Antigenicity, Function, and Conformation of Synthetic Oligopeptides Corresponding to Amino-Terminal Sequences of Wild-Type and Mutant Matrix Proteins of Vesicular Stomatitis Virus

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The matrix (M) protein of vesicular stomatitis virus (VSV) has a major antigenic determinant (epitope 1) that maps to a region extending from amino acids 19 through 43 and transcription-inhibition activity that maps to the first 43 N-terminal amino acids (J. R. Ogden, R. Pal, and R. R. Wagner, *J. Virol.* 58:860-868, 1986). The M protein of temperature-sensitive mutant *tsO23*(III) is devoid of epitope 1 and transcription-inhibition activity and substitutes glutamic acid for glycine at amino acid 21 as well as having amino acid substitutions at positions 111 and 227 (K. Morita, R. Vanderloef, and J. Lenard, *J. Virol.* 61:256-263, 1987). We undertook to map more precisely epitope 1 and the transcription-inhibition region of VSV M protein by means of synthetic oligopeptides generated by an automated solid-phase protein synthesizer. A pentadecapeptide designated PI(*wt*, Gly₂₁), corresponding to amino acids 17 to 31 of wild-type (*wt*) M protein, strongly bound monoclonal antibody MAb2 (directed to epitope 1); however, an analogous pentadecapeptide with glutamic acid substituted for glycine at position 21, designated PII(*tsO23*, Glu₂₁), completely failed to recognize MAb2. Polyclonal antibody raised in rabbits immunized with PI(*wt*, Gly₂₁) reacted strongly with *wt* M protein, the homologous pentadecapeptide, and, to a lesser extent, PII(*tsO23*, Glu₂₁). Anti-PII(*tsO23*, Glu₂₁) failed to recognize PI(*wt*, Gly₂₁) or *wt* M protein. Anti-PI(*wt*, Gly₂₁) competed efficiently for binding of MAb2 to *wt* M protein and was as effective as MAb2 in reversing inhibition of VSV transcription by *wt* M protein. Neither PI(*wt*, Gly₂₁) nor PII(*tsO23*, Glu₂₁) exhibited any ability to inhibit VSV transcription. However, a lysine-rich oligopeptide, PIII(Met₁-Leu₂₀), corresponding to the first 20 N-terminal amino acids of *wt* M protein, and polylysine itself did inhibit VSV transcription, albeit much less efficiently than native *wt* M protein. Monospecific polyclonal antibody directed to the 20-mer oligopeptide PIII(Met₁-Leu₂₀) reversed transcription inhibition by M protein in a dose-dependent manner almost identical to that of anti-PI(*wt*, Gly₂₁) and epitope 1-specific MAb2. Examination by circular dichroism spectropolarimetry revealed significant differences in the conformation of the two pentadecapeptides attributable to the Gly→Glu amino acid substitution at position 21.

The enveloped, negative-strand RNA virus vesicular stomatitis virus (VSV) is the prototype of the family *Rhabdoviridae* (23). Of the five virally coded proteins in the VSV virion, two are membrane associated. The externally oriented spike glycoprotein (G) recognizes host cell receptors (19) and is the type-specific antigen which gives rise to and reacts with neutralizing antibody (4, 11). The matrix (M) protein lines the inner surface of the virion membrane (26) and resides in close association with the ribonucleoprotein (RNP) core of the assembled virion (14). The M protein also functions as a negative effector of VSV transcription (3, 6-8, 25). Monoclonal antibodies (MAbs) directed to the VSV M protein have been shown to bind to four topologically discrete M protein epitopes (17). An MAb to one of these antigenic determinants (epitope 1) selectively reverses M protein inhibition of VSV transcription (16). Partial and complete trypsin digests and Western blotting (immunoblotting) have provided evidence that localizes epitope 1 to a region of the M protein extending from amino acids 18 to 43 (15).

The M proteins of temperature-sensitive (*ts*) VSV mutants in complementation group III lose their ability to inhibit VSV transcription (4, 25). We have been particularly interested in one of these group III VSV mutants, *tsO23*, because its M protein completely loses its ability to bind MAb

directed to epitope 1 but retains binding affinity for MAbs to epitopes 2 and 3; three other group III *ts* mutants have M proteins that retain epitope 1 antigenic determinants (16). The M genes of these group III *ts* mutants have been sequenced by Morita et al. (12), who identified frequent nucleotide changes, often leading to amino acid substitutions, at widely variable locations of mutant and revertant M genes. Of particular interest to us were the three amino acid substitutions in the *tsO23* M protein: Gly-21→Glu, Leu-111→Phe, and His-227→Tyr. The Gly-21→Glu amino acid substitution was unique among the M protein *ts* mutants and is within the Lys-18-Lys-43 region of wild-type M protein where epitope 1 appears to be located (15).

The studies performed to date suggest that the amino-terminal domain of the VSV M protein may also contain sequences responsible for its inhibition of viral transcription as well as the antigenic sequences for binding the MAb that reverses inhibition of transcription (16). The data further imply that amino acid substitutions in *tsO23* M protein induce a conformational change in the M protein that leads to loss of MAb recognition and concurrent loss of transcription-inhibition activity. To further characterize the epitope 1 MAb-binding site and to locate the M protein domain that imparts the biological function of transcription inhibition, synthetic oligopeptides corresponding to sequences in the amino-terminal domain of the VSV M protein were generated and tested for their antigenic, transcription-inhibition, and conformational properties.

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MATERIALS AND METHODS

Cells and virus. The Indiana serotype of the Orsay wild-type (*wt*) strain of VSV and its *ts* mutant *tsO23* (complementation group III) were originally provided by A. Flamand, Faculté des Sciences, Université de Paris-Sud, Orsay, France. The San Juan strain of VSV-Indiana was originally obtained from the U.S. Agriculture Research Center, Beltsville, Md. Plaque-purified virus was used to infect baby hamster kidney (BHK-21) cells at a multiplicity of 0.1 PFU per cell, as described previously (9). Bullet-shaped virions were harvested by differential, rate-zonal, and equilibrium centrifugations, and the purified virus was stored in phosphate-buffered saline (PBS), pH 7.4, at a concentration of 2 to 4 mg/ml at -70°C .

Isolation of M protein. As described previously (24), the M protein from *wt* VSV (Orsay strain) was isolated from purified virions by exposing the virus to 1% aqueous Triton X-100 containing 10 mM Tris hydrochloride, pH 8.0, and 250 mM NaCl. The RNPs were removed by centrifugation at $240,000 \times g$ for 2 h at 4°C , and the M protein was purified by column chromatography on Whatman P-11 phosphocellulose.

Isolation of RNP cores. Transcriptionally active RNP cores were isolated from whole virions as described previously (16). Briefly, VSV (0.5 mg/ml) was disrupted in 1% aqueous Triton X-100 containing 10 mM Tris hydrochloride (pH 8.0) and 250 mM NaCl for 30 min at room temperature. The RNP cores, largely devoid of M protein, were then pelleted by centrifugation at $189,000 \times g$ for 2 h through a 50% glycerol pad. The pelleted RNP cores were resuspended in 10 mM Tris hydrochloride buffer, pH 7.5, and used immediately for transcription assays. Alternatively, RNP cores associated with M protein (RNP/M) were isolated from whole virus (1 mg/ml) by dialysis overnight against 10 mM Tris hydrochloride (pH 7.5), and the virus was disrupted with 60 mM octylglucopyranoside (Calbiochem, La Jolla, Calif.) in the same salt-free buffer. The RNP/M cores were pelleted by centrifugation in an SW50.1 rotor at 45,000 rpm for 2 h through a 50% glycerol pad.

Transcription assays. In a procedure described by Pal et al. (16), with certain modifications, RNP cores were added to a reaction mixture containing 7.5 mM MgCl_2 , 10 mM Tris hydrochloride, pH 8.0, 1 mM dithiothreitol, 1 mM each ATP, CTP, and GTP, and 0.1 mM UTP supplemented with 10 to 50 μCi of [α - ^{32}P]UTP. The final concentration of RNP cores was 180 $\mu\text{g}/\text{ml}$, and NaCl was included in the reaction mixture at a concentration of 0.08 M. The reactions were initiated by addition of the RNP cores to the reaction mixture. The reactions were allowed to proceed for 2 h at 31°C and quenched by the addition of 0.1 volume of 500 mM Tris hydrochloride, pH 8.0, containing 1.5 M NaCl and 50 mM EDTA. The extent of RNA synthesis in trichloroacetic acid (TCA)-precipitated material was then determined by liquid scintillation spectrometry. Each reaction was carried out in duplicate. The procedure used for RNP/M core transcriptional assays was the same, except that NaCl was omitted from the reaction mixture.

Preparation of polyclonal antibody to peptide-BSA conjugates. Synthetic oligopeptides prepared with the aid of an automatic solid-phase synthesizer and purified by high-pressure liquid chromatography were supplied by the University of Virginia Protein and Nucleic Acid Research Facility. The peptides were coupled to bovine serum albumin (BSA) with glutaraldehyde according to the protocol described by Morrow and Dasgupta (13). Two-month-old New Zealand White

rabbits were immunized by multiple-site percutaneous injection of 500 to 800 μg of each peptide-BSA conjugate emulsified in complete Freund adjuvant. Control serum was obtained from each rabbit prior to immunization. Booster injections containing 250 μg of BSA-coupled peptide were administered at 4, 7, and 10 weeks after primary immunization. Exsanguination was carried out 11 weeks after initial immunization. Clarification of whole blood was accomplished by allowing coagulation, followed by centrifugation at $18,000 \times g$ for 20 min at 4°C . Anti-BSA immunoglobulins were removed by albumin-linked Sepharose affinity chromatography (13).

Western and dot blot analysis. As described previously (17), proteins of disrupted virions (1 μg) were subjected to electrophoresis through a 12.5% polyacrylamide slab gel containing 0.1% aqueous sodium dodecyl sulfate (SDS). The separated proteins were transferred to nitrocellulose sheets (Schleicher & Schuell Inc., Keene, N.H.) by the method of Towbin et al. (21). Alternatively, isolated M protein, peptide-BSA conjugates, or free peptides were spotted directly onto the nitrocellulose. The sheets were then exposed to MAb or polyclonal antibody (immunoglobulin G [IgG], 10 $\mu\text{g}/\text{ml}$) in 50 mM Tris hydrochloride, pH 7.4, containing 10 mM NaCl, 5 mM EDTA, 0.25% gelatin, and 0.05% Nonidet P-40. After being rinsed, the sheets were incubated with 1 μCi of ^{125}I -labeled staphylococcal protein A (33 mCi/mg) per ml. The extent of antibody binding was determined by autoradiography and laser densitometry.

Competitive binding assays. Antibody and antigen competitive binding assays were carried out by enzyme-linked immunosorbent assay (ELISA). In brief, 0.2 μg of antigen in PBS (50 μl total volume) was added to wells of a polyvinyl chloride 96-well microtiter plate (Flow Laboratories, Beltsville, Md.). After overnight incubation, the wells were rinsed with 10 mM Tris hydrochloride, pH 8.0, containing 0.05% Tween-20 and 0.01% thimerosal. All wells were then treated with 100 μl of 10% gamma globulin-free horse serum or with 3% milk protein in 10 mM Tris hydrochloride, pH 7.4, and 150 mM NaCl. After 60 min at 37°C , the wells were rinsed. Mouse MAb or rabbit polyclonal antibody was then added to the wells in PBS (25 to 125 ng, 100 μl total volume). After incubation with antibody for 2 h at room temperature, the wells were rinsed. The extent of specific antibody binding was then determined by adding 50 μl of rabbit anti-mouse IgG conjugated to alkaline phosphatase or goat anti-rabbit IgG conjugated to alkaline phosphatase (Zymed Laboratories, Burlingame, Calif.). After 30 min at room temperature, the wells were rinsed, and the colorimetric reagent *p*-nitrophenylphosphate (0.05 mg) in 100 mM 2-amino-2-methyl-1,3-propanediol, pH 10 (50 μl total volume), was added to each well. Color was developed for 30 to 60 min at 37°C , and the optical density of each well was determined at 502 nm in a Titertech ELISA reader. The extent of antibody binding to immobilized antigen against which the antibody was raised was taken as a 100% response. In all cases, the immobilized antigen was present in vast excess relative to the amount of antibody present.

RESULTS

Synthetic oligopeptides corresponding to N-terminal regions of *wt* and *tsO23* M proteins. Previous studies in this laboratory suggested that epitope 1 and the transcription-inhibitory activity of *wt* VSV M protein are both located at the N-terminal region. The evidence for this hypothesis is based on the following observations. (i) MAb2 directed to epitope

FIG. 1. Amino acid sequences of the M protein N-termini of the VSV-Indiana (Orsay strain) *wt* and group III mutant *tsO23*, and synthetic oligopeptides corresponding to *wt* amino acid sequences 17 to 31 [PI(*wt*, Gly₂₁)], to *tsO23* mutant amino acid sequences 17 to 31 [PII(*tsO23*, Glu₂₁)], and to *wt*-mutant sequences 1 to 20 [PIII(Met₁-Leu₂₀)]. The synthetic oligopeptides were prepared under the supervision of Jay Fox of the University of Virginia Protein and Nucleic Acid Research Center with the aid of an automated solid-phase peptide synthesizer. The peptides were purified by high-performance liquid chromatography and sequenced to ensure their validity.

1 readily reverses VSV transcription inhibition by *wt* M protein (16); (ii) both epitope 1 and the transcription-inhibitory activity are located within the first 43 N-terminal amino acids of *wt* M protein, based on evidence that both functions are lost or markedly diminished by trypsin cleavage at Lys-43 (15, 17); (iii) epitope 1 is located between amino acids 19 (Lys) and 43 (Lys), because binding of MAb2 to M protein occurs only when this amino acid sequence is retained after partial tryptic cleavage at Lys-18 or Lys-19 (15); (iv) the intact M protein of mutant *tsO23* is completely devoid of epitope 1 and transcription-inhibitory activity (16); and (v) sequencing the M gene of *tsO23* reveals an amino acid substitution (Gly→Glu) at residue 21 (10), a finding we have confirmed by direct amino acid sequencing (unpublished data). Later sequencing data by Morita et al. (12) also identified other amino acid substitutions in the M protein of *tsO23* at position 111 (Leu_{*wt*}→Phe_{*tsO23*}) and at position 227 (His_{*wt*}→Tyr_{*tsO23*}). However, data based on transient expression of M protein genes with site-directed mutations cloned in pBSM13 (phage M13 plasmid with multiple cloning sites and T3 and T7 polymerase promoters), and the T7 polymerase-vaccinia virus system reveal that amino acid position 111 is the *ts* site of *tsO23* and position 21 is the site which determines altered antigenicity and probably transcription inhibition (Y. Li, L. Luo, R. M. Snyder, and R. R. Wagner, submitted for publication).

With these findings in hand, we set out to design and test synthetic oligopeptides corresponding to certain N-terminal amino acid sequences of VSV *wt* and *tsO23* M proteins. A particular aim was to determine whether the Gly→Glu substitution at amino acid 21 results directly in loss of the antigenic determinant (epitope 1) independently of amino

acid substitutions elsewhere in the M protein. Figure 1 illustrates the relevant amino acid sequences extending from Met-1 to Met-33 for M protein with glycine (Gly) at position 21 of *wt* M protein and with glutamic acid (Glu) at position 21 for *tsO23* M protein. Also shown are amino acid sequences of synthetic oligopeptides prepared with the aid of an automatic solid-phase peptide synthesizer: a pentadecapeptide, designated PI(*wt*, Gly₂₁), corresponds to *wt* sequence Ser-17-Thr-31, with a glycine at position 21; another pentadecapeptide, PII(*tsO23*, Glu₂₁), prepared with identical Ser-17-Thr-31 sequences except for a glutamic acid at position 21; and a third oligopeptide, designated PIII(Met₁-Leu₂₀), which corresponds to the first 20 amino acids of the VSV M protein. As described in Materials and Methods, polyclonal antibodies were raised in rabbits hyperimmunized with PI(*wt*, Gly₂₁), PII(*tsO23*, Glu₂₁), and PIII(Met₁-Leu₂₀) coupled to BSA and are designated anti-PI(*wt*, Gly₂₁), anti-PII(*tsO23*, Glu₂₁), and anti-PIII(Met₁-Leu₂₀), respectively.

Comparative and competitive binding of monoclonal and polyclonal antibodies to whole M protein and to synthetic oligopeptides. The synthetic oligopeptides and their corresponding polyclonal (but monospecific) antibodies provide a basis for confirming partial evidence for the location of epitope 1 on the Gly-21 *wt* M protein and its absence on the Glu-21 *ts*O23 M protein previously investigated with MABs (15, 16). To this end, MAb2 (directed to epitope 1) and MAB3 (directed to epitope 2) were tested for their ability to bind *wt* M protein, synthetic oligopeptides PI(*wt*, Gly₂₁), PII(*ts*O23, Glu₂₁), and PIII(Met₁-Leu₂₀), and a control G protein synthetic oligopeptide. Polyclonal antibodies anti-PI(*wt*, Gly₂₁), anti-PII(*ts*O23, Glu₂₁), and anti-PIII(Met₁-Leu₂₀) were also tested for their ability to bind *wt* M protein and the four

FIG. 2. Relative binding affinity of monoclonal and polyclonal antibodies to whole *wt* M protein and to synthetic oligopeptide antigens corresponding to *wt* or *tsO23* amino acid sequences. As described in Materials and Methods, the extent of antigen-antibody interaction was determined by ELISA with 0.2 μ g of M protein or oligopeptide immobilized in wells of microtiter plates. After nonspecific protein-binding sites were blocked with gamma globulin-free horse serum or with 3% milk protein, each of the two MABs, polyclonal antibodies, or normal rabbit serum (50 μ l, 50 μ g/ml) was added to wells containing one of the five antigens: *wt* M protein, peptide PI(*wt*, Gly₂₁), peptide PII(*tsO23*, Glu₂₁), peptide PIII(Met₁-Leu₂₀), or control peptide corresponding to the G protein sequence. The extent of antibody binding was determined by adding alkaline phosphatase-linked rabbit anti-mouse IgG or goat anti-rabbit IgG for the colorimetric *p*-nitrophenylphosphate assay, as described in Materials and Methods. Binding at the 100% level for each antibody represents the optimal response observed for each immobilized antigen used for raising that specific antibody.

synthetic oligopeptides. Initial studies by Western blotting revealed that MAb2 and anti-PI(*wt*, Gly₂₁) bound strongly to *wt* M protein and to PI(*wt*, Gly₂₁) but not to PII(*tsO23*, Glu₂₁) (data not shown). Moreover, anti-PI(*wt*, Gly₂₁) competitively inhibited binding of MAb2 to *wt* M protein and to oligopeptide PI(*wt*, Gly₂₁), indicating that polyclonal anti-PI(*wt*, Gly₂₁) is directed to epitope 1. However, MAb2 did not compete as effectively for anti-PI(*wt*, Gly₂₁) binding sites on *wt* M protein or on PI(*wt*, Gly₂₁), indicating that the polyclonal antibody recognized antigenic determinants on the pentadecapeptide PI(*wt*, Gly₂₁) other than epitope 1 (data not shown).

Figure 2 shows the comparative reactivity of monoclonal and polyclonal antibodies with *wt* M protein and four synthetic oligopeptides as determined by ELISA. MAb2 (directed to epitope 1) clearly reacted with oligopeptide PI(*wt*, Gly₂₁) but only to a level representing 68% of its reactivity with whole *wt* M protein. Strikingly, MAb2 exhibited only background reactivity with oligopeptides PII(*tsO23*, Glu₂₁), PIII(Met₁-Leu₂₀), and the G protein control. MAb3 (directed to epitope 2) reacted only with native *wt* M protein and not at all significantly with any of the oligopeptides. Polyclonal anti-PI(*wt*, Gly₂₁) reacted almost as well (94%) with whole *wt* M protein as it did with the immunogenic oligopeptide PI(*wt*, Gly₂₁). Anti-PI(*wt*, Gly₂₁) also reacted with pentadecapeptide PII(*tsO23*, Glu₂₁) but at a much reduced level of 31%. Of considerable interest and somewhat unexpectedly, anti-PII(*tsO23*, Glu₂₁) exhibited significant reactivity only with its immunogenic pentadecapeptide PII(*tsO23*, Glu₂₁) but gave only background titers on reaction with *wt* M protein or PI(*wt*, Gly₂₁). The polyclonal serum raised against peptide PIII(Met₁-Leu₂₀) failed to recognize either pentadecapeptide PI(*wt*, Gly₂₁) or PII(*tsO23*, Glu₂₁) but did bind *wt* M protein to a level 53% of that of the inducing antigenic peptide PIII(Met₁-Leu₂₀). Background levels are shown by ELISA titers of normal rabbit serum with M protein and the four oligopeptides (Fig. 2).

These data reveal that the antigenic determinant recognized by epitope 1-specific MAb2 is located, at least in part, on *wt* M protein between amino acids 17 and 31. The amino acid substitution Gly-21 $\rightarrow$ Glu in the pentadecapeptide PI(*wt*, Gly₂₁), corresponding to the amino acid sequence Ser-17-Thr-31 of *tsO23* M protein, resulted in complete loss of epitope 1. Polyclonal antibody raised against pentadecapeptide PI(*wt*, Gly₂₁) reacted with the same antigenic region on *wt* M protein recognized by MAb2 and showed reduced cross-reactivity with pentadecapeptide PII(*tsO23*, Glu₂₁), which itself exhibited no antigenic relationship to *wt* M protein or its epitope 1 amino acid sequence, Ser-17-Thr-31. These data confirm the critical role of Gly-21 in the epitope 1 specificity of *wt* M protein of VSV.

We also tested by Western blot analyses the ability of polyclonal antibodies raised to Ser-17-Thr-31 pentadecapeptides PI(*wt*, Gly₂₁), PII(*tsO23*, Glu₂₁), and PIII(Met₁-Leu₂₀) to recognize *wt* and *tsO23* M proteins. In these experiments, proteins of SDS-disrupted VSV-Indiana (San Juan and Orsay strains) and *tsO23* were separated on 12.5% polyacrylamide-SDS gels, electroblotted onto nitrocellulose, and exposed to MAb2 (epitope 1), anti-PI(*wt*, Gly₂₁), anti-PII(*tsO23*, Glu₂₁), or anti-PIII(Met₁-Leu₂₀) prior to binding of ¹²⁵I-labeled staphylococcal protein A and autoradiography. As previously described (17), MAb2 reacted strongly with *wt* M protein but not at all with *tsO23* M protein. On the other hand, anti-PI(*wt*, Gly₂₁) exhibited strong reactivity with both *wt* and *tsO23* M proteins, whereas anti-PII(*tsO23*, Glu₂₁) completely failed once again to react with either *wt* or *tsO23* M proteins (data not shown). The anti-PIII(Met₁-Leu₂₀) serum reacted with both *wt* and *tsO23* M proteins to the same extent. These data appear to confirm the ELISA studies (Fig. 2) showing that anti-PI(*wt*, Gly₂₁) recognizes sequences in the *wt* M protein region Ser-17-Thr-31 other than epitope 1. Quite strikingly, however, the *tsO23* M protein substitution of glutamic acid for glycine at position 21 of the pentadecapeptide Ser-17-Thr-31 not only resulted

FIG. 3. Reciprocal competition of MAb2 (directed to epitope 1) and anti-PI(*wt*, Gly₂₁) for binding to antigenic determinants on *wt* M protein and pentadecapeptide PI(*wt*, Gly₂₁). Purified VSV M protein (0.2 μ g) or pentadecapeptide PI(*wt*, Gly₂₁) (0.2 μ g) was immobilized in wells of microtiter plates for ELISA determinations. (A) Constant amount (225 μ g) of MAb2 IgG mixed with increasing concentrations of rabbit anti-PI(*wt*, Gly₂₁) IgG was added to M protein or peptide PI(*wt*, Gly₂₁), and the amount of MAb2 IgG bound was measured by ELISA with alkaline phosphatase-linked rabbit anti-mouse IgG antibody. (B) Constant amount of polyclonal anti-PI(*wt*, Gly₂₁) IgG mixed with increasing concentrations of MAb2 IgG was added to M protein or peptide PI(*wt*, Gly₂₁), and the amount of anti-PI(*wt*, Gly₂₁) IgG bound was measured by ELISA with alkaline phosphatase-linked goat anti-rabbit IgG antibody.

in loss of epitope 1 but also completely ablated the immunogenicity of the pentadecapeptide.

The ability of polyclonal anti-PI(*wt*, Gly₂₁) to compete with MAb2 (to epitope 1) for antigenic binding sites on *wt* M protein and pentadecapeptide PI(*wt*, Gly₂₁) was tested by reciprocal cross-reacting ELISA titrations. In these experiments, 0.2 μ g of *wt* M protein or pentadecapeptide PI(*wt*, Gly₂₁) was immobilized in wells of microtiter plates, to which was then added 100- μ l amounts of 225 μ g of anti-PI(*wt*, Gly₂₁) IgG mixed with serial dilutions of 0 to 225 μ g of MAb2 IgG or 225 μ g of MAb2 IgG mixed with serial dilutions of 0 to 225 μ g of anti-PI(*wt*, Gly₂₁) IgG. The extent of anti-PI(*wt*, Gly₂₁) IgG binding was measured by ELISA with alkaline phosphatase-linked goat anti-rabbit IgG, and that of MAb2 IgG was measured by ELISA with alkaline phosphatase-linked rabbit anti-mouse IgG.

Figure 3 compares the relative ability of anti-PI(*wt*, Gly₂₁) to compete with MAb2 and of MAb2 to compete with anti-PI(*wt*, Gly₂₁) for binding to *wt* M protein and to pentadecapeptide PI(*wt*, Gly₂₁). As shown in Fig. 3A, the binding of MAb2 to *wt* M protein was decreased by 40 to 50% by increasing concentrations of polyclonal anti-PI(*wt*, Gly₂₁), whereas the binding of MAb2 to pentadecapeptide PI(*wt*, Gly₂₁) was almost completely blocked by increasing concentrations of anti-PI(*wt*, Gly₂₁). As anticipated from the foregoing studies, the binding of anti-PI(*wt*, Gly₂₁) to *wt* M protein was only partially blocked by increasing concentrations of MAb2, and the presence of MAb2 had virtually no effect on the binding of anti-PI(*wt*, Gly₂₁) to the pentadecapeptide PI(*wt*, Gly₂₁) (Fig. 3B). These experiments confirm the hypothesis that the polyclonal antibody recognizes antigenic determinants other than those of epitope 1 and competes more effectively for M protein and pentadecapeptide binding sites than does the epitope 1-specific monoclonal antibody MAb2. Conversely, the lower efficiency of MAb2 competitive binding is surely due to the presence in polyclonal anti-PI(*wt*, Gly₂₁) of antibodies to multiple antigenic determinants.

Comparative effects of monoclonal and polyclonal antibodies on inhibition of VSV transcription by *wt* M protein. It is well established that endogenous VSV transcription is inhibited by M proteins of *wt* virus but not by M proteins of group

III *ts* mutants (3, 6–8, 25). Moreover, Pal et al. (16) have recently shown that MAb2 directed to epitope 1 of M protein reverses this inhibition of VSV transcription by *wt* M protein, whereas MABs directed to epitope 2 or 3 do not reverse transcription inhibition but even slightly enhance M protein transcription inhibition. It was of interest to determine whether polyclonal anti-PI(*wt*, Gly₂₁), anti-PII(*ts*O23, Glu₂₁), and anti-PIII(Met₁-Leu₂₀) would affect VSV transcription-inhibition by *wt* M protein. To this end, in vitro transcription assays were performed with VSV RNP cores containing a full complement of attached M protein (RNP/M cores), as described in Materials and Methods. To these transcription-inhibited RNP/M cores were added increasing concentrations of purified IgG from monoclonal or polyclonal antibodies, and incorporation of [α -³²P]UTP into TCA-precipitable mRNA was measured in a complete in vitro transcription mixture.

Figure 4 clearly shows that increasing concentrations of MAb2, anti-PI(*wt*, Gly₂₁), and anti-PIII(Met₁-Leu₂₀) were equally effective in reversing the RNP transcription-inhibitory activity of *wt* M protein. In sharp contrast, polyclonal anti-PII(*ts*O23, Glu₂₁) IgG had no effect at concentrations up to 200 μ g/ml, and MAB3 slightly increased the M protein-inhibitory activity. This experiment reveals that polyclonal antibody to amino acid sequence Ser-17–Thr-31 of *wt* M protein reverses transcription inhibition by *wt* M protein in the same manner as does epitope 1-specific MAB. The fact that antibody directed to the first 20 N-terminal amino acids also reversed transcription inhibition indicates that the effect is not epitope specific but does tend to implicate the entire N-terminal region of the M protein. The similarity in the dose-response curves for MAB2, anti-PI(*wt*, Gly₂₁), and anti-PIII(Met₁-Leu₂₀) suggests a common mechanism for their reversal of M protein transcription inhibition.

Effect on VSV transcription of synthetic oligopeptides corresponding to M protein amino acid sequences. In vitro transcription by RNP cores essentially devoid of M protein is markedly inhibited by reconstitution with free M protein (3); this inhibition by exogenous M protein can be reversed by MAB2 directed to epitope 1 (16). Protease cleavage studies suggest that the N-terminal region of *wt* VSV M protein may contain amino acid sequences responsible, at

FIG. 4. Reversal of inhibition of viral transcription by MABs (MAB2 or MAB3) and polyclonal anti-PI(*wt*, Gly₂₁), anti-PII(*ts*O23, Glu₂₁), or anti-PIII(Met₁-Leu₂₀) antibodies. VSV RNPs with attached M protein (RNP/M cores) were isolated as described in Materials and Methods. Antibody in the concentrations indicated was added in 10 mM Tris hydrochloride buffer (pH 8.0). The reactions were initiated by adding RNP/M cores (180 μg/ml) and allowed to proceed for 2 h at 31°C, when quenching was accomplished by adding 0.1 volume of the Tris hydrochloride buffer containing 1.5 M NaCl and 30 mM EDTA, as well as carrier yeast RNA (50 μg/ml). RNA synthesis was determined by precipitation with ice-cold 10% TCA, followed by filtration onto glass fiber disks and quantitation by liquid scintillation spectrometry. The extent of RNA transcription by RNP/M cores in the presence of increasing antibody concentrations is shown for epitope 1-specific MAB2 (●), epitope 2-specific MAB3 (■), polyclonal anti-PI(*wt*, Gly₂₁) (○), anti-PII(*ts*O23, Glu₂₁) (Δ), anti-PIII(Met₁-Leu₂₀) (▲), and normal rabbit serum (□).

least to some degree, for downregulating the transcription-inhibitory activity of M protein (15). These observations, coupled with the finding that anti-PIII(Met₁-Leu₂₀) could also reverse M protein inhibition of transcription, made it of interest to determine whether synthetic oligopeptides with amino acid sequences corresponding to those at two locations in the N-terminal region of M protein could influence transcription by RNP cores. For this purpose, RNP cores essentially devoid of M protein were prepared from VSV virions exposed to 1% Triton X-100 and 250 mM NaCl, as described in Materials and Methods, and used in a complete in vitro transcription reaction system. To samples of the RNP cores were added increasing concentrations of either M protein, the two Ser-17-Thr-31 pentadecapeptides PI(*wt*, Gly₂₁) and PII(*ts*O23, Glu₂₁), or the oligopeptide PIII(Met₁-Leu₂₀) in order to determine their effects on RNA synthesis as measured by incorporation of [α -³²P]UTP. Because of the high lysine content (40%) of peptide PIII(Met₁-Leu₂₀), it was also of interest to test the inhibitory activity of polylysine; this latter experiment could provide evidence on the possibility that the lysine-rich region of the M protein inhibits transcription by reacting with the polyribophosphate backbone of the VSV genome.

Figure 5 shows that increasing concentrations (up to 132 μg/ml) of the pentadecapeptides PI(*wt*, Gly₂₁) and PII(*ts*O23, Glu₂₁) had no significant effect on VSV RNP transcription. Of considerable interest was the observation that the oligopeptide PIII(Met₁-Leu₂₀) inhibited RNP transcription increasingly to a level ~50% of that of the RNP controls when

FIG. 5. Comparative inhibition of VSV transcription by synthetic oligopeptides PI(*wt*, Gly₂₁) (■), PII(*ts*O23, Glu₂₁) (Δ), PIII(Met₁-Leu₂₀) (▲), and polylysine (○). RNP cores free of M protein were prepared from VSV virions as described in Materials and Methods and added to a complete in vitro transcription reaction mixture (50 μl total volume). As described in the legend to Fig. 4, transcription reactions were initiated by adding RNP cores (180 μg/ml) with vigorous shaking to ensure complete suspension. Synthetic oligonucleotides or purified M protein were included in the reaction mixtures at the concentrations indicated. After incubation for 2 h at 31°C, the reactions were quenched. The results of transcription inhibition are recorded as percent incorporation of [³²P]UMP into viral mRNA in the presence versus absence (controls) of synthetic oligopeptides or M protein.

the 20-mer oligopeptide concentration was 132 μg/ml, as did the polylysine. This finding suggests that the lysine-rich N-terminus of *wt* M protein provides an amino acid sequence that inhibits VSV transcription. It should also be noted that the mass of the 20-mer oligopeptide PIII(Met₁-Leu₂₀) represents less than 10% of that of the intact M protein and was therefore used at a molar concentration about 11 times higher than that required for the 229-amino-acid M protein to inhibit transcription by 70% (data not shown). Clearly, the Met-1-Leu-20 oligopeptide was far less effective as a transcription inhibitor than the native M protein. Conceivably, the downstream amino acid sequence Ser-17-Thr-31 provides a site for binding of M protein to the RNP core, permitting greater transcription-inhibitory activity than that of the oligopeptide Met-1-Leu-20. Such an hypothesis may also explain the ability of both MAB2 and anti-PI(*wt*, Gly₂₁) to reverse inhibition of transcription by *wt* M protein. Further proof of this hypothesis must await the availability of a pure oligopeptide corresponding to the first 31 amino acids (Met-1-Thr-31) at the N-terminus of *wt* M protein.

Comparative oligopeptide conformation determined by CD. The striking differences in antigenic properties between the M proteins of *wt* VSV and *ts*O23, as well as their corresponding Ser-17-Thr-31 pentadecapeptides, strongly suggest a conformational change caused by the Gly→Glu substitution at amino acid 21. No other explanation would seem to account for the complete loss of epitope 1 in the M proteins of *ts*O23 and PII(*ts*O23, Glu₂₁) or the marked immunogenic differences between PI(*wt*, Gly₂₁) and PII(*ts*O23, Glu₂₁). In a collaborative study with Matthew Pincus and his colleagues, we predicted, by computation of energy minimization, the conformational changes expected from a Gly→Glu substitution at amino acid 21 of a dodecapeptide extending from

FIG. 6. CD spectropolarimetry of pentadecapeptides PI(*wt*, Gly₂₁) and PII(*tsO23*, Glu₂₁). The spectra were obtained with a JASCO J-600 spectropolarimeter at an instrument time constant of 2 s, a scan speed of 20 nm/min, and a band width of 1 nm. The oligopeptide spectra were obtained at a temperature of $25 \pm 2^\circ\text{C}$ in 25 mM NaPO₄ buffer (pH 7.4) containing 150 mM NaCl. The results were calculated as the mean residual molar ellipticity ($[\theta] \times 10^6$) for seven spectral scans for each peptide.

Lys-15 through Pro-26, corresponding to amino acid sequences of *wt* and *tsO23* M proteins. These computations result in a predicted alteration from a β -bend in the Gly dodecapeptide to an α -helix or random coil in the Glu dodecapeptide propagating through residues 20 and 21 (2). With this prediction in mind, it was of interest to compare by circular dichroism (CD) spectropolarimetry the spectra of the two Ser-17–Thr-31 pentadecapeptides PI(*wt*, Gly₂₁) and PII(*tsO23*, Glu₂₁).

Figure 6 shows that the CD spectra of these two pentadecapeptides were indeed quite different. The results, calculated as the mean residual ellipticity, $[\theta] \times 10^6$, for each pentadecapeptide, showed that PI(*wt*, Gly₂₁) had a somewhat larger ellipticity, with the extreme blue shift of 7.8 nm compared with PII(*tsO23*, Glu₂₁). By using the computer algorithm of K. D. Williams (Virginia Commonwealth University, Richmond, Va.), our CD spectral data predicted that pentadecapeptide PI(*wt*, Gly₂₁) contains ~50% helix and ~35% β structure, whereas PII(*tsO23*, Glu₂₁) appears to be predominantly a random coil (~85%) with some helical structure.

These studies tend to support a major conformational change in this region of the M protein undergoing Gly→Glu substitution at amino acid 21. It is wise to keep in mind, however, that CD spectra measure only the time-average conformation of these oligopeptides and may not necessarily reflect the conformation adopted by the same amino acid sequence present internally in the complete protein. The CD spectra do demonstrate, however, that a single amino acid substitution in an otherwise unrestrained short peptide sequence can have a profound effect on the allowed conformations and may well explain the observed alterations in antigenicity and function of both the oligopeptides and the native proteins.

DISCUSSION

When glutamate was present at position 21, M protein was no longer recognized by epitope 1-specific MAb2. As expected, the synthetic pentadecapeptide PI(*wt*, Gly₂₁) was recognized quite well by MAb2, but the corresponding glutamate-containing pentadecapeptide was not. This finding

is consistent with the idea that a glutamate substitution at position 21 induces a new conformation in both the synthetic pentadecapeptide and presumably the virion M protein. It was of interest that the polyclonal antibody raised against peptide PI(*wt*, Gly₂₁) recognized peptide PII(*tsO23*, Glu₂₁) to a limited extent. This finding is consistent with the presence of common antigenic determinants present on PI(*wt*, Gly₂₁) and PII(*tsO23*, Glu₂₁) that are unaffected by the amino acid substitution. The polyclonal antibody raised by immunization with pentadecapeptide PII(*tsO23*, Glu₂₁), on the other hand, did not recognize peptide PI(*wt*, Gly₂₁) at all, nor did it recognize *wt* M protein.

In a recent study on the theoretical effects of amino acid substitutions at position 21 of the M protein, it was proposed that substitution of a glutamate at position 21 should profoundly affect the least-energy conformation of a dodecapeptide corresponding to amino acid sequence Lys-15–Pro-26 of the M protein (2). The glycine at position 21 can adopt a conformation that leads to a stable β -bend-type structure, whereas the least-energy conformation of the Glu-21-containing dodecapeptide assumes either an unstable α -helix or a random coil. Interestingly, the Gly-21-containing dodecapeptide can adopt a conformation very similar to that of the Glu-21 dodecapeptide with little energy cost, whereas the most stable conformation of the Gly-containing dodecapeptide is disallowed when a glutamate is present at position 21 (2). Assuming that the structure of the two dodecapeptides is relatively dynamic, it is reasonable to assume that the Gly-containing peptide would spend some period of time in the higher-energy conformation that is similar to that of the least-energy conformation of the Glu-containing peptide. Thus, a polyclonal antibody to peptide PI(*wt*, Gly₂₁) might well contain immunoglobulin molecules that recognize peptide PII(*tsO23*, Glu₂₁). Since peptide PII(*tsO23*, Glu₂₁) apparently cannot adopt a conformation similar to that of peptide PI(*wt*, Gly₂₁), less anti-PII(*tsO23*, Glu₂₁) antibody would be expected to be available to recognize peptide PI(*wt*, Gly₂₁) than PII(*tsO23*, Glu₂₁). This could also explain why polyclonal antibody directed to peptide PI(*wt*, Gly₂₁) recognized VSV *tsO23* M protein. However, one would also expect from this reasoning that some of the polyclonal immunoglobulin raised against pentadecapeptide PII(*tsO23*, Glu₂₁) would recognize that portion of peptide PI(*wt*, Gly₂₁) in the higher-energy conformation. Since virtually no recognition was observed when polyclonal anti-PII(*tsO23*, Glu₂₁) was tested for binding to *wt* M protein, peptide PI(*wt*, Gly₂₁), or *tsO23* M protein, it may be that the least-energy conformation of peptide PII(*tsO23*, Glu₂₁) is not present in the *ts* M protein or in the glutaraldehyde cross-linked pentadecapeptide PII(*tsO23*, Glu₂₁)–BSA conjugate.

The pentadecapeptides were also compared by CD spectroscopy to see whether differences in conformation could be detected. As shown in Fig. 6, the peptides did assume quite different conformations in solution. The extent of secondary structure associated with each spectra was determined by using a computer algorithm. The extent to which secondary structure exists in oligopeptides has not been fully resolved, although examples of oligopeptides with structure have been reported (5, 20). The observations by CD spectropolarimetry that the two pentadecapeptides PI(*wt*, Gly₂₁) and PII(*tsO23*, Glu₂₁) assumed significantly different conformations under physiological conditions, along with the immunologic data, seem to indicate that a single Gly→Glu substitution in otherwise identical sequences produces a profound effect in the conformation adopted by the Ser-17–Thr-31 pentadecapeptide. Further physicochemical charac-

terization will be required, however, to determine the precise conformational changes that occur.

The fact that the *tsO23* M protein not only lost the ability to bind MAb2 but also was defective in its ability to inhibit viral transcription suggests that the conformational change associated with the Gly→Glu conversion also interferes with its recognition of transcribing RNP cores. This observation, coupled with the finding that antibody which bound to the amino-terminal domain of the *wt* M protein also reversed the inhibition of transcription, suggests that it is indeed the amino-terminal domain of the M protein that mediates the downregulation of viral transcription. This supposition was supported by the finding that the polyclonal antibody directed against pentadecapeptide PI(*wt*, Gly₂₁) not only recognized the *wt* M protein but also reversed its inhibition of transcription. It was of interest that when assayed directly for their ability to inhibit transcription, only peptide PIII(Met₁-Leu₂₀) showed inhibitory activity. Even though a much higher molar ratio of PIII(Met₁-Leu₂₀) to RNP than of M to RNP was required for the same level of inhibition, it is clear that the corresponding N-terminal region of the M protein must be involved in the transcriptional regulation process. The observation that polylysine also inhibited VSV transcription suggests that the polycationic nature of the amino terminus of M protein may be an important factor in its inhibition of transcription by interaction with the phosphate backbone of the viral genome.

Morita et al. (12) recently described a revertant of the VSV *ts* mutant *tsO23*, the M protein of which retains glutamic acid at position 21 but still recovers much of its ability to inhibit viral transcription at a high M/RNP ratio. The M protein of this revertant (*tsO23r1*) underwent a Phe→Leu substitution at amino acid position 111, reverting to the *wt* Leu-111 configuration. This finding is not inconsistent with the observations reported here, to the effect that the M protein region primarily responsible for transcription inhibition is the lysine-rich amino terminus. In a series of 25 spontaneous revertants of group III *ts* mutants, Morita et al. (12) reported mutations in these revertants ranging from amino acids 64 to 215, equivalent to 60% of the *wt* M protein. Although selected for loss of the *ts* phenotype, the M proteins of these revertants differed widely in their ability to inhibit transcription. The Gly-21→Glu-21 substitution in the *tsO23* M protein results in local perturbation in conformation, leading to loss in epitope 1, which may also alter the M protein site for recognition of the RNP transcription function. The Leu→Phe substitution at position 111 of the *tsO23* M protein may also serve to alter the conformation of the lysine-rich amino terminus. These discrepancies may be resolved by studies in progress on site-directed mutations of the M gene cloned in vaccinia virus expression vectors; these studies have already revealed that epitope 1 is dependent on glycine at amino acid 21 and that the *ts* phenotype of *tsO23* is caused by the Leu-111→Phe-111 substitution, as predicted by Morita et al. (12). However, all these experiments indicate that the *ts* phenotype of *tsO23* is unrelated to restriction in expression of epitope 1 or transcription inhibition.

Further studies will be required to delineate the exact mechanisms by which M protein inhibits viral transcription. That a single amino acid substitution can have a profound effect on biological function has been observed in other systems. For example, the P21 protein encoded by the *ras* oncogene is nontransforming when position 12 of the protein contains a glycine. Amino acid substitutions at this position induce a cell-transforming activity in the protein (1, 18).

Clearly, in the case of the M protein, the Gly→Glu substitution at position 21 has a profound effect on both immunogenic and transcription-regulatory properties of the M protein. The evidence presented here also suggests that these alterations in biological properties originate in an altered conformation brought about by a single amino acid substitution.

ACKNOWLEDGMENTS

We thank Jay Fox for assistance in peptide synthesis, CD spectroscopy, and useful discussions on the conformation of peptides. We also thank Ruth Snyder and Oneida Mason for assistance.

This research was supported by Public Health Service grants AI-11112 and AI-21652 from the National Institute of Allergy and Infectious Diseases and by grant MV-9 from the American Cancer Society.

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