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Glycoprotein B of Herpes Simplex Virus Type 1 Oligomerizes through the Intermolecular Interaction of a 28-Amino-Acid Domain

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Herpes simplex virus type 1 glycoprotein B (gB) is an envelope component that plays an essential role in virus infection. The biologically active form of gB is an oligomer that contributes to the process of viral envelope fusion with the cell surface membrane, resulting in viral penetration and initiation of the replication cycle. In previous studies, two discontinuous sites for oligomer formation were identified: a nonessential upstream site located between residues 93 and 282 and an essential downstream site located between residues 596 and 711. In this study, in vitro-transcribed and -translated gB test molecules were used to characterize the more active essential membrane-proximal domain. A series of gB test polypeptides mutated in this downstream oligomerization domain were assayed for their abilities to form oligomers with a mutant gB capture polypeptide containing the analogous wild-type domain. Detection of oligomers was achieved by coimmunoprecipitation of two gB mutant molecules by using a monoclonal antibody specific for a hemagglutinin epitope tag introduced into the coding sequence of the capture polypeptide. Analysis of the immune-precipitated products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated that the downstream oligomerization domain resided within residues 626 to 676. This region was further resolved into two segments, residues 626 to 653 and 653 to 675, each of which was independently sufficient to form oligomers. However, residues 626 to 653 provided for a stronger interaction between gB monomers. Moreover, this stretch of 28 amino acids was shown to form oligomers when introduced into the carboxy-terminal region of gB monomers lacking this domain at the normal site, thus indicating that this domain was functionally independent of its natural location within the gB molecule. Further analysis of the sequence within residues 596 to 653 by using mutant test polypeptides altered in individual amino acids revealed that cysteines 9 and 10, located at positions 596 and 633, respectively, were not required for oligomer formation but contributed to dimer formation and/or stabilization. The results of this study suggest that oligomerization of gB monomers is induced by interactions between contiguous residues localized within the ectodomain near the site of molecule insertion into the viral envelope membrane.

Herpes simplex virus type 1 (HSV-1) specifies at least 11 glycoproteins, designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM (3, 33, 34, 41, 54; for reviews, see references 59 and 61). Glycoproteins B, D, H, and L have each been demonstrated to be essential for virus replication in tissue culture (10, 18, 33, 39, 40). The gB homologs constitute the most highly conserved protein among herpesvirus glycoproteins, suggesting a common essential function in the herpesvirus life cycle, namely, membrane fusion events such as those required for virus penetration and cell-to-cell spread of the virus from infected to adjacent noninfected cells (8, 47, 49, 56). Glycoprotein B is also a potent immunogen capable of inducing both neutralizing antibody and virus-specific cytotoxic T cells (23, 24, 29, 30, 44), and information on the antigenic structure of gB in relation to its functional domains has been reported (11, 43).

Glycoprotein B is composed of 904 amino acids which include a cleavable signal sequence of 30 residues, a 697-residue external domain, a 68-residue hydrophobic transmembrane domain containing three putative segments traversing the membrane bilayer (where the third segment is proposed to specify the anchor sequence) (55), and an extensive cytoplasmic domain of 109 residues located at the carboxy terminus of the protein (6, 7, 50). The role of the cytoplasmic domain in cell-cell fusion is supported by the mapping of *syn* mutations within this region (4, 26). HSV-1 gB contains 6 consensus sequence sites for N-linked glycosylation and 10 cysteine residues that are highly conserved among gB homologs (50). These structures are likely to be important for the overall intramolecular organization of the molecule.

The biologically active form of gB is a multimer (9, 13, 31, 60). This conclusion is based on the observation that mutant polypeptides coexpressed with their wild-type counterparts lead to a decrease in infectious virus (9). Since defective forms of gB inhibit complementation of gB null mutants by the wild-type gB molecule, mutant forms exhibit the property of negative transdominance. However, this phenomenon is dependent on the ability of a mutant molecule to form hetero-oligomers with the wild-type molecule, indicating that the gB molecule in its simplest active form is a dimer. The oligomer-forming domain has been localized between amino acid residues 463 and 791 (9). Later studies further determined that the region which contributed to the assembly, conformation, and stability of gB dimers resides within residues 630 to 720 (53). This region lies within the ectodomain and functions in oligomerization independently of the hydrophobic transmembrane sequence and the intracellular carboxy terminus (2, 53). Highlander et al. (31) identified two sites of oligomerization by using a coimmunoprecipitation assay of polypeptides expressed from cotransfected Vero cells; these included an upstream site located between residues 93 and 282 and a downstream site located between residues 595 and 711. However, Cai et al. (9) showed

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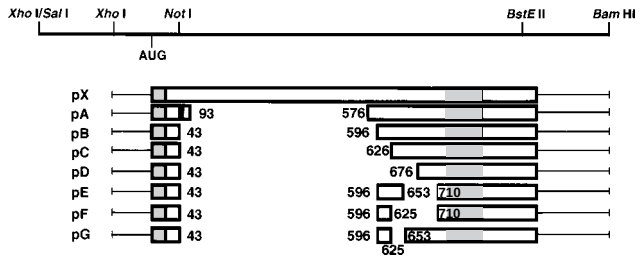

FIG. 1. Structures of gB mutant plasmids pA through pG. The region of the HSV-1 genome encoding glycoprotein gB present in the plasmid pKBXX (10) is shown at the top. Relevant restriction sites and the translation start site are indicated. The second *Bst*EII site specific for KOS DNA (16) resides within the deleted sequences of pA to pG and is not shown in the diagram. pX encodes a 3.46-kb HSV-1 fragment that includes the 2.7-kb gB-coding sequence shown. The hemagglutinin epitope tag (■) was introduced into pA following residue 41 to detect oligomer formation. gB-coding sequences specified by the plasmids are indicated by open rectangles, and the numbers refer to codons present at the boundaries of the deletions. The signal peptide (left) is represented by a cross-hatched box, and the transmembrane domain (right) is represented by a gray rectangle.

that deletion of the downstream site (residues 616 to 711) eliminated the ability of the mutant gB molecule to inhibit complementation, suggesting that the upstream site provided only a weak interaction between gB monomers and was not sufficient to compete with interactions imparted by the membrane-proximal site. Moreover, a virus mutant containing a gB gene with a deletion of the downstream site-coding sequence was not able to form infectious virus and did not form oligomers when infected-cell extracts were analyzed by sedimentation (17). This virus did, however, interfere with complementation when grown in a cell line capable of expressing wild-type gB. This effect was attributed to competitive interference with the processing of the wild-type molecule rather than hetero-oligomer formation with the gB mutant protein expressed from the viral genome.

In this study, we further defined the membrane-proximal site by using a coimmunoprecipitation assay involving gB mutant polypeptides cotranslated in vitro. The results of this study demonstrated that two oligomerization domains were located between residues 626 and 675; these domains were independently sufficient to produce oligomers, but residues 626 to 653 demonstrated stronger oligomerization properties. Moreover, these residues (626 to 653) represent a moveable domain capable of restoring the oligomerization phenotype to an oligomer-deficient polypeptide. Site-directed mutagenesis experiments that altered individual residues showed that the cysteines at positions 596 and 633 were not required for oligomerization but suggested that these amino acids stabilized the oligomer-forming site and/or increased the efficiency of formation of the oligomers.

MATERIALS AND METHODS

Plasmid construction. Restriction sites relevant to the present study, in the region of the glycoprotein B gene of HSV-1 strain KOS, are shown in Fig. 1. The gB transcription start site is 33 nucleotides upstream of the *Xho*I site (48). Therefore, transfer of the *Xho*I-*Bam*HI fragment to the *Sal*I-*Bam*HI sites of pGEM-3 (Promega, Madison, Wis.) places the gB-coding sequence under the control of the SP6 promoter contained within pGEM-3, providing a means for in vitro transcription of gB message, which can be subsequently translated and analyzed for oligomerization.

Previous studies showed that amino acid residues 43 to 595 are not required for oligomer formation, since gB polypeptides with these residues deleted formed hetero-oligomers when cotranslated in the presence of a gB molecule with residues 93 to 576 deleted and this latter truncated molecule also formed an hetero-oligomer with the wild-type molecule (31). Therefore, most of the plasmids used in the present study were derived from pKΔ5C, which had amino acid residues 43 to 595 deleted, since the oligomerization-forming domain was still intact. pKΔ5C also encodes six amino acids not present in the mature gB sequence because of prior linker insertions between codons 43 and 595 (9, 31). The *Xho*I-*Bst*EII fragment of pKΔ5C was further modified to remove the coding sequence for these amino acids by using a PCR overlap extension technique that requires four oligonucleotides as primers (32). One primer was complementary to the coding strand upstream of the *Xho*I site, and a second primer was complementary to the noncoding strand downstream of the *Bst*EII site. A pair of complementary internal primers was designed to delete the desired sequence. Application of this method with pKΔ5C, for example, used internal primers which encoded gB residues 39 to 43 and 596 to 598. Each complementary primer was used with the appropriate 5′- or 3′-terminal oligonucleotides in PCRs that generated 5′ and 3′ DNA fragments having complementary ends at residues 39 to 43 and 596 to 598. The PCR products were gel purified and annealed in a subsequent reaction in which the 3-OH of each strand served as a primer for the synthesis of the complementary strand. The resulting product was amplified further by PCR, enzymatically digested with *Xho*I-*Bst*EII, and cloned into pKΔ5C previously digested with the same enzymes. The modified gB sequence in pKΔ5C was then excised as an *Xho*I-*Bam*HI fragment and ligated into *Sal*I-*Bam*HI-digested pGEM-3 to generate a new plasmid designated pB. Plasmids pC, pD, pE, pF, pG, pB(C633S), and pB(C596S/C633S) were derived from pB by using the PCR overlap extension technique.

Plasmid pA was constructed as follows. The *Xho*I-*Bam*HI fragment from plasmid pKΔSs (31) was cloned into pGEM-3. To detect the presence of oligomers, complementary oligonucleotides encoding a nonapeptide epitope of influenza virus hemagglutinin (65) were inserted in frame at a unique *Not*I site between codons 41 and 42 (10) of the gB sequence. The correct orientation of the epitope tag sequence was determined by DNA sequence analysis. It had been previously shown that sequences corresponding to codons 43 to 92 could be deleted from gB without loss of function (17a). Plasmid pH was constructed by cloning the *Xho*I-*Bam*HI fragment from pKΔ4B (9) into pGEM-3. The pI plasmid was generated by PCR overlap extension from pC and lacks in addition nucleotides coding for residues 654 to 710. The gB-coding sequence from pI was cloned into pGEM-7 and subjected to oligonucleotide-directed in vitro mutagenesis with the Bio-Rad (Richmond, Calif.) mutagenesis kit to create pI(C633S), which has a substitution of a serine for a cysteine at position 633. Plasmids pAC and pHC were generated by cloning the PCR product coding for residues 626 to 653 of pB into the *Bst*EII sites (after residue 875) of pA and pH, respectively. Plasmids pAC(C633S) and pHC(C633S) were generated like pAC and pHC except that pB(C633S) was used as the PCR template. The mutations in all plasmids were sequenced by using the Sanger-Nicklen-Coulson Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). DNA restriction and DNA-modifying enzymes (New England BioLabs, Inc., Beverly, Mass.) and PCR reagents (Perkin-Elmer Cetus, Norwalk, Conn.) were used according to the manufacturer's specifications.

Preparation and analysis of gB mutant polypeptides. In vitro transcription was performed on 10 μg of linearized templates by using SP6 RNA polymerase (Promega). Forty nanograms of in vitro-derived transcript was translated in a rabbit reticulocyte lysate (nuclease treated) supplemented with canine pancreatic microsomal membranes (Promega) in the presence of [^{35}S]methionine (NEN Dupont). After translation, or after cotranslation of the two RNA species, the translation reaction mixture was divided into three parts: one was used directly for electrophoresis, one was immunoprecipitated with a gB-specific polyclonal antibody (a gift of Thomas Holland, Wayne State University), and one was immunoprecipitated by using the monoclonal antibody (MAb) 12ca5-j produced in response to the hemagglutinin nonapeptide (BAbCO, Richmond, Calif.). Immunoprecipitation was performed in a buffer consisting of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40 (Sigma, St. Louis, Mo.), and 0.5 mM tosyl lysyl chloromethyl ketone (TLCK) (Sigma). The immune complexes were incubated with protein A-Sepharose (Sigma) for 1 h, and the resulting complex was centrifuged and washed five times with 600 μl of buffer. The pellet was resuspended in Laemmli loading buffer (38), boiled for 2 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12 or 17% polyacrylamide) with 1.2% *N,N′*-diallyltartardiamide (Bio-Rad) as a cross-linker. After electrophoresis, the gel was fixed, treated with En^{3}Hance solution (NEN-Dupont), vacuum dried, and exposed to X-OMAT-XAR film (Kodak, Rochester, N.Y.). For the analysis of the presence of disulfide bridges involved in dimer formation by gB mutant polypeptides, individually translated gB mutant polypeptides were solubilized in loading buffer in the presence or absence of beta-mercaptoethanol and subjected to SDS-PAGE. After electrophoresis, the gels were treated as described above.

Sedimentation analysis of gB polypeptides. Vero cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Gibco/BRL Laboratories, Grand Island, N.Y.). Confluent monolayers of Vero cells in 60-mm-diameter dishes were infected at a multiplicity of infection of 10 with wild-type (KOS) virus or with a gB mutant virus with residues 43 to 595 deleted (KΔ5C) (9, 31). At 6 h postinfection, the cells were labeled with 250 μCi of [^{35}S]methionine (NEN Dupont) for a period of 10 min (pulse) and either harvested directly or washed and incubated for 1 h (chase) in fresh complete medium before being harvested. Cells were harvested by scraping the monolayer in 1 ml of Tris-saline buffer, pelleted, and resuspended in 200 μl of lysis buffer (0.5 mM Tris-HCl [pH 6.8], 150 mM NaCl, 1% Triton X-100 [Sigma]) supple-

mented with 1 mM TLCK (Sigma). The cell suspension was lysed on ice for 10 min before centrifugation for 15 min at maximal speed in a table microcentrifuge at 4°C. Supernatants were layered on top of an ultracentrifuge tube previously filled with 10.8 ml of an 8 to 20% (wt/wt) sucrose gradient prepared in the lysis buffer described above. Centrifugation was carried out at 4°C for 25 h (KOS) or 54 h (KΔ5C) at 40,000 rpm in an SW41 rotor. After centrifugation, fractions were collected, immunoprecipitated with a pool of gB-specific MAbs (43), and analyzed by SDS-PAGE. After electrophoresis, the gels were treated as described above.

RESULTS

In vitro assay for gB oligomer formation. The present study was undertaken to finely map the downstream oligomerization site localized between residues 596 and 711. Throughout this study a coimmunoprecipitation assay was used to evaluate oligomer formation. In this assay, two plasmids encoding gB products were transcribed and translated in vitro, and the resulting gB polypeptides were tested for their ability to oligomerize. Most plasmids used in the present study encode gB residues 1 to 43 (of which residues 1 to 30 specify the signal sequence), a portion of the extracellular domain consisting of residues 596 to 726, the transmembrane domain (residues 727 to 795), and the cytoplasmic domain (residues 796 to 904) (Fig. 1). One product was wild type with respect to the oligomerization domain (capture molecule), and the other expressed a truncated or mutated form of this domain (test molecule). Moreover, the capture and test products contained a common antigenic site within the cytoplasmic domain of the molecule, whereas the capture molecule was engineered to contain a unique epitope derived from the hemagglutinin protein of influenza virus. Thus, following cotranslation, both products could be immunoprecipitated with the anti-cytoplasmic domain antibody (Abc), while only the capture molecule could be immunoprecipitated by the anti-hemagglutinin epitope antibody (Abn). If on cotranslation of the capture and test products, the mutated gB polypeptides formed oligomers, then immunoprecipitation of the capture product with the Abn antibody would coprecipitate the test product. Coprecipitation of the test product was then taken as evidence for oligomer formation and provided a method for analyzing the roles of specific gB sequences in this interaction. This technique is illustrated in Fig. 2 for the plasmids pA and pB.

For Fig. 2, the in vitro-transcribed RNA from each plasmid which contains a downstream oligomer sequence was translated in an in vitro translation system with a rabbit reticulocyte lysate either separately or together in the presence of [^{35}S]methionine and canine microsomal membranes. Omission of these membranes led to the absence of translated products, whereas their presence led to full-length translated polypeptides exhibiting endo-β-*N*-acetylglucosaminidase sensitivity (data not shown). The gB products were analyzed by SDS-PAGE following immune precipitation with antibody that recognized epitopes present in both polypeptides (Abc) to visualize the products from both plasmids or with antibody to the influenza virus hemagglutinin epitope (Abn), present only on the capture polypeptide, to determine whether oligomer formation occurred. The translation product of pA was immunoprecipitated by the Abn antibody (Fig. 2B, lane 2), since it contained the influenza virus hemagglutinin epitope, as well as by the Abc antibody (lane 3). The translated product could also be detected without immunoprecipitation (Fig. 2B, lane 1). As expected, the translated product of pB was immunoprecipitated by the Abc antibody (Fig. 2B, lane 6) but not by the Abn antibody (lane 5), since this site was absent from the translated product of pB. However, following cotranslation of pA and pB (Fig. 2B, lanes 7 to 9), the pB polypeptide was coprecipitated by the antibody specific for the hemagglutinin epitope (lane 8).

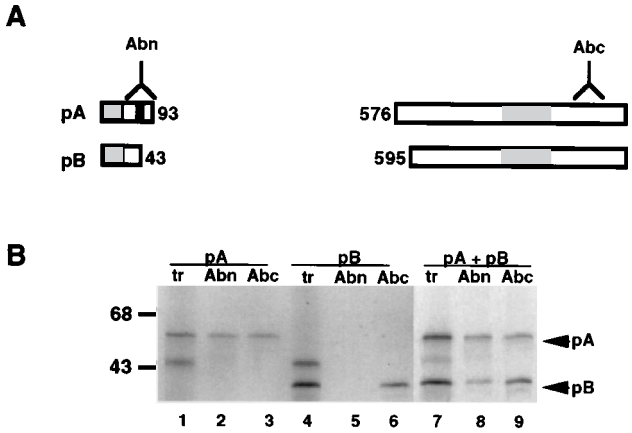

FIG. 2. In vitro assay for oligomer formation. (A) Antibody recognition sites present in the polypeptides encoded by plasmids pA and pB. The antibodies are designated Abc (specific for the cytoplasmic domain of gB) and Abn (specific for the hemagglutinin epitope tag [■]). pB encodes a polypeptide that is recognized only by Abc antibody. Proteins are represented as described for Fig. 1. (B) Translation products analyzed directly (tr) or following immune precipitation with antibodies by SDS-PAGE. Individual translation products are shown for pA (lanes 1 to 3) and pB (lanes 4 to 6) and for the cotranslated products of pA and pB (lanes 7 to 9). The mobilities of polypeptides encoded by pA and pB are indicated by arrows on the right, while the mobilities (in thousands) of radioactively labeled marker polypeptides are indicated on the left.

Thus, the polypeptide containing the Abn antibody epitope (pA product) captured the epitope-deficient polypeptide (pB product) because of the interaction between the two molecules. This was taken as evidence of formation of oligomers between the pA and pB polypeptides. This assay was then used to further define sequences which were essential for oligomer formation by the gB polypeptides.

In addition to the expected products of pA (Fig. 2B, lane 1) and pB (lane 4) or the cotranslated product of both mutant gB polypeptides (lane 7), a band at approximately 42 kDa was detected. The labeling of this band is thought to result from a tRNA-dependent but ribosome-independent addition of methionine to a preexisting protein present in the reticulocyte lysate (35) and not antigenically related to gB since it was not immunoprecipitated by the Abc antibody (Fig. 2B, lanes 3, 6 and 9) or by any other anti-gB MAbs described by our group (reference 43 and data not shown).

Localization of the gB oligomer-forming domain. The in vitro-transcribed RNAs from plasmids pB through pG were each cotranslated with RNA from pA, and the polypeptides were analyzed for oligomer-forming ability (Fig. 3). The sequences encoded by pB (residues 596 through 904) were sufficient for coimmunoprecipitation by Abn antibody, which is specific for the hemagglutinin epitope tag present only in the pA polypeptide (Fig. 2 and Fig. 3, lane 2). The translation product of pC (containing residues 626 through 904) was also coimmunoprecipitated by the Abn antibody (Fig. 3, lane 4), while the translation product of pD (containing residues 676 through 904) was not immunoprecipitated (lane 6). These data indicated that a structure formed by residues 626 through 675 was important for oligomer formation.

It has been previously shown by Highlander et al. (31) that a polypeptide encoded by pKΔ4B (9) containing residues 1 to 43 and 711 to 904 did not contribute to oligomer formation. Plasmids pE and pF were constructed such that they encode residues present in pKΔ4B and the sequence or a portion of the sequence between residues 596 and 653. The translation product of pE (containing residues within pKΔ4B plus residues

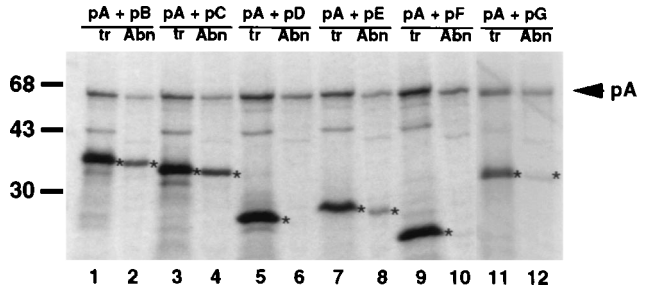

FIG. 3. Mapping of the downstream oligomerization domain of HSV-1 gB. Each of the RNA species encoded by plasmids pB through pG (see Fig. 1) were cotranslated in the presence of pA, and the translation products were analyzed directly (tr) or following immunoprecipitation with the antibody specific for the hemagglutinin epitope encoded only by pA (Abn). The mobility of the pA-encoded polypeptide is shown at the right, and the positions of the polypeptides encoded by pB through pG are indicated by asterisks. Numbers on the left indicate the mobilities (in thousands) of radioactively labeled marker polypeptides.

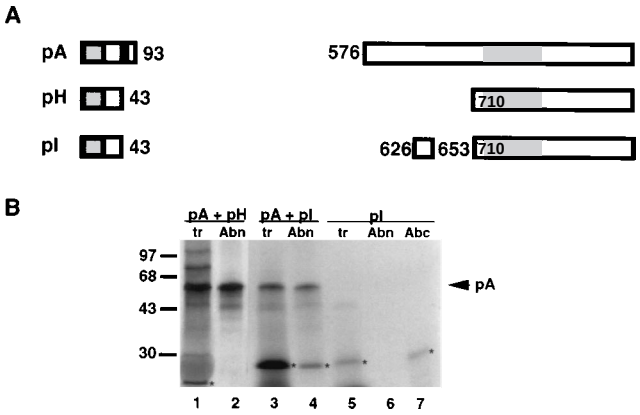

FIG. 4. Amino acids 626 to 653 of gB are capable of effecting gB oligomer formation. (A) Structures of the mutant gB plasmids pA, pH, and pI. Proteins are represented as described for Fig. 1. (B) pA was cotranslated in presence of pH (lanes 1 and 2) and of pI (lanes 3 and 4). The cotranslated polypeptides were analyzed directly (tr) (lanes 1 and 3) or following immunoprecipitation with Abn antibody (lanes 2 and 4). pI polypeptide translated in absence of pA polypeptide was analyzed directly (lane 5) or after immunoprecipitation with Abn (lane 6) or Abc (lane 7) antibody. The arrow on the right side indicates the mobility of the pA product, and the asterisks show the mobilities of the pH and pI translation products. Numbers on the left indicate the mobilities (in thousands) of radioactively labeled marker polypeptides.

596 to 653) was immunoprecipitated by Abn antibody when cotranslated in the presence of pA RNA (Fig. 3, lane 8); however, the pF translation product (containing residues within pKΔ4B plus residues 596 to 625) cotranslated in presence of pA RNA (lane 10) was not coimmunoprecipitated. These results indicated that residues 596 to 625 were not sufficient for oligomerization, while residues 626 to 653 appeared to contain the gB oligomer-forming domain. However, the translated product of pG (containing residues within pB with the additional deletion of residues 626 to 652) was coprecipitated by the Abn antibody when cotranslated in the presence of pA RNA (Fig. 3, lane 12) but in very low abundance, suggesting that residues 653 to 676 also contain amino acids capable of inducing oligomer formation. As visualized in Fig. 3, the size of each of the mutant polypeptides was consistent with the predicted size deduced from the coding sequence present in the mutant gB constructs. SDS-PAGE analysis of the coimmunoprecipitated products demonstrated that all mutant proteins were precipitated by the Abc antibody and, as expected, that none of these smaller deletion mutant polypeptides were immunoprecipitated by the Abn antibody when translated in absence of pA RNA (data not shown).

In order to verify that residues 626 to 653 were sufficient for oligomerization, their coding sequences were inserted into plasmid pH to generate pI. As described in Materials and Methods, pH and pKΔ4B encode the same gB residues, and sequences within pKΔ4B did not inhibit complementation (9) or produce an oligomer-forming product in transient assays (31). As expected, the translation product of pH was not coimmunoprecipitated by the Abn antibody when cotranslated in presence of the pA polypeptide (Fig. 4B, lane 2). However, the translation product of pI was immunoprecipitated by the Abn antibody (lane 4). When pI RNA was translated in the absence of pA RNA, this polypeptide was not immunoprecipitated by the Abn antibody (Fig. 4B, lane 6) but was immunoprecipitated by the Abc antibody (lane 7). These results demonstrated that the sequence encompassing residues 626 to 653 was sufficient to promote oligomer formation.

Residues 626 to 653 inserted into the cytoplasmic domain of two gB mutant molecules are sufficient to cause oligomerization. The experiments described above demonstrated that amino acid residues 626 to 653 were sufficient for oligomer formation (Fig. 4). To determine if these sequences were active in a manner independent of their position in the gB molecule, this domain was inserted into the cytoplasmic domain of gB and tested for oligomerization. The sequence encoding residues 626 to 653 was inserted between the codons for amino acids 875 and 876 of plasmids pA and pH (Fig. 5A). In order to maintain the reading frame, two amino acids were added between residue 875 of pA/pH and residue 626 of the inserted

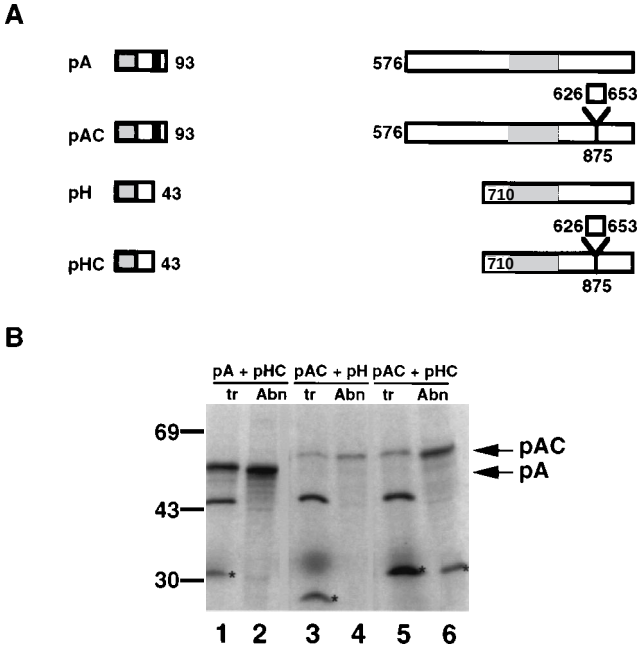

FIG. 5. Residues 626 to 653 inserted into the gB cytoplasmic domain of two constructs are sufficient for oligomer formation. (A) Structures of plasmids pA, pAC, pH, and pHC, displaying the insertion of residues 626 to 653 into the cytoplasmic domain of pA and pH. Proteins are represented as described for Fig. 1. (B) Cotranslation product analysis before (tr) and after (Abn) Abn antibody immunoprecipitation. The mobilities of the pA and pAC polypeptides are indicated at the right, and the mobilities of pH (lane 3) and pHC (lanes 1 and 5) are indicated by asterisks. Numbers on the left indicate the mobilities (in thousands) of radioactively labeled marker polypeptides.

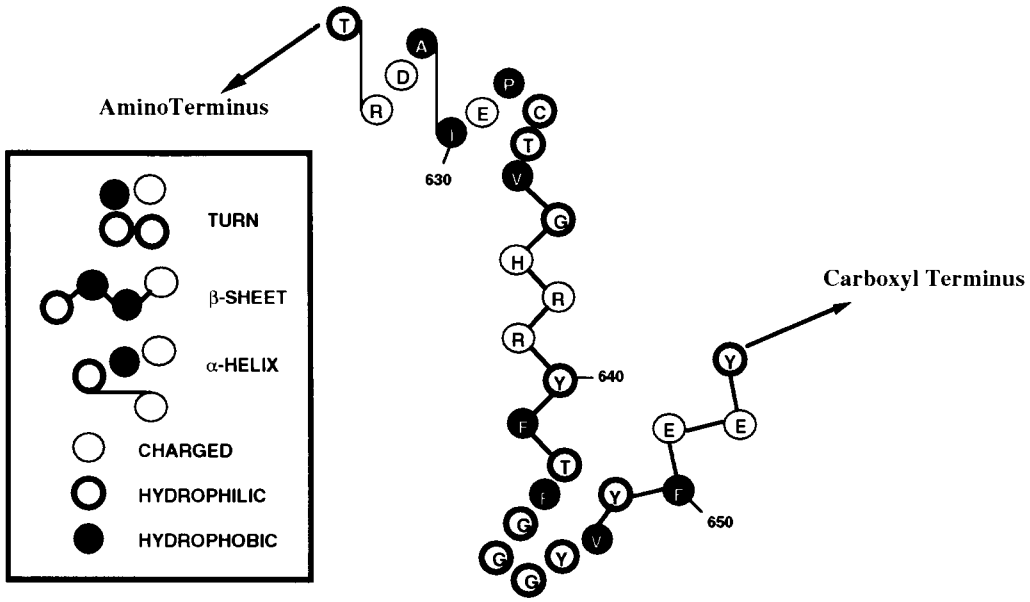

FIG. 6. Predicted secondary structure of the gB sequence between residues 626 and 653. The Chou-Fasman and Garnier-Osguthorpe-Robson parameters were used to predict the structural features of the oligomerization domain of gB. The amino acids are numbered in 10s, and the standard one-letter amino acid code is used. The arrows indicate the directions of the carboxyl and amino termini of the represented gB sequence in the gB protein.

sequence (valine and threonine), while one amino acid was added between residue 653 of the insert and residue 876 of pA/pH (leucine). The cotranslation product combinations tested are indicated at the top of Fig. 5B. We previously demonstrated that the polypeptide encoded by pH (Fig. 4), which does not encode the oligomer-forming domain, was not coprecipitated by the Abn antibody when cotranslated in presence of the pA polypeptide. In cotranslation experiments when a cytoplasmic domain oligomerization site was present on only one of the two polypeptides, there was no detectable coprecipitation product (Fig. 5B, lanes 2 and 4). However, when both of the recombinant gB molecules containing the oligomer-forming domain in their cytoplasmic domains (pAC and pHC) were tested for oligomerization, coprecipitation was observed (Fig. 5B, lane 6). These same results were also obtained when the oligomerization domain was inserted after residue 824 (*Mlu*I site) of pH and pA polypeptides (data not shown). These observations indicated that residues 626 to 653 represent a moveable domain that is sufficient to induce oligomer formation.

Substitution of serine for cysteine at position 633 reduces oligomerization. Secondary structure analysis of the sequence of 28 residues located within residues 626 to 653 did not reveal any putative leucine zipper motif or other known motifs characteristic of protein-protein interactions in dimerization (52). However, a beta sheet (residues 634 to 643) followed by a turn (residues 643 to 647) and an alpha helix (residues 649 to 657) was indicated by the Chou-Fasman (12) and Garnier-Osguthorpe-Robson (27) parameters for secondary protein structure predictions (28) (Fig. 6). Moreover, within the gB mutant polypeptide encoded by pI only one cysteine (at position 633) is present in the entire molecule. This cysteine is located within the oligomerization domain (Fig. 6) and could be involved in cystine residue formation during oligomerization. To examine the potential role of cysteine 633 in oligomer formation, cysteine 633 was replaced by a serine by site-directed mutagenesis, the resulting mutant polypeptide, pI(C633S), was analyzed for oligomer formation with the pA polypeptide, and the oligomer-forming capability was compared with that of the pA and unmodified pI polypeptides. The gB polypeptides encoded by pI were cotranslated in the presence of the pA polypeptide (Fig. 7B, lane 1) and coimmunoprecipitated as expected by Abc and Abn antibodies (lanes 2 and 3, respectively). The cotranslated product of pA and pI(C633S) (Fig. 7B, lane 4) was immunoprecipitated by the Abc antibody (lane 5); however, the Abn antibody-immunoprecipitated products (lane 6) displayed a twofold decrease (quantified by densitometry) in oligomerization. This decrease is based on comparing the ratio of the Abn antibody-immunoprecipitated product of the pI and pA polypeptides (Fig. 7B, lane 3) with the ratio of the

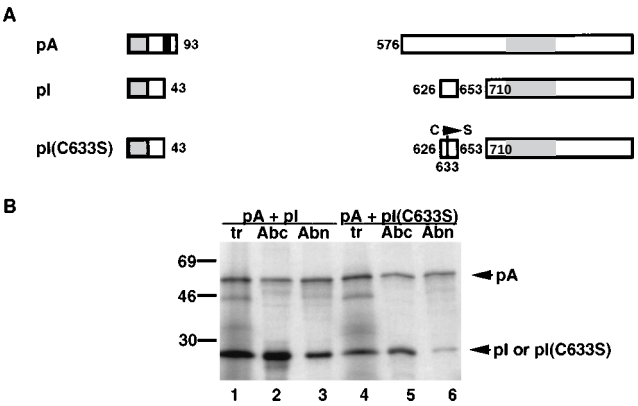

FIG. 7. Substitution of a serine for cysteine at position 633 impairs the ability of gB polypeptides to form oligomers. (A) Structures of the gB mutant polypeptides pA, pI, and pI(C633S), displaying the substitution of a serine for cysteine at position 633 in the pI polypeptide. Proteins are represented as described for Fig. 1. (B) pA was cotranslated in presence of pI (lanes 1 to 3) and of pI(C633S) (lanes 4 to 6). The cotranslated polypeptides were analyzed directly (tr) (lanes 1 and 4) or following immunoprecipitation with Abc (lanes 2 and 5) or Abn (lanes 3 and 6) antibody. The arrows on the right indicate the mobilities of the pA and pI [or pI(C633S)] polypeptides. Numbers on the left indicate the mobilities (in thousands) of radioactively labeled marker polypeptides.

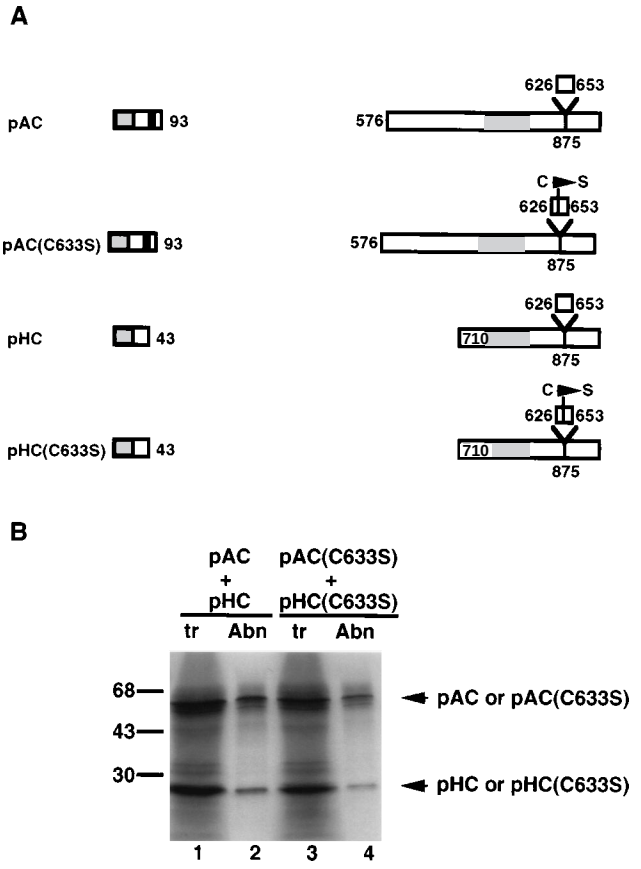

FIG. 8. Substitution of a serine for cysteine at position 633 of residues 626 to 653 inserted into the gB cytoplasmic domain of pA and pAC reduces the ability of gB polypeptides to form oligomers. (A) Structures of the plasmids pAC, pAC(C633S), pHC, and pHC(C633S), displaying the locations of the single amino acid substitution introduced into the oligomerization domain (residues 626 to 653) previously inserted into the cytoplasmic domains of pA and pH. Proteins are represented as described for Fig. 1. (B) Cotranslation product analysis before (tr) and after (Abn) Abn antibody immunoprecipitation. The arrows on the right indicate the mobilities of pAC [or pAC(C633S)] and pHC [or pHC(C633S)]. Numbers on the left indicate the mobilities (in thousands) of radioactively labeled marker polypeptides.

pI(C633S) and pA polypeptides (lane 6). These results suggest that the cysteine at position 633 is not required for oligomerization but stabilizes and/or increases the efficiency of oligomer formation by the gB mutant polypeptides.

The same substitution of a serine for a cysteine at position 633 was performed with the pAC- and pHC-encoded polypeptides, in which the oligomerization domain encoding residues 626 to 653 was introduced into the carboxy-terminal domain of the molecule. The structures of the plasmids used for this experiment are displayed in Fig. 8A. As demonstrated previously (Fig. 5), the polypeptides encoded by pAC and pHC formed oligomers as visualized by the presence of the pHC polypeptide in the Abn antibody-immunoprecipitated products (Fig. 8B, lane 2). Substitution of a serine for cysteine at position 633 within the oligomerization domain introduced into the carboxy-terminal domains of pAC and pHC also reduced oligomerization by about 50% (Fig. 8B, lane 4). These results confirmed those shown in Fig. 7 for the pI and pI(C633S) polypeptides, in which the substitution of serine for cysteine at position 633 reduced but did not completely disrupt the oligomerization properties of the two polypeptides. Again, the reduction in oligomerization suggested that the cysteine at position 633 might play a role in the efficiency of oligomer formation and/or in stabilizing the complex.

Role of disulfide bridge formation in the oligomerization properties of the gB mutant polypeptides. Since the substitution of a serine for the only cysteine present into the entire molecule encoded by pI decreased oligomer formation (Fig. 7), it was anticipated that an intermolecular disulfide bridge between cysteines located on different molecules played a role in oligomer formation or maintenance of the complex. To confirm the possible role of intermolecular disulfide bridge oligomerization, the pI and pI(C633S) polypeptides were individually translated and subjected to SDS-PAGE analysis under reducing and nonreducing conditions (Fig. 9). The translated product of pI was resolved as a monomer under reducing conditions (lanes 1 and 2) independently of the boiling step but was resolved as a mixture of monomers and dimers under nonreducing conditions (lanes 3 and 4). However, as expected, the translated product of pI(C633S), which did not contain cysteine, was detected only as a monomer under either reducing (Fig. 9, lanes 5 and 6) or nonreducing (lanes 7 and 8) conditions. These results suggested that a disulfide bridge was formed between the two cysteines at position 633 located on different molecules of pI. However, this intermolecular disulfide bridge cannot by itself account for the oligomer-forming property of the pI polypeptide, since the polypeptides encoded by pI(C633S) or pHC(C633S), in which the cysteine was replaced by serine, still formed oligomers with the products of the pA or pAC(C633S) polypeptides, respectively (Fig. 7 and 8). The presence of the monomeric form of the pI polypeptide detected under nonreducing conditions (Fig. 9, lanes 3 and 4) suggested that the majority of the pI polypeptides were not in a dimeric complex. However, when the translation was carried out with a lower concentration of the microsomal membranes (data not shown), dimerization was more efficient, suggesting that the presence of the monomeric forms of the pI polypeptide might be attributable to a low density of gB molecules in the microsomal membrane. An extra band migrating at 50 kDa in lane 3 of Fig. 9 is of unknown origin but was frequently observed in samples that were not boiled or reduced.

Cysteines having free disulfide groups are more active in forming disulfide bonds than cysteines already covalently

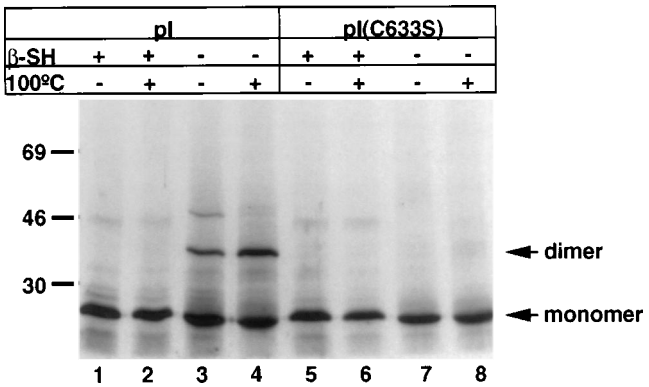

FIG. 9. Effect of beta-mercaptoethanol on the dimerization of pI- and pI(C633S)-encoded polypeptides. The translated products of pI (lanes 1 to 4) and pI(C633S) (lanes 5 to 8) were solubilized in loading buffer in presence (lanes 1, 2, 5, and 6) or absence (lanes 3, 4, 7, and 8) of beta-mercaptoethanol (β-SH). Samples were either boiled (100°C) for 2 min (lanes 2, 4, 6, and 8) or directly subjected (lanes 1, 3, 5, and 7) to SDS-PAGE. The arrows indicate the positions of the monomeric and dimeric forms of the gB mutant polypeptides. Numbers on the left indicate the mobilities (in thousands) of radioactively labeled marker polypeptides.

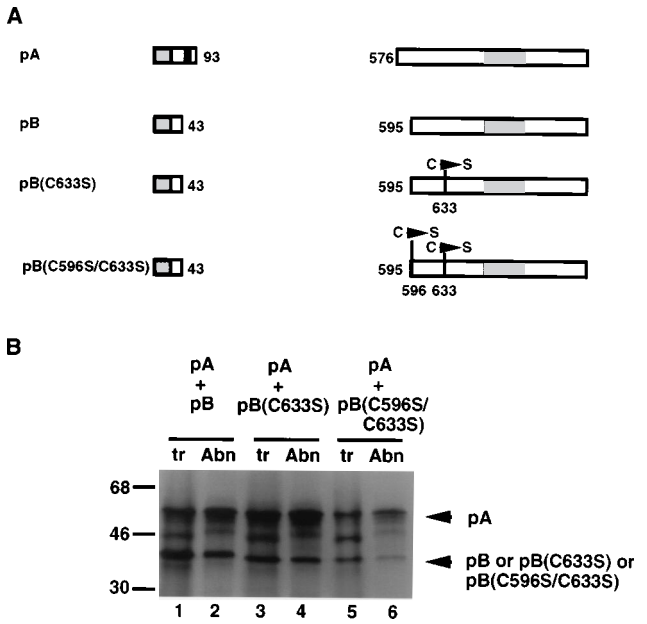

FIG. 10. Effect of a single substitution of a serine for cysteine at position 633 and a double substitution of serines for cysteines at positions 596 and 633 on oligomerization properties of pB polypeptide with pA polypeptide. (A) Structures of the gB mutant polypeptides pA, pB, pB(C633S), and pB(C596S/C633S), displaying the substitutions of a serine for cysteine at position 596 and/or 633 in the pB polypeptide. Proteins are represented as described for Fig. 1. (B) pA polypeptide was cotranslated in presence of pB (lanes 1 and 2), pB(C633S) (lanes 3 and 4), and pB(C596S/C633S) (lanes 5 and 6). The cotranslated polypeptides were analyzed directly (tr) (lanes 1, 3 and 5) or following immunoprecipitation with Abn antibody (Abn) (lanes 2, 4, and 6). The arrows on the right indicate the mobilities of pA and pB [or pB(C633S) or pB(C596S/C633S)]. Numbers on the left indicate the mobilities (in thousands) of radioactively labeled marker polypeptides.

linked through their sulfide groups (15). The intermolecular disulfide bridges observed between the two polypeptides translated from pI could thus represent more-reactive molecules in terms of their potential for covalent bonding. Cysteines 9 and 10 (positions 596 and 633, respectively) have been suggested to form disulfide bridges as determined by mass spectrometry and amino acid sequencing analysis of gB peptides generated by trypsin digestion under nonreducing conditions and isolated by reverse-phase high-performance liquid chromatography (5a). Whether these disulfide bridges were inter- or intramolecular had not been determined. In order to determine the role of cysteine 10 as an intra- or intermolecular disulfide-bounded partner in oligomerization, we analyzed the oligomerization properties with the translated product of pA of a gB polypeptide containing cysteines 9 and 10 (pB) and two other polypeptides in which one cysteine [pB(C633S)] or two cysteines [pB(C596S/C633S)] have been replaced by a serine residue (Fig. 10). The structures of the polypeptides used in this assay are displayed in Fig. 10A. The polypeptide translated from pB contains cysteines 9 and 10 (positions 596 and 633, respectively), whereas the polypeptide translated from pB(C633S) has a substitution of serine for cysteine at position 633 and the polypeptide translated from pB(C596S/C633S) has serine substituted for both cysteines at positions 596 and 633. The coprecipitation analysis of these three gB mutant polypeptides in the presence of the pA polypeptide and Abn antibody (Fig. 10B) demonstrated oligomer formation. The gB polypeptide encoded by pA was cotranslated in the presence of pB (Fig. 10B, lanes 1 and 2), pB(C633S) (lanes 3 and 4), or pB(C596S/C633S) (lanes 5 and 6). As previously demonstrated (Fig. 2 and 3), the product of the pA and pB polypeptides formed oligomers as judged by the presence of the pB polypeptide in the immunoprecipitated product (Fig. 10B, lane 2). The cotranslated product of pA and pB(C633S) was also immunoprecipitated by the Abn antibody (Fig. 10B, lane 4) with an efficiency (quantified by densitometry) similar to, and in some experiments slightly higher than, that of the products of the pA and pB polypeptides. However, the immunoprecipitation product of the cotranslated polypeptides from pA and pB(C596S/C633S) (Fig. 10B, lane 6) demonstrated a decrease of 61% in the oligomerization properties of these two polypeptides compared with the oligomerization properties of pA and pB polypeptides. These results demonstrated that the cysteines at positions 596 and 633 were not essential for oligomer formation, since oligomerization between the polypeptides encoded by pA and pB(C596S/C633S) was observed. These cysteines appeared to play a role, however, in formation and/or stabilization of the complex, since a decrease in oligomerization between the pA and pB(C596S/C633S) polypeptides compared with the oligomerization between the pA and pB encoded polypeptides was observed. Moreover, the results suggested that substitution of a serine at position 633 for a cysteine may increase oligomer formation through possible intermolecular disulfide bridges formed between cysteine 596 of the two polypeptides.

The role of cysteines 9 and 10 in the formation of disulfide bridges was then analyzed by SDS-PAGE under reducing and nonreducing conditions (Fig. 11). The translated products of the three gB mutant polypeptides were resolved as expected as monomers under reducing conditions (Fig. 11, lanes 1, 3, and 5). As expected, the product of pB(C596S/C633S) did not form dimers under nonreducing conditions, since there are no cysteines in this mutant polypeptide (Fig. 11, lane 6). As predicted, the polypeptide encoded by pB(C633S) formed intermolecular disulfide bridges to form homodimers as demonstrated by the presence of a band migrating at twice the molecular size of the monomer under nonreducing conditions (Fig. 11, lane 4). However, under these conditions, the translated product of pB migrated also as a dimer (Fig. 11, lane 2) but to a lesser extent than the product of pB(C633S) (lane 4), suggesting that an equilibrium between the monomeric (intramolecular disulfide bridges) and the dimeric (intermolecular disulfide bridges) forms of the molecule might be estab-

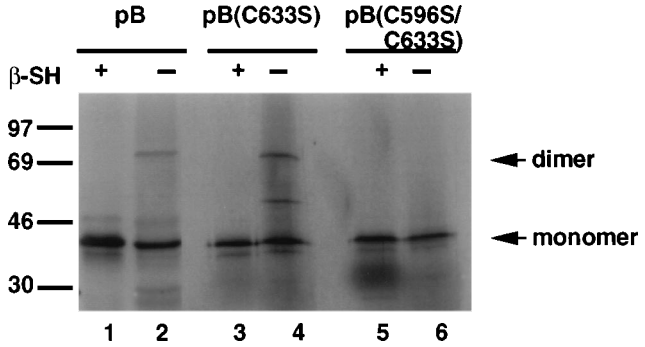

FIG. 11. Effect of beta-mercaptoethanol on the dimerization properties of pB and its cysteine-substituted forms. The translated products of pB (lanes 1 and 2), pB(C633S) (lanes 3 and 4), and pB(C596S/C633S) (lanes 5 and 6) were solubilized in loading buffer in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of beta-mercaptoethanol (β-SH). The arrows at the right indicate the positions of the monomeric and dimeric forms of the gB mutant polypeptides. Numbers on the left indicate the mobilities (in thousands) of radioactively labeled marker polypeptides.

lished when both cysteines are present. As also observed in Fig. 9, an extra band of unknown origin migrating at 50 kDa is seen in Fig. 11.

In addition to that for cysteine, four other single amino acid substitutions were individually introduced into pB (arginine 599 to glutamate, glycine 615 to proline, phenylalanine 641 to tyrosine, and tyrosine 653 to proline) and examined in the oligomerization assay. None of these substitutions had an effect on oligomerization with the pA polypeptide (data not shown).

Dimerization of wild-type or mutant gB polypeptides occurs after translation. To determine if the wild-type gB molecule (KOS) or the mutant gB molecule with residues 43 to 595 deleted (KΔ5C) was translated as monomers prior to self-association to form dimers, Vero cells were infected with KOS or KΔ5C virus and the monomeric and dimeric forms of gB were analyzed by sedimentation analysis of gB polypeptides. Infected-cell extracts were sedimented in sucrose gradients (18- and 54-h centrifugations for KOS and KΔ5C, respectively) and fractionated, and each fraction was immunoprecipitated with a pool of gB-specific MAbs for subsequent SDS-PAGE analysis. Most of the radioactivity associated with the full-length monomeric gB polypeptides was detected in fraction 5, while fraction 8 contained the dimeric form (chase) (Fig. 12A). A comparative sedimentation analysis with proteins with known molecular masses indicated that fractions 5 and 8 contained polypeptides of 100 and 200 kDa, respectively. As demonstrated earlier (31), these results suggested that wild-type gB was translated as a monomer that subsequently formed dimers rather than with dimerization occurring during the process of translation. Similar pulse-chase experiments performed with KΔ5C-infected cells (Fig. 12B) also demonstrated that gB was first translated as monomers (fractions 7 and 8) and that this was followed by self-association to form dimers (fractions 8 and 9). For the mutant molecules, the monomeric and dimeric forms were predicted to have approximate molecular masses of 45 and 80 kDa, respectively.

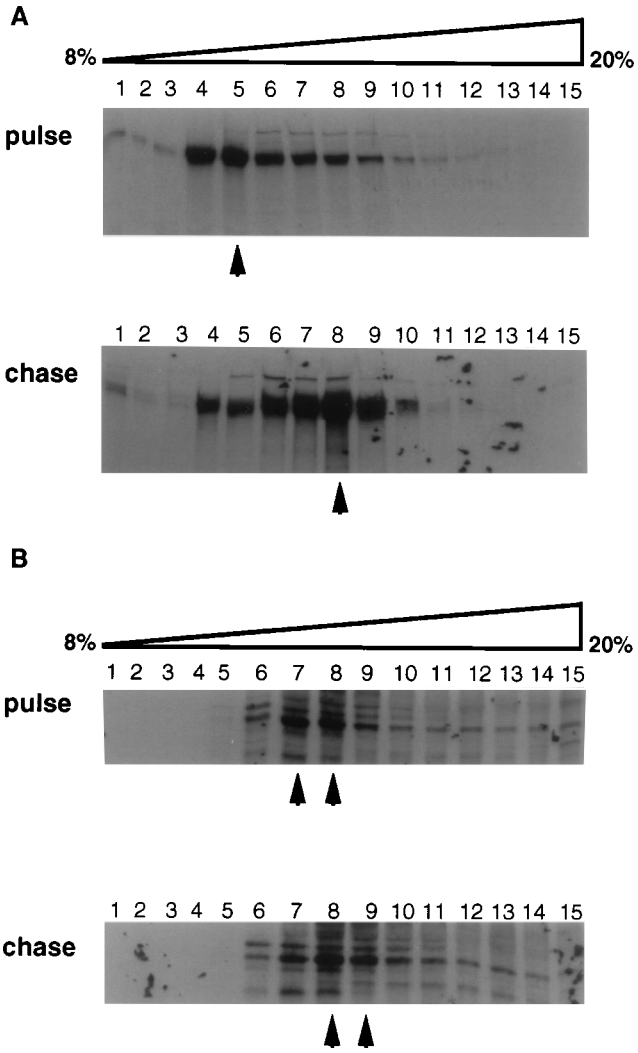

FIG. 12. Sedimentation analysis of [^{35}S]methionine-labeled gB polypeptides from infected cells. KOS (A)- and KΔ5C (B)-infected cells were pulse-labeled at 6 h postinfection and lysed immediately (pulse) or after 1 h of chase (chase) before being layered onto sucrose gradients. After sedimentation, fractions were collected and treated with a pool of gB MAbs, and the immune complexes were analyzed by SDS-PAGE. The arrows at the bottom of each panel indicate the fraction(s) containing the highest concentration of gB polypeptides.

DISCUSSION

A number of viral membrane proteins examined to date form either hetero-oligomers, such as G1 and G2 of Uukuniemi virus (51) and E1, E2, and E3 of Semliki Forest virus (36), or homo-oligomers, such as gB of HSV-1, cytomegalovirus, or pseudorabies virus (5, 31, 64), hemagglutinin of influenza virus (14), and envelope glycoproteins of human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus (20, 21, 57). One of the proposed explanations for the oligomerization of viral glycoproteins is the increased diversity of the three-dimensional structures without a concomitant need to increase genetic coding information (19). Since the oligomer can possess biological activities, such as ion channels or receptor binding function, not achieved by their respective monomers, an understanding of the functional biology of these molecules depends in part on information regarding their oligomeric forms.

In this study, we developed an in vitro oligomerization assay in order to further delineate the downstream oligomerization site of gB. The development of an in vitro system was necessary because analysis of oligomerization by the complementation inhibition assay used previously depended not only on the oligomerization properties of the protein but also on glycoprotein processing (17). Highlander et al. (31) also used a co-immunoprecipitation assay to score the oligomerization properties of cotranslated gB mutant polypeptides expressed in Vero cells following superinfection with a gB null virus to stimulate plasmid gene expression. That assay was more difficult to control, since the results depended on the efficiency of transfection, which can vary from experiment to experiment.

In our current in vitro assay, [^{35}S]methionine-labeled, co-translated gB mutant polypeptides were immunoprecipitated with an antibody that recognized an amino acid sequence unique to one of the two translated polypeptides. We reasoned that if oligomerization occurred, the test polypeptide lacking the antibody-specific epitope would be contained in the precipitate because of its oligomeric association with the epitope-bearing capture polypeptide. After denaturation, both polypeptides could be visualized by SDS-PAGE. A series of mutant polypeptides with a portion of the oligomerization domain of gB deleted were produced and tested for their abilities to oligomerize with the epitope-tagged capture molecule possessing the entire oligomerization domain. The N-terminal and C-terminal boundaries of the oligomerization site were each defined by the polypeptide encoded by pC (lacking residues 43 to

625) and the polypeptide encoded by pD (lacking residues 43 to 676); the polypeptide encoded by pC produced oligomers with the polypeptide encoded by pA, while that encoded by pD did not. This sequence of 49 residues can be divided into two segments (residues 626 to 653 and 653 to 675), each of which was sufficient to induce oligomerization. The weaker association with the capture molecule of the polypeptides with residues 626 to 653 deleted suggested that these residues were more critical than residues 653 to 676 in the oligomer-forming interaction.

We demonstrated that residues 626 to 653 were also able to restore the oligomerization properties of an oligomer-deficient gB molecule when inserted in the ectodomain of this mutated polypeptide and after residue 824 or 875 within the carboxy-terminal portion of the gB molecule. This sequence of 28 amino acid residues when inserted into the ectodomain of an oligomer-deficient gB polypeptide (pI) was located 63 residues closer to the transmembrane domain than in the wild-type molecule, and insertion of these residues into the carboxy-terminal portion of a molecule transposed the oligomerization domain from one side to the other of the membrane bilayer forming the microsomal membranes. Taken together, these data suggest that residues 626 to 653 represent a moveable domain capable of inducing oligomerization of two molecules. These results do not exclude, however, the possibility that other regions of gB are capable of either stabilizing the oligomerization complex or bringing these domains into close proximity in order to facilitate their association.

Secondary structure analysis of the sequence of 28 residues located within residues 626 to 653 did not reveal any putative leucine zipper motif or other known motifs characteristic of protein-protein interactions in dimerization (52). However, the low surface probability value for these 28 residues (28) combined with an unfavorable free energy for forming an interface with water (62) suggests that they form a hydrophobic-like region and thus are internally situated within the molecule.

Of the 10 cysteines that are conserved among all gB homologs (6, 22, 25, 37, 42, 45, 58), one cysteine (residue 633) resides within the strong 28-residue oligomerization domain (residues 626 to 653). We demonstrated that conversion of this cysteine to a serine residue when inserted either in the ectodomain or in the carboxy-terminal portion of the molecule reduced oligomer formation by approximately 50%. These results demonstrated that the cysteine residue at position 633 was not essential for oligomer formation but contributed to oligomer formation or stability.

Analysis of the nature of the disulfide bridges involved in the oligomerization of the pI-encoded molecule revealed the presence of intermolecular disulfide bridges. Since our oligomerization assay involved the use of mutant polypeptides, it is possible that these alterations induced nonphysiological oligomer-forming interactions such as intermolecular disulfide bridges. By mutating only one cysteine, for example, a natural intramolecular disulfide bridge could be disrupted, thereby promoting the formation of intermolecular disulfide bridges following oligomerization. In order to investigate this possibility, we substituted serine for both cysteines contained within the oligomer-forming domain. The removal of cysteine at positions 596 and 633 did not, however, prevent oligomerization, indicating that disulfide bonding was not essential to this activity. Moreover, the pB polypeptide containing both cysteines resulted in the production of a mixture of monomeric and dimeric forms favoring the monomer, while the pB(C633S) polypeptide containing only one cysteine increased the relative concentration of the dimeric form. This may indicate that a transition from intramolecular to intermolecular disulfide bridges occurs soon after gB synthesis. It has been reported previously that gB oligomers could be converted to monomers after boiling of the glycoprotein complex under nonreducing conditions (13). On the one hand, these results could be taken as evidence for the absence of intermolecular disulfide bridges linking the gB monomers in the oligomeric state. On the other hand, our results suggested that intermolecular disulfide bridges may form between gB monomers following oligomer formation. These differences in experimental findings might be explained in several ways. First, our assay employed mutant polypeptides with a considerable portion of the gB molecule deleted, which may have affected the arrangement of the cystine residues compared with that in the wild-type molecule. However, cysteines 9 and 10, the only cysteines present in our gB test molecules, appear to form either inter- or intramolecular disulfide bridges in the wild-type molecule (5a) (see Results), indicating that our test constructs may not have aberrant bonds. Second, it has been demonstrated that boiling proteins at 100°C can induce β-elimination of cystine residues in the pH range of 4 to 8 and that proteins with several cysteine residues undergo reshuffling of -S-S- bonds within minutes (63). This paper suggests that the original report of gB monomer formation after boiling of the gB protein in the absence of a reducing agent may not provide definitive evidence that intermolecular disulfide bridges play no role in dimer formation.

In summary, our data suggest that gB may be translated as a monomer having intramolecular disulfide bonds which subsequently self-associate through a hydrophobic-like interaction to form a dimer. Dimerization may lead to altered disulfide bridges involving cysteines on the two monomer units, resulting in more-stable homodimers through intermolecular disulfide bond formation. Analysis of the monomeric and dimeric forms of wild-type or mutant gB polypeptides radiolabeled in virus-infected cells by using a pulse-chase design provided results consistent with this conclusion, inasmuch as monomers appeared to be chased into dimers. A similar rearrangement of disulfide bonds during maturation of the Sindbis virus E1 glycoprotein (1, 46) has been reported, which may contribute to virus disassembly during the initial stages of infection. Whether a similar process takes place during HSV-1 infection remains to be determined. A deeper understanding of the role of disulfide bridging in oligomerization and gB function in infection awaits further studies in which the mutant gB genes are introduced into the virus and tested for their abilities to support virus replication.

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REFERENCES

1. **Abell, B., and D. T. Brown.** 1993. Sindbis virus membrane fusion is mediated by reduction of glycoprotein disulfide bridges at the cell surface. J. Virol. **67:** 5496–5501.
2. **Ali, M. A.** 1990. Oligomerization of herpes simplex virus glycoprotein B occurs in the endoplasmic reticulum and a 102 amino acid cytoplasmic domain is dispensable for dimer assembly. Virology **178:**588–592.
3. **Baines, J. D., and B. Roizman.** 1993. The UL10 gene of herpes simplex virus 1 encodes a novel glycoprotein, gM, which is present in the virion and in the plasma membrane of infected cells. J. Virol. **67:**1441–1452.
4. **Bond, V. C., S. Person, and S. C. Warner.** 1982. The isolation and charac-

terization of mutants of herpes simplex virus type 1 that induce cell fusion. J. Gen. Virol. **61:**245–254.
5. **Britt, W. J., and L. G. Vugler.** 1992. Oligomerization of human cytomegalovirus major envelope glycoprotein complex gB (gp55-116). J. Virol. **66:** 6747–6754.
5a. **Burke, R. L.** Personal communication.
6. **Bzik, D. J., C. DebRoy, B. A. Fox, N. E. Pederson, and S. Person.** 1986. The nucleotide sequence of the gB glycoprotein gene of HSV-2 and comparison with the corresponding gene of HSV-1. Virology **155:**322–333.
7. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology **133:**301–314.
8. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. **62:**2596–2604.
9. **Cai, W., S. Person, C. DebRoy, and B. Gu.** 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1: an analysis of linker insertion mutants. J. Mol. Biol. **201:**575–588.
10. **Cai, W., S. Person, S. C. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61:**714–721.
11. **Chapsal, J. M., and L. Pereira.** 1981. Characterization of epitopes on native and denatured forms of herpes simplex virus glycoprotein B. Virology **164:** 427–434.
12. **Chou, P. Y., and G. D. Fasman.** 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47:**45–148.
13. **Claesson-Welsh, L., and P. G. Spear.** 1986. Oligomerization of herpes simplex virus glycoprotein B. J. Virol. **60:**803–806.
14. **Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius.** 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. J. Cell Biol. **103:**1179–1191.
15. **Creighton, T. E.** 1984. Dusulfide bond formation in proteins. Methods Enzymol. **107:**305–329.
16. **DeLuca, N., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gB (VP7). Virology **122:**411–423.
17. **Desai, P., F. L. Homa, S. Person, and J. C. Glorioso.** 1994. A genetic selection method for the transfer of HSV-1 glycoprotein B mutations from plasmid to the viral genome: preliminary characterization of transdominance and entry kinetics of mutant viruses. Virology **204:**312–322.
17a. **Desai, P., and S. Person.** Unpublished results.
18. **Desai, P. J., P. A. Schaffer, and A. C. Minson.** 1988. Excretion of noninfectious virus particles lacking glycoprotein H by temperature sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity. J. Gen. Virol. **69:**1147–1156.
19. **Doms, R. W., R. A. Lamb, J. K. Rose, and A. Helenius.** 1993. Folding and assembly of viral membrane proteins. Virology **193:**545–562.
20. **Earl, P. L., R. W. Doms, and B. Moss.** 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. Proc. Natl. Acad. Sci. USA **87:**648–652.
21. **Earl, P. L., R. W. Doms, and B. Moss.** 1992. Multimeric CD4 binding exhibited by human and simian immunodeficiency virus envelope protein dimers. J. Virol. **66:**5610–5614.
22. **Eberle, R., and D. Black.** 1991. The simian herpesvirus SA8 homologue of the herpes simplex virus gB gene: mapping, sequencing, and comparison to the HSV gB. Arch. Virol. **118:**67–86.
23. **Eberle, R., and S.-W. Mou.** 1983. Relative titers of antibodies to individual polypeptide antigens of herpes simplex virus type 1 in human sera. J. Infect. Dis. **148:**436–444.
24. **Eberle, R., S.-W. Mou, and J. A. Zaia.** 1985. The immune response to herpes simplex virus: comparison of the specificity and relative titers of serum antibodies directed against viral polypeptides following primary herpes simplex virus type 1 infections. J. Med. Virol. **16:**147–162.
25. **Ellinger, K., F. Neipel, L. Foà-Tomasi, G. Campadelli-Fiume, and B. Fleckenstein.** 1993. The glycoprotein B homologue of human herpesvirus 6. J. Gen. Virol. **74:**495–500.
26. **Gage, P. J., M. Levine, and J. C. Glorioso.** 1993. Syncytium-inducing mutations localize to two discrete regions within the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B. J. Virol. **67:**2191–2201.
27. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120:**97–120.
28. **Genetics Computer Group.** 1994. Program manual for the Wisconsin package. Genetics Computer Group, Madison, Wis.
29. **Ghiasi, H., R. Kaiwar, A. B. Nesburn, and S. L. Wechler.** 1992. Expression of glycoprotein B of herpes simplex virus type 1. Virus Res. **22:**25–39.
30. **Glorioso, J. C., C. H. Schroder, G. Kumel, M. Szczesiul, and M. Levine.** 1984. Immunogenicity of herpes simplex virus glycoprotein gC and gB and their role in protective immunity. J. Virol. **50:**805–812.
31. **Highlander, S. L., W. F. Goins, S. Person, T. C. Holland, M. Levine, and J. C. Glorioso.** 1991. Oligomer formation of the gB glycoprotein of herpes simplex virus type 1. J. Virol. **65:**4275–4283.
32. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
33. **Hutchinson, L., H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A. C. Minson, and D. C. Johnson.** 1992. A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. **66:**2240–2250.
34. **Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F. L. Graham, and D. C. Johnson.** 1992. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. **66:**5603–5609.
35. **Jackson, R. J., and T. Hunt.** 1983. Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. Methods Enzymol. **96:**50–74.
36. **Kielian, M., S. Jungerwirth, K. U. Sayad, and S. DeCandido.** 1990. Biosynthesis, maturation, and acid activation of the Semliki Forest virus fusion protein. J. Virol. **64:**4614–4624.
37. **Kongsuwan, K., C. T. Prideaux, M. A. Johnson, M. Sheppard, and K. J. Fahey.** 1991. Nucleotide sequence of the gene encoding infectious laryngotacheitis virus glycoprotein B^1. Virology **184:**404–410.
38. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
39. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62:**1486–1494.
40. **Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer.** 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology **115:**149–160.
41. **MacLean, C. A., L. M. Robertson, and F. E. Jamieson.** 1993. Characterization of the UL10 gene product of herpes simplex virus type 1 and investigation of its role *in vivo*. J. Gen. Virol. **74:**975–983.
42. **Maeda, K., T. Horimoto, J. Norimine, Y. Kawaguchi, K. Tomonaga, M. Niikura, C. Kai, E. Takahashi, and T. Mikami.** 1992. Identification and nucleotide sequence of a gene in feline herpesvirus type 1 homologous to the herpes simplex virus gene encoding the glycoprotein B. Arch. Virol. **127:** 387–397.
43. **Marlin, S. D., S. L. Highlander, T. C. Holland, M. Levine, and J. C. Glorioso.** 1986. Antigenic variation (*mar* mutations) in herpes simplex virus glycoprotein B can induce temperature-dependent alterations in gB processing and virus production. J. Virol. **59:**142–153.
44. **Meignier, B., T. M. Jourdier, B. Norrild, L. Pereira, and B. Roizman.** 1987. Immunization of experimental animals with reconstituted glycoprotein mixture of herpes simplex virus 1 and 2: protection against challenge with virulent virus. J. Infect. Dis. **155:**921–930.
45. **Misra, V., R. Nelson, and M. Smith.** 1988. Sequence of a bovine herpesvirus type-1 glycoprotein gene that is homologous to the herpes simplex gene for the glycoprotein gB. Virology **166:**542–549.
46. **Mulvey, M., and D. T. Brown.** 1994. Formation and rearrangement of disulfide bonds during maturation of the Sindbis virus E1 glycoprotein. J. Virol. **68:**805–812.
47. **Navarro, D., P. Paz, and L. Pereira.** 1992. Domains of herpes simplex virus 1 glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion. Virology **186:**99–112.
48. **Pederson, N. E., S. Person, and F. L. Homa.** 1992. Analysis of the gB promoter of herpes simplex virus type 1: high-level expression requires both an 89-base-pair promoter fragment and a nontranslated leader sequence. J. Virol. **66:**6226–6232.
49. **Peeters, B., N. de Wind, M. Hooisma, F. Wagenaar, A. Gielkens, and R. Moormann.** 1992. Pseudorabies virus envelope glycoproteins gp50 and gII are essential for virus penetration, but only gII is involved in membrane fusion. J. Virol. **66:**894–905.
50. **Pellet, P. E., K. G. Kousoulas, L. Pereira, and B. Roizman.** 1985. Anatomy of the herpes simplex virus 1 strain F glycoprotein B gene: primary sequence and predicted protein structure of the wild type and of monoclonal antibody-resistant mutants. J. Virol. **53:**243–253.
51. **Persson, R., and R. F. Pettersson.** 1991. Formation and intracellular transport of a heterodimeric viral spike protein complex. J. Cell Biol. **112:**257–266.
52. **Phizicky, E. M., and S. Fields.** 1995. Protein-protein interactions: methods for detection and analysis. Microbiol. Rev. **59:**94–123.
53. **Qadri, I., C. Gimeno, D. Navarro, and L. Pereira.** 1991. Mutations in conformation-dependent domains of herpes simplex virus 1 glycoprotein B affect the antigenic properties, dimerization, and transport of the molecule. Virology **180:**135–152.
54. **Ramaswamy, R., and T. C. Holland.** 1992. In vitro characterization of the HSV-1 UL53 gene product. Virology **186:**579–587.
55. **Rasile, L., K. Ghosh, K. Raviprakash, and H. P. Ghosh.** 1993. Effects of deletion in the carboxy-terminal hydrophobic region of herpes simplex virus glycoprotein gB on intracellular transport and membrane anchoring. J. Virol. **67:**4856–4866.
56. **Rauh, I., and T. C. Mettenleiter.** 1991. Pseudorabies virus glycoproteins gII and gp50 are essential for virus penetration. J. Virol. **65:**5348–5356.
57. **Rey, M.-A., B. Krust, A. G. Laurent, L. Montagnier, and A. G. Hovanessian.** 1989. Characterization of human immunodeficiency virus type 2 envelope

glycoproteins: dimerization of the glycoprotein precursor during processing. J. Virol. **63:**647–658.
58. **Riggio, M. P., A. A. Cullinane, and D. E. Onions.** 1989. Identification and nucleotide sequence of the glycoprotein gB of equine herpesvirus 4. J. Virol. **63:**1123–1133.
59. **Roizman, B., and A. Sears.** 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsh, J. L. Melnick, and T. P. Monath (ed.), Virology, 2nd ed. Raven Press, New York.
60. **Sarmiento, M., and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex virus. IV. Conformation of the virion glycoprotein designated VP7 (B2). J. Virol. **29:**1159–1167.
61. **Spear, P. G.** 1993. Membrane fusion induced by herpes simplex virus, p. 201–232. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Boca Raton, Fla.
62. **Tanford, C.** 1980. The hydrophobic effect: formation of micelles and biological membranes. John Wiley & Sons, Inc., New York.
63. **Volkin, D. B., and A. M. Klibanov.** 1987. Thermal destruction processes in proteins involving cystine residues. J. Biol. Chem. **262:**2245–2950.
64. **Whitbeck, J. C., L. J. Bello, and W. C. Lawrence.** 1988. Comparison of the bovine herpesvirus 1 gI gene and the herpes simplex virus type 1 gB gene. J. Virol. **62:**3319–3327.
65. **Wilson, I. A., H. L. Niman, R. A. Houghten, A. R. Cherenson, M. L. Connolly, and R. A. Lerner.** 1984. The structure of an antigenic determinant in a protein. Cell **37:**767–778.