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The Export Pathway of the Pseudorabies Virus gB Homolog gII Involves Oligomer Formation in the Endoplasmic Reticulum and Protease Processing in the Golgi Apparatus

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The pseudorabies virus gII gene shares significant homology with the gB gene of herpes simplex virus type 1. Unlike gB, however, gII is processed by specific protease cleavage events after the synthesis of its precursor. The processed forms are maintained in an oligomeric complex that includes disulfide linkages. In this report, we demonstrate the kinetics of modification, complex formation, and subsequent protease processing. In particular, we suggest that gII oligomer formation in the endoplasmic reticulum is an integral part of the export pathway and that protease cleavage occurs only after oligomers have formed. Furthermore, through the use of glycoprotein gene fusions between the gIII glycoprotein and the gII glycoprotein genes of pseudorabies virus, we have mapped a functional cleavage domain of gII to an 11-amino-acid segment.

Herpesvirus envelope glycoproteins are found both in the envelope of the virus particle and in most of the infected-cell membranes. It is not yet clear whether the proteins at either location (virus or cell membranes) have the same organization or function. Nevertheless, it has been established that these glycoproteins play essential roles in the infectious process (adsorption and penetration of virus), viral pathogenesis, and viral interaction with the immune system of the host (34). Analysis of the synthesis, assembly, and export of these important viral proteins through the exocytic pathway of the host cell is an active research area.

It is well known that most mature plasma membrane proteins in eucaryotic cells are homo- or heterooligomers and that their functions are intimately related to their quaternary structure (2). From work with vesicular stomatitis virus G protein (5, 14), the influenza virus hemagglutinin protein (9, 37), and the Rous sarcoma virus envelope protein (7), it has been established that oligomerization (in the above cases, trimerization) occurs early after synthesis in the rough endoplasmic reticulum (ER). Moreover, in these and other studies it is clear that oligomerization is an obligatory event prior to transport to the Golgi apparatus, since the cell has also evolved specific mechanisms to recognize unfolded or nonoligomerized proteins and retain them in the ER (2).

In general, the nature of oligomerization of herpesvirus membrane proteins, the kinetics of quaternary structure formation and the relationship of this structure to function are not well understood. Herpes simplex virus type 1 (HSV-1) gB has been characterized in considerable detail and has been shown to exist as a dimer (4, 6, 30, 34), but little is known of the kinetics of oligomerization and the structure of the dimer. Sarmiento and Spear (30) provided convincing evidence that the gB dimer is unusually stable, being resistant to dissociation by either sodium dodecyl sulfate (SDS) or reducing agents. The gB glycoprotein is highly conserved among herpesviruses and, in many, is proteolytically processed (11, 13, 17, 20–22, 24, 31, 33, 35). In the cases in which the structures of the gB homologs were examined, the cleaved molecules were thought to be held together in a complex involving disulfide linkages (11, 13, 17, 20, 33). The gB homologs also share an unusual characteristic in that unlike most other herpesvirus glycoproteins tested, mature protein often contains mixed endoglycosidase H-sensitive and resistant N-linked sugar modifications.

Our laboratory has been studying the envelope glycoproteins of the swine herpesvirus, pseudorabies virus (PRV). Previously, we established that the PRV gII gene shared 62% DNA homology with the HSV-1 gB gene and 50% amino acid homology at the primary amino acid level. Many regions of complete identity were observed, and we speculated that the secondary and higher-order structures of gII and gB would be quite similar. One notable difference was that unlike the HSV-1 gB protein, PRV gII is processed by protease cleavage. By sequence comparison, we noted a block of nonhomology containing several basic amino acids that were predicted to constitute the protease processing site (25).

In this report, we discuss our initial work showing that the gII protein is initially synthesized as a monomer in the ER, rapidly converted to an oligomer (presumably a dimer), and then transported as a dimer to the Golgi, in which it receives complex sugar modifications before being proteolytically processed. The processed forms are maintained in a highly stable complex that cosediments in a sucrose gradient with the 110-kilodalton (kDa) dimer. This complex is maintained by disulfide linkages and possibly hydrophobic interactions (11, 17, 30; see below). Finally, through construction of hybrid glycoprotein genes, we have defined an 11-amino-acid segment of the gII protein as a functional cleavage domain. When a synthetic oligomer encoding these residues is inserted in an unrelated PRV glycoprotein gene, the resulting hybrid protein is cleaved.

MATERIALS AND METHODS

Cells and virus. The PK15 cells and the Becker strain of PRV (PRV-Be) have been described previously (26).

Construction of pALM77 and pALM82. All *Escherichia coli* strains have been described previously (27), and plasmids were constructed by standard recombinant DNA techniques. The PRV gII sequences were derived from plasmid

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pALM45 (25) or were prepared on an Applied Biosystems DNA synthesizer. The gIII sequences were derived from plasmid pALM3 (28). pALM3 and pALM45 were digested with *Sac*I, and the appropriate fragments were ligated and transformed into *E. coli* NF1829. The resulting plasmid, pALM77, contains a gene fusion with the first 156 codons of the gIII gene fused in frame to 168 codons from the gII gene (containing the putative protease-processing domain), which is fused in frame to the last 189 codons of gIII. In this report, we refer to this "tribrid" gene as a gIII-gII gene fusion.

pALM82 was constructed to contain DNA sequences encoding the 11 amino acids corresponding to PRV gII residues 494 to 504 (25) inserted into an *Sac*I site of the PRV gIII gene. A synthetic DNA oligomer (39 base pairs) was synthesized and inserted into the *Sac*I sites of pALM15 that contains 3.6 kilobases of PRV DNA including the entire gIII gene (28). The 39-base-pair linker sequence encodes 11 gII amino acids and 2 duplicated residues from PRV gIII. The linker was designed to contain a *Bgl*II restriction enzyme site not present in the parental sequence as an aid in identifying the isolated mutants. The *Bgl*II site encompasses codons 502 and 503 but does not change the amino acid sequence (see Fig. 4). A plasmid was isolated that contained the synthetic oligomer in the correct orientation inserted at the second *Sac*I site of the PRV gIII gene (see Fig. 4) and was designated pALM82.

Construction of PRV mutants. Recombinant viruses were obtained by using the calcium phosphate cotransfection and gene replacement techniques described previously (10, 28). Immunoreactivity of plaques, as detected by immunoperoxidase staining (black-plaque assay), was used to identify mutant viruses as described previously (8, 12, 32). pALM77 was cotransfected with PRV-Be DNA. A plaque that did not react with the PRV-Be-specific monoclonal antibody M1 in the black-plaque assay was purified and designated PRV77. pALM82 was cotransfected with DNA from PRV10, a virus that lacks gIII (28). A plaque reacting with the M1 antibody in the black-plaque assay was purified, and the resulting virus was designated PRV82.

Steady-state labeling and immunoprecipitation of PRV glycoproteins. In steady-state radiolabeling experiments, PK15 cells were infected at a multiplicity of infection (MOI) of 10 with either PRV-Be, PRV77, or PRV82 and grown throughout a 16-h infection in medium containing 55 μCi of [^{3}H]glucosamine per ml. Experiments involving fucose labeling were done as described for glucosamine labeling but contained 55 μCi of [^{3}H]fucose per ml. Infected-cell, virion, and medium fractionation of PRV-infected PK15 cells was performed as previously described (1, 29). The preparation of infected-cell extracts and the immunoprecipitation procedure have been described previously (27).

Antibody reagents. The antisera used in these studies included mouse monoclonal antibody M1 (reactive with the mature form of gIII but not the gIII precursor) (11), goat polyvalent 282 antiserum raised against a denatured *E. coli*-produced Cro-gIII fusion protein (reactive with native and denatured gIII protein) (29), goat polyvalent B67 antiserum raised against an *E. coli*-produced β-galactosidase fusion protein containing amino acids 312 to 431 of gIII (reactive only with denatured gIII protein), and goat polyvalent 284 antiserum raised against immunoaffinity-purified gII protein (reactive with native and denatured forms of the gII glycoprotein).

Pulse-chase analysis. The pulse-chase procedure was performed as described previously (29). Briefly, PK15 cells were infected at an MOI of 10 with PRV-Be, PRV77, or PRV82. At 5.5 h post infection the cells were starved for cysteine. For pulse-chase analysis at 20°C, the cells were shifted to 20°C and all the medium was maintained at 20°C for the rest of the experiment. At 6 h postinfection a radioactive pulse with 100 μCi of [^{35}S]cysteine per ml was administered for 2 min, the radiolabel was removed, and the cells were incubated in media containing excess nonradioactive cysteine for various times. At the desired chase times, the monolayers were harvested and the samples were immunoprecipitated with either 282 or 284 antiserum.

Sedimentation in sucrose gradients. Sedimentation in sucrose gradients was done essentially as described by Hampl et al. (11) and Sarmiento and Spear (30) with minor modifications. Briefly, a series of plates were pulsed and chased as described above. At various times the infected cells were solubilized in 1% Triton X-100–150 mM NaCl–50 mM Tris (pH 7.5) on ice for 10 min. Next they were scraped, and the nuclei were pelleted at 11,750 × *g* for 5 min at 4°C. The supernatant was layered onto an 11-ml 5 to 15% sucrose gradient containing 0.1% Triton X-100, 150 mM NaCl, and 50 mM Tris (pH 7.5) and sedimented in an SW41 rotor at 196,000 × *g* for 20 h at 4°C. Fractions from each gradient were used as sources for immunoprecipitation with 284 antiserum.

Polyacrylamide gel analysis. All immunoprecipitates were loaded onto SDS–10% polyacrylamide slab gels. Electrophoresis was performed as described previously (27). Fluorography was conducted with sodium salicylate (3) and was followed by autoradiography.

RESULTS

Kinetic relationship and cellular localization of the species in the gII complex. It is established that three forms of gII are found in the virus envelope (11). Hampl et al. (11) refer to these forms as gIIa (125 kDa), gIIb (74 kDa), and gIIc (58 kDa). Using a different set of calibrating markers, we calculated the apparent molecular masses of these forms to be 110, 68, and 55 kDa, respectively (25). In addition, a fourth species (of 100 kDa) was found only in infected cells. It has been postulated that the 68- and 55-kDa forms arise by protease cleavage of the 110-kDa form (17), but the kinetic (precursor-product) relationship and cellular location during the synthesis and export of these four species of the gII glycoprotein during synthesis have not been established. This was accomplished by pulse-chase experiments at 37 and 20°C. The 20°C temperature has been shown to slow export from the ER to the Golgi apparatus and to block cell surface localization of exported proteins (19). As a control, we monitored the kinetics of export of the well-characterized glycoprotein gIII of PRV (29).

The pulse-chase experiments were performed as described and referenced in Materials and Methods. The results of this analysis are shown in Fig. 1. Previously, it had been shown that the 74-kDa species of gIII is the precursor synthesized in the ER that rapidly chases to the mature 92-kDa species carrying Golgi-derived modifications (29). With these criteria, the kinetics of export from the ER to the Golgi were slowed by 45 min when the temperature was reduced to 20°C (Fig. 1B). As expected, at 20°C the extent of the chase of the gIII 74-kDa species to the 92-kDa species was not as efficient as seen at 37°C; 74-kDa forms could be observed even as long as 240 min after the chase.

Unlike gIII, full-length gII was not detected in 2 min. We presume that the various species of immunoprecipitable gII are incomplete polypeptides with different degrees of N-

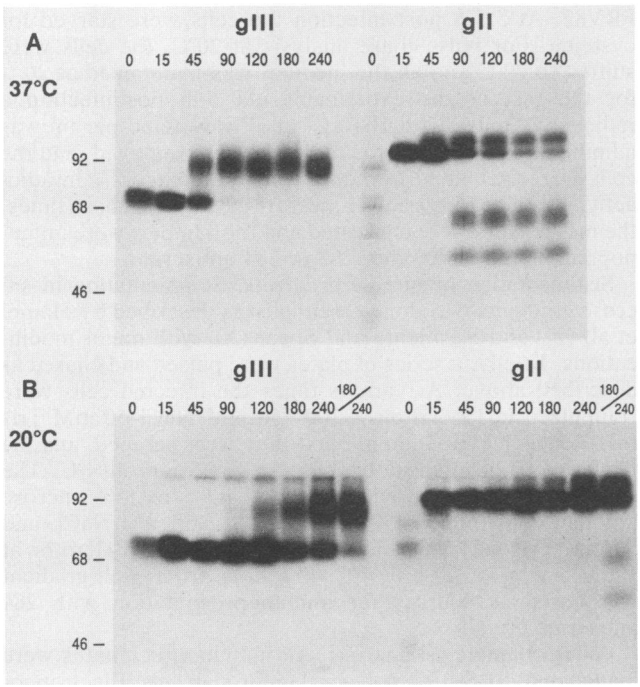

FIG. 1. Pulse-chase analysis of gIII and gII species from PRV-Be-infected cells at 37°C (A) or 20°C (B). PK15 cells were infected with PRV-Be at an MOI of 10 and incubated at 37°C. At 5.5 h postinfection the infected cells were depleted of their intracellular pools of cysteine. Also at this time, half of the plates were shifted to 20°C. At 6 h postinfection, cells were pulse-labeled with [^{35}S] cysteine for 2 min and then incubated in the presence of excess nonradioactive cysteine. At the times indicated above each lane, the cells were harvested and lysed, and portions were used for immunoprecipitation with 282 (gIII-specific) or 284 (gII-specific) antiserum. The lanes labeled 180/240 indicate that at 180 min postpulse, a plate was shifted from 20 to 37°C until 240 min. Immunoprecipitates were then resolved on an SDS–10% polyacrylamide gel and visualized by fluorography. Molecular mass standards are indicated to the left.

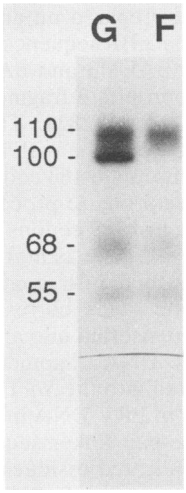

FIG. 2. Immunoprecipitation of gII labeled with either [^{3}H]glucosamine (G) or [^{3}H]fucose (F) from PRV-Be-infected PK15 cells. PK15 cells were infected at an MOI of 10 with PRV-Be and labeled with 55 μCi of either [^{3}H]glucosamine or [^{3}H]fucose from 1 to 16 h postinfection. At this time the cells were harvested and lysed, and portions were used for immunoprecipitation with gII-specific 284 antiserum. Immunoprecipitates were resolved on SDS–10% polyacrylamide gels and visualized by fluorography. The molecular masses of the gII species are indicated in kilodaltons to the left of the gel.

linked sugar addition. The reason for this phenomenon is not completely understood and may only reflect the size of the gII protein compared to the gIII protein. Nevertheless, after 15 min of chase at 37°C, a distinct 100-kDa form of gII was apparent. This species chased to the 110-kDa form with kinetics similar to those observed for the conversion of the 74-kDa precursor to mature 92-kDa gIII protein at 37°C. That is, the 92-kDa form of gIII was not detected at 15 min but was clearly present at 45 min, and, similarly, the 110-kDa form of gII was not detected at 15 min but was clearly present at 45 min. These results are consistent with the hypothesis that the 100-kDa form of gII is synthesized initially in the ER and converted to the 110-kDa form in the Golgi (see below). By 90 min of chase at 37°C, the 110-kDa form of gII was cleaved into the 55- and 68-kDa forms. The pulse-chase experiment at 20°C provided clear evidence that the 55- and 68-kDa forms were produced very late in the export pathway (Fig. 1B). At 20°C, the formation of 110-kDa gII from the 100-kDa precursor was delayed (but not blocked) at essentially the same rate and to the same extent as the delay in modification of the gIII 74-kDa precursor to the 92-kDa form. This was in contrast to the appearance of the 55- and 68-kDa species. The treatment at 20°C essentially blocked formation of the 68- and 55-kDa gII proteins. Thus, the cleavage event must occur late in the export pathway, presumably in the Golgi, but only after the formation of the 110-kDa species.

An important control is shown in the 20°C panel (Fig. 1B, lanes 180/240), when at 180 min after the chase, one plate was shifted from 20°C to 37°C until 240 min after the chase. The appearance of the 55- and 68-kDa forms was observed clearly, demonstrating that the 20°C treatment was reversible and had not adversely affected the export and cleavage pathway.

Assignments of locations of the various forms in the ER and Golgi apparatus by kinetic experiments were strengthened by the results of sugar-labeling experiments. In particular, it is known that many glycoproteins are modified by fucose addition via a Golgi enzyme. Labeling of any gII species by fucose would indicate that it had passed through the Golgi during the export process. Glucosamine, on the other hand, is added to glycoproteins initially in the ER via cotranslational N-linked glycosylation. Therefore, we labeled infected cells with either [^{3}H]glucosamine or [^{3}H]-fucose and examined the gII family of glycoproteins by immunoprecipitation (Fig. 2). As expected, all four gII species (110, 100, 68, and 55 kDa) were labeled with glucosamine. Fucose, however, did not label the 100-kDa species but labeled the 110-, 68-, and 55-kDa gII forms. These data further support the proposal that the 100-kDa precursor is localized only to the ER and early Golgi compartments, whereas the 110-, 68-, and 55-kDa species are further along in the export pathway and have passed through the Golgi compartment where fucose is added.

Kinetics of gII dimerization and processing. Sarmiento and Spear (30) suggested that gB (and no other HSV-1 glycoproteins examined) formed oligomers, and they provided technical insight to the analysis of such structures. Given the significant primary sequence homology, it seemed likely that PRV gII would also form oligomers. However, since gII is also proteolytically processed, we were particularly inter-

ested in the kinetics of oligomerization of precursors and products and the structure of the final cleaved products. To determine whether gII assembles into an oligomeric structure, we used a pulse-chase format to monitor gII molecules during export and processing. At various times after a 2-min pulse, chase samples were solubilized in 1% Triton X-100, layered on a 5 to 15% sucrose gradient containing 0.1% Triton X-100, and sedimented at 4°C overnight as described in Materials and Methods. Molecular mass markers were run in a parallel gradient. It should be noted that no reducing agents were used during the solubilization and sedimentation. The gradient was then fractionated, and samples were immunoprecipitated with polyvalent gII-specific 284 antiserum. Immunoprecipitates were boiled in sample buffer (containing β-mercaptoethanol) and electrophoresed through a 10% polyacrylamide gel as described in Materials and Methods. This analysis allowed us to observe not only the structures of primary products of synthesis, but also the structures of products that assemble as export continues. The denaturing gel electrophoresis analysis enabled us to deduce the composition of the complexes so formed.

The results of this analysis are presented in Fig. 3. The sedimentation positions of molecular mass markers in kilodaltons are indicated at the top of the figure. After 10 min of chase, the majority of labeled gII sedimented as a monomer of approximately 100 kDa. By 30 min of chase, most of these monomers chased to a position of about 200 kDa. We presume that these are dimers of the 100-kDa protein. It is important to note that a small quantity of 110-kDa species was observed at this chase point and that it sedimented at about 200 kDa, not at the 100-kDa monomer position (Fig. 3B, fraction 7). After 60 min of chase, the 55- and 68-kDa processed forms of gII appeared, but only in fractions sedimenting as predicted for gII dimers. It is noteworthy that the 110-, 68-, and 55-kDa species appeared only in the dimer fraction and not in the monomer fraction. Under these conditions, there is no evidence for higher-order oligomers.

When purified virions labeled with [^{3}H]glucosamine for 16 h were solubilized in Triton X-100 and the glycoproteins were analyzed, a similar picture emerged. No form of gII sedimented in the monomer position. Furthermore, no 100-kDa precursor was detected, but significant quantities of the 110-, 68-, and 55-kDa dimer complex were observed (data not shown).

As a technical note, Triton X-100 was chosen as the solubilizing detergent after we initially attempted to solubilize gII with octyl glucoside as described by Einfeld and Hunter (7) for sedimentation of Rous sarcoma virus envelope glycoprotein. We found that when infected cells were solubilized with octyl glucoside, gII floated at the top of the gradient and did not sediment. In contrast, the gIII glycoprotein sedimented identically when it was solubilized in either Triton X-100 or octyl glucoside.

Defining a gII protease cleavage domain. Robbins et al. (25) predicted the protease cleavage site of gII to be within residues 498 to 502 based on sequence comparison with HSV-1 gB and known processing sites of other gB homologs (13, 33). The oligomeric structure of gII prompted us to investigate the topology required for cleavage. We used the technique of gene fusion to determine whether a defined segment of gII could be transferred to another protein and confer the ability to be proteolytically processed. Briefly, we inserted segments of the gII gene predicted to contain the cleavage domain into the nonessential gIII gene by using techniques described previously (28). Infectious viruses carrying these gIII-gII fusion genes were tested to see whether

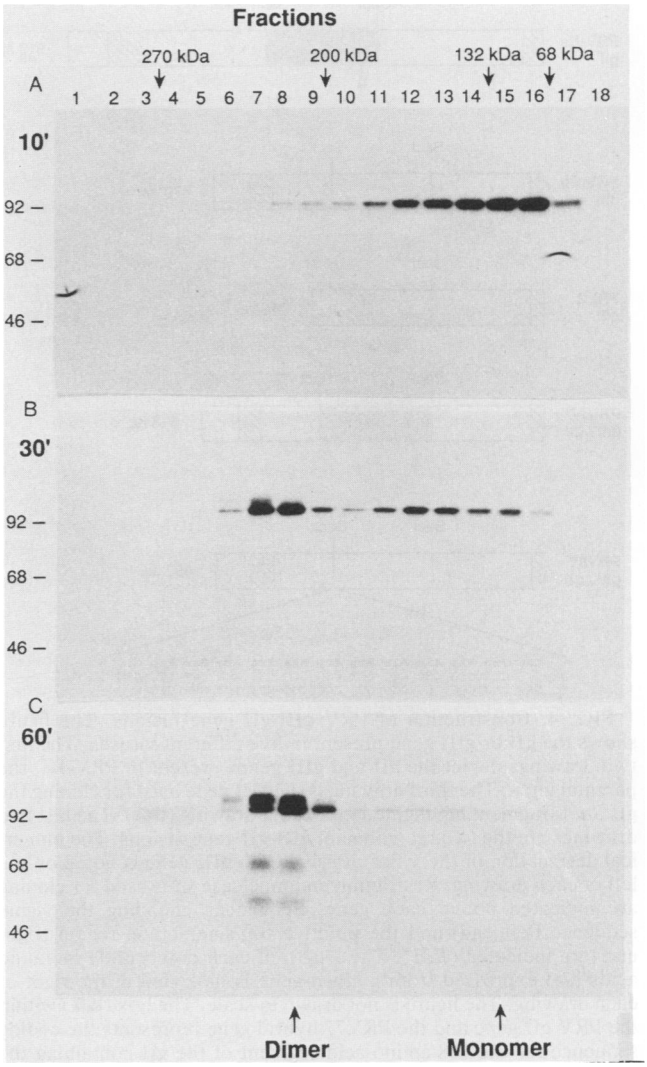

FIG. 3. Gradient fractionation of PRV gII. PK15 cells were infected at an MOI of 10. The cells were pulse-labeled at 6 h postinfection and chased for 10 (A), 30 (B), or 60 (C) min as indicated to the left of the panels. The cells were solubilized in 1% Triton X-100 and fractionated by sedimentation through a 5 to 15% sucrose gradient containing 0.1% Triton X-100. The fractions were immunoprecipitated with the gII-specific polyvalent 284 antiserum. The immunoprecipitates were resolved on an SDS–10% polyacrylamide gel and visualized by fluorography. Molecular mass markers were sedimented on a parallel sucrose gradient, and their relative positions are indicated across the top panel. The positions of the molecular mass standards (in kilodaltons) resolved on the polyacrylamide gel are indicated to the left of each panel. The relative positions of the expected monomers and dimers of gII are indicated at the bottom.

the novel hybrid proteins were cleaved. The details of construction of the two mutants are described in Materials and Methods. Two mutant viruses, PRV77 and PRV82, were constructed, each carrying a hybrid gIII-gII gene. PRV77 expresses a gIII-gII fusion protein containing the first 156 amino acids of gIII, 168 amino acids of gII, and the carboxy-terminal 189 amino acids of gIII. PRV82 expresses a gIII protein with an insertion of 13 amino acids containing the putative gII-processing domain at the second *Sac*I site of gIII at amino acid 291. The 13-amino-acid insertion contains

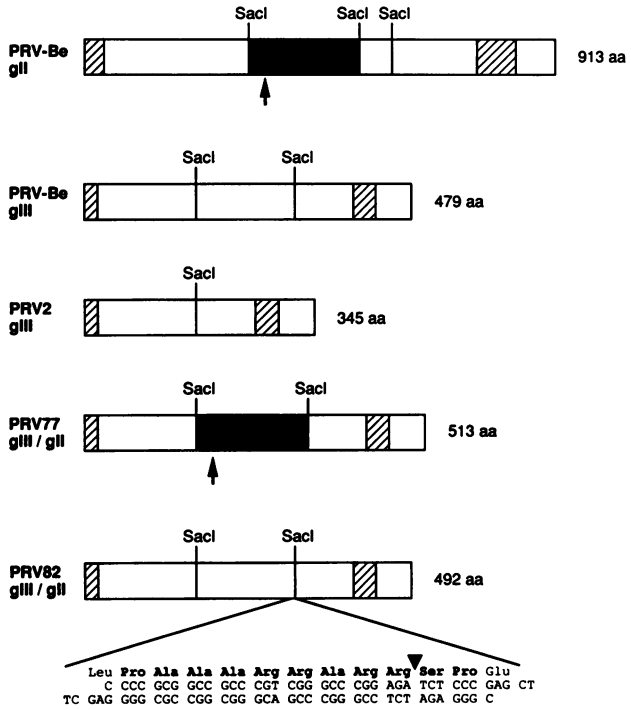

FIG. 4. Construction of PRV gIII-gII gene fusions. The figure shows the gII or gIII gene present in five different viruses. The first two drawings depict the gII and gIII genes present in PRV-Be, the parental virus. The third drawing is the gIII gene used for cloning the gII *Sac*I fragment highlighted in the first drawing (■). The last two drawings are the two recombinant gIII-gII gene fusions. The numerical designation of the virus carrying each gIII gene is noted on the left of each drawing. Restriction endonuclease sites used for cloning are indicated above each gene. Sequences encoding the signal sequence (left end) and the putative transmembrane region (right end) are indicated (▨). The length of each polypeptide in amino acids (aa) expressed from a given gene is indicated at the right of each drawing. The figure is not drawn to scale. The box (■) within the PRV gII gene and the PRV77 hybrid gene represents the coding sequence for the 168-amino-acid segment of the gII containing the protease cleavage site (indicated by the arrow). The DNA sequence of the synthetic oligonucleotides inserted at the second *Sac*I site of the parental gIII gene is indicated below PRV82. The amino acids shown in bold type encompass residues 494 to 504 of the parental gII gene containing the gII protease cleavage site (▼).

the 11 amino acids corresponding to PRV gII residues 494 to 504 as well as 2 amino acids encoded by the *Sac*I linkers. The second *Sac*I site in the gIII gene was chosen because it was located in a hydrophilic region of gIII predicted to be on the surface of the protein in its tertiary conformation and therefore likely to be available for protease processing (data not shown). Relevant regions of these viruses are shown in Fig. 4. Purification of nucleocapsid DNA extracted from cells infected with PRV77 or PRV82 and subsequent restriction enzyme digests followed by Southern blot analysis verified that the correct insertions were obtained (data not shown).

Protease processing of the gIII-gII fusion proteins. PK15 cells were infected at an MOI of 10 with PRV-Be, PRV77, or PRV82 and radiolabeled continuously with [^{3}H]glucosamine until 16 h postinfection. At this time the infected cells and media were separated by centrifugation into three fractions: infected cells washed free of medium and free virions, virions released into the medium, and virion-free medium. Proteins were immunoprecipitated with gIII-specific antibodies M1, 282 antiserum, and B67 antiserum, as well as the gII-specific 284 antiserum. The B67 antiserum is critical to this analysis. It was produced in goats against an *E. coli* expressed β-galactosidase fusion protein containing amino acids 312 to 431 of gIII and is therefore reactive only with this carboxy-terminal region of gIII. In this analysis we were able to determine, first, whether processing of the fusion proteins occurred by the appearance of smaller gIII-specific proteins, and second, the location of the various proteins so produced (infected cells, virus envelope, or media). Since the processing of the hybrid proteins should separate each protein into an amino-terminal segment with no membrane anchor and a carboxy-terminal segment containing a membrane anchor, we would expect the amino-terminal segment to appear in the media and not in virions. Conversely, we would expect the carboxy-terminal segment to be found in virions but not in media. All forms should be found in the infected cell. It is important to note that the localization phenotype assumes that the cleavage products are not held together and are free to move. Since the cleavage products of the parental gII protein are held together by strong interactions, this is an important consideration. In particular, this might be a problem with PRV77, in which 168 amino acids of gII are fused to gIII, but is unlikely with PRV82, in which only 11 amino acids (no cysteines) from gII are fused to gIII.

The results of this analysis are shown in Fig. 5. In Fig. 5A, C, and D the left lanes marked Be represent gIII proteins from parental virus (PRV-Be) infection immunoprecipitated by three different antisera against gIII. Lanes 1 and 2 show polyvalent 282 antiserum, when the extract was either denatured by boiling (lane 2) or not denatured (lane 1) prior to immunoprecipitation. Boiling increased the apparent reactivity of the mature 92-kDa protein with 282 antiserum. Lane 3 shows reactivity with the carboxy-terminal-specific B67 antiserum (samples were boiled prior to immunoprecipitation), and lane 4 shows reactivity with the monoclonal antibody M1 (samples were not boiled prior to immunoprecipitation). The lane number and corresponding antibodies are identical in the subsequent lanes in each panel. The last three lanes in Fig. 5A and C (lanes marked 5) are immunoprecipitations by polyvalent 284 gII antisera of samples from infections by PRV-Be (lanes Be), PRV77 (lanes 77), and PRV82 (lanes 82). These lanes serve as controls for the efficiency of infection by each virus.

The parental virus (PRV-Be) is considered first where no cleavage of gIII is expected. By 16 h postinfection the control PRV-Be-infected cells (Fig. 5A) contained significant amounts of the 74-kDa precursor (Fig. 5A, Be, lanes 1, 2, and 3) and 92-kDa mature form (Fig. 5A, Be, lanes 1, 2, 3, and 4). Note that the M1 monoclonal antibody recognized predominantly the mature but not the precursor species. A significant amount of the mature 92-kDa form, but not the 74-kDa precursor, was packaged in virus envelopes (Fig. 5C, Be, lanes 1, 3, and 4). The media also contained a small quantity of the 92-kDa species at this late time after infection (Fig. 5D, Be, lanes 1, 3, and 4). 284 antiserum recognized the predicted family of gII proteins in infected cells (Fig. 5A; cf. Fig. 2 and 3). Note that in virions (Fig. 5C), the 100-kDa gII precursor is not detected as expected.

Infection by the two gIII-gII hybrid viruses revealed a completely different profile of gIII-specific proteins. We expected these fusion proteins to have characteristics similar to that of gIII: a precursor from and a higher-molecular-mass mature form. We observed not only these species, but also

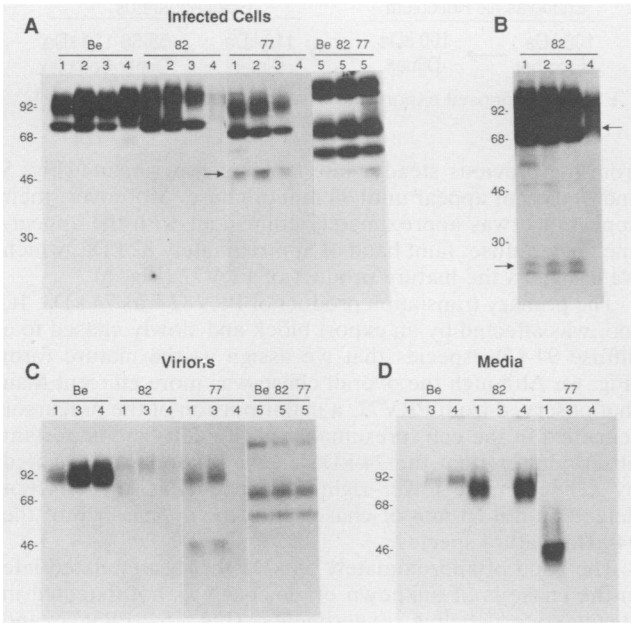

FIG. 5. Localization of glycoprotein gIII and gIII-gII fusion proteins. PK15 cells were infected at an MOI of 10 and labeled continuously for 16 h with [^{3}H]glucosamine. At 16 h postinfection, samples were separated by centrifugation into infected cells free of extracellular virions (A and B), virions (C), and medium fractions free of extracellular virions (D). After detergent lysis of each fraction, samples of each were used for immunoprecipitation. Immunoprecipitates were resolved on an SDS–10% polyacrylamide gel, and fluorography was used to visualize the ^{3}H-labeled polypeptides. The infecting virus strain and the protein immunoprecipitated are indicated across the top of each panel: Be, PRV-Be; 82, PRV82; 77, PRV77. In the last three lanes of panels A and C, samples from each of the three infections were reacted with the polyvalent 284 gII antiserum, with the infecting virus indicated over each lane. Panel B is an overexposure of the gIII-specific immunoprecipitation of PRV82-infected cells from the corresponding lanes in the left panel (82, lanes 1, 2, 3, and 4). The molecular mass standards (in kilodaltons) are shown at the left of each panel. The numbers above each lane refer to a specific antiserum: lanes 1, 282 antiserum with samples not boiled prior to immunoprecipitation; lanes 2, 282 antiserum with sample boiled in the presence of DTT and SDS prior to immunoprecipitation; lanes 3, B67 antiserum with samples boiled in the presence of DTT and SDS prior to immunoprecipitation; lanes 4, anti-gIII monoclonal antibody M1 with samples not boiled prior to immunoprecipitation; lanes 5, polyvalent 284 gII antiserum with samples not boiled prior to immunoprecipitation. The arrows in panels A and B indicate the novel gIII-specific proteins thought to be derived by protease processing.

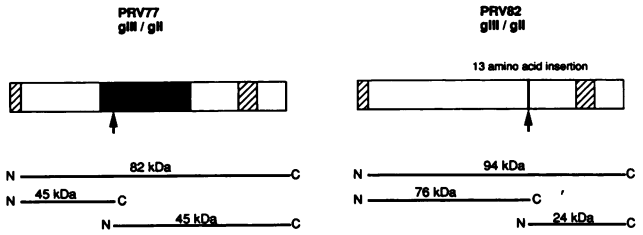

FIG. 6. Representation of the gIII-gII fusion proteins and cleavage products expressed by PRV77 and PRV82. The rectangular boxes depict the gIII-gII fusion proteins. The putative 22-amino-acid signal peptide and the predicted membrane-spanning region of the proteins are represented by ▨ at the left and right end, respectively. Other symbols: ■, gII insertion; ▲, putative cleavage site. The first line beneath the boxes represents unprocessed mature protein (N and C stand for the amino- and carboxy-terminal ends, respectively). The predicted cleavage products for each are indicated beneath each mature protein, with their observed apparent molecular mass in kilodaltons indicated.

novel forms of the gIII-gII fusion proteins. We will argue that these novel forms are consistent with proteolytic cleavage of the fusion proteins. Figure 6 is a representation of the two expected fusion proteins and their products as determined by the experiments described below. Note that this figure depicts only the mature forms and does not indicate the precursors.

Infected cells from PRV77 infection contained proteins of 82, 68, and 45 kDa (Fig. 5A, 77, lanes 1, 2, and 3). We show below by pulse-chase analysis that the 68-kDa form is the primary product of translation and the 82-kDa form is the mature form. We discovered that two gIII species comigrated with an apparent molecular mass of 45 kDa. These two proteins can be distinguished by differential reactivity with B67 antiserum that reacts with epitopes present in residues 312 to 341 of gIII (Fig. 5C and D, 77, lanes 3). It should also be noted that the gIII protein expressed by PRV77 did not react with monoclonal antibody M1 (Fig. 5C and D, 77, lanes 4). This was an expected result, since PRV2 (a virus carrying a gIII deletion corresponding to the same deletion found in PRV77) did not react with the M1 monoclonal antibody. In the gII-specific reactions with 284 antiserum (Fig. 5A, lanes 5), normal gII proteins were observed as expected. Note that in PRV77-infected cells, faint immunoreactivity that may correspond to the gIII-gII fusion proteins was observed (Fig. 5A, 77, lane 5).

Infected cells from a PRV82 infection contained new gIII-reactive proteins of approximately 94 and 74 kDa (Fig. 5A, 82, lanes 1, 2, 3, and 4). Two additional gIII reactive proteins of 24 and 76 kDa protein were observed upon overexposure (Fig. 5B, lanes 1, 2, and 3, denoted by arrows). Note that both the 94- and 76-kDa proteins were apparent (Fig. 5B, lane 4) after immunoprecipitation with the mouse monoclonal antibody M1, which recognizes only the mature form of wild-type gIII. The 74-kDa form recognized by 282 and B67 sera (Fig. 5, 82, lanes 1, 2, and 3) is not the 76-kDa species recognized by monoclonal antibody M1. Finally, it should be noted that after overexposure of the infected cells from the PRV-Be infection, no protein of 24 kDa was detected (data not shown).

The best evidence that proteolytic cleavage was responsible for the novel forms of the gIII-gII fusion proteins comes from analysis of the medium fractions. If the cleaved products are free to dissociate, we would expect to find the amino-terminal products secreted into the media, since they lack their anchor sequence. The PRV82 76-kDa protein, barely detected in infected cells with M1 monoclonal antibody, was predominant in the media and reacted strongly with 282 antiserum and the M1 monoclonal antibody (Fig. 5D, 82, lanes 1 and 4) but not with the B67 antiserum (Fig. 5D, 82, lane 3). This result implied that the 76-kDa secreted species did not contain the carboxy-terminal membrane anchor of gIII, but retained the M1 conformational epitope characteristic of mature gIII. Similarly, a 45-kDa protein produced by PRV77 was also predominant in the media but reacted only with 282 antiserum and not with B67 or M1 antibodies (Fig. 5D, 77, lanes 1, 3, and 4). This form must also lack the membrane anchor domain of gIII.

We would expect that virion envelopes would retain the fusion proteins anchored in membranes, and we might also

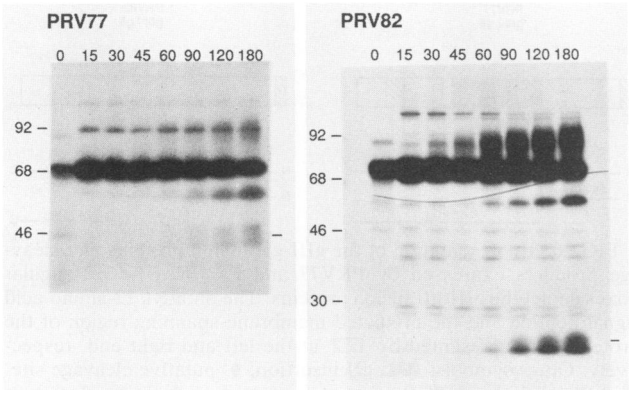

FIG. 7. Pulse-chase analysis of gIII-gII fusion proteins from PRV77- and PRV82-infected cells. PK15 cells were pulse-labeled at 6 h postinfection and chased for the times indicated at the top of each panel (in minutes). The infecting virus strain is indicated at the top left of each panel. Molecular mass standards (in kilodaltons) are indicated to the left of each panel. The dash on the right of each panel indicates the novel cleavage products.

expect a virion location for the processed forms if they contained a membrane anchor. The signals, if any, that are responsible for location of an envelope protein to the virion are not known, so lack of virion location must be carefully interpreted. PRV77 virions contained novel 82- and 45-kDa proteins reactive with gIII antisera (Fig. 5C, 77, lanes 1, 3, and 4). PRV82 virions contained barely detectable quantities of two novel proteins that were reactive with gIII antiserum and had apparent molecular masses of 94 and 76 kDa (Fig. 5C, 82, lanes 1, 3, and 4). Unexpectedly, the 76-kDa protein found in PRV82 virions was apparently the same species found in the media, since it reacted with both 282 antiserum and M1 antibody, but not with B67 antiserum. Therefore, even though this protein must lack a membrane anchor, it is present in virions. The small (24-kDa) protein seen in PRV82-infected cells, presumably derived from the carboxy terminus, was not detectable in either the virus envelope or the medium (data not shown).

Pulse-chase analysis of the fusion proteins. The steady-state labeling experiments in the previous section suggested that the fusion proteins were indeed cleaved. In this section, we test the prediction that such cleavage is accomplished by a Golgi protease. If the smaller proteins were produced by action of this enzyme, the novel forms would appear late in the export pathway. Moreover, these forms should coincide with or follow the appearance of the Golgi-modified form of the fusion protein. The basic assumptions for this experiment are that products observed in the pulse are by definition localized to the ER and products appearing late in the chase with slower electrophoretic mobility have passed into the Golgi compartments. To determine the kinetics of processing, we performed pulse-chase experiments at 37°C as described in Materials and Methods. The samples were immunoprecipitated with the gIII specific 282 serum.

Results of these experiments are shown in Fig. 7. The in vivo primary translation product of PRV77 was 68 kDa in that it was the first form synthesized in 2 min. We assume, but have not proved, that this form contains N-linked high-mannose sugar modifications. Unlike the PRV-Be precursor, the 68-kDa precursor of PRV77 was inefficiently chased to the 82-kDa mature form, suggesting that much of the primary translation product is retained in the ER. The important fact is that the 45-kDa cleavage products predicted

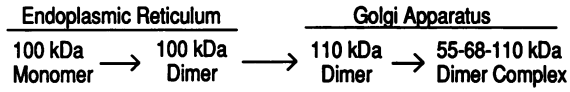

FIG. 8. Proposed export and assembly pathway for PRV gII.

from the previous steady-state labeling experiments (Fig. 5 and 6) did not appear until 90 min of chase. Moreover, their appearance was approximately coincident with the appearance of a diffuse, faint band of approximately 82 kDa, which we assign as the mature product of PRV77 (Fig. 6).

The primary translation product of PRV82 was 74 kDa. It, too, was affected by an export block and slowly chased to a diffuse 94-kDa species that we assign as the mature form (Fig. 6). Although the overall chase was more efficient than that observed from PRV77, a major portion of the precursor remained in the cell, presumably in the ER. The important observation is that the 24-kDa cleavage product (indicated by a line on the lower right of Fig. 7 [Fig. 6]) was not detected until 60 min of chase, after the appearance of the 94-kDa mature species.

The band of approximately 58 kDa that accumulated late in the chase is of unknown origin, but was not detected in steady-state labeling experiments (Fig. 5, PRV77 and PRV82). Finally, it should be noted that the novel 76-kDa cleavage product identified during steady-state labeling by its presence in the media is obscured by the primary translation product in this analysis and that the kinetics of its appearance cannot be determined.

We found that insertion of the 39-base-pair linker in gIII in the opposite orientation resulted in synthesis of a fusion protein that also had an export defect but did not give rise to the proposed cleavage products found for PRV82 (data not shown).

From these experiments, we conclude that the new gIII-specific proteins observed in steady-state labeling experiments arise from a late event in the export pathway, most probably cleavage of the hybrid proteins by a Golgi protease. Cleavage of both hybrid proteins is inefficient, owing in part to the small amount of hybrid protein exported out of the ER. This follows from the observation that the primary translation products of both gIII-gII hybrid proteins chased slowly to the higher-molecular-mass mature species. This effect is probably compounded further, since gII protein itself is not cleaved with 100% efficiency. For example, even in purified virions, we find significant quantities of 110-kDa uncleaved dimers (Fig. 5C, lanes 5).

DISCUSSION

The data presented here define the pathway of export, assembly, and processing of the gII glycoprotein of PRV and give some insight into the protein structure required for proteolytic cleavage. Since the gII protein shares significant homology with HSV-1 gB and a variety of other herpesvirus gB homologs, it is likely that this pathway will be similar in most respects for the gB homologs of herpesviruses in general.

The salient features of this pathway are shown diagrammatically in Fig. 8. gII synthesis initiates on the rough ER, where the 100-kDa monomeric form of gII is the primary product of synthesis. We have shown that this 100-kDa form can be reduced in apparent molecular mass by treatment with endoglycosidase H, suggesting that nascent chains cotranslationally acquire N-linked core glycosylation (data not shown). Shortly after synthesis, the 100-kDa mono-

mers are assembled into oligomers (presumably homodimers) of 100-kDa subunits in the ER. These dimers are exported to the Golgi apparatus for further modifications, which include the addition of fucose. These modifications result in dimers with 110-kDa subunits. Following sugar modification of the 110-kDa dimer, it is cleaved by a Golgi protease into 68 and 55-kDa species that remain tightly bound in a complex with sedimentation characteristics of the 110-kDa dimer. Cleavage does not seem to be 100% efficient, since 110-kDa dimers are found not only in infected cells after several hours of chase following a 2-min pulse (Fig. 1 and 3), but also in purified virions.

The precise composition of the mature dimers is not known. It is striking that the processed form of gII continues to sediment as a dimer. It is also clear that significant quantities of the 110-kDa form sediment as a dimer. It may be that each mature, processed dimer contains an uncleaved subunit of 110 kDa and cleaved subunits of 68 and 55 kDa. Alternatively, both chains of the dimer may be cleaved but are held together by strong interactions, most probably including disulfide linkages. It is also possible that there are at least two populations of mature dimers: uncleaved dimers of 110-kDa subunits and cleaved dimers held together by strong interactions. If two populations of dimers exist, it would be interesting to determine whether both populations are functional. Further work is necessary to determine the absolute composition of the dimers both in the virions and in the infected cells.

The dimer complexes of gII are remarkable for their similarity to those observed for HSV-1 gB by Sarmiento and Spear (30). We have determined that, like HSV-1 gB dimers, gII dimers are held together by both disulfide bonds and hydrophobic interactions (data not shown). Briefly, Triton X-100-solubilized extracts were treated at 37°C for 30 min with one of the following treatments: SDS, dithiothreitol (DTT), and DTT plus SDS. The treatment was carried out on infected-cell lysates prior to sedimentation in the sucrose gradients. Treatment with SDS alone did not disrupt the dimers. Treatment with DTT resulted in a shift of some 100-kDa dimers to monomers. Combined treatment with DTT plus SDS resulted in total disruption of the dimers into their monomeric forms of 110, 100, 68, and 55 kDa. It may be that a population of the 100-kDa dimers (presumably in the ER) is held together only by disulfide bonds, suggesting that the disulfide bonds are formed prior to interactions that require DTT, SDS, and heat for disruption. The protein domains necessary for dimer formation are under investigation in a variety of laboratories. For example, Manservigi et al. (18) have found that the hydrophobic transmembrane domain is required for dimer formation of HSV-1 gB. It will be interesting to see whether these domains are retained in the many gB homologs and whether they have interchangeable functions.

This work also made progress in defining a functional cleavage domain of gII: what amino acid sequence is required to create the specific substrate for gII-specific cleavage. We also hoped to learn something of the required structure of the cleavage substrate. Our kinetic experiments indicated that the substrate for cleavage was the 110-kDa dimer, but it was not clear whether this structure was necessary or sufficient.

When these experiments were initiated, we did not know the precise cleavage site for gII. However, comparing the deduced amino acid sequences and knowing the processing sites of varicella-zoster virus and cytomegalovirus gB homologs (13, 31), we were able to direct our attention to the Arg-Ser residues at positions 502 and 503 as the possible cleavage site. Our first objective was to determine the minimal domain or cassette that would contain the correct sequence and topology for processing, and our second objective was to determine whether this processing cassette could be used to bring about specific cleavage of an unrelated glycoprotein. Briefly, our approach was to take segments of the gII gene and clone them into the well-characterized, nonessential gIII gene with the idea that the resulting hybrid protein should be processed by the same cellular protease that cleaves gII. Such an approach could be complicated by several issues, including the following. (i) gIII is a very different protein from gII in that it is highly modified and does not appear to form oligomers (our unpublished observations). (ii) The hybrid protein may not be exported to the same compartment as gII and may not see the cellular protease. (iii) The hybrid protein may not fold properly, so that the protease cleavage site is inaccessible or does not form correctly. (iv) The hybrid protein may have an export defect due to improper folding and may not be exported to the Golgi for processing. The last is a common problem that we have observed with gIII hybrid proteins (36). Despite these potential problems, we believe that the fusion proteins expressed by PRV77 and PRV82 are indeed cleaved by a protease acting late in the export pathway.

Two gII-gIII fusion proteins were constructed for this analysis. One (expressed by PRV77) contained 168 amino acids of gII encoding the putative protease-processing domain replacing the central 134 amino acids of gIII. The other (expressed by PRV82) contained the complete gIII protein with only 11 additional amino acids of gII containing the putative processing domain. Steady-state labeling experiments indicated the presence of novel gIII-specific proteins expressed by PRV77 and PRV82. Pulse-chase experiments demonstrated which forms corresponded to the primary product of translation (precursor) and clearly demonstrated that the novel products arose late in export, consistent with their being formed by a late event in the Golgi apparatus. We concluded that the both fusion proteins had an export defect because both primary translation products were inefficient in attaining the characteristic shift in molecular mass characteristic of Golgi modifications. We suggest that the origin of these novel proteins can best be explained by specific protease processing directed to a sequence encoded in the gII DNA carried by both PRV77 and PRV82 (Fig. 6). Specific cleavage is suggested primarily by the location of the putative cleavage products and reaction with the B67 antisera (carboxy-terminal specific). First, we suggest that PRV77 produces a hybrid protein whose mature unprocessed form has an apparent molecular mass of 82 kDa. It is cleaved into two species of approximately 45 kDa each. One of these species is secreted into the media (presumably the amino-terminal segment lacking the transmembrane anchor), and the other is retained in membranes and can be localized in virus envelopes. Similarly for PRV82, we suggest that the full-length mature protein has an apparent molecular mass of 94 kDa and is cleaved into a 76-kDa form that is secreted into the media (the amino-terminal portion lacking the transmembrane anchor) The carboxy-terminal 24-kDa protein was observed in infected cells only after long exposure and was detected neither in the media nor in the virus envelope. It is not clear whether the protein is present below our level of detection or is absent in virions.

The results with PRV82 reveal that the 11 amino acids from gII, P-494 AAARRARRS P504 confer two phenotypes to the gIII-gII hybrid protein. First, the hybrid protein is

defective in export from the ER to the Golgi apparatus, and, second, the protein is processed by a late Golgi event. We know that the export defect is not a property of the gII sequence but rather of the insertion itself at this *Sac*I site. When the 39-base-pair linker was inserted in the reverse orientation, a hybrid protein was expressed that had the same export defect (data not shown). Significantly, no processing of the hybrid protein expressed by this virus occurred. Therefore, we suggest that these 11 amino acids of gII contain a functional processing domain of gII. These results imply that the actual substrate for gII cleavage is not necessarily a structure created by the 110-kDa dimer but, rather, is probably inherent in the short segment of amino acids surrounding the cleavage site. The precise site of cleavage for gII is now known to be between R502 and S503 (U. Wolfer, personal communication; M. Riviere, personal communication).

Although the processing of many gB homologs has been reported (13, 21, 22, 24, 31, 33), it is curious that HSV gB is not processed. The significance of the protease cleavage and the resulting protein complex is not well understood. For example, for viruses whose gB protein is cleaved, it is not known whether processing is absolutely required for virus infectivity. Mutant viruses defective in processing, drugs that inhibit the Golgi protease, transient-expression experiments, and cell lines expressing mutant gII proteins are among the experiments in progress to understand the significance of processing.

The processing enzymes for cytomegalovirus and bovine herpesvirus type 1 are of cellular origin (21, 33). It is likely that the other gB homologs are processed by the same enzyme, since the known or predicted cleavage sites all contain basic residues, often two or more arginines. Although the actual protease has not been identified, possible candidates can be suggested. The enkephalin-generating, trypsinlike Golgi-associated enzyme from bovine chromaffin granules has been reported to process peptides containing an arginine-arginine pair as well as lysine-arginine dipeptides (15, 16). It is also possible that gII and the other gB homologs are processed by the same enzyme that cleaves certain retrovirus envelope glycoproteins. For example, four retroviruses, feline leukemia virus, human T-cell lymphotropic virus types I and II, and bovine leukemia virus, all possess arginine residues at the cleavage sites of their glycosylated envelope proteins (23). Further work is needed to identify and localize this important herpesvirus glycoprotein-processing enzyme.

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