Disulfide-Bonded Discontinuous Epitopes on the Glycoprotein of Vesicular Stomatitis Virus (New Jersey Serotype)

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Intrachain disulfide bonds between paired cysteines in the glycoprotein (G) of vesicular stomatitis virus (VSV) are required for the recognition of discontinuous epitopes by specific monoclonal antibodies (MAbs) (W. Keil and R. R. Wagner, *Virology* 170:392–407, 1989). Cleavage by *Staphylococcus aureus* V8 protease of the 517-amino-acid VSV-New Jersey G protein, limited to the glutamic acid at residue 110, resulted in a protein (designated G_{V8}) with greatly retarded migration by polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions. By Western blot (immunoblot) analysis, protein G_{V8} was found to lose discontinuous epitope IV, which maps within the first 193 NH₂-terminal amino acids. These data, coupled with those obtained by PAGE migration of a vector-expressed, truncated protein (G₁₋₁₉₃) under reducing and nonreducing conditions, lead us to postulate the existence of a major loop structure within the first 193 NH₂-terminal amino acids of the G protein, possibly anchored by a disulfide bond between cysteine 108 and cysteine 169, encompassing epitope IV. Site-directed mutants in which 10 of the 12 cysteines were individually converted to serines in vaccinia virus-based vectors expressing these single-site mutant G proteins were also constructed, each of which was then tested by immunoprecipitation for its capacity to recognize epitope-specific MAbs. These results showed that mutations in NH₂-terminal cysteines 130, 174, and, to a lesser extent, 193 all resulted in the loss of neutralization epitope VIII. A mutation at NH₂-terminal cysteine 130 also resulted in the loss of neutralization epitope VII, as did a mutation at cysteine 108 to a lesser extent. Both epitopes VII and VIII disappeared when mutations were made in COOH-distal cysteine 235, 240, or 273, the general map locations of epitopes VII and VIII. These studies also reveal that distal, as well as proximal, cysteine residues markedly influence the disulfide-bond secondary structure, which ostensibly determines the conformational structure of the VSV-New Jersey G protein required for presentation of the major discontinuous epitopes recognized by neutralizing MAbs.

The virion membrane glycoprotein (G) of vesicular stomatitis virus (VSV) serves as a model system for studying the synthesis, processing, and membrane insertion of eucaryotic glycoproteins (19, 22). The G protein is also both the major organ for recognition and adsorption of VSV to the surface of host cells (1, 3) and the serotypic viral antigen that gives rise to and reacts with neutralizing antibodies (9). Bricker et al. (2) have raised monoclonal antibodies (MAbs) that recognize at least nine independent epitopes on the G protein of the New Jersey serotype of VSV (VSV-NJ). By expressing deletion mutants and chimeras of recombinant G proteins, we have mapped the binding affinity of conformation-dependent, neutralizing MAbs to the central region of the 517-amino-acid VSV-NJ G protein, extending from amino acid residues 193 to 289 (8). Also demonstrated in these studies was the existence of a conformational but nonneutralization region (designated epitope IV) within the first 193 NH₂-terminal amino acids. The reduced form of the VSV-NJ G protein could not recognize neutralizing and some nonneutralizing MAbs reactive with conformational epitopes, indicating that disulfide bonds play a key role in defining the discontinuous epitopes of the VSV-NJ G protein (8). As defined by Laver et al. (10), the term discontinuous “is a

more accurate description of nonlinear epitopes since they are assembled from several different portions of the polypeptide chain.”

VSV glycoproteins of both serotypes have 12 highly conserved cysteine residues (5). To date, no data on the location of disulfide bonds between paired cysteine residues that determine the secondary structure defining the discontinuous conformational epitopes of VSV-NJ G protein have been provided. To this end, we performed two types of experiments designed to locate the likely pairs among the 12 cysteine residues that would define the discontinuous epitopes for recognition of neutralizing MAbs. In the first experiments, we located the potential disulfide-forming cysteine residues in a limited protease-digested peptide reactive by Western blotting (immunoblotting) with specific MAbs and mapped by NH₂-terminal amino acid sequencing. In the second experimental approach, we located cysteine residues and specific epitopes in unreduced truncated segments or site-directed mutants of VSV-NJ G protein expressed by a vaccinia virus vector and immunoprecipitated by epitope-specific MAbs.

MATERIALS AND METHODS

Viruses and cells. The VSV-NJ serotype (Ogden strain) propagated in BHK-21 cells was isolated and purified as previously described (6, 16). The vaccinia virus recombinant expressing the bacteriophage T7 RNA polymerase gene (4) was kindly supplied by Bernard Moss and was propagated in CV-1 monkey kidney cells in the presence of Dulbecco

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modified Eagle medium supplemented with 10% fetal bovine serum.

V8 protease digestion of VSV glycoprotein. The VSV-NJ G protein was isolated from virions as previously described (18). Briefly, purified virions were resuspended in 1 ml of 50 mM Tris-HCl buffer (pH 8.0) at a concentration of 0.5 to 1.0 mg/ml and exposed for 1 h at room temperature to the dialyzable nonionic detergent β -octylglucoside (Calbiochem, La Jolla, Calif.). After removal of the nucleocapsids by centrifugation at $150,000 \times g$, the supernatant fluid was dialyzed for 12 to 14 h at 4°C against repeated changes of 10 mM Tris-HCl (pH 7.8), and the detergent-free G protein was concentrated to a protein concentration of 2 to 3 mg/ml in Centricon (Amicon).

Time-limited fragmentation of the VSV-NJ G protein was conducted with TN buffer (100 mM NaCl, Tris-HCl [pH 7.5]) in the presence of 0.1% sodium dodecyl sulfate (SDS) (final concentration). The standard protease reaction was carried out with appropriate amounts of *Staphylococcus aureus* V8 protease (Pierce) diluted in a final 50- μ l volume of TN buffer containing 10 to 15 μ g of purified G protein. Protease digestion was terminated after a period of incubation by adding 20 μ l of $3 \times$ TN buffer to each sample, and each sample was then loaded onto polyacrylamide-SDS gels for electrophoretic separation of the resulting peptides. These V8-generated peptides were blotted onto nitrocellulose filters (Millipore) and, where appropriate, NH_2 -terminal amino acids were sequenced with the aid of an Applied Biosystems 470A Sequencer (7).

Plasmids. The basic vector used to express the entire or truncated VSV-NJ G protein, as well as its site-directed mutants, was pWK9 (8), which was derived from the vector pTF7IHB-1 containing the phage T7 promoter and terminator sequences constructed by Fuerst et al. (4). The pWK9 plasmid is actually a hybrid, since the N-terminal 43 amino acids were derived from the G gene of VSV-Indiana (VSV-Ind) and are virtually identical to the comparable amino acid sequences of VSV-NJ; this pWK9 plasmid was used because its expression is far superior to that of the recombinant VSV-NJ G gene (8). Expression of this plasmid and similar pTF plasmids requires coinfection with the vaccinia virus recombinant vTF1-6,2, which expresses the phage T7 polymerase. To create a truncated pWK9 G gene expressing the first 193 amino acids of the VSV-NJ G protein (G_{1-193}), we created an *Nco*I site overlapping the ATG codon at position 1 of the coding region of pWK9 by employing polymerase chain reaction technology (Gene Amp; U.S. Biochemicals). The truncated G gene was excised from this recombinant by exposure to restriction enzymes *Nco*I and *Pst*I, and the resulting 576-bp fragment was inserted into the identical site of the cap-independent vector pTM1, also driven by the phage T7 polymerase promoter (17), kindly supplied by Bernard Moss. This new plasmid was designated pPG7 and expressed the truncated VSV-NJ G protein G_{1-193} . A polypeptide expressing the first 411 amino acids of the VSV-NJ G protein was also constructed. This construct was derived from the VSV-NJ G gene plasmid pNJ-G2 of Gallione and Rose (5) by cloning a *Pst*I-*Acc*I fragment (blunt ended in the *Acc*I site with T4 polymerase before treatment with *Pst*I) into the *Pst*I-*Stu*I sites of plasmid pPG7 described above; this construct was designated pPG8 and expressed the truncated VSV-NJ G protein G_{1-411} .

A vector expressing the carboxy-terminal amino acids 193 through 517 of the VSV-NJ G protein was constructed from plasmid pCL5, which was originally made by cloning a *Pst*I fragment of plasmid pNJ-G2 of Gallione and Rose (5). This

*Pst*I fragment, coding for amino acids 193 to 517 of the VSV-NJ G protein, was inserted into the *Pst*I site of plasmid pF2,7-SSV, a vaccinia virus expression vector in which the oligonucleotide coding for the 16-amino-acid signal sequence (plus four additional amino acids) of the VSV-Ind G protein is directly upstream from a unique *Pst*I site (7a). Therefore, this pCL5 plasmid expresses a hybrid protein containing the signal sequence and four extra amino acids of the VSV-Ind G protein followed by the carboxy-terminal 325 amino acids (residues 193 to 517) spanning the central and carboxy-terminal thirds of the VSV-NJ G protein.

Site-directed Cys $\rightarrow$ Ser mutants. Site-directed mutagenesis was carried out by the double-mutant technique with plasmid Select 1 (pS1) (Altered Sites; Promega) carrying the coding sequences for a major VSV glycoprotein fragment (pS1-470). The base-mismatched synthetic oligonucleotide primers were designed to convert either TGT to AGT or TCG to TCC, providing mutations in the VSV-NJ G gene that substitute a serine residue (Ser) for each of the cysteines (Cys) on the VSV-NJ G protein (24). Briefly, the *Eco*RI-*Sac*I DNA fragment excised from plasmid pWK9 was cloned into the identical restriction enzyme sites of plasmid pS1 to give pS1-470. Single-stranded DNA coding for this G protein fragment was obtained by transforming *Escherichia coli* JM109 with pS1-470 in medium containing tetracycline (15 μ g/ml) and helper phage R408. The heteroduplex double-stranded DNA produced by extension with T7 polymerase after the annealing of both primer oligonucleotides (one conferring ampicillin resistance and the other creating the required mutation in the Cys codon) was used to transform the repair-minus strain *E. coli* BMH71-18 *mutS* (25). After the transformants were grown in Luria-Bertani medium containing ampicillin and a second round of transformation in JM109 was performed, minipreps of different colonies were screened by differential dot blot hybridization (26) and by sequencing (20) to confirm the presence of the mutations. In our hands, the average efficiency of this method was close to 50%. We were unable, however, to construct site-directed mutants of cysteine residues at positions 15 and 75 under conditions that readily provided mutants for the other 10 cysteine positions. Poor binding of these two mutant oligonucleotides, probably based on the presence of the secondary structure in the single-stranded DNA templates, may be a possible explanation for the failure to create these mutants. Plasmid DNA preparations of 10-ml cultures from each confirmed mutant clone were treated with the appropriate restriction enzymes, and the fragments bearing the respective mutations were electroeluted from agarose gels and ligated to the appropriate restriction sites in the expression vector pWK9.

MAbs. As previously described in detail (2, 6), MAbs directed to nine specific epitopes of the VSV-NJ G protein were prepared from hybridomas in BALB/c mice. Used in these studies in the form of immunoglobulin G from ascites fluids purified on protein A-Sepharose columns were MAb 7 (epitope III), MAb 9 (epitope IV), MAb 14 (epitope VII), and MAb 15 (epitope VIII). The partial mapping of these epitopes to regions of the VSV-NJ G protein has been previously described (8).

Transient expression and labeling of VSV-NJ glycoproteins. CV-1 cells were grown to 80% confluence in 35-mm-diameter plates (3×10^5 cells) and infected at a multiplicity of infection of 10 with recombinant vaccinia virus vTF1-6,2. After 30 min of adsorption, the inoculum was removed and a mixture of CsCl-purified plasmids (10 μ g) and Lipofectin (20 μ g; Bethesda Research Laboratories) in 1 ml of OptiMEM

was added to the monolayer and incubated for another 3.5 h at 37°C. At 4 h postinfection, the medium was brought to a final concentration of 10% fetal bovine serum, and the cells were incubated for another 6 h at the same temperature. At 10 h postinfection, the medium was removed and replaced with 0.5 ml of modified minimal essential medium containing 1/10 the regular amount of cold methionine and 260 μ Ci of [35 S]methionine per ml (ICN; 1,100 Ci/mM). The incubation was continued for another hour at 37°C and was followed by a brief washing of the monolayer with phosphate-buffered saline buffer and resuspension of the cells in HO buffer (20 mM Tris-HCl [pH 7.8], 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride) in the cold at a ratio of approximately 0.2 ml per plate (or 1.5×10^6 cells per ml).

Immunoprecipitation and Western blot analysis. Immunoprecipitation of G protein components from infected or transfected cells was performed after the lysis of cells in HO buffer followed by the removal of nuclei and debris by centrifugation at $15,000 \times g$ for 10 min. As previously described (6), the cytoplasmic fluids were divided into identical fractions, each of which was then incubated for 10 to 12 h at 4°C with individual epitope-specific MAbs. Protein A-Sepharose beads (Sigma) were added to a final concentration of 1%, and the samples were placed on a rocking platform for incubation at room temperature for an additional 4 h. The protein A-Sepharose immune complexes were pelleted by centrifugation at $15,000 \times g$ for 3 min and washed once with SALT buffer (50 mM Tris-HCl [pH 7.8], 1 M NaCl) and twice with RIPA buffer (20 mM Tris-HCl [pH 7.8], 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS); the immune complexes were resuspended in Laemmli sample buffer for analysis of the 35 S-labeled G proteins or its fragments by SDS-polyacrylamide gel electrophoresis (PAGE). The dilutions of MAbs used for optimal immunoprecipitation were as follows: 1:300 for MAb 7, 1:200 for MAb 9, 1:20 for MAb 14, and 1:50 for MAb 15. No SDS was used in the immunoprecipitation reaction mixtures in order to maximize the reactivity of the conformational epitopes.

As described by Keil and Wagner (8), Western blot analyses were performed with unlabeled proteins separated by PAGE and transferred by electroblotting onto nitrocellulose sheets, which were exposed to the appropriate dilutions of the individual MAbs. The sheets were then incubated with 125 I-labeled staphylococcal protein A (Amersham) and autoradiographed with screen enhancement.

RESULTS

Degrees of reduction and proteolysis determine PAGE migration of VSV-NJ G protein. As an initial step in determining the influence of disulfide bonds on conformation, the G protein of VSV-NJ was exposed to increasing concentrations of a reducing agent or a protease before its migration during PAGE under reducing or nonreducing conditions was determined. In the first experiment, VSV-NJ virions suspended in sample buffer containing 3% SDS were subjected to electrophoresis on 12.5% polyacrylamide gels containing either no reducing agent or concentrations of β -mercaptoethanol varying from 0.01 to 0.5 M. In the second experiment, purified VSV-NJ G protein in TN buffer containing 0.1% SDS was exposed to varying concentrations of *S. aureus* V8 protease to provide ratios of V8 protease to G protein of 1:100 to 1:10 (wt/wt) prior to PAGE in the absence of β -mercaptoethanol or any other reducing agent.

FIG. 1. Comparative electrophoretic migrations in SDS-polyacrylamide gels of the VSV-NJ G protein exposed to increasing concentrations of β -mercaptoethanol (MSH) reducing agent (A) or V8 protease (B), illustrated by a stick model of the G protein (C) showing the map locations of cysteine residues (●), the major V8 protease cleavage site (↑) with relevant amino acid sequences, and the N-linked carbohydrate chain at residue 179 (○) and the approximate map locations of epitopes IV, VII, and VIII. (A) Purified VSV-NJ virions were resuspended in sample buffer containing 3% SDS and aliquots exposed to increasing concentrations of β -mercaptoethanol. The VSV proteins are labeled unreduced G protein (G_U), reduced G protein (G_R), L, N, and M; lane 1 shows marker 43-kDa ovalbumin and 27-kDa carbonic anhydrase. (B) Purified VSV-NJ G protein was resuspended in proteolysis buffer (see Materials and Methods) at a protein concentration of 200 μ g/ml and distributed in aliquots of 50 μ l. Each G protein sample was incubated for 5 h at 37°C with increasing concentrations of *S. aureus* V8 protease to yield ratios of V8 protease to G protein of 1:100 (lane 3), 1:50 (lane 4), 1:20 (lane 5), and 1:10 (lane 6). After incubation with V8 protease, 15 μ l of 3 $\times$ gel-loading buffer without reducing agents was added to each of the G protein samples, which were boiled for 5 min before analysis by electrophoresis in SDS-12.5% acrylamide gels. Lane 2 shows the control glycoprotein incubated in the absence of protease (G), and lane 1 shows the markers shown in panel A. Both gels were stained with Coomassie blue dye.

Figure 1 shows the effects of increasing concentrations of β -mercaptoethanol or V8 protease on the migration of VSV-NJ G protein in polyacrylamide gels under reducing or nonreducing conditions, as well as a stick model of the G protein to illustrate the locations of cysteines, certain epitopes, and the primary V8 protease cleavage site (Fig. 1C). Clearly illustrated in Fig. 1A is the gradually slower

PAGE migration of G protein under conditions of increasing concentrations of β -mercaptoethanol, whereas the reducing agent exerts no significant effect on the mobilities of other VSV-NJ proteins. This gel-retardation effect under increased reducing conditions is a well-known phenomenon for this and other glycoproteins attributable to the phased reduction of the six potential disulfide bonds that serve to stabilize the highly compact polypeptide structure that causes rapid gel migration (21).

Figure 1B shows the electropherogram profile of products generated by limited and progressive digestion of VSV-NJ G protein by V8 protease under nonreducing conditions. After the exposure of G protein to V8 protease at V8/G ratios of 1:100 or 1:50 (Fig. 1B, lanes 3 and 4), a band designated G_{V8} that migrates more slowly than the untreated G protein appears (Fig. 1B, lane 2). At increasing concentrations of V8 protease at V8/G ratios of 1:20 and 1:10 (Fig. 1B, lanes 5 and 6), low-molecular-weight G peptides appear, with the concomitant disappearance of the high-molecular-weight G protein.

The retarded migration of G protein resulting from limited V8 protease digestion (Fig. 1B, lane 3), similar to that resulting from limited β -mercaptoethanol reduction (Fig. 1A, lane 5), suggests that V8 protease preferentially cleaves at one site in a major loop of the G protein anchored by at least one disulfide bond that makes this a tightly packed region of the G protein. The assumption leading to this hypothesis is that cleavage at a single site in such a major loop could result in relaxation of the three-dimensional structure with consequently retarded electrophoretic mobility. In support of such a hypothesis, we found by amino acid sequence analysis of the G_{V8} protein that V8 protease generated two distinct amino-terminal regions at residue 1 and residue 111; these two partial amino acid sequences are shown in Fig. 1C. This finding is consistent with that of a V8 protease cleavage site between glutamic acid (E-110) and alanine (A-111). The sequence data in Fig. 1C indicate that the glutamic acid residue at position 110 is a more accessible target site for V8 protease cleavage than the other 10 amino-proximal or 24 carboxy-distal glutamic acid and aspartic acid residues in the VSV-NJ G protein. Confirming the preferential V8 protease cleavage site at E-110 was the finding that electrophoresis of the G_{V8} protein under reducing conditions revealed the disappearance of the slowly migrating G_{V8} protein and the appearance of a rapidly migrating (40-kDa) peptide (data not shown), ostensibly representing the region of G protein from amino acids 111 to 517. Low yields prevented us from detecting the smaller peptide after the reduction of G_{V8} or determining the amino acid sequence of the 40-kDa peptide. However, these results are quite compatible with the existence of a major loop structure anchored by a disulfide bond in a region flanked by a more compact, tightly coiled structure.

For future reference, Fig. 1C also shows the relative positions on the stick model of the 12 cysteines and three discontinuous epitopes (IV, VII, and VIII) in relation to the putative V8 cleavage site in the amino-proximal two-thirds of the VSV-NJ G protein.

Comparative affinity of G_{V8} for binding epitope-specific MAbs. It was of interest to determine whether the single cleavage site at Glu-110 made by 0.1 μ g of V8 protease would alter the conformation of G protein sufficiently to abolish specific conformational antigenic determinants (epitopes). As previously described (8) and as summarized in the legend to Fig. 1, MAb 9 directed to epitope IV binds to a region roughly within the first 193 N-terminal amino acids

FIG. 2. Western blot analysis of VSV-NJ G protein partially cleaved by V8 protease (G_{V8}). Purified G protein (10 μ g) was cleaved at Glu-110 by incubation for 5 h at 37°C with 0.1 μ g of V8 protease in TN buffer containing 0.1% SDS (see the legend to Fig. 1). Uncleaved (G) and G_{V8} proteins were then made 1% with respect to SDS, boiled for 5 min, and subjected to electrophoresis on 12.5% polyacrylamide-SDS gels under nonreducing conditions. The electrophoresed G and G_{V8} proteins were then blotted onto a nitrocellulose sheet and tested for their abilities to bind MAb 9 (directed to epitope IV, lanes 1 and 2) or MAb 15 (directed to epitope VIII, lanes 3 and 4), as detected by 125 I-labeled staphylococcal protein A and autoradiography. Lanes: 1 and 3, untreated glycoprotein (G); 2 and 4 (overexposed), protease-treated glycoprotein (G_{V8}).

of intact VSV-NJ G protein. On the other hand, neutralizing MAbs directed to epitopes VII, VIII, and V bind to the central region of the intact G protein, roughly between amino acids 193 and 289.

To determine whether the conformationally altered G_{V8} protein loses the capacity to bind these MAbs, purified VSV-NJ G protein (10 μ g) was exposed to 0.1 μ g of V8 protease (V8/G ratio, 1:100), as shown in Fig. 1B, lane 3. This G_{V8} protein and control uncleaved G protein were subjected to SDS-PAGE in the absence of β -mercaptoethanol and blotted onto nitrocellulose filters. The binding of each epitope-specific MAb was determined as outlined in Materials and Methods by the addition of 125 I-labeled protein A followed by autoradiography.

MAb 11 (directed to epitope V) and MAb 14 (directed to epitope VII) reacted as well with G_{V8} protein as they did with intact, faster-migrating G protein (data not shown). Figure 2 reveals that MAb 8 (directed to epitope IV) fails to bind to V8 protease-nicked G_{V8} but binds well to the unnickd control G protein. In contrast, MAb 15 (directed to epitope VIII) retains its capacity to bind the G_{V8} protein. As noted, both MAb 8 and MAb 15 reacted with residual G protein undigested by the limited 0.1 μ g of protease V8. Also of note in Fig. 2 are the more rapidly migrating bands that bind MAb 8 and MAb 15; these bands are presumably V8 protease fragments of G protein that have not yet been characterized.

These data indicate that cleavage at Glu-110 disrupts the conformation of VSV-NJ G protein within the domain of epitope IV. These results also suggest that altered conformation resulting from the interruption of G protein between amino acids 110 and 111 does not affect the disulfide-bond secondary structure that could influence neutralization (discontinuous) epitopes further downstream.

Plasmids expressing truncated fragments of VSV-NJ G protein and their recognition by epitope-specific MAbs. The preceding experiments and those previously published (8) indicate that neutralization epitopes VII, VI, VIII, and V are located carboxy distal to amino acid residue 193 of VSV-NJ

FIG. 3. PAGE analysis of disulfide bonds (A) and epitopes (B) present in truncated G proteins expressed in a vaccinia virus recombinant system. CV-1 cells were transfected with plasmids pPG7, expressing truncated protein G_{1-193} ; pPG8, expressing G_{1-411} ; pCL5, expressing $G_{193-517}$; or pWK9, expressing the entire G protein, each labeled with [^{35}S]methionine (see Materials and Methods). (A) Truncated proteins G_{1-411} (first three lanes) and G_{1-193} (last three lanes) were immunoprecipitated with MAb 9 (directed to epitope IV), whereas truncated protein $G_{193-517}$ (middle three lanes) was immunoprecipitated with MAb 8 (directed to epitope III). The [^{35}S]labeled immunoprecipitates of each of the three truncated G proteins were resuspended in SDS-containing gel buffers and treated with β -mercaptoethanol (MSH) at final concentrations of 0, 3, or 25 mM before the analysis of electrophoretic mobility in a 15% polyacrylamide-SDS gel. (B) The [^{35}S]methionine-labeled proteins expressed by pWK9 expressing complete G (upper third of panel B), by pCL5 expressing $G_{193-517}$ (middle third of panel B), or by pPG7 expressing G_{1-193} (lower third of panel B) were immunoprecipitated with MAb 8 directed to epitope III (lane 1), with MAb 9 directed to epitope IV (lane 2), with MAb 14 directed to epitope VII (lane 3), or with MAb 15 directed to epitope VIII (lane 4). The [^{35}S]labeled immunoprecipitates were analyzed by electrophoresis on 15% polyacrylamide-SDS gels followed by autoradiography.

G protein. However, the question of whether cysteine residues located within the first 193 N-terminal amino acids form disulfide bonds critical to the three-dimensional structure of VSV-NJ G protein essential for recognition of discontinuous epitopes by neutralizing MAbs remains. To this end, we constructed plasmids driven by the vaccinia virus-expressed T7 polymerase that express truncated VSV-NJ G protein fragments encompassing the first 193 amino acids (G_{1-193}), the first 411 amino acids (G_{1-411}), and amino acids 193 to 517 ($G_{193-517}$). G_{1-193} contains cysteine residues 15, 75, 84, 108, 130, 169, 174, and 193, whereas $G_{193-517}$ contains the carboxy-distal cysteine residues 193, 235, 240, 273, and 304. The truncated peptide G_{1-411} contains all 12 cysteine residues of the VSV-NJ G protein (see Materials and Methods). These truncated proteins were expressed in CV-1 cells transfected with plasmids pPG7 (expressing G_{1-193}), pPG8 (expressing G_{1-411}), and pCL5 (expressing $G_{193-517}$). As described in Materials and Methods, the truncated proteins were biologically labeled with [^{35}S]methionine and immunoprecipitated with epitope-specific MAbs before analysis on SDS-polyacrylamide gels under nonreducing or reducing conditions (with β -mercaptoethanol).

Figure 3 depicts a composite of a series of experiments

designed to compare the PAGE mobilities of plasmid-expressed truncated proteins G_{1-411} , $G_{193-517}$, and G_{1-193} under reducing and nonreducing conditions, as well as the capacity of these plasmid-expressed truncated G proteins to recognize epitope-specific MAbs. Figure 3A shows that truncated protein G_{1-411} , which contains all 12 cysteine residues, reacts to increasing concentrations of β -mercaptoethanol by progressively retarded migration on SDS-polyacrylamide gels in a manner identical to that of native VSV-NJ G protein (Fig. 1A). Similarly, the amino-terminal truncated protein G_{1-193} , which contains the first eight cysteine residues, also responds to β -mercaptoethanol by progressively retarded migration during SDS-PAGE. In sharp contrast, exposure to β -mercaptoethanol of $G_{193-517}$, which contains the carboxy-distal five cysteine residues, resulted in no detectable shift in SDS-PAGE migration.

These effects of a reducing agent on the migration of plasmid-expressed truncated regions strongly suggest the presence of at least one major loop flanked by a stabilizing disulfide bond(s) located exclusively within the first 193 NH_2 -terminal amino acids of the VSV-NJ G protein. By the same token, the cysteine residues located in the carboxy-distal 325 amino acids apparently do not create a looplike structure, since reduction of $G_{193-517}$ does not influence its migration during SDS-PAGE. This, of course, does not rule out disulfide bonds forming short loop structures distal to residue 193.

Although neutralization epitopes VII and VIII had previously been mapped to a region between amino acids 193 and 289 of the VSV-NJ G protein (8), it was important to determine whether the cysteine residues located within the NH_2 -proximal 193 amino acids are essential for the conformational integrity of these epitopes. To this end, CV-1 cells were transfected with pWK9, pCL5, or pPG7 expressing the entire VSV-NJ G protein, $G_{193-517}$, or G_{1-193} , respectively, and labeled with [^{35}S]methionine as described in Materials and Methods. The reactivities of these expressed proteins with epitope-specific MAbs were determined by immunoprecipitation of the cytoplasmic contents with MAb 8 (epitope III), MAb 9 (epitope IV), MAb 14 (epitope VII), and MAb 15 (epitope VIII) followed by SDS-PAGE and autoradiography. The electropherogram data shown in Fig. 3B reveal that all four epitopes are present in the entire G protein expressed by pWK9, as determined by immunoprecipitation with each of the four MAbs (Fig. 3B, upper panel). In sharp contrast, protein $G_{193-517}$ (expressed by pCL5) was immunoprecipitated only by MAb 8 directed to epitope III (Fig. 3B, lane 1, middle panel); epitope III is a continuous linear epitope located between amino acids 299 and 317 (8). Quite convincingly, G_{1-193} expressed by pPG7 (Fig. 3B, lane 2, lower panel) was precipitated only by MAb 9 directed to epitope IV, which had previously been mapped to a region between amino acids 80 and 183 of the VSV-NJ G protein (8).

The most significant finding in the experiment recorded in Fig. 3B is the failure of MAb 14 and MAb 15 to react with the truncated protein $G_{193-517}$, which was expected from previous experiments (8) to contain the amino acid sequences comprising epitopes VII and VIII. Clearly, these neutralization discontinuous epitopes are not located in the first N-terminal 193-amino-acid sequence of the VSV-NJ G protein. One can only conclude that at least a portion of the first 193-amino-acid sequence must complement sequences downstream to form discontinuous epitopes VII and VIII. In all likelihood, cysteine residues in the G_{1-193} fragment are required to form disulfide bonds with cysteines present in the $G_{193-517}$ fragment to produce the G protein secondary and

three-dimensional structures for recognition of the conformational epitopes VII and VIII by MAb 14 and MAb 15, respectively.

Effect of cysteine→serine site-directed mutations on MAb recognition of discontinuous epitopes. We next attempted to determine which of the 12 cysteine residues are responsible for the configuration of the VSV-NJ G protein that results in recognition by MAbs of neutralization discontinuous epitopes VII and VIII. To this end, we created site-directed mutants in each of 10 codons that would substitute a serine for a cysteine in the VSV-NJ G protein expressed by pWK9. In order to create individual Cys→Ser mutants at centrally located cysteines 235, 240, 273, and 304 by site-directed mutagenesis as described in Materials and Methods, we cloned site-directed mutated fragments derived from pS1-G470 into identical restriction sites in pWK9. Five Cys→Ser mutations were created at NH₂-terminal cysteines at positions 84, 108, 130, 169, and 174 by cloning *EcoRI*-*PstI* fragments from mutated pS1-G470 into identical restriction sites of the expression vector pWK9. The *EcoRI* site is located 12 bases upstream from the ATG start codon, and the *PstI* site is at base 576 of the G gene, corresponding to amino acid Cys-193. The Cys→Ser mutation at amino acid residue 193 consisted of a G-to-C substitution at base 574, thus removing the unique G gene *PstI* site and requiring the use of an *NcoI* site at base 638 to clone an *EcoRI*-*NcoI* fragment from the pS1 Cys₁₉₃→Ser mutant into identical restriction sites of pWK9. The 10 Cys→Ser mutations in pWK9 were designated pSER 309, pSER 273, pSER 240, pSER 235, pSER 193, pSER 174, pSER 169, pSER 130, pSER 108, and pSER 84. All attempts to create Cys→Ser mutations at the first two NH₂-terminal cysteines failed, probably owing to the internal secondary structure preventing annealing of the primer oligonucleotides.

The 10 individual Cys→Ser mutated VSV-NJ G proteins were expressed in CV-1 cells transfected with each plasmid along with the T7 polymerase recombinant vaccinia virus vTF1-6,2. The transfected CV-1 cells were labeled with [³⁵S]methionine for 1 h at 9 h posttransfection, after which the cells were lysed with detergent-containing HO buffers (see Materials and Methods). Extracts of the transfected cells were then exposed to MAbs directed to epitopes III, IV, VII, and VIII. The immunoprecipitated mutant ³⁵S-labeled G proteins were washed, resuspended in mercaptoethanol-containing gel loading buffer, analyzed by SDS-PAGE, and autoradiographed as described in Materials and Methods.

Figure 4 shows the electropherograms of the 10 [³⁵S]methionine-labeled G proteins with individual Cys→Ser mutations that had been recognized by immunoprecipitation with MAbs directed to four separate epitopes. Also shown in Fig. 4 is a stick model of the VSV-NJ G protein with the approximate map locations of epitopes III, IV, VII, and VIII, as well as the amino acid locations of each Cys→Ser mutation aligned with a chart summarizing the immunoprecipitability of each G protein mutant by each epitope-specific MAb. The standard 100% immunoprecipitation shown in the summary dot diagram in Fig. 4B is based on the reaction with each MAb of wild-type G protein expressed by pWK9. As shown in the electropherograms and in the summary dot diagram, none of the eight Cys→Ser mutations influenced the 100% reactivity of the expressed mutant G proteins with MAb 8 directed to the linear (nonconformational and non-neutralization) epitope III. Even the mutation of Cys-304 did not affect the MAb reactivity of linear epitope III, previously mapped between amino acids 299 and 317 (8). On the other

hand, reactivity with MAb 9, directed to nonneutralization but conformational epitope IV, mapped roughly to amino acids 1 to 193, was markedly reduced by mutations at cysteine residues 108 and 169 and to a somewhat lesser extent (15 to 50%) by mutations at cysteines 84 and 174. These data indicate that two disulfide bonds within the first 193 amino acids of the VSV-NJ G protein provide the conformation required for the integrity of epitope IV. Clearly, mutations of cysteines carboxy distal to residue 193 do not influence the antigenicity of epitope IV.

The electropherograms and summary dot diagram in Fig. 4 also show that single-site mutations at cysteines 235, 240, and 273 have a marked effect on the binding of neutralizing MAbs 14 and 15, directed to epitopes VII and VIII, respectively, which map within the region of amino acids 193 to 289. Unlike the case with epitope IV, mutations in cysteines outside their mapping region markedly influence the integrity of epitopes VII and VIII. As shown in Fig. 4, a Cys→Ser mutation at residue 130 completely abolishes the reactivity of the expressed mutant G protein with MAb 14 and MAb 15, directed to epitopes VII and VIII, respectively. This finding would make cysteine 130 a candidate for forming the NH₂-terminal disulfide bond that ostensibly anchors the loop structure essential for the expression of epitopes VII and VIII. However, a mutation at cysteine 174 also ablates epitope VIII without altering the reactivity of MAb 14 directed to epitope VII. In addition, mutations at Cys-108 and Cys-193 exhibit partial (15 to 50%) decreases in the binding affinities of epitopes VII and VIII for their respective MAbs 14 and 15. These data collectively suggest that the discontinuous and neutralization epitopes VII and VIII depend, at least to some extent, on the conformation of G protein provided by disulfide bonds emanating from cysteine residues amino proximal to the region at which these epitopes map.

DISCUSSION

Our laboratory has undertaken the mapping of the antigenic determinants of the type-specific G protein of VSV, particularly those of VSV-NJ, which is the primary etiologic agent of enzootic and epizootic diseases in cattle and swine (15). Despite being handicapped by not having a three-dimensional crystallographic structure of the G protein, we started by raising MAbs directed to nine nonoverlapping epitopes, many of which mapped by crude protease-digestion techniques to the central third of the 517-amino-acid VSV-NJ G protein (2). The map locations of the epitopes were greatly refined by determining the binding affinities of epitope-specific MAbs to expressed deletions, truncations, and chimeras of the VSV-Ind and VSV-NJ G proteins (8). The general map locations of neutralization epitopes V, VI, VII, and VIII were confirmed by the binding of MAbs to neutralization-escape mutants of VSV-NJ and sequencing the mutant G genes (11). Additional confirmatory data were obtained by sequencing G gene mutants spontaneously arising in diseased cattle and swine (12). Experiments were also performed to determine the effect, if any, of glycosylation on the conformational G protein epitopes of VSV-NJ grown in the presence of tunicamycin (6). These experiments suggested that the loss of antigenic determinants on unglycosylated G protein was probably due to aberrant folding leading to altered conformation, presumably resulting from the mismatched formation of disulfide bonds in G proteins defective in N-linked glycosylation, as proposed by Machamer and Rose (14).

FIG. 4. Comparative recognition of epitopes III, IV, VII, and VIII in single-site Cys→Ser G mutants as determined by immunoprecipitation with specific MAbs of plasmid-expressed mutant G proteins labeled with [³⁵S]methionine analyzed by SDS-PAGE autoradiographs for each numbered cysteine mutant (A) and summarized by a dot diagram aligned with a G protein stick model showing the positions of cysteine mutations and epitope map locations (B). (A) Ten different Cys→Ser mutants in the G gene of pWK9 were transiently expressed in CV-1 cells coinfecting with the vaccinia virus T7 polymerase recombinant vTF1-6,2 and labeled with [³⁵S]methionine for 1 h at 9 h posttransfection. As described in Materials and Methods, cell lysates were then prepared and divided into four equal samples for immunoprecipitation with MAbs; the G protein immune complexes were then boiled in SDS-containing sample buffer for analysis by electrophoresis on 15% polyacrylamide-SDS gels under reducing conditions and autoradiographed. For each numbered cysteine mutant transfectant, the G protein was immunoprecipitated by MAb 8 (epitope III, lane 1), MAb 9 (epitope IV, lane 2), MAb 14 (epitope VII, lane 3), or MAb 15 (epitope VIII, lane 4). Control lanes (C) signify exposure of cell lysate to MAb 10 of VSV-Ind G protein which does not cross-react with the VSV-NJ G protein. (B) G protein stick model showing the relative distribution of the numbered cysteine residues and the proposed binding sites of MAbs directed to epitopes III, IV, VII, and VIII. The dot diagram summarizes the comparative reactivity of each individual mutant G protein with each of the four MAbs (●, >50% binding; ○, <15% binding; ⊙, intermediate [15 to 50%] binding). The evaluation of MAb binding to mutant proteins is based on the results of five independent experiments. The binding affinities of MAbs to mutant proteins were scored by using a baseline of 100% for binding to wild-type G protein expressed by pWK9, shown in Fig. 3. The protein bands comigrating in the region of G protein appear to be of cellular origin.

These background studies led us to embark on the experiments reported here, which were designed to determine the contribution of each cysteine residue to disulfide bond formation, resulting in the correct secondary structure and conformation required for recognition by MAb's of certain VSV-NJ G protein epitopes. A somewhat unexpected finding of these studies resulted from limited digestion with *S. aureus* V8 protease, which converted purified G protein to a more slowly migrating form (G_{V8}) on PAGE; amino acid sequencing revealed that G_{V8} was cleaved at a single site between Glu-110 and Ala-111. This retarded gel migration of G_{V8} under nonreducing conditions resembled the slow migration of uncleaved G protein under reducing conditions, suggesting the presence of a major loop structure in the vicinity of Glu-110 flanked by a disulfide-bonded compact

structure, possibly between cysteines at residues 108 and 169. A highly significant finding was the complete failures of MAb 14 and MAb 15 to recognize the expressed carboxy-distal 325 amino acids in truncated G₁₉₃₋₅₁₇, despite ample evidence that these neutralization epitopes are located between amino acids 193 and 289 (8). These data, as well as those obtained by analysis of the protease-cleaved protein G_{V8}, make more cogent the postulated existence of an extended loop structure originating from a cysteine amino proximal to residue 110 and extending to a cysteine downstream from the cysteine at residue 193. Such a disulfide bond, encompassing an extended peptide loop, could well influence the three-dimensional structure of the VSV-NJ G protein required for exposing the discontinuous epitopes VII and VIII carboxy distal to amino acid 193.

The disappearance of neutralization epitopes VII and VIII was noted in expression vectors of G protein with site-specific mutants upstream and downstream of amino acid 193. As might be expected from their map locations between residues 193 and 289 (8), cysteine mutations at residues 235, 245, and 273 resulted in the loss of epitopes VII and VIII. In addition, however, a mutation of Cys-130 in the putative proximal loop structure also resulted in the disappearance of both neutralization epitopes, VII and VIII. Also resulting in the disappearance of epitope VIII were mutations in Cys-174 and possibly in Cys-193. Therefore, it seems likely that the putative loop structure, possibly resulting from a disulfide formed by cysteines 108 and 169, has a major influence on the three-dimensional structure of the VSV-NJ G protein required for the expression of neutralization epitopes located carboxy distal to residue 193. In contrast, none of the cysteine mutations downstream from residue 193 influenced the expression of epitope IV, presumably located in the proximal loop structure. The data suggest differences in the two neutralization epitopes, since the reactivity of epitope VIII is compatible with that of a three-dimensional structure dependent on at least two disulfide bonds, possibly formed between Cys-130 and Cys-174 and any combination of the cysteines at positions 193, 235, 240, and 273. However, unlike that of epitope VIII, the MAb reactivity of epitope VII is not significantly affected by mutations at cysteine residue 169, 174, or 193, thus making less likely a hypothesis that Cys-130 forms a disulfide bond with one of the three cysteine residues at position 169, 174, or 193 located close to the N-glycosylation site at asparagine 179 (6). The most feasible model, therefore, would seem to be the presence of a major loop formed by a disulfide bond between Cys-130 and one of the more centrally located cysteines at residue 235, 240, or 273. This latter model also envisions minor loops composed of disulfide bonds formed by cysteine residues within the putative major loop, thus explaining the loss of epitope VIII by mutations in these cysteines.

Studies of other viruses have shown that mutations resulting in Cys→Ser substitutions in glycoproteins can result in profound antigenic or functional alterations in herpes simplex virus (24) and human immunodeficiency virus (23). These studies on the glycoproteins of herpes simplex virus, human immunodeficiency virus, and VSV raise the question of whether a mutation of a single cysteine residue not only results in the loss of a single disulfide bond but also causes nascent glycoproteins to undergo aberrant disulfide bonding among other cysteine residues. Machamer et al. (13) have shown, for example, that failure of the VSV-Ind glycoprotein to form correct intrachain disulfide bonds can result in the formation of stable disulfide bonds, with cellular glycoproteins being formed concurrently. Such an observation raises the question of whether there was aberrant disulfide bonding intrachain or with cellular glycoproteins in our studies with VSV-NJ G mutants, resulting in the loss of specific epitopes.

Indirect experimental evidence argues against aberrant disulfide bond formation as the explanation for the loss of epitopes in expressed mutant VSV-NJ G proteins with single Cys→Ser substitutions as follows. (i) Similar epitopes are lost in the partially digested glycoprotein G_{v8}, as occurs with Cys→Ser substitutions in the same regions. (ii) The map locations assigned to the discontinuous epitopes altered by Cys→Ser substitutions are quite compatible with the locations of these epitopes in expressed truncated G proteins G₁₋₁₉₃ and G₁₉₃₋₅₁₇ (Fig. 3). (iii) The results of immunoprecipitation experiments shown in Fig. 4 clearly demonstrate

that a mutation in Cys-108 affects only epitope IV, whereas a mutation in the adjacent cysteine (Cys-130) results in the loss of epitope VIII but not that of epitope IV. (iv) Although defective glycosylation of glycoproteins and aberrant disulfide bond formation seem to be closely related events (24), we had previously shown that the failure to glycosylate nascent VSV-NJ G protein at asparagine 179 results in the loss of epitope VII but not that of the closely adjacent epitope VIII (6). Our experiments reported here show the opposite effect, i.e., the loss of epitope VIII but not that of epitope VII when mutations were made in Cys-174 and Cys-193 quite close to the glycosylation site at residue 179.

Clearly, crystallographic studies to define the three-dimensional structure of VSV-NJ G protein are required to confirm or modify the partial model proposed here, which is largely based on putative cysteine pairing and disulfide bond secondary structure.

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REFERENCES

1. Bishop, D. H. L., P. Repik, J. F. Obijeski, N. F. Moore, and R. R. Wagner. 1975. Restitution of infectivity to spikeless vesicular stomatitis virus by solubilized viral components. *J. Virol.* **16**:75–84.
2. Bricker, B., R. M. Snyder, J. W. Fox, W. A. Volk, and R. R. Wagner. 1987. Monoclonal antibodies to the glycoprotein of vesicular stomatitis virus (New Jersey serotype): a method for preliminary mapping of the epitopes. *Virology* **161**:533–540.
3. Cartwright, B., P. Talbot, and F. Brown. 1970. The proteins of biologically active subunits of vesicular stomatitis virus. *J. Gen. Virol.* **7**:267–272.
4. Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**:8122–8126.
5. Gallione, C. J., and J. K. Rose. 1983. Nucleotide sequence of a cDNA clone encoding the entire glycoprotein from the New Jersey serotype of vesicular stomatitis virus. *J. Virol.* **46**:162–169.
6. Grigera, P. R., M. M. Mathieu, and R. R. Wagner. 1991. Effect of glycosylation on the conformational epitopes of the glycoprotein of the vesicular stomatitis virus (New Jersey serotype). *Virology* **180**:1–9.
7. Hunkapiller, M. H., R. M. Hewick, W. J. Dryer, and L. E. Hood. 1983. High sensitivity sequencing with a gas phase sequencer, p. 399–413. In C. H. W. Hirs and S. N. Timasheff (ed.), *Methods in enzymology*, vol. 91. Academic Press, New York.
- 7a. Keil, W. Unpublished data.
8. Keil, W., and R. R. Wagner. 1989. Epitope mapping by deletion mutants and chimeras of two vesicular stomatitis virus glycoprotein genes expressed by a vaccinia virus vector. *Virology* **170**:392–407.
9. Kelley, J. M., S. U. Emerson, and R. R. Wagner. 1972. The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody. *J. Virol.* **10**:1231–1235.
10. Laver, W. G., G. M. Air, R. G. Webster, and S. S. Smith-Gill. 1990. Epitopes on protein antigens: misconceptions and realities. *Cell* **61**:553–556.
11. Luo, L., Y. Li, R. M. Snyder, and R. R. Wagner. 1988. Point mutations in glycoprotein gene of vesicular stomatitis virus (New Jersey serotype) selected by resistance to neutralization

- by epitope-specific monoclonal antibodies. *Virology* **163**:341–348.
12. Luo, L., Y. Li, and R. R. Wagner. 1990. Spontaneous mutations leading to antigenic variations in the glycoproteins of vesicular stomatitis virus field isolates. *Virology* **174**:70–78.
 13. Machamer, C. E., R. W. Doms, D. G. Bole, A. Helenius, and J. K. Rose. 1990. Heavy chain binding protein recognizes incompletely disulfide bonded forms of vesicular stomatitis virus G protein. *J. Biol. Chem.* **265**:6879–6883.
 14. Machamer, C. E., and J. K. Rose. 1988. Influence of new glycosylation sites on expression of vesicular stomatitis G protein at the plasma membrane. *J. Biol. Chem.* **263**:5948–5964.
 15. Mason, J. 1986. Proceedings of an international conference on vesicular stomatitis. U. S. Commission for the Prevention of Foot-and-Mouth Disease, Mexico City.
 16. McSharry, J. J., and R. R. Wagner. 1971. Lipid composition of purified vesicular stomatitis virus. *J. Virol.* **7**:59–70.
 17. Moss, B., O. Velroy-Stein, T. Mizukami, W. A. Alexander, and T. R. Fuerst. 1990. New mammalian expression vectors. *Nature (London)* **348**:91–92.
 18. Petri, W. A., and R. R. Wagner. 1979. Reconstitution into liposomes of the glycoprotein of vesicular stomatitis virus by detergent dialysis. *J. Biol. Chem.* **254**:4313–4316.
 19. Rose, J. K., and R. W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell. Biol.* **4**:257–288.
 20. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
 21. Scheele, G., and R. Jacoby. 1982. Conformational changes associated with proteolytic processing of presecretory proteins allow glutathione-catalyzed formation of native disulfide bonds. *J. Biol. Chem.* **257**:12277–12282.
 22. Stephens, E. B., and R. W. Compans. 1988. Assembly of animal viruses at cellular membranes. *Annu. Rev. Microbiol.* **42**:489–516.
 23. Tschachler, E., H. Buchow, R. C. Gallo, and M. S. Reitz. 1990. Functional contribution of cysteine residues to the human immunodeficiency virus type 1 envelope. *J. Virol.* **64**:2250–2259.
 24. Wilcox, W. C., D. Long, D. L. Sodora, R. J. Eisenberg, and G. H. Cohen. 1988. The contribution of the cysteine residues to antigenicity and extent of processing of herpes simplex virus type 1 glycoprotein D. *J. Virol.* **62**:1941–1947.
 25. Zell, R., and H. J. Fritz. 1987. DNA mismatch-repair in *Escherichia coli* counteracting the hydrolytic deamination of 5-methyl-cytosine residues. *EMBO J.* **6**:1809–1815.
 26. Zoller, M. J., and M. Smith. 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. *Nucleic Acids Res.* **10**:6487–6500.