 14 amino acids from the gIII C terminus (Table 1). As shown in Fig. 6, the gIII protein made by PRV1009 was defective in both the rate and extent of processing, indicating that the deletion did not relieve the PRV1005 gIII-processing defect. Conversion of the PRV1009 gIII precursor to the mature form did not occur until about 60 min into the chase period. The rate of processing, expressed as the time at which 50% of the precursor is converted to the mature form ($T_{1/2}$), was greater

than 120 min, and the extent of processing was also impaired. After 120 min of chase, only 40% of the gIII precursor was converted to the mature species.

Since we were unable to reverse the export defect of the PRV1005 gIII protein by removing most of the hydrophobic amino acids, the cytoplasmic tail sequences themselves may be responsible for the observed defective export in the absence of a membrane anchor.

Conserved sequence upstream of membrane retention domain affects protein processing of gIII proteins with synthetic polyleucine transmembrane domains. PRV1006 expresses an apparently normal gIII protein even though the protein contains a synthetic membrane anchor domain consisting essentially of polyleucine. We noted that at least 8 hydrophobic or neutral amino acids still remained in PRV1006 (residues 436 to 443) and that these residues (YDASPASV) were highly conserved in five of the seven sequenced gC homologs (11). The conserved nature of these sequences implied that they might have a functional role in the hydrophobic region. As described previously, PRV1009 contains a mutant gIII gene lacking essentially all the hydrophobic domain, including the YDASPASV sequence (amino acids 436 to 467). This mutant protein was defective in both the rate and extent of processing and also had a significant defect in membrane retention (Fig. 6). We constructed PRV1010 by replacing the deleted sequence in PRV1009 with 20 leucine residues and 1 isoleucine residue (PRV1010 [Table 1]). We expected that PRV1010 would express a gIII protein with a functional membrane anchor domain and normal export kinetics as we had previously seen with PRV1006.

Unexpectedly, the PRV1010 mutant protein was defective in both the rate and extent of processing (Fig. 7). The $T_{1/2}$ for conversion of precursor to mature gIII was greater than 2 h, compared with 40 min for the gIII protein expressed by PRV1006 (Table 2). Since the gIII protein expressed by PRV1006, which contains a replacement of only 23 hydrophobic amino acids with 1 isoleucine and 20 leucine residues, did not exhibit an export defect, we predicted that all or part of the sequence YDASPASV would be required for gIII to form an export-competent phenotype. However, when we deleted these eight residues from otherwise wild-type gIII, we observed no defect at all in export kinetics or localization
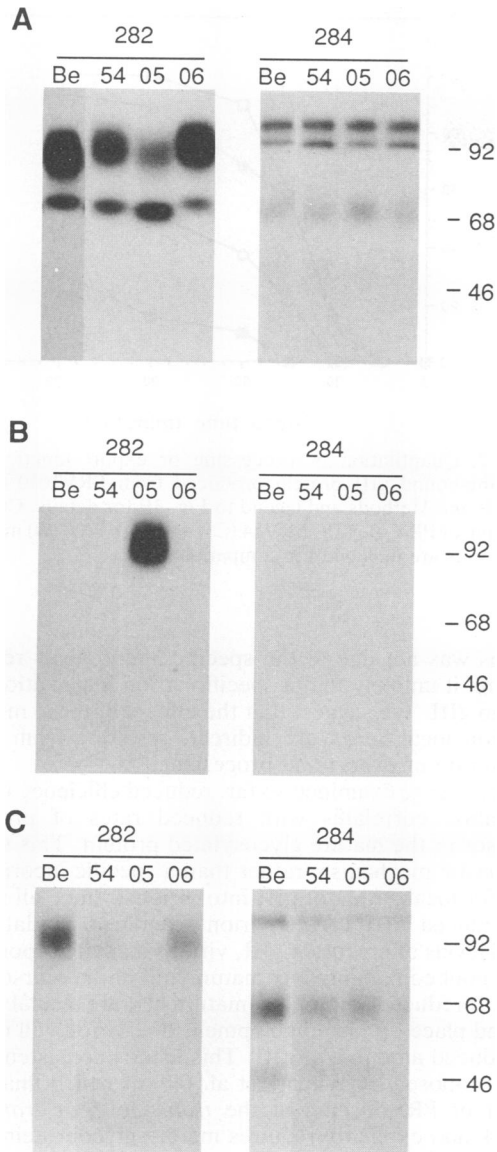
FIG. 4. Localization of wild-type and mutant forms of glycoprotein gIII during steady-state radiolabeling conditions. See Materials and Methods and legend to Fig. 2 for details. Fractions are indicated at upper left. (A) Cells, (B) medium, and (C) virions. The antisera used (282 and 284) are indicated across the top. The following infecting virus stains are indicated above each lane: Be, PRV-Be; 54, PRV54; 05, PRV1005; and 06, PRV1006. The molecular mass markers (in kilodaltons) are shown at the right of each panel.
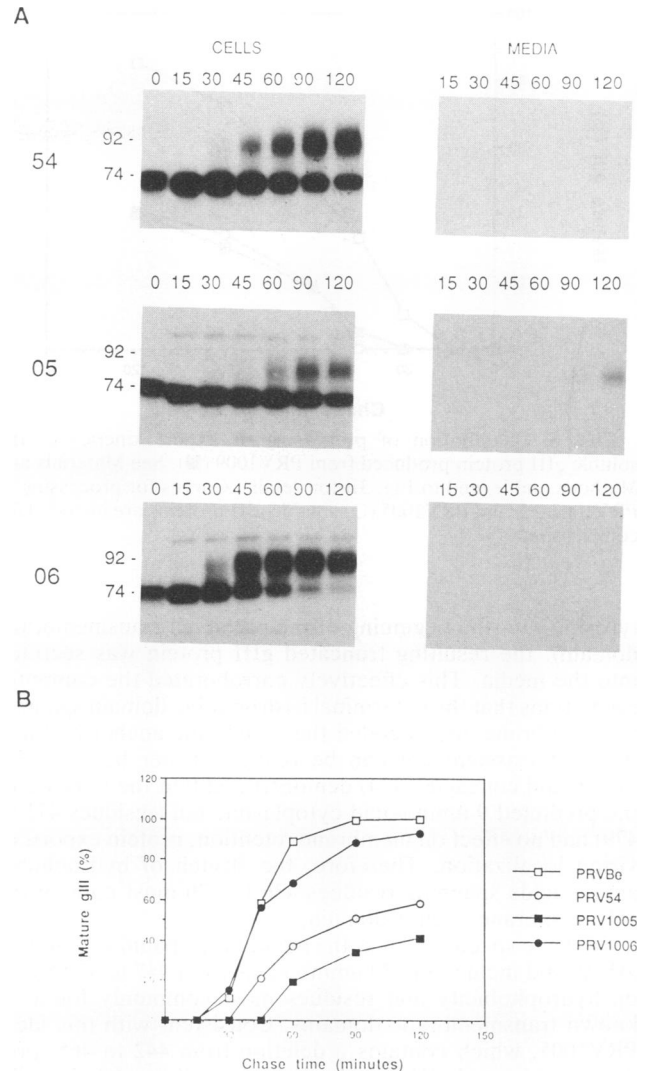

FIG. 5. Pulse-chase analysis of mutant forms of glycoprotein gIII produced from PRV54 (54), PRV1005 (05), and PRV1006 (06) and quantitation. See Materials and Methods and legend to Fig. 3 for details. The chase time (in minutes) is indicated above each lane. The infecting viral strain and the precursor (74-kDa) and mature (92-kDa) forms of gIII are indicated at the left of each panel.

(unpublished observations). At this time, we can only conclude that the YDASPASV sequence is important for export and localization of gIII proteins with polyleucine synthetic transmembrane anchor domains.

DISCUSSION

The gIII envelope glycoprotein of PRV and its homologs contains a large C-terminal hydrophobic domain of 35 amino acids. It is generally accepted that this domain contains a transmembrane region that spans and anchors the protein in a lipid bilayer. Until this report, experimental validation of this prediction was lacking. To span a 3.0-nm lipid bilayer,

the membrane anchor domain of a protein need only be 20 to 24 hydrophobic amino acids arranged in an alpha-helical structure (9). Proteins that span membranes typically contain a stretch of 24 hydrophobic amino acids bordered by positively charged amino acids (3, 25, 38, 39). The gIII glycoprotein is thought to span the membrane once, but the stretch of 35 residues between the residues at positions 435 and 471 is considerably longer than required (Table 1). Our goal in this study was to define the membrane anchor domain by constructing defined mutations in this region. Those mutants secreting gIII into the media were considered to have membrane anchor defects. We also gained some insight into additional functions of the hydrophobic region by measuring the rate of export and the ability of mutant proteins to be localized to virus particles. The phenotypes of the mutant proteins are summarized in Table 2.

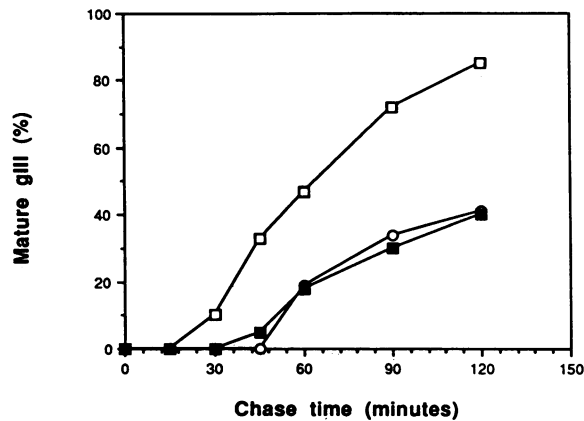When a site-directed nonsense mutation was created at

FIG. 6. Quantitation of processing or export kinetics of the soluble gIII protein produced from PRV1009 (■). See Materials and Methods and legend to Fig. 3B for details. Curves for processing of PRV1007 (□) and PRV1005 (○) soluble gIII proteins are included for comparison.
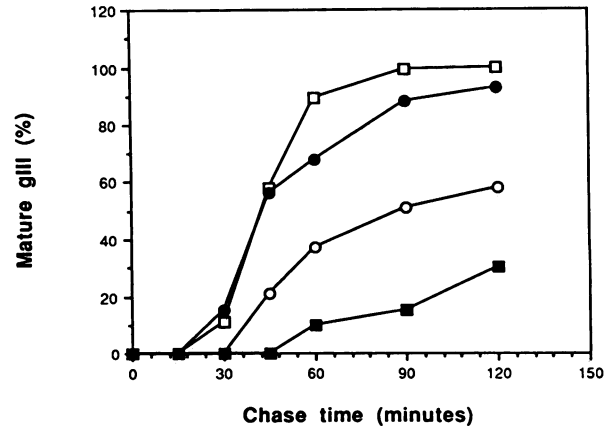

FIG. 7. Quantitation of processing or export kinetics of the membrane-bound gIII protein produced from PRV1010 (■). See Materials and Methods and legend to Fig. 3B for details. Curves for processing of PRV-Be (□), PRV54 (○), and PRV1006 (●) membrane gIII proteins are included for comparison.

tyrosine 436 (the beginning of the predicted transmembrane domain), the resulting truncated gIII protein was secreted into the media. This effectively corroborated the computer predictions that the C-terminal hydrophobic domain spanned the membrane and encoded the membrane-anchoring function. This assignment can be refined further because Solomon and colleagues (34) demonstrated that the removal of the predicted 9-amino-acid cytoplasmic tail (residues 471 to 479) had no effect on membrane retention, protein export, or virion localization. Therefore, the stretch of hydrophobic amino acids spanning residues 436 to 470 must contain the gIII membrane anchor domain.

We have speculated that the membrane-spanning region of gIII would include the 24 amino acids from 447 to 470 based on hydrophobicity and residues most commonly found in known transmembrane domains. Consistent with this idea, PRV1005, which contains a deletion from 442 to 465, produced a secreted gIII protein. Paradoxically, a deletion of 8 amino acids from the center of this region (PRV54) had little or no effect on membrane anchoring, suggesting that adjacent hydrophobic amino acids could compensate. For this to be correct, membrane anchoring should not depend on specific sequences.

A significant finding was that functional membrane anchoring did not depend on the presence of specific amino acids. As noted above, PRV1005 contained a 23-amino-acid deletion resulting in secretion of the gIII protein. Replacement of the 23 hydrophobic gIII amino acids with a synthetic transmembrane domain consisting of 1 isoleucine and 20 leucine residues restored essentially wild-type membrane anchor function (PRV1006). We concluded that the polyleucine tract was sufficient for membrane anchor function in the gIII protein.

In our experience, gIII proteins lacking membrane anchor function are not found in virus particles. For example, PRV1007 virions contain no detectable gIII protein. However, some mutations within the gIII membrane anchor domain that did not overtly affect membrane retention had significant effects on localization to virions (e.g., PRV54 and PRV1010; data not shown). Since the 23-amino-acid polyleucine replacement mutant (PRV1006) was not defective in virion localization, we can conclude that the virion localization defect exhibited by the PRV54 and PRV1010 gIII

proteins was not due to the specific amino acids removed. We think it unlikely that a specific virion localization signal exists in gIII. We suggest that the effects of these mutations on virion localization are indirect, resulting from the decreased rate of export and processing.

In every case examined so far, reduced efficiency of virion localization correlates with reduced rates of processing precursor to the mature glycosylated protein. This suggests a stochastic mechanism rather than a specific incorporation signal for localization of gIII into virions. Since all mutants with reduced gIII in the virion examined to date make normal levels of precursor gIII, virions must incorporate gIII from a pool containing only mature and not precursor forms of gIII. If reduced amounts of mature gIII are available at the time and place of final envelopment, the virion will incorporate reduced amounts of gIII. This idea is consistent with a model proposed by Whealy et al. (40) in which final envelopment of PRV occurs at the trans-Golgi or trans-Golgi network and explicitly requires mature glycoproteins at the site of envelopment.

We do not yet understand why some of the secreted and nonsecreted proteins made by the transmembrane mutants have different rates of processing and export. It is clear that correct tertiary and quaternary structures are required to make most membrane glycoproteins competent for transport out of the ER (7, 8, 13, 17, 22, 28, 31, 43). Proteins that fail to fold correctly are generally retained in the ER and are eventually degraded (16, 21, 28). The rules for export of membrane-bound proteins have been established from studies of proteins that require oligomerization before transport, such as the influenza virus hemagglutinin precursor and the vesicular stomatitis virus G glycoproteins. The gIII envelope glycoprotein does not seem to form oligomers by associating with itself or with any other proteins (unpublished observations). It is possible, however, that membrane-bound monomeric proteins like gIII also require proper tertiary structure for efficient export.

Clearly, the composition of the hydrophobic or transmembrane domain plays a role in determining export or processing efficiency of membrane-bound gIII. PRV1006 and PRV1010 provide a striking example of this. Both mutants have functional, synthetic polyleucine transmembrane do-

TABLE 2. Phenotypes of wild-type and mutant gIII proteins[a]

Viral strain	Mutation	Processing rate ($T_{1/2}$) (min)	Extent of processing (% processed)	Membrane anchoring	Virus envelope
PRV-Be	None	40	100	+++	+++
PRV1007	Amber at 436	65	85	−	−
PRV54	9-amino-acid deletion	85	60	+++	+
PRV1005	23-amino-acid deletion	>120	40	−	−
PRV1006	20-amino-acid polyleucine	40	93	+++	+++
PRV1009	31-amino-acid deletion	>120	40	−	−
PRV1010	20-amino-acid polyleucine	>120	30	+++	+

[a] Rate and extent of processing values were obtained from pulse-chase analysis quantitation curves. Membrane anchoring phenotypes are represented as +++ for membrane bound and − for soluble. Virion envelope localization phenotypes are represented as follows: +++, wild-type levels in virions; (+, reduced levels in virions; −, not detected in virions. The membrane anchoring and virus envelope localization of gIII proteins expressed by PRV1009 and PRV1010 are included in the table even though data are not shown.

mains, but only PRV1010 is defective in export. The only apparent significant difference is that PRV1010 is deleted for the entire hydrophobic region while PRV1006 retains eight gIII residues from this region (YDASPASV, residues 436 to 443). This unexpected observation directed our attention to these amino acids since they were highly conserved in five of seven sequenced gIII homologs (11). When we deleted these amino acids in the wild-type gIII gene, the resultant protein was indistinguishable from wild-type gIII in all respects (unpublished observations). Apparently, this conserved sequence upstream of the membrane retention domain is required for efficient export of a protein with a polyleucine transmembrane domain but has no effect if the protein contains a wild-type gIII transmembrane region. Further work is necessary to understand this phenomenon.

PRV54 presents another example of how composition of the transmembrane region affects export but not membrane-anchoring function. This mutant expresses a gIII protein with an 8-amino-acid deletion in the central portion of the C-terminal hydrophobic segment. The gIII protein expressed by PRV54 is not defective in membrane anchoring but is significantly reduced in rate of export as measured by posttranslational processing. Since the predicted membrane anchor domain could be replaced with nonspecific hydrophobic amino acids (PRV1006), the export defect exhibited by PRV54 cannot be due to the specific amino acids that were removed. Perhaps the export defect is elaborated when new amino acids upstream or downstream of the deletion are used to span the membrane. This abnormal sequestering of sequences in the lipid bilayer may well affect protein structure which in turn may affect protein export and localization.

The export or processing defects of mutants lacking a functional membrane anchor are noteworthy. Conversion of the PRV1007 truncated gIII precursor to the mature species was about 25 min slower than processing of wild-type gIII. Processing of the two gIII deletion mutants produced from PRV1005 and PRV1009 was at least 55 min slower than the truncated gIII protein of PRV1007. It is possible that the differential export rates of the PRV1007, PRV1005, and PRV1009 gIII proteins are due to varying degrees of gIII misfolding. This would imply that the hydrophobic domain is required for gIII to acquire the proper conformation for efficient export. Additionally, this suggests that different forms of soluble misfolded protein exist that are affected in their rates of processing. An alternative explanation would be that the slight reduction in the rate of export of the PRV1007 gIII protein compared with that of wild-type gIII is simply due to bulk fluid flow, which is predicted to be slower than the bulk outward flow of membranes (7, 14, 31, 36), and that the much greater reductions in the kinetics of PRV1005

and PRV1009 gIII export are due to misfolding. Since the only difference between these proteins is the number of amino acids in the C terminus, this possibility would imply that all or part of the C-terminal sequence (IMATCVYY RQAGP) must be causing the export defect of the PRV1005 and PRV1009 gIII proteins. Since the cytoplasmic tail is normally sequestered from the ectodomain of gIII by the transmembrane domain, these tail sequences might adversely affect gIII structure, thereby dramatically decreasing export. Further work is necessary to determine which tail residues, if any, are involved in this export defect.

In conclusion, through genetic analysis we localized a region of the gIII protein required for membrane retention, efficient processing, and virion localization of the PRV gIII envelope glycoprotein. Our work strongly supports the prediction that residues 447 to 470 contain the membrane anchor domain. This domain is not sequence specific since a stretch of polyleucine confers normal membrane retention, virion localization, and processing kinetics to a deletion mutant lacking these functions. Furthermore, we suggest that the hydrophobic region encodes more than the membrane anchor domain since both efficiency of export and localization to virions are affected by mutations in this region. We speculate that efficient export and processing of gIII require proper protein conformation, which is dependent on the overall composition of the hydrophobic domain. Furthermore, we propose that efficient localization of membrane-bound gIII to virions requires efficient gIII export and processing.

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