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Analysis of Pseudorabies Virus Glycoprotein gIII Localization and Modification by Using Novel Infectious Viral Mutants Carrying Unique *Eco*RI Sites

J. PATRICK RYAN, MARY E. WHEALY, ALAN K. ROBBINS, AND LYNN W. ENQUIST*

Central Research and Development Department, E. I. du Pont de Nemours and Co., Experimental Station E328/B13, Wilmington, Delaware 19898

Received 30 March 1987/Accepted 19 June 1987

We have constructed two pseudorabies virus (PRV) mutants, each with a unique *Eco*RI restriction site in the nonessential gIII envelope glycoprotein gene. Since no natural PRV isolate has been reported to contain *Eco*RI sites, the isolation and single-step growth curve analysis of these mutants established that PRV can carry such a site with little ill effect in tissue culture. Virus carrying these defined mutations produced novel gIII proteins that enabled us to begin functional assignment of protein localization information within the gIII gene. Specifically, one viral mutant contained an in-frame synthetic *Eco*RI linker sequence that was flanked on one side by the first one-third of the gIII gene and on the other side by the last one-third of the gene. The resulting protein lacked the middle one-third of the parental species, including five of eight putative N-linked glycosylation signals, but was still glycosylated and found in enveloped virions; it was not secreted into the medium. A second viral mutant contained an in-frame synthetic *Eco*RI linker sequence that additionally specified a nonsense codon at position 158, producing a gIII protein that was glycosylated and secreted into the medium; the fragment was not found in enveloped virions. By endoglycosidase and pulse-chase analyses, we established a precursor-product relationship between the various forms of gIII expressed in the parental and mutant strains, and perhaps determined certain features of the gIII protein that are required for its efficient export within the cell.

Our laboratory has been studying the envelope glycoproteins of the swine herpesvirus, pseudorabies virus (PRV) (26–28). PRV, being a typical herpesvirus, acquires its envelope as capsids bud from the nucleus of the infected cell (3, 31). These budding events into the perinuclear space are thought to occur at inner nuclear membrane "patches," where the virus-encoded glycoproteins are the only proteins present (3). The immature particles, whose glycoproteins have received primary N-linked, high-mannose glycosylation, then enter the lumen of the endoplasmic reticulum. At this stage, egressing virions apparently share the constitutive protein export pathway of the host cell with discrete viral glycoproteins that have been synthesized at the rough endoplasmic reticulum. Evidence exists that both discrete and virion-associated glycoproteins then travel to elements of the Golgi apparatus, where a number of modifications can occur, including alteration of the N-linked sugar side chains to the complex variety, attachment of O-linked oligosaccharides, acylation by fatty acids, and sulfation (17, 18, 31). From experiments with either herpes simplex virus (HSV) (16) or PRV (20) and the ionophore monensin, the egressing virion apparently enters the Golgi apparatus as an intact structure. Monensin, which blocks protein transport from the Golgi apparatus to the cell surface, eliminates cell surface-associated virus particles, as observed by thin-section electron microscopy, and leads to the recovery of virions whose envelope glycoproteins contain an immature, high-mannose form of N-linked glycosylation. From the Golgi apparatus, the individual glycoproteins and virus particles must then travel, perhaps both by vesicular transport, to the plasma membrane, where they either are incorporated and presented on the cell surface (discrete species) or are released from the cell (mature, infectious virions).

We have demonstrated previously that glycoprotein gIII is a PRV envelope component that can be deleted with no apparent effect on virus growth in tissue culture (28). In this report, we present an approach with a cloned gIII gene containing a known deletion mutation used as a vehicle to introduce defined insertions into infectious virus. Our rationale for constructing these mutants was twofold. First, because the Becker strain of PRV contains no *Eco*RI restriction sites within its 150-kilobase-pair (kbp) genome (3), we wanted to establish that PRV could harbor *Eco*RI sites with no detrimental effect to viral growth and gene expression in tissue culture. Second, we sought to use the mutants so constructed to analyze the structure and function of PRV glycoprotein gIII. In particular, our goal was to initiate studies on the relationship of the two predominant glycosylated forms of the gIII protein, how they are transported in the infected cell, how they are localized in specific cell membranes, and the role they play in assembly of virus envelopes.

MATERIALS AND METHODS

Cells and virus. The PK15 cells and the Becker strain of PRV (PRV-Be, our wild-type strain) have been described (27).

Construction of gIII mutants. The general techniques used in this report are described by Robbins et al. (28). Cotransfections were done exactly as described with the gIII-specific monoclonal antibody M1 as a selective and screening agent. Mutant virus were named by the number of the pALM plasmid that was used to make it. For example, PRV1 contains the mutation carried by pALM1; PRV4 carries the mutation specified by pALM4; and so forth.

* Corresponding author.

Since no isolate of PRV is known to contain a natural *Eco*RI restriction site (3), we were unsure whether such sequences could be tolerated by the virus. Therefore, we introduced the restriction sites into a previously characterized mutant gIII allele (28) and crossed the new constructions into PRV-Be, selecting for a mutant gIII phenotype. The general approach was first to add synthetic *Eco*RI linkers to the unique *Sac*I site in a derivative of the bacterial expression plasmid pALM2, where a 402-bp in-frame deletion had been constructed previously (28) and then to introduce the linker/deletion mutations into PRV-Be by cotransfection. Selection was for viruses exhibiting resistance to the gIII monoclonal antibody M1, a phenotype characteristic of PRV2, a gIII mutant carrying just the 402-bp in-frame *Sac*I deletion (28).

To facilitate the construction and analysis of gIII derivatives containing *Eco*RI linkers, we removed the unique *Eco*RI site present in the vector portion of pALM2 by digesting the plasmid with *Eco*RI, filling in the ends with the Klenow fragment of DNA polymerase I in the presence of all four deoxynucleotides, and then rejoining the ends by blunt-end ligation. This derivative of pALM2 was called pALM7.

The plasmids pALM1 and pALM4 were constructed by inserting the following synthetic DNA linkers at the unique *Sac*I site of pALM7:

```
in pALM1:        5'        C GAA TTC G AG CT 3'
                 3' TC GA G CTT AAG C       5'
          Codon:   156 157                292

in pALM4:        5'        C TAG AAT TCA GAG CT 3'
                 3' TC GAG ATC TTA AGT C       5'
          Codon:   156 157 TER
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Both linkers maintain the reading frame of the truncated gIII protein, as indicated by the spacing, and also introduce an *Eco*RI site (5'-GAATTC-3') bracketed by *Sac*I sites. The linker in pALM1 adds 4 extra amino acids to the truncated protein produced by pALM7. The linker in pALM4 introduces an in-frame nonsense codon (5'-TAG-3') immediately after codon 157 in the truncated protein produced by pALM7.

The linkers were synthesized on an Applied Biosystem DNA synthesizer with phosphoramidite chemistry and purified by reverse-phase high-performance liquid chromatography. Each phosphorylated linker was then ligated to *Sac*I-digested pALM7 DNA with T4 DNA ligase.

The structure of each plasmid was verified by restriction analysis and, if possible, protein production. Verification of the linker mutation in pALM1 and pALM4, and of the deletion mutation in pALM2, was obtained by DNA sequencing by the dideoxy sequencing method as modified by Zagursky et al. (34). The sequencing primer, 5'-CGTCACG-TTCACCAC-3', was annealed to the noncoding strand of purified double-stranded plasmid DNA 3' to the unique *Sac*I site of each plasmid.

For all of the procedures described, the pALM plasmids were maintained and propagated in *Escherichia coli* NF1829 (26).

DNA and RNA analysis. The techniques of Southern and Northern blot analysis as well as in vitro translation used are described and referenced in Robbins et al. (28).

Single-step growth curves. For each time point to be studied, PK15 cells in 60-mm dishes were infected at 37°C with either PRV-Be or mutant virus at a multiplicity of infection (MOI) of 5. For one dish of each set, the contents were fractionated and titers were determined (see below) immediately following a 1-h incubation period. For the rest of the dishes following this period, virus was removed, and the monolayers were washed with phosphate-buffered saline (PBS) and overlaid with Dulbecco modified Eagle medium (DMEM) containing 2% heat-inactivated fetal bovine serum (maintenance medium). Subsequently, virus titers were determined at 2-h intervals continuing through 15 h of infection; a 24-h postinfection time point titer was also determined. For each time point, the culture medium from an infection was used to rinse the infected-cell monolayer by vigorous pipetting and was then removed. The monolayer was further rinsed with PBS and harvested by scraping into maintenance medium. Both fractions were briefly sonicated before titers were determined by standard means.

Antibody reagents and immunoprecipitation procedure for PRV glycoproteins. The antisera used in these studies included: mouse monoclonal M1 (reactive with native but not denatured gIII) (13); mouse monoclonals M2 and M3 (reactive with native but not denatured gII) (13); and two sera prepared against a denatured, *E. coli*-produced Cro-gIII fusion protein (rabbit polyvalent 490 serum [28] and goat polyvalent 282 serum, each reactive with denatured gIII antigen).

The preparation of cell extracts and the immunoprecipitation procedure have been described (27).

Infected-cell, virion, and medium fractionation. PK15 cells were infected at an MOI of 10 with either PRV-Be or each of the mutant viruses and labeled continuously throughout a 16-h infection at 37°C with maintenance medium containing 50 μCi of [^{3}H]glucosamine per ml as described previously (28). After this time, the culture medium from each plate was retained and used to isolate virions by the purification scheme described by Ben-Porat et al. (2). The purified preparation was lysed in RIPA buffer (10 mM Tris, pH 7.8, 150 mM NaCl, 1% Trition X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]), and an amount corresponding to roughly one-fifth of the total virus released was used in each of a series of immunoprecipitations with antibodies M1, 490, M2, and M3.

Culture medium fractions were obtained after removal of cell debris by centrifugation. Supernatants were further clarified by centrifugation at 20,000 × *g* through a 30% sucrose pad to remove virions. Roughly one-tenth of the original volume was used in each immunoprecipitation with antibodies M1, 490, M2, and M3.

After removal of the culture medium, infected-cell monolayers were washed with PBS and lysed in RIPA buffer. After clarification by ultracentrifugation, one-tenth of each cell extract was processed for immunoprecipitation with each of the antibodies M1, 490, M2, and M3.

EndoH and endoF treatment. Endoglycosidase H (endoH) and endoglycosidase F (endoF) (New England Nuclear Corp.) were used according to the specifications provided. Substrate for the enzymes was gIII immunoprecipitated from infected cells that had been steady-state radiolabeled for 2 h with 100 μCi of [^{35}S]cysteine per ml.

Pulse-chase analysis of PRV gIII proteins in infected cells. PK15 cells in 60-mm dishes, one for each chase point to be taken, were infected at an MOI of 10 with either PRV-Be or each of the mutant viruses. After a 1-h adsorption period at 37°C, the virus suspension was removed, and the monolayers were washed once with PBS, overlaid with maintenance medium, and then incubated at 37°C. Thirty minutes before the radioactive pulse was administered, the maintenance medium was removed, and the monolayers were washed once with PBS and then overlaid with modified Eagle

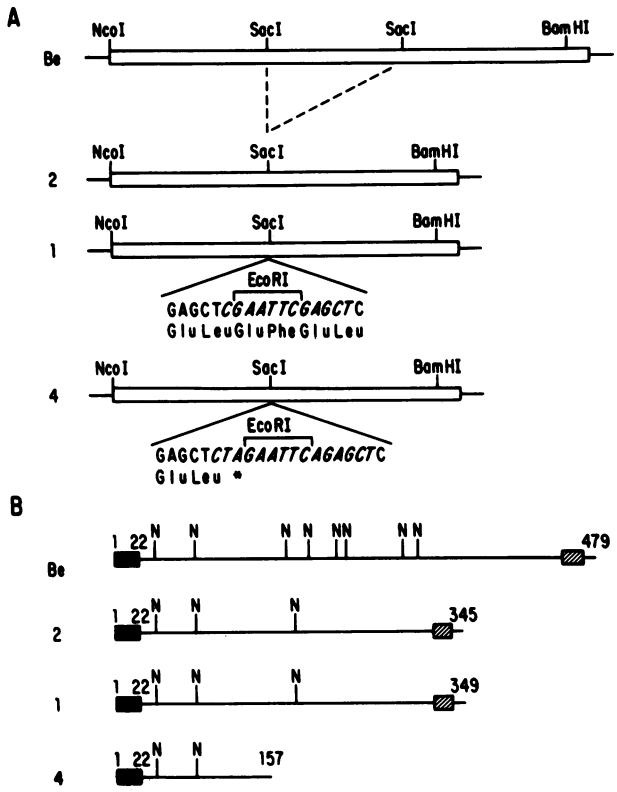

FIG. 1. Map of the parental and mutant gIII genes and depiction of the encoded glycoproteins. (A) The gIII coding region is represented by an open box. Relevant restriction endonuclease sites characterized previously (26, 28) are shown; the *Nco*I site contains the translational start site for gIII, while the *Bam*HI site lies midway through the sequences that encode the putative membrane-spanning region of the protein. The dashed lines indicate the loss of an internal *Sac*I fragment from the parental copy of the gene and the subsequent generation of a unique *Sac*I restriction site in the PRV2 gIII gene. For each of the gIII genes harbored by mutants PRV1 and PRV4, the inserted linker sequence is indicated in italics; certain of the gIII flanking sequences are also shown. In each case, the *Eco*RI site within each linker sequence is bracketed. In addition, the encoded amino acids are shown below each sequence; an asterisk indicates the site of translation termination. (B) The predicted mature portion of each gIII glycoprotein is indicated by a solid line. The putative 22-amino-acid signal peptide of each species is depicted as a solid box, and the predicted membrane-spanning region of the proteins is represented by a hatched box. Vertical lines ending with N indicate predicted sites for N-linked glycosylation. The length of each polypeptide (in number of amino acids) is indicated at the carboxy terminus of each protein. Abbreviations: Be, PRV Becker; 2, PRV2; 1, PRV1; 4, PRV4. See text and reference 26 for additional details.

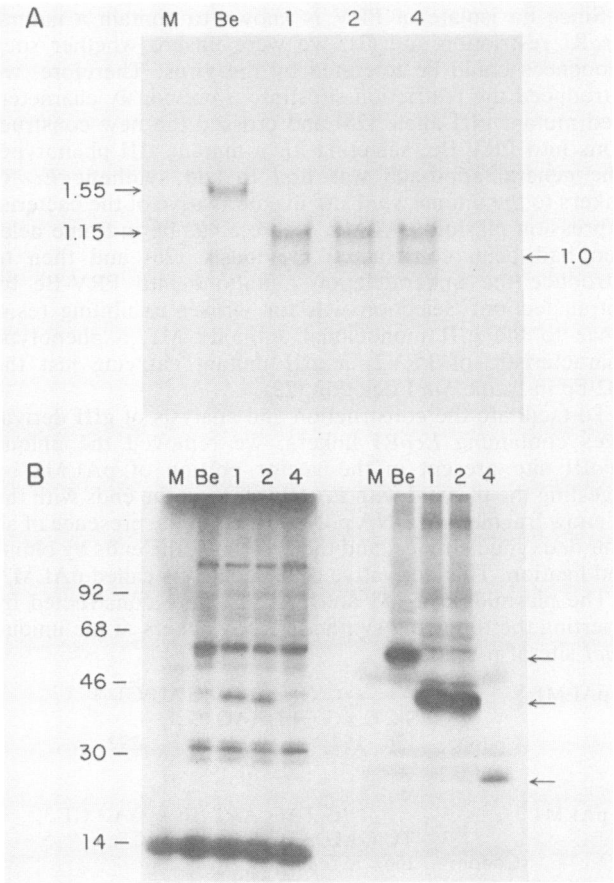

FIG. 2. Northern blot analysis and in vitro translation of RNA extracted from uninfected and parental and mutant PRV-infected PK15 cells. (A) Total RNA was extracted from mock-infected and PRV-infected cells at 16 h postinfection. Equal amounts of RNA were fractionated on an agarose-formaldehyde gel and transferred to nitrocellulose for subsequent hybridization. The blot was hybridized with ^{32}P-labeled plasmid DNA from pALM15, a plasmid that contains a 3.6-kb insert of PRV DNA carrying the gIII gene and flanking regions (28). RNA sizes were estimated by using 16S, 18S, 23S, and 28S rRNA molecules as standards. In the autoradiogram, the gIII-specific bands are indicated (in kilobases) at left. A band that hybridized to PRV sequences present in pALM15 that flank the gIII coding region is indicated (in kilobases) at right. This species migrated independently of any gIII alterations and served as a control in the experiment (28). (B) Total RNA from mock-infected and PRV-infected cells was harvested at 16 h postinfection, and equal amounts were translated in a rabbit reticulocyte system. A portion of the products was immunoprecipitated with 282 serum, and both the reaction mix (left side) and the immunoprecipitate (right side) of each sample were fractionated on a 7 to 17% SDS-polyacrylamide gradient gel, followed by fluorography. Migration of the molecular mass markers (in kilodaltons) is indicated at left. Arrows at right indicate the migration of the gIII products. Lanes: M, mock infected; Be, PRV-Becker-infected; 1, PRV1-infected; 2, PRV2-infected; 4, PRV4-infected. See Materials and Methods and reference 28 for further details.

medium lacking cystine, methionine, and fetal bovine serum (prepared from a Select-Amine kit; Gibco Laboratories). After depletion of the intracellular cysteine pools, medium was removed, the monolayers were washed once with PBS, and a radioactive pulse with 100 μCi of [^{35}S]cysteine per ml was performed in cystine-free medium at the times referred to in Results. After 2 min at 37°C, the pulse medium was removed, the monolayers were washed once with PBS, and then either the contents of the plate were harvested (zero chase point; see below for harvesting details) or the monolayers were overlaid with maintenance medium containing 20-fold-normal concentrations of cystine and methionine. Incubation was at 37°C for the chase times indicated for each experiment.

In standard pulse-chase experiments, time point samples were harvested by first removing the culture medium and washing the monolayers once with PBS and then scraping

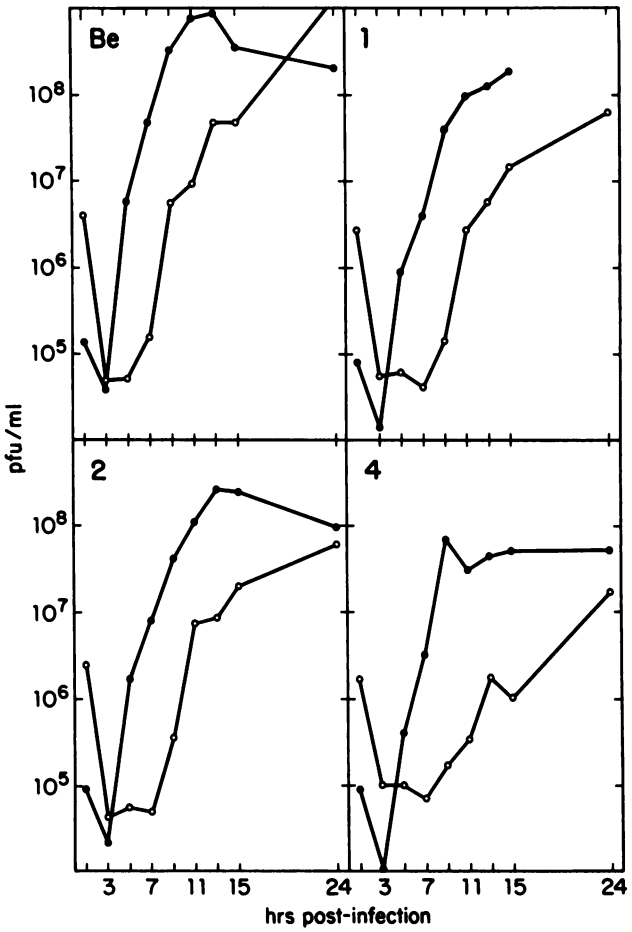

FIG. 3. Single-step growth curves of PRV-Be and mutant viruses. PK15 cells were infected with PRV strains at an MOI of 5 and incubated at 37°C. At 1, 3, 5, 7, 9, 11, 13, 15, and 24 h postinfection, plates were harvested, and the virus titers of the cell and medium fractions were determined separately and plotted. The infecting virus strain is indicated in the upper left corner of each plot. Symbols: ●, cell-associated virus; ○, virus found in the medium. See Materials and Methods for details.

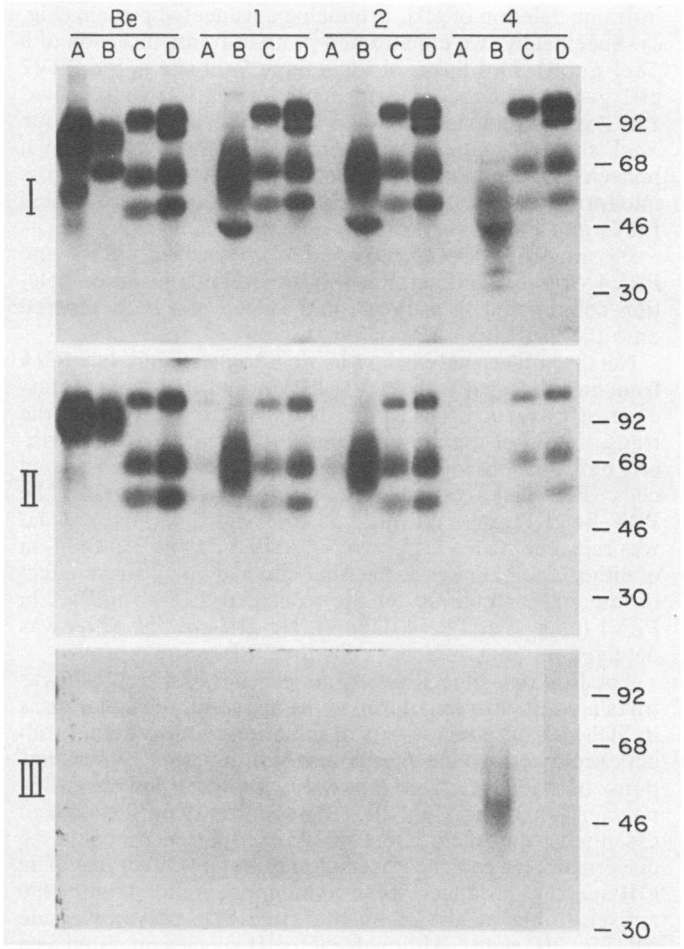

FIG. 4. Localization of wild-type and mutant forms of glycoprotein gIII during steady-state radiolabeling conditions. PK15 cells were infected at an MOI of 10 with parental or mutant virus and labeled throughout a 16-h period with [^{3}H]glucosamine. Immediately following the radiolabeling, the contents of each 100-mm dish were divided into cell, virion, and medium fractions, and representative amounts of each fraction were used in an immunoprecipitation procedure with four different antibodies. The immunoprecipitates were resolved on a 7 to 17% SDS-polyacrylamide gradient gel, and fluorography was used to allow visualization of the ^{3}H-labeled polypeptides. The infecting virus strain is indicated across the top. Fractions are indicated at left: cells (I), virions (II), and medium (III). Antibodies used were anti-gIII monoclonal antibody M1 (lanes A), anti-gIII polyclonal antibody 490 (lanes B), anti-gII monoclonal antibody M2 (lanes C), and anti-gII monoclonal antibody M3 (lanes D). The positions of molecular mass standards (in kilodaltons) are shown at right. See Materials and Methods and reference 28 for details.

the monolayers into PBS. The cell suspension was then added to an equal volume of 2× RIPA buffer, and the lysed cell extract was clarified by ultracentrifugation as described previously (27). One-tenth of the total volume was then used in an immunoprecipitation procedure with 282 serum.

In pulse-chase experiments in which the medium fractions were assayed for gIII content, the infected-cell monolayers were treated as just described. However, culture medium was retained, and the virions and cell debris were removed by centrifugation at 20,000 × *g* through a 30% sucrose pad (2). One-fourth of the total medium volume was removed from above the pad and used in an immunoprecipitation procedure with 282 serum.

Polyacrylamide gel analysis. All immunoprecipitates were loaded onto SDS-polyacrylamide gels. Electrophoresis was performed as described previously (27), with either 10 or 7 to 17% gradient gels as noted. Fluorography was conducted with sodium salicylate (5), followed by autoradiography. Autoradiograms resulting from pulse-chase experiments were scanned with an LKB densitometer interfaced with an AT&T microcomputer.

RESULTS

Mutant construction. Details of the two constructions are presented in Materials and Methods. In general, *Eco*RI linker sequences were introduced into a plasmid copy of a gIII deletion allele (28) and then cotransfected into PRV-Be as previously described (11, 28). Viruses expressing mutant gIII proteins were detected by a "black plaque" assay through their nonreactivity with a monoclonal antibody that recognizes wild-type gIII (13, 14, 30). The net outcome of these manipulations was the generation of two derivatives of PRV2, a virus described previously (28) that carries a 402-bp

in-frame deletion of gIII, producing a truncated protein (Fig. 1). Specifically, we constructed (i) an in-frame insertion of a *Sac*I-*Eco*RI-*Sac*I linker at the unique *Sac*I site in the PRV2 gIII gene that added 4 extra amino acids to the truncated PRV2 gIII protein and (ii) an in-frame insertion at the unique *Sac*I site of a similar *Sac*I-*Eco*RI-*Sac*I linker carrying a nonsense codon immediately following codon 157. The virus mutants carrying these insertion mutations were called PRV1 and PRV4, respectively.

By analyzing DNA extracted from purified PRV1 and PRV4 virions, we determined that the *Eco*RI insertion/deletion constructed in each plasmid indeed had been crossed onto the virus (data not shown).

Northern blot analysis and in vitro translation of late RNA from cells infected with PRV1, PRV2, and PRV4. The presence of novel *Eco*RI sequences did not interfere with the transcription of the mutant genes, as determined by Northern blot analysis of total cytoplasmic RNA from infected cells (Fig. 2A). As reported by Robbins et al. (28), the PRV-Be gIII transcript was 1.55 kilobases (kb) (lane Be) and was replaced with a 1.15-kb RNA in PRV2 (lane 2). Addition of either *Eco*RI linker at the *Sac*I site had no apparent effect on the size or quantity of the truncated 1.15-kb mRNA in PRV1 (lane 1) or PRV4 (lane 4). No gIII-specific RNA was detected in mock-infected cells (lane M).

Total cytoplasmic RNA from cells infected for 16 h was translated in vitro to determine the apparent molecular mass in SDS-polyacrylamide gels of the primary translation products produced by the *Eco*RI insertion mutants. When portions of each reaction mix were analyzed on an SDS-polyacrylamide gel (Fig. 2B, left), the overall profiles looked essentially the same, i.e., no gross differences could be discerned between the parental and mutant infections. The gIII-specific products were immunoprecipitated with 490 antiserum and analyzed on the same SDS-polyacrylamide gel (Fig. 2B, right). Although each gIII species migrated as a larger protein than its predicted molecular mass, the relative migration of each in vitro-translated product was as expected. The gIII species encoded by PRV1 (lane 1) and PRV2 (lane 2) were of essentially the same molecular mass and smaller than the wild-type polypeptide (lane Be). Still smaller was the in vitro-translated product from a PRV4 infection, a gIII "amber fragment" representing the first one-third of the wild-type protein (lane 4). The discrepancy in predicted molecular mass from that observed under these electrophoresis conditions is probably due to the primary sequences being rich in proline, alanine, and valine residues (26).

Single-step growth curves of gIII mutants in PK15 cells. As shown previously for several PRV gIII mutants, virus strains lacking wild-type gIII in their envelopes grow to somewhat lower titers after infection of PK15 cells (19, 28). This point is illustrated in Fig. 3, in which a virus growth curve involving PRV-Be and mutant strains is depicted. PK15 cells were infected with 5 PFU per cell, and titers in the infected cells and culture medium were determined independently at 2-h intervals to determine the number of plaque-forming particles present in each fraction (see Materials and Methods). The overall pattern for each mutant infection was similar to that found for the parental PRV-Be infection, although a lower number of PFU was obtained for each mutant throughout the time course. The most significant differences between the parental infection and infections with each mutant strain were found in the culture medium fractions. Each mutant virus exhibited a significantly longer lag in the initial increased appearance of infectious virions in the culture medium. Moreover, while more PRV-Be infectious particles were eventually found in the medium than associated with the cell, none of the mutant virus infections led to such a distribution of PFU. These results support our previous suggestion that PRV gIII mutants may be defective in viral release or may be more fragile than parental PRV in the absence of stabilizing membranes (19).

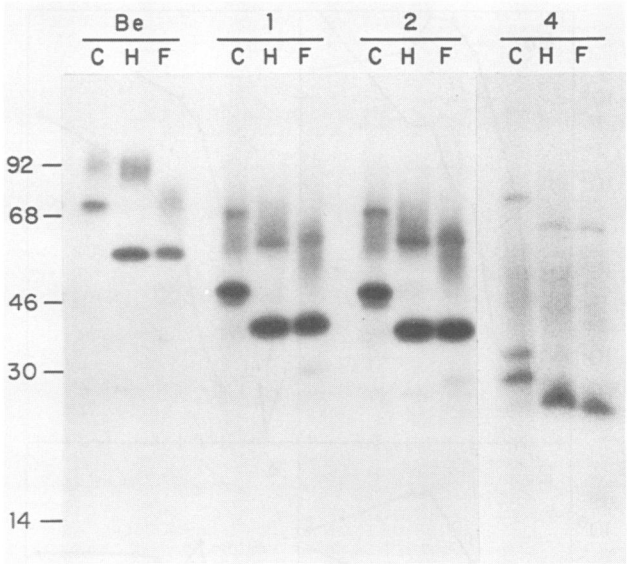

FIG. 5. Endoglycosidase treatment of wild-type and mutant forms of glycoprotein gIII. PK15 cells were infected with PRV strains at an MOI of 10 and then radiolabeled with [^{35}S]cysteine from either 4 to 6 h postinfection (PRV-Be-infected cells) or 6 to 8 h postinfection (PRV1-, PRV2-, or PRV4-infected cells). Immediately following the radiolabeling period, monolayers were harvested and gIII species were immunoprecipitated with 282 serum. The immunoprecipitates were then left untreated (lanes C), treated with endoH (lanes H), or treated with endoF (lanes F) prior to fractionation on 7 to 17% SDS-polyacrylamide gradient gels and visualization by fluorography. The infecting virus strain is indicated across the top. The position of molecular mass standards (in kilodaltons) are shown at left. Note that the gIII species resulting from a PRV4 infection were resolved on a separate gel from those species resulting from either PRV-Be, PRV1, or PRV2 infections. See Materials and Methods for details.

Localization of two forms of gIII during infections with mutant viruses. PK15 cells were infected (MOI, 10) with either PRV-Be or each of the mutant viruses and radiolabeled continuously for 16 h with [^{3}H]glucosamine, as previously outlined (28). The contents of 100-mm dishes were separated into three fractions: infected cells, virions released into the medium, and medium less virions. Immunoprecipitations from representative amounts of each fraction were then performed with the gIII-specific antibodies M1 and 490 as well as the gII-specific antibodies M2 and M3 (13, 28). The latter two antibodies recognize another PRV envelope glycoprotein, gII (13, 22, 23), and the immunoprecipitated product served as a control for determining the relative amount and integrity of each fraction analyzed on SDS-polyacrylamide gels (Fig. 4).

For each infection, two forms of the gIII glycoprotein were found within the infected cell (Fig. 4, panel I). As observed before (19, 28), the polyclonal 490 serum immunoprecipitated a 74-kilodalton (kDa) species and a more diffuse 92-kDa form from parental PRV-infected cells (lane B under Be). In contrast, the monoclonal antibody M1 did not

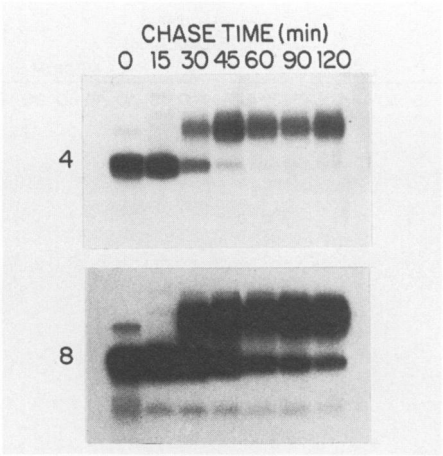

FIG. 6. Pulse-chase analysis of wild-type gIII species at 4 and 8 h postinfection. PK15 cells were infected with PRV-Be at an MOI of 10 and incubated at 37°C. Thirty minutes prior to radiolabeling, the infected cells were depleted of their intracellular pools of cysteine and methionine. At either 4 h (top) or 8 h (bottom) postinfection, cells were pulse-labeled with [^{35}S]cysteine for 2 min and then incubated in the presence of excess nonradioactive cystine and methionine. At the times indicated above each lane, the cells were harvested, and the gIII species were immunoprecipitated with 282 serum. Immunoprecipitates were then resolved on a 10% SDS–polyacrylamide gel and visualized by fluorography. See Materials and Methods for details.

recognize the 74-kDa form; however, in addition to the 92-kDa species, this antibody appeared to bring down a smaller, approximately 58-kDa glycoprotein that may be gIII related (lane A under Be). Results from infections with either PRV1 or PRV2 were identical. For each mutant, a 43-kDa species, migrating as a sharp band on the SDS-polyacrylamide gel, and a more diffuse 68-kDa form were detected with 490 serum (lanes B under 1 and 2). From PRV4-infected cells, this serum recognized a discrete 35-kDa form of the gIII amber fragment and also a diffuse 56-kDa species (lane B under 4). As expected, none of the altered gIII glycoproteins reacted with monoclonal antibody M1 (lanes A); each mutant virus had been identified in a black plaque assay through its nonreactivity with this antibody.

For each of the gIII species encoded by PRV-Be, PRV1, and PRV2, only the larger, more diffuse form of the glycoprotein was immunoprecipitated from virions (Fig. 4, panel II, lanes A and B under Be and lanes B under 1 and 2). This outcome was in agreement with a previous result reported for wild-type gIII (13) and occurred despite the removal of the middle one-third of the wild-type sequences from PRV1- and PRV2-encoded gIII. In contrast, no form of the gIII protein encoded by PRV4 could be detected in the mutant virus envelope with the polyclonal 490 serum (lane B under 4).

In only one instance was gIII detected in an infected cell culture medium. A diffuse, approximately 56-kDa glycoprotein from the PRV4-infected cell medium was specifically immunoprecipitated with 490 serum (Fig. 4, panel III, lane B under 4). This protein was the glycosylated amber fragment, representing only the first one-third of the wild-type protein and lacking a predicted transmembrane anchor sequence.

For every virus analyzed, the control gII glycoprotein maintained a parental PRV-Be profile (13, 25, 28). The M3 monoclonal antibody recognized four forms of this glycoprotein in the infected cell (Fig. 4, panel I, lanes D). However, only the three forms that were immunoprecipitated by the M2 monoclonal antibody were found associated with virions (panel II, lanes C). In no case were any of the forms found in the infected-cell medium (panel III, lanes C and D). Note that, compared with a parental PRV infection, no differences in the amount of gII could be discerned in either the infected-cell or virion fractions obtained from mutant virus infections.

EndoH and endoF analysis of gIII encoded by PRV-Be and mutant strains. Both wild-type and mutant gIII species exhibited a characteristic profile in SDS-polyacrylamide gel analysis, namely a sharp band representing a low-molecular-mass species along with a diffuse high-molecular-mass form. These forms usually represent different states of glycosylation (6).

The N-linked glycosylation states of gIII encoded by PRV-Be and each of the mutant viruses are shown in Fig. 5 with the glycosidases endoH and endoF. EndoH has been shown to cleave only the high-mannose form of N-linked side chains (acquired as the nascent chain crosses the rough endoplasmic reticular membrane), while endoF has been shown to remove both the high- and complex-mannose forms (7, 32). The latter species is generated in the Golgi apparatus through a modification of the high-mannose form. Sensitivity of the glycoprotein to the enzymes can be detected through a shift in the migration of the protein on SDS-polyacrylamide gels.

Infected cells were continuously radiolabeled with [^{35}S]cysteine from either 4 to 6 h (PRV-Be) or 6 to 8 h (PRV1, -2, and -4) postinfection, and the infections were immediately terminated. Glycoprotein gIII was immunoprecipitated from each infected cell extract with anti-gIII 282 serum and was then treated with either endoH or endoF (see Materials and Methods).

When the immunoprecipitated glycoproteins were not treated with the glycosidases, a characteristic pattern for each allelic form of gIII was obtained from each infection by SDS-polyacrylamide gel analysis (Fig. 5, lanes C). Note that the gIII pattern that resulted from a PRV4 infection revealed two lower-molecular-mass forms (30 and 35 kDa) along with the larger diffuse species (approximately 56 kDa). The existence of a doublet for the lower-molecular-mass species was in fact found for all of the allelic forms of gIII, including the wild type. However, the better separation achieved on SDS-polyacrylamide gels with the small gIII protein expressed by PRV4 enhanced the resolution of the doublet form. After treatment with endoH (Fig. 5, lanes H), the migration of the diffuse, higher-molecular-mass forms of gIII remained essentially unchanged, indicating that these forms were Golgi- or post-Golgi-localized glycoproteins. However, all of the discrete lower-molecular-mass doublet forms shifted to a single faster-migrating species on SDS-polyacrylamide gels. The change in apparent molecular mass indicated that these species were pre-Golgi localized and ranged from a loss of approximately 16 kDa for wild-type gIII to a loss of about 2 to 7 kDa for the gIII amber fragment. This range most likely reflects the difference in the number of N-linked sugar side chains that are present on each structure. The gel migration of the digested polypeptides was very similar to the gel migration observed for the corresponding in vitro translation products (Fig. 2B).

Treatment with endoF had a similar effect on the migration of the lower-molecular-mass forms of the various gIII glycoproteins (Fig. 5, lanes F). Moreover, this enzyme cleaved each of the higher-molecular-mass species into a faster-migrating but still diffuse form. The altered migration was not as obvious for the gIII product of PRV4. Still, endoF

digestion maintained the relative separation of the low- and high-molecular-mass polypeptides that was observed for the control, untreated samples (lanes C). The implication of this observation will be addressed in the Discussion. An unexpected finding was that from each of the mutant virus infections, a discrete band of approximately 74 kDa could be observed in the untreated sample. This band shifted in migration after either endoH or endoF treatment. The nature of this glycoprotein will be considered in the Discussion.

Pulse-chase analysis of wild-type gIII at 4 and 8 h postinfection. A relationship having been established between the precursor 74-kDa (pre-Golgi-localized) and mature 92-kDa (Golgi- or post-Golgi-localized) forms of wild-type gIII, it was of interest to determine the rate at which the precursor was converted to the mature form. This would indicate the time of transit for wild-type gIII from its site of synthesis to the Golgi apparatus. The experiment used a pulse-chase format involving PK15 cells that had been infected at an MOI of 10 with PRV-Be (see Materials and Methods). Preliminary studies had indicated that gIII synthesis could be detected by pulse-radiolabeling as early as 4 h postinfection, while maximum gIII expression was achieved by 8 h postinfection (data not shown). Both time points were chosen to initiate a 2-min pulse of infected cells with [^{35}S]cysteine, followed by chase periods of 0, 15, 30, 45, 60, 90, and 120 min in the presence of excess nonradioactive cystine. Chase point samples were harvested by removing the culture medium and lysing the infected cells in RIPA buffer.

An important finding was that the kinetics of gIII conversion were different depending on the time after infection at which the pulse was administered (Fig. 6). For both the 4-h (top panel) and 8-h (lower panel) postinfection experiments, only the 74-kDa doublet form of gIII was immunoprecipitated with 282 serum immediately following the 2-min pulse. A minor, discrete band of about 90 kDa was present on the SDS-polyacrylamide gel after each pulse. This band was absent in the 15-min chase sample, and its significance is uncertain. By 30 min of chase, both profiles contained mature, 92-kDa gIII. After 60 min of chase, virtually all of the 4-h-postinfection pulse-labeled gIII was found in the diffuse, mature form. In contrast, roughly 25% of the radiolabel was still found in the 74-kDa species after 60 min in the 8-h-postinfection pulse-chase profile, as judged by densitometry conducted on a lighter exposure of the autoradiogram. Both profiles revealed that little if any further conversion of the endoH-sensitive form to the endoH-resistant form occurred after 90 min of chase.

A band representing a protein of about 58 kDa was faintly visible in the 8-h-postinfection profile but was demonstrably absent in the 4-h-postinfection profile, even after overexposure of the autoradiogram (data not shown). This protein did not accumulate or chase with time, but rather appeared to be a stable, primary translation product. It is probably the same polypeptide as that found in the monoclonal antibody M1-immunoprecipitated sample of [^{3}H]glucosamine-labeled wild-type gIII shown in Fig. 4, and would therefore be a glycoprotein. However, further work is necessary to clarify its relationship to gIII.

Pulse-chase analysis of gIII resulting from infections with PRV1, PRV2, and PRV4. The steady-state labeling analysis of the truncated forms of gIII produced by PRV1 and PRV2 implied that the mutant proteins were essentially like wild-type gIII in their ability to be processed and localized to virion envelopes (Fig. 4, panels I and II). The following set

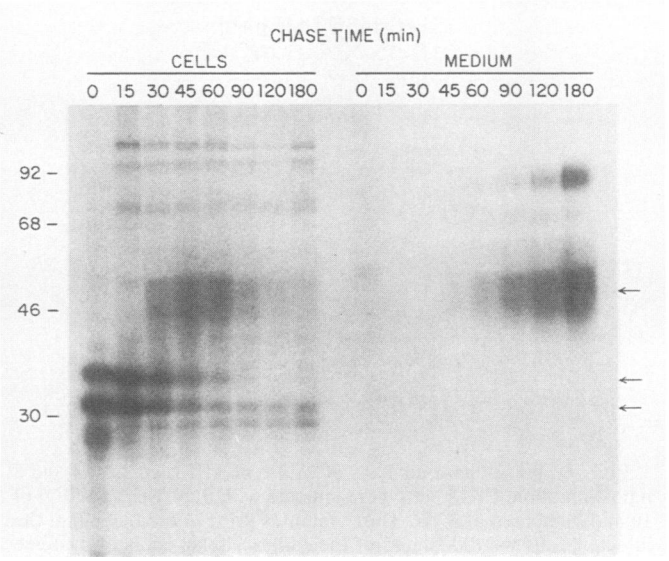

FIG. 8. Pulse-chase analysis of PRV4-encoded gIII species. PK15 cells were pulse-labeled at 6 h postinfection. At the termination of each chase period, gIII was immunoprecipitated with 282 serum from both the cell and medium fractions. The left portion of the autoradiogram shows immunoprecipitated gIII obtained from infected cells, while the right portion shows gIII immunoprecipitated from the culture medium. The positions of molecular mass standards (in kilodaltons) are indicated at left. The arrows at the right indicate the positions of known PRV4-encoded gIII species. See Materials and Methods and the legend to Fig. 6 for details.

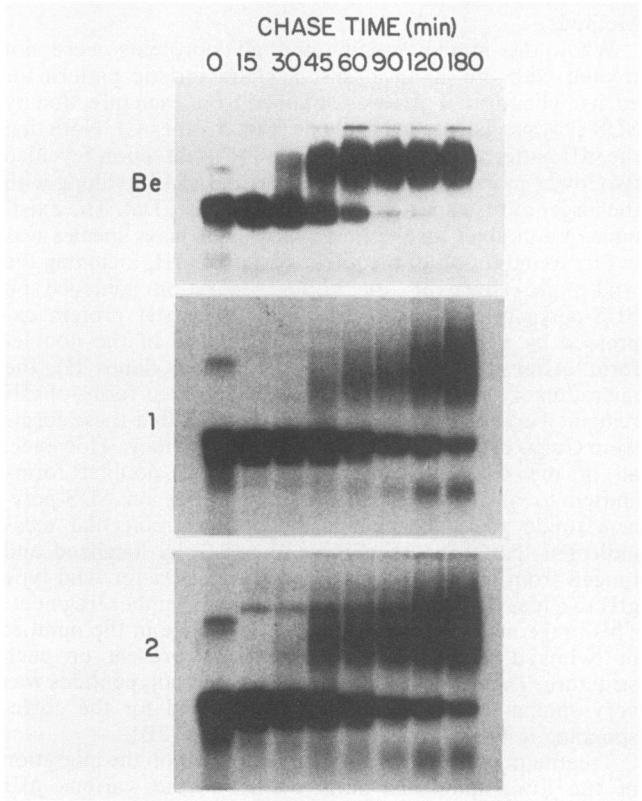

FIG. 7. Pulse-chase analysis of gIII species from PRV-Be-, PRV1-, and PRV2-infected cells. PK15 cells were pulse-labeled at 6 h postinfection. The infecting virus strain is indicated at left. See Materials and Methods and the legend to Fig. 6 for details.

of experiments indicated, first, that these mutant proteins had different processing kinetics than wild-type gIII and, second, that the processing kinetics of the PRV4 amber fragment provided insight into various pathways used by the gIII protein in infected cells.

The export kinetics of the various truncated forms of gIII were determined in infections involving PRV1, -2, and -4. Infected PK15 cells were pulse-labeled for 2 min at 6 h postinfection in the hopes of ensuring an equivalent stage of infection between the parent PRV-Be and each of the mutants, since mutant infections progressed more slowly than parental infections (Fig. 3). After incubation in the presence of excess nonradioactive cystine for 0, 15, 30, 45, 60, 90, 120, and 180 min, each chase point sample was harvested in two fractions. Infected-cell fractions were lysed in RIPA buffer, while culture medium fractions were retained after any released virions were removed (see Materials and Methods). Chase samples from both fractions were immunoprecipitated with 282 serum, followed by SDS-polyacrylamide gel analysis.

The profile obtained for wild-type gIII (top panel, Fig. 7) closely resembled that observed after 4 h of infection (Fig. 6, top panel). The export kinetics for the truncated forms of gIII encoded by PRV1 and PRV2 (Fig. 7, middle and bottom panels, respectively) were indistinguishable from one another. Each zero-chase sample was analogous to the wild type. However, unlike the wild-type profile, neither mutant form of gIII was processed to the mature species by 30 min of chase. Rather, for both mutants, an additional 15 min of chase was required before the processed form of gIII could be detected. Two further differences between the wild-type and mutant export kinetics were discerned. Virtually all of the wild-type gIII precursor was converted to the mature form, with no additional chase occurring after 90 min. In contrast, effective conversion of the truncated gIII precursors occurred throughout the 3-h chase period, but a significant fraction of each precursor remained unprocessed at the end of 3 h.

No form of gIII reactive with 282 serum was detected in the cell culture medium from the PRV-Be or either of the mutant virus infections, even after 3 h of chase (data not shown).

The export kinetics of the gIII protein encoded by PRV4 are shown in Fig. 8. This abbreviated form of gIII contained only two cysteine residues, as opposed to eight contained within the wild-type protein. Therefore, the autoradiogram was exposed considerably longer than those shown for the other allelic forms of gIII, resulting in more background signal. Immediately following the 2-min pulse, only the approximately 30-kDa and 35-kDa polypeptides that constitute the precursor doublet were specifically immunoprecipitated. As was found for a wild-type infection, a mature form of the gIII product could be detected in the cell fraction by 30 min of chase. Concomitant with the appearance of the 56-kDa mature form, the amount of the 35-kDa precursor species decreased, indicating its conversion to the mature form. The amount of the mature species within the infected cell increased throughout 60 min of chase, but then began to decline, until very little of the 56-kDa protein was detected after 180 min.

As the levels of the cell-associated mature form of the PRV4-encoded gIII protein decreased, 282 serum specifically immunoprecipitated a polypeptide from the cell culture medium (Fig. 8) whose 56-kDa molecular mass was the same as that for the [^{3}H]glucosamine-labeled species immunoprecipitated from the medium fraction shown in Fig. 4. The released mature form was apparent by 45 to 60 min of chase and appeared to increase in amount throughout the 3-h chase period. No form of the precursor species was detected in the culture medium at any point during the chase period. However, two diffuse bands migrating at apparent molecular masses of about 68 and 90 kDa were visible on the polyacrylamide gel in lanes representing gIII immunoprecipitations from late chase points. The relationship of these forms to gIII is unclear.

DISCUSSION

In this report, we established that PRV can carry an *Eco*RI site with few apparent ill effects in tissue culture, even though no natural isolate of PRV reported to date has such a site. We introduced two *Eco*RI linker insertions into the unique *Sac*I site in the gIII gene of pALM7 and transferred these mutations into PRV, creating a set of viruses with mutant gIII genes for analysis. Each of the alterations had at least one common trait: the insertions and in-frame deletions were at the same position in the gene, simplifying the interpretation of results. The ability to insert unique *Eco*RI sites into this large DNA virus has proven useful in the insertional mutagenesis of gIII (19) and may lead to the construction of novel cloning vehicles.

None of the mutant viruses have significant defects in their ability to propagate as infectious particles, although the titers of mutant stocks are consistently lower than those of PRV-Be. The most consequential differences between parental and mutant virus infections can be found in the number of PFU found in the culture medium; all of the gIII mutants exhibited a definite defect in both the rate and extent of release. These results support the hypothesis that PRV gIII mutants may be defective in either viral release or envelope stability. No compensatory changes in gIII mutant envelopes were detected (unpublished observations).

The three mutations described here enabled us to begin a detailed analysis of gIII processing in PRV-infected cells. From endoH analyses, we concluded that for each allelic form of gIII the sharp, lower-molecular-mass band observed on SDS-polyacrylamide gels represented a pre-Golgi-localized protein since all of the N-linked sugar side chains present were endoH sensitive. Under certain conditions, the pre-Golgi form of gIII for each mutant migrated as a doublet on SDS-polyacrylamide gels. In each case, the doublet was converted by endoH to a single species; a similar observation has been made for the gC (formerly gF) glycoprotein of HSV-2 (35). Perhaps this doublet represents an inherent deficiency in the N-linked glycosylation of the nascent gIII polypeptide chain. Because the gIII amber fragment exhibited this phenomenon, it may be that one of the first two consensus sites for N-linked sugar addition (Asn-X-Ser/Thr at amino acids 40 and 84) is not a readily accessible substrate and is at times unmodified within the population of gIII glycoproteins present in the infected cell.

In all cases, the heterogeneous, high-molecular-mass form of gIII was shown by endoH and endoF analyses to contain the complex-mannose variety of N-linked side chains, indicating that these proteins were in or had passed through the Golgi apparatus. However, we could also conclude from the endoF analyses that the modification(s) leading to the marked shift in gIII migration from the sharp lower-molecular-mass forms to the diffuse higher-molecular-mass forms was unrelated to the N-linked sugar side chains whose structure was assayed. Similar results have been obtained for HSV-1 glycoprotein gC (17, 33), for which it was dem-

onstrated that the altered migration pattern of the endoH-resistant species was due to addition of O-linked sugar side groups in the Golgi apparatus. A similar circumstance for gIII seems likely but has not been proven.

In agreement with the localization assignments derived from endoH and endoF digestions, only the mature species of each allelic form of gIII could be identified in either virions (PRV-Be, -1, and -2) or culture medium (PRV4). Since the truncated forms of gIII expressed by PRV1 and PRV2 were localized correctly to the virion envelope, we can conclude the following. First, the glycoproteins, despite missing the middle one-third of the wild-type sequence, must contain sufficient export signals to allow their targeting to and anchoring in the correct orientation within the inner nuclear membrane from which the viral envelope is derived. Second, the mutant glycoproteins are not excluded from the envelope structure, where it has been reported that only virus-encoded proteins reside (3). Apparently, the rather significant deletions introduced into the gIII gene have not altered any structure in the protein important for its recognition as a virion component.

We cannot conclude that the gIII species encoded by PRV1 and PRV2 are fully export competent as discrete polypeptides. Once localized to an inner nuclear membrane "patch," subsequent maturation of the precursor species to a mature, post-Golgi form is perhaps only accomplished passively through its residence within an otherwise wild-type egressing virion. This possibility, however, cannot be true for the truncated gIII protein encoded by PRV4. This abbreviated polypeptide was not found associated in any detectable manner with the virus yet was released in significant amounts into the culture medium. This suggests that the first one-third of the gIII glycoprotein contains sufficient information to allow export to and release from the infected-cell surface. In addition, the results support the existence of a membrane-anchoring function carboxy terminal to the deletion endpoint found in the PRV1 and PRV2 gIII species (amino acid 292), consistent with previous predictions made by methods such as that of Kyte and Doolittle (8, 21, 26). The release into the culture medium of viral envelope proteins after removal of their transmembrane domains has been well documented (4, 10, 29). These experiments indicate that a membrane-anchoring function is necessary, although perhaps not sufficient, for incorporation into a virion envelope. Requirements for specific incorporation into the viral envelope remain to be defined.

While the fractionation results indicated that perhaps each of the allelic forms of gIII contained protein export signals, the findings gave no indication of the efficiency with which each protein was localized. Wild-type pulse-chase kinetics were established by analyzing PRV-Be-infected cells at 4 h postinfection. The experiments revealed a rapid, nearly 100% efficient chase of the pre-Golgi form to the mature species. If the radioactive pulse was administered later in infection (8 h postinfection), the efficiency of export decreased, with precursor gIII still present after 2 h of chase. Nevertheless, the overall rate was qualitatively similar both early and late. This difference in efficiency but not rate may reflect a general deterioration of the host population's export machinery due to advancing stages of infection, with a certain percentage of the gIII glycoproteins entering "dead-end" export pathways harbored by essentially dead cells. Alternatively, or perhaps in addition, the decreased efficiency may reflect an increased proportion of the radiolabeled gIII residing in immature viral envelopes at late times; the budding process from the nucleus may significantly slow the apparent export kinetics of the assayed glycoprotein (15). In either case, the pulse-chase format should assay a "mixed" population of wild-type gIII, including polypeptides that are components of egressing virions as well as polypeptides synthesized and exported as discrete entities. Since approximately 0.1 PFU was associated with the cell at 4 h after infection, a significant fraction of the gIII assayed was probably in the form of discrete polypeptides. However, by 8 h after infection, a 300-fold increase in the number of cell-associated PFU occurred, so that the distribution of gIII at this time was difficult to determine. Similar alternatives were considered by Johnson and Smiley (15) in determining the export kinetics of the gD glycoprotein of HSV-2 in cells constitutively expressing the protein. In that study, the rapid kinetics observed for the gD species produced by a stable cell line was significantly slowed after infection with HSV-1. Whether the slowed rate was due to incorporation of the cell-encoded gD into viral envelopes or to a general deterioration of the host cells could not be determined.

In addition to reduced efficiency of export, the truncated gIII glycoproteins produced by PRV1 and PRV2 had slower rates of transit from their site of synthesis to the Golgi apparatus. If these glycoproteins can be exported as discrete polypeptides, this suggests that the primary export impairment probably results from a problem with the host machinery recognizing the altered gIII substrate rather than a defect within some fraction of the machinery itself, as considered above. In contrast, the truncated protein synthesized in PRV4-infected cells maintained wild-type export kinetics and was apparently recognized by the host as a constitutively secreted protein and not simply released from the cell in an indirect manner. Perhaps owing to its relatively short length and soluble nature, the amber fragment became committed to export before achieving an export-defective conformation.

The time of transit from the Golgi apparatus to the cell surface was qualitatively established for the truncated gIII glycoprotein of PRV4 by determining the time after pulse labeling in which the protein first appeared in the culture medium. Determining the rate at which the anchored forms of gIII encoded by PRV-Be, PRV1, and PRV2 reached the cell surface has not been as straightforward. However, preliminary results from experiments with PRV-Be-, PRV1-, or PRV2-infected cells, in which only surface-exposed proteins were reacted with either protease or gIII-specific antibodies, indicate that the mature form of wild-type and mutant gIII is localized to the surface of infected cells (unpublished observations).

In several instances after immunoprecipitation with gIII-specific antisera, additional bands were observed on SDS-polyacrylamide gels that proved unique to the experimental conditions.

(i) A 58-kDa glycoprotein, immunoprecipitated with both monoclonal antibody M1 and polyclonal 282 serum, was observed only in experiments with PRV-Be in which the radiolabel was present relatively late in infection. At present, it is not known whether this is a gIII-related polypeptide or another protein associated with gIII only in sufficient amounts to be detected late in infection. The possibility that the protein is a degradation product of gIII does not seem likely since it was present at late times immediately following a 2-min pulse-radiolabeling.

(ii) An endoH-sensitive protein of approximately 74 kDa was detected in immunoprecipitations of gIII from cells infected with PRV1, -2, or -4. This glycoprotein is unlikely to be gIII related since its apparent mass remained virtually the

same whether it was identified in PRV4-infected cells or PRV1- or PRV2-infected cells. It may have been present in all gIII immunoprecipitations with 282 serum but obscured by comigration with wild-type gIII. The 74-kDa protein may be analogous to the previously described immunoglobulin heavy-chain-binding protein, BiP (12), although this protein was glycosylated and BiP is not. It has been suggested that BiP binds to malfolded mutant proteins in the rough endoplasmic reticulum, preventing their entrance into the export pathway (9). At this time, it is not known whether the 74-kDa protein is specifically complexed to gIII or is nonspecifically precipitated.

(iii) In pulse-chase experiments for each allelic form of gIII, the zero chase point contained a faint but distinct band that migrated as approximately 10 kDa larger than the precursor gIII. The protein was absent by the 15-min chase point. Because the size and appearance of this band (being discernable as a doublet whenever the precursor was found to be so) were dependent on the precursor of the gIII allele studied, it is most likely gIII related. Perhaps a small percentage of the newly synthesized precursor can be immunoprecipitated while still complexed to other, as yet unidentified, proteins.

(iv) Two heterogeneous species of higher molecular mass than the secreted, mature form of the gIII protein encoded by PRV4 accumulated with time in the medium in pulse-chase experiments involving PRV4-infected cells. We cannot rule out the possibility that the largest of the two forms is gX, a 90-kDa released glycoprotein of PRV (1, 24) that may be nonspecifically immunoprecipitated because of its abundance in the culture medium. Alternatively, the two higher forms may represent some multimer of the mature gIII amber fragment.

The results presented here indicate that the nonessential gIII glycoprotein of PRV can function as a useful model system not only for herpesvirus glycoprotein structure and function studies, but also for the functional analysis of eucaryotic protein localization signals. The facile in vitro engineering of mutations into infectious virus greatly extends the utility of the gIII system, since the mutant protein can be studied in an infected cell. Moreover, the novel egress pathway used by herpesviruses in general may lead to insights into the protein export process that might not be realized through the study of other nonherpesvirus proteins.

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