Immunogenicity of an Aphthovirus Chimera of the Glycoprotein of Vesicular Stomatitis Virus

PABLO R. GRIGERA,^{1,2} MERCEDES GARCIA-BRIONES,¹ OSVALDO PERIOLO,¹ JOSE L. LA TORRE,¹
AND ROBERT R. WAGNER^{2*}

Centro de Virología Animal (CEVAN-CONICET), 1414 Capital Federal, Buenos Aires, Argentina,¹ and Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908²

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An oligodeoxynucleotide coding for amino acids 139 through 149 of antigenic site A (ASA) of the VP₁ capsid protein of the foot-and-mouth disease virus C3 serotype (FMDV C3) was inserted into three different in-frame sites of the vesicular stomatitis virus New Jersey serotype (VSV-NJ) glycoprotein (G) gene cDNA present in plasmid pKG97 under control of the bacteriophage T7 polymerase promoter. Transfection of these plasmids into CV1 cells coinfecting with the T7 polymerase-expressing vaccinia virus recombinant vTF1-6,2 resulted in expression of chimeric proteins efficiently reactive with both anti-FMDV and anti-VSV G antibodies. However, *in vitro* translation of transcripts of these VSV-G/FMDV-ASA chimeric plasmids resulted in proteins that were recognized by anti-G serum but not by anti-FMDV serum, indicating a requirement for *in vivo* conformation to expose the ASA antigenic determinant. Insertion of DNA coding for a dimer of the ASA unidecapeptide between the VSV-NJ G gene region coding for amino acids 160 and 161 gave rise to a chimeric ASA-dimer protein designated GF2d, which reacted twice as strongly with anti-FMDV antibody as did chimeric proteins in which the ASA monomer was inserted in the same position or two other G-gene positions. For even greater expression of chimeric VSV-G/FMDV-ASA proteins, plasmid pGF2d and a deletion mutant pΔGF2d (G protein deleted of 324 C-terminal amino acids) were inserted into baculovirus vectors expressing chimeric proteins GF2d-bac and ΔGF2d-bac produced in Sf9 insect cells. Mice vaccinated with three booster injections of 30 μg each of partially purified GF2d-bac protein responded by enzyme-linked immunosorbent assay with FMDV antibody titers of 1,000 units, and those injected with equivalent amounts of ΔGF2d-bac protein showed serum titers of up to 10,000 units. Particularly impressive were FMDV neutralizing antibody titers in serum of mice vaccinated with ΔGF2d-bac protein, which approached those in the sera of mice vaccinated with three 1-μg doses of native FMDV virions. Despite excellent reactivity with native FMDV, the anti-ΔGF2d-bac antibody present in vaccinated mouse serum showed no capacity to bind to sodium dodecyl sulfate (SDS)-denatured FMDV virions and only minimal reactivity with VP₁ protein by Western blotting (immunoblotting) after SDS-polyacrylamide gel electrophoresis. It was also shown in a competitive binding assay that a synthetic ASA unidecapeptide, up to concentrations of 200 μg/ml, was quite limited in its ability to inhibit binding of anti-ΔGF2d-bac antibody to native FMDV virions. These results suggest that the chimeric VSV-G/FMDV-ASA proteins mimic the capacity of FMDV to raise and react with neutralizing antibodies to a restricted number of ASA conformations present on the surface of native FMDV particles.

The glycoprotein (G) of vesicular stomatitis virus (VSV) is a potent immunogen that confers protection against infection with homologous virus in animals ranging from mice to cattle (12, 17, 24). Both B- and T-cell epitopes present on the VSV G protein are believed to play critical roles in promoting resistance to infection after convalescence from infection or vaccination with G protein (12). Although the crystal structure of the G protein remains to be determined, immunological and biochemical data have given indirect evidence on the structure of the B-cell epitopes and the topography of the protein. For example, binding sites of four independent neutralizing monoclonal antibodies (MAbs) have been mapped to a region between amino acids 193 and 289 of the G protein of the VSV New Jersey serotype (VSV-NJ), suggesting the existence of a major antigenic region exposed within the central segment of the 517-amino-acid protein (16). In addition, gel electrophoresis and amino acid sequence analyses after protease V8 exposure of purified VSV-NJ G protein led us to postulate the

existence of a second exposed loop region located upstream and flanking amino acid 110 (13). This latter region is also the location of the disulfide bond-dependent discontinuous epitope IV; unlike the more centrally located discontinuous epitopes between amino acids 193 to 289, epitope IV is not neutralizing and is shared by G proteins of both the Indiana and New Jersey serotypes (16). Burkhart et al. (6) have demonstrated the existence of at least three helper T-cell epitopes in the sequence of the VSV G protein (Indiana serotype) that are believed to be critical for the expansion and prolongation of the anti-G-specific B-cell response in mice (19). The excellent humoral response induced by isolated G protein in cattle suggests, in addition, that helper T cells play a critical role in the production of neutralizing antibody in these animals (24).

The mapping of two putative exposed areas on the surface of the VSV-NJ G protein, as well as the likely presence of T-cell epitopes within certain sequences, prompted us to investigate whether the G protein would be a suitable immunogenic carrier for a short heterologous amino acid sequence inserted into a G-protein chimera. An 11-mer oligopeptide containing the sequence from amino acids 139 through 149 of the VP₁ capsid protein of foot-and-mouth disease virus (FMDV; C3 serotype) was chosen for this purpose. This peptide represents the pri-

* Corresponding author. Mailing address: Department of Microbiology, School of Medicine, Box 441, University of Virginia, Charlottesville, VA 22908. Phone: (804) 924-1948. Fax: (804) 982-1071. Electronic mail address: rrw@virginia.edu.

mary amino acid sequence of antigenic site A (ASA) located at the G-H loop of the VP₁ protein, which is exposed, according to crystallographic data, on the surface of the FMDV capsid (19, 28). This sequence contains an arginine-glycine-aspartic acid (RGD) motif, highly conserved among the different FMDV serotypes; the RGD motif is presumably involved in the integrin-mediated attachment of FMDV virions to the cellular membrane and has been postulated to be a main target of neutralizing antibodies arising in the infected host (2, 10, 26). As in the case of VSV-induced immunity, neutralizing antibodies play a key role in the highly complex mechanism of protection against FMDV challenge of animals following vaccination or recovery from infection (30). The ASA sequence in synthetic peptides apparently fails to mimic the ASA conformation present in the virion and seems to lack appropriate T-helper cell epitopes, both properties necessary for induction of an efficient humoral response protecting against FMDV infection (31, 34).

Construction of recombinant proteins presenting the relevant target sequence in the correct configuration and aligned with appropriate T-helper epitopes has become critical for vaccine design (9a) and therefore seems a proper approach for improving the performance of the ASA sequence as an effective anti-FMDV immunogen (11). We report here the *in vitro* and *in vivo* expression of chimeric polypeptides by recombinant VSV-G/FMDV-ASA vectors as well as the analysis of their reactivities with anti-FMDV specific antibodies. Also evaluated is the capacity of these VSV-G/FMDV-ASA chimeras to induce in mice the production of antibodies that neutralize the infectivity of FMDV.

MATERIALS AND METHODS

Cells and viruses. Growth of CV1 cells in monolayer cultures and production of the vaccinia virus recombinant expressing the bacteriophage T7 polymerase (vTF1-6.2; a gift of Bernard Moss) have been described previously (13). Native FMDV used as an antigen for the enzyme-linked immunosorbent assay (ELISA) and neutralization studies was obtained by infecting BHK-21 cell monolayers with the FMDV C3 serotype as described by Denoya et al. (7). Conditions for infection, cloning, and harvesting of recombinant baculovirus grown in *Spodoptera frugiperda* (Sf9) cells were carried out as described by O'Reilly et al. (35). The VSV-NJ serotype (Ogden strain) propagated in BHK-21 cells was isolated and purified as previously described (14). Baculovirus *Autographa californica* was grown in Sf9 insect cells at 29°C.

Polyclonal and monoclonal antibodies. Anti-FMDV serum was prepared by immunizing rabbits with 50 µg of purified C3 serotype FMDV virions resuspended in Freund's complete adjuvant followed by boosters with the same amount of antigen resuspended in Freund's incomplete adjuvant at 15 and 30 days after the initial inoculation. A similar protocol using purified VSV-NJ glycoprotein was carried out to obtain the anti-G polyclonal serum. In both cases, specific antibody activity in sera collected at day 45 was monitored by ELISA and by Western blot (immunoblot) analysis. Preparation and characterization of MA9, directed to epitope IV of the VSV-NJ G protein, have been described in detail elsewhere (5, 13).

Chimeric plasmid constructs. For insertion into three unique sites of the VSV-NJ G gene, three pairs of partially complementary synthetic oligonucleotides were prepared, each with specific restriction sites and coding for the undecapeptide ARRGLAHLAT of the ASA of the VP₁ protein of the FMDV C3 Resende serotype (25). Each oligonucleotide pair was independently annealed, extended with Klenow polymerase, treated with the appropriate restriction enzymes, and cloned separately into the corresponding restriction enzyme sites on the G gene present in plasmid pKG97. Plasmid pKG97 resulted from the cloning of the VSV-NJ G coding sequence of plasmid pWK9 (16) into the cap-independent plasmid pTM1 flanked by the T7 polymerase promoter and terminator sequences (32) (kindly supplied by Bernard Moss). The sequences of oligonucleotides 1 to 6 (restriction site sequences underlined) are as follows:

- 1, 3'-TACCAGCGCAGCCGCGGTGATCTGGCTCATCTAGCTA-5'
- 2, 3'-GCATTGTTAAGCATGTGACAGTAGCTAGATGAGCC-5'
- 3, 3'-TGGATCGATGCTCGCCGCGGTGATCTGGC-5'
- 4, 3'-ACCATCGATCAGTAGAGCCAGATCAGCCGCG-5'
- 5, 3'-AGTGTCTGCACTGCAGCCGCGGTGATCTGGCTCATCTAGCTA-5'
- 6, 3'-TAACCATGGACGCATGTGACAGTAGCTAGATGAGCC-5'

The double-stranded DNA product that resulted from the Klenow polymerase extension of the partially annealed paired oligonucleotides 1 and 2 was incubated in the presence of *Hin*PI and *Hpa*I and extracted, and the blunt ends were ligated

into the *Hin*PI-*Hpa*I-treated plasmid pKG97 to give plasmid pGF1. This VSV-G/FMDV-ASA chimeric plasmid was used to transform *Escherichia coli* DH5α (Bethesda Research Laboratories) competent cells; screening for production of the desired constructs was carried out by *Sac*II digestion of miniprep DNAs from 1-ml cultures of picked colonies. The *Sac*II restriction site (CCGCGG) is exclusively present in the inserted ASA coding sequence. Primer extension sequencing verified the presence of the FMDV ASA sequence, herein designated position 1, replacing a G-gene coding tract containing codons from amino acids 44 to 59 and in frame with the rest of the G-gene coding sequence.

The same strategy was followed to insert the FMDV ASA coding sequence into two downstream G-gene sites designated position 2 (between G-gene codons 160 and 161), to give plasmid pGF2, or by the ASA gene replacing the G-gene coding DNA tract for amino acids 194 through 212 at designated position 3 (plasmid pGF3). In the case of pGF2, the Klenow enzyme-extended pair of annealed oligonucleotides 3 and 4 was treated with *Cla*I and ligated to *Cla*I-treated-plasmid pWK9 purified from Dam⁻ *E. coli* GM33. After transformation and analysis of pGF2 clones, we select two plasmids (pXIII and pXIV) containing one and two copies, respectively, of the ASA gene insert in frame with the rest of the G-gene coding sequence. The monomeric and dimeric VSV-G/FMDV-ASA chimeric sequences were then independently moved into pTM1 plasmids designated pGF2m for the ASA monomer and pGF2d for the ASA dimer. For construction of pGF3, use of *Pst*I and *Nco*I restriction enzymes to insert the ASA sequences downstream in the G gene was followed by cloning steps similar to those described for constructing pGF1. As can be deduced from the oligonucleotide sequence, the FMDV ASA replacements at positions 1 and 3 have three extra amino acids, AHA at residues 150 to 152 of the VP₁ sequence that is not present in position 2.

Transient expression in vTF1-6.2-infected CV1 cells. Experimental conditions for expression of the chimeric plasmids in CV1 cells were identical to those already described (13). Briefly, monolayers of CV1 cells (35-mm-diameter plates) were infected with recombinant vaccinia virus vTF1-6.2 and lipotransfected with 10 µg of the appropriate plasmid for 3 h. The cells were further incubated for another 6 h in the presence of medium containing 10% fetal calf serum and then labeled with [³⁵S]methionine (150 µCi/ml) for 1 h in methionine-free medium. After the labeling period, cells were washed with phosphate-buffered saline (PBS), lysed in radioimmunoprecipitation assay buffer, and processed for immunoprecipitation as described previously (13).

Production of baculovirus VSV-G/FMDV-ASA recombinants and their expression in Sf9 cells. An *Eag*I-*Eco*RI fragment was excised from the VSV-G/FMDV-ASA coding region of the vaccinia vector pGF2d and ligated into compatible restriction enzyme sites of baculovirus vector pAcSG2 (PharMingen) to construct plasmid pGF2d-bac. A baculovirus recombinant (designated vGF2d-bac) was obtained by cotransfection of Sf9 cells with linearized baculovirus DNA and pGF2d-bac assisted by Lipofectin (Bethesda Research Laboratories). Baculovirus recombinants that produce plaques on Sf9 cell monolayers were picked and tested in 24-well culture plates for the capacity to synthesize in Sf9 cells chimeric VSV-G/FMDV-ASA protein as determined by reactivity with polyclonal anti-VSV/G serum in immunodot blot and Western blot assays. A deletion mutant (designated ΔGF2d-bac) was also created by *Pst*I treatment of plasmid pGF2d-bac, which removed the G-gene nucleotides coding for the COOH-terminal 324 amino acids, leaving intact the coding region for the NH₂-terminal amino acids of the VSV G gene and the FMDV ASA sequence between amino acids 160 and 161; fused at the COOH terminus were 26 additional amino acids coded by the baculovirus transfer vector pAcSG₂. This plasmid, designated ΔGF2d-bac, cotransfected into Sf9 cells with linearized baculovirus DNA gave rise to baculovirus recombinant ΔGF2d-bac, which also expressed a protein that reacted strongly with anti-VSV G and anti-FMDV antibodies.

Chimeric proteins GF2d-bac and ΔGF2d-bac were prepared by infecting spinner cultures of Sf9 cells with vGF2d-bac and ΔGF2d-bac at multiplicities of 10 to 20 PFU per cell at 29°C. Cells harvested at 5 days postinfection were pelleted by low-speed centrifugation and lysed by treatment for 5 min at room temperature with SF buffer (50 mM Tris [pH 7.4], 100 mM NaCl, 10% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride). Cell extracts were centrifuged at 2,000 rpm for 10 min at 4°C; the majority of the recombinant proteins was present in the insoluble pellet, which was resuspended in 1 volume of PBS and then in 2 volumes of 3% sodium dodecyl sulfate (SDS) for 10 min at 100°C with vortexing. After clarification for 10 min in an Eppendorf Microfuge, the cell extracts were lyophilized twice, resuspended, and stored in PBS buffer in preparation for inoculation of mice. The GF2d-bac and ΔGF2d-bac proteins were quantitated by Western blot and digital scanning densitometry analysis using a known amount of VSV-NJ G protein as a standard. Recovery of ΔGF2d-bac was consistently at least twice that of GF2d-bac. As judged by scanning densitometry, the concentration of ΔGF2d-bac in Sf9-infected cells was 2 to 5% of total cellular protein; similar estimates were difficult for quantitation of GF2d-bac because of large amounts of 55- to 60-kDa cellular proteins. Chimeric proteins were purified by elution from anti-VSV G antibody-Sepharose columns.

ELISA. Anti-FMDV rabbit serum was used as capture antibody in a 1/5,000 dilution in carbonate fixing buffer; this procedure was followed by washing with PBS, blocking with bovine serum albumin (BSA)-containing buffer, and incubation with appropriate dilutions of FMDV whole virions as the source of native viral antigen. Dilutions of FMDV-immune and control mouse sera, as well as test sera, were then added; the mixtures were incubated overnight at 4°C and washed,

and then anti-mouse immunoglobulin-peroxidase conjugates (Jackson ImmunoResearch) were added in 1/5,000 dilution. The chromogen was 2,2'-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) (36).

Vaccination of mice. Mice 2 months of age were injected intraperitoneally (i.p.) with 10 or 30 μ g of either GF2d-bac or Δ GF2d-bac protein (or with variable amounts of synthetic peptides), each mixed with Freund's complete adjuvant. Booster injections were given i.p. at days 10 and 20 with the same doses of antigen emulsified in Freund's incomplete adjuvant. Mice were bled at day 35, and anti-FMDV titers measured by ELISA. The titration of neutralizing antibody present in serum of vaccinated mice was performed by plaque reduction assays of FMDV plated on BHK cell monolayers as described by Bricker et al. (5). Staining of the cell monolayer with crystal violet was carried out 2 days after FMDV infection. Sera from mice inoculated with 3 doses of 1 μ g of FMDV were used as positive controls, and sera of mice injected with adjuvant alone served as negative controls.

In vitro expression of chimeric polypeptides. Purified plasmids pKG97, pGF1, pGF2m, pGF2d, and pGF3 were independently transcribed by T7 polymerase and translated in the presence of [35 S]methionine, using the TnT coupled reticulocyte lysate system (Promega) in the presence or absence of a canine pancreatic microsomal membrane preparation. Aliquots of the lysate (undiluted or diluted 1:10) were analyzed by electrophoresis on 15% polyacrylamide minigels and exposed to X-ray films for the appropriate time after fluorography with Resolution mix.

RESULTS

In vitro expression of VSV-NJ G-gene chimeras coding for the FMDV ASA sequence. As described in Materials and Methods, the oligonucleotide sequence coding for the antigenic site A FMDV unidcapeptide was inserted into three separate in-frame sites of the VSV-NJ G-gene plasmid pKG97. These chimeric plasmids were expressed in vitro, using the coupled transcription-translation TnT expression system in the presence of [35 S]methionine and in the presence or absence of canine pancreatic microsomal membrane. The radiolabeled in vitro translation products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

Figure 1 shows stick models of the FMDV ASA sequence inserted into three positions at three unique restriction enzyme sites of pKG97 as well as the SDS-PAGE autoradiographs of the coupled transcription-translation products. The FMDV-ASA unidcapeptide cDNA is inserted into position 1 at the NH₂ terminus between G-gene codons 43 to 60 to form the chimeric plasmid pGF1, between amino acid codons 160 and 161 to form the chimeric plasmid pGF2, and between amino acid codons 193 and 213 in the central region of the G gene to form chimeric plasmid pGF3 (Fig. 1A). In the case of position 2 at the *Clal* site of the VSV-NJ G gene, we inserted both a monomer of the FMDV ASA unidcapeptide designated pGF2m or a tail-to-head dimer of the ASA unidcapeptide to form a chimera designated pGF2d. It should be noted that position 2 of the VSV-NJ G protein is located within a sequence thought to form an exposed loop created by a disulfide bond probably anchored by cysteine residues 108 and 169 of the VSV-NJ G protein (13).

Figure 1B shows the electropherograms of 35 S-labeled proteins synthesized in vitro by the control G-protein-expressing plasmid pKG97 (lane 1) and the four chimeric VSV-G/FMDV-ASA proteins transcribed by phage T7 polymerase and translated on reticulocyte ribosomes in the absence of canine pancreatic microsomal membrane. As noted, each of the chimeric VSV-G/FMDV-ASA proteins exhibited electrophoretic mobility very similar to that of the wild-type G protein except for the G/ASA-dimer product expressed by pGF2d, the mobility of which was slightly retarded (lane 3).

Figure 1C shows the difference in the electrophoretic mobilities of in vitro-transcribed and -translated VSV-G/FMDV-ASA chimeric and control G proteins expressed by pKG97, pGF2d, and pGF3 in the presence or absence of canine pancreatic microsomal membrane. Clearly, the control and chi-

FIG. 1. Stick models of the coding sequence for ASA of the FMDV VP₁ protein inserted in-frame in three sites of the VSV G-gene expression vector pKG97 (A), SDS-PAGE electropherograms of chimeric proteins synthesized in vitro by coupled transcription-translation (B), and comparative SDS-PAGE migration of chimeric proteins synthesized in vitro in the presence or absence of canine pancreatic microsomal membranes (C). Double-stranded DNA with nucleotide sequences coding for the 11 amino acids of ASA (black boxes) were inserted into the restriction enzyme sites shown for the VSV-NJ G-gene plasmid pKG97 with a bacteriophage T7 polymerase promoter signal (T7p) to create plasmids pGF1, pGF2m, and pGF3 with the ASA monomer inserted into three distinct positions (1, 2, and 3) of the G gene and pGF2d with the ASA dimer inserted at the *Clal* site between G-gene codons 160 and 161 (position 2), as was the case for the ASA monomer in pGF2m. Oligonucleotide fragments excised from pKG97 from codons 45 to 60 by *HinfI-HpaI* and from codons 193 to 210 by *PstI-NcoI* were replaced by the oligonucleotide sequence coding for ASA to give rise to plasmids pGF1 and pGF3, respectively. Plasmids pKG97, pGF1, pGF2m, pGF2d, and pGF3 were transcribed by phage T7 polymerase and translated in a TnT reticulocyte cell-free translation system for 60 min at 30°C in the presence of [35 S]methionine (200 μ Ci/ml). Acetone-precipitated aliquots were dried, resuspended in PAGE sample buffer, subjected to electrophoresis on SDS-15% polyacrylamide minigels, and autoradiographed (lanes 1 to 5 in panel B illustrate the translational products of pKG97, pGF1, pGF2d, pGF2m and pGF3, respectively). Panel C shows the autoradiographs after SDS-PAGE of the [35 S]methionine-labeled products of in vitro-transcribed/translated plasmids pKG97, pGF2d, and pGF3 in the absence (-) or presence (+) of canine pancreatic microsomal membranes (cpmm).

meric G proteins migrate more slowly by SDS-PAGE when they are glycosylated during in vitro translation in the presence of canine pancreatic microsomal membranes.

Immunoprecipitability of in vitro- and in vivo-synthesized VSV-G/FMDV-ASA chimeras by antibodies directed to VSV G and FMDV proteins. It was of interest to determine whether the in vitro-synthesized glycosylated VSV-G/FMDV-ASA chimeras could assume the correct conformation that would allow their antigenic determinants to be recognized by anti-G and anti-FMDV antibodies, or whether in vivo synthesis is essential for correct conformation and antigenic recognition. To this end, VSV-G/FMDV-ASA chimeric proteins were synthesized and labeled with [35 S]methionine in the coupled transcription-translation TnT system in the presence of canine pancreatic microsomal membrane, or in [35 S]methionine-labeled CV1 cells infected with vTF1-6,2 and transfected with plasmid pWK97, pGF1, pGF2m, pGFd, or pGF3. The chimeric pro-

FIG. 2. Comparative immunoprecipitability by polyclonal anti-VSV-G and anti-FMDV sera and by MAb 9 of plasmid-expressed VSV-NJ G protein and VSV-G/FMDV-ASA chimeric proteins synthesized in vitro (A) or in vivo (B and C) and analyzed by SDS-PAGE. As shown in panel A, coupled T7 polymerase transcription and reticulocyte lysate translation of pKG97 (lanes 1 and 6), pGF1 (lanes 2 and 7), pGF2m (lanes 3 and 8), pGF2d (lanes 4 and 9), and pGF3 (lanes 5 and 10) were carried out in a cell-free microsome-containing TnT system in the presence of [35 S]methionine and extracted in radioimmunoprecipitation assay buffer (100 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.1% sodium deoxycholate, 50 mM Tris [pH 8.0]). After clarification by low-speed centrifugation, the 35 S-labeled proteins were immunoprecipitated by either anti-G serum or anti-FMDV serum, each diluted 1:200, and analyzed on SDS-15% polyacrylamide slab gels and autoradiography. For in vivo synthesis of VSV G or chimeric VSV-G/FMDV-ASA proteins, CV1 cell monolayers were infected with the T7 phage polymerase-synthesizing vaccinia virus recombinant vTF1-6.2, transfected with the G protein- or VSV-G/FMDV-ASA protein-expressing plasmids for 10 h at 37°C, and pulsed for 1 h with [35 S]methionine (150 μ Ci/ml). The CV1 cells were then washed, suspended in radioimmunoprecipitation assay buffer, and clarified by centrifugation for 5 min in a Microfuge. The 35 S-labeled proteins in these clarified lysates were then immunoprecipitated as described above with either anti-G or anti-FMDV polyclonal serum or by MAb 9 directed to G-protein epitope IV. Panel B shows autoradiographs of VSV-G or VSV-G/FMDV-ASA proteins precipitated by anti-G or anti-FMDV serum after expression by pKG97 (lanes 1 and 6), pGF1 (lanes 2 and 8), pGF2m (lanes 3 and 9), pGF2d (lanes 4 and 10), pGF3 (lanes 5 and 11), and mutant pGF3 (Δ gly₁₄₃) with glycine deleted from the RGD motif of ASA (lanes 6 and 12). Panel C shows SDS-PAGE autoradiographs of 35 S-labeled proteins synthesized in CV1 cells transfected with pKG97 (lanes 1 and 6), pGF1 (lanes 2 and 7), pGF2m (lanes 3 and 8), pGF3d (lanes 4 and 9), and pGF3 (lanes 5 and 10) and immunoprecipitated with either anti-FMDV serum or MAb 9 (epitope [Ep] IV of G protein).

teins synthesized in vitro or in vivo were tested for the capacity to be immunoprecipitated by rabbit polyclonal anti-G serum, by anti-G MAb 9 directed to G-protein epitope IV, or by polyclonal anti-FMDV rabbit serum strongly reactive with the FMDV VP₁ protein and its ASA sequence (37). The 35 S-labeled immunoprecipitates were identified and analyzed by SDS-PAGE and autoradiography.

Figure 2 shows SDS-PAGE autoradiographs comparing the immunoprecipitabilities of in vitro- and in vivo-glycosylated VSV-G/FMDV-ASA chimeric proteins. As noted in Fig. 2A, polyclonal anti-VSV G serum efficiently immunoprecipitated all the in vitro-glycosylated chimeric proteins synthesized on templates pGF1, pGF2m, pGF2d, and pGF3 (lanes 2 to 5); the

wt VSV-NJ G protein synthesized by pKG97 was also readily precipitated by polyclonal anti-G serum (lane 1). In sharp contrast to their recognition by polyclonal anti-G serum, none of the four in vitro-glycosylated VSV-G/FMDV-ASA chimeric proteins was immunoprecipitated by the hyperimmune anti-FMDV polyclonal serum (lanes 7 to 10).

Apparently, glycosylation alone is not sufficient to provide the conformation required to expose the ASA sequence to anti-FMDV antibody. We had previously shown that chaperones or other posttranslational factors may be required for correct disulfide-bonded conformation of the VSV-NJ G protein, which can present conformational epitopes even when the G protein is not glycosylated in tunicamycin-treated cells (14, 29). Since sequencing data certified the in-frame insertion of the FMDV ASA coding sequences in all four VSV-G/FMDV-ASA chimeric proteins, this result strongly suggests the existence of conformational restrictions preventing the exposure of the ASA epitope(s) on the surface of the chimeric polypeptides. The failure of the in vitro-synthesized chimeras to recognize anti-FMDV serum is probably due to either the complete absence or the aberrant formation of disulfide bonds under these experimental conditions. Either deficiency is highly likely to influence the way the chimeric polypeptides fold in the absence of endoplasmic reticulum, thus leading to conformations that preclude exposure of the ASA sequence and its binding to specific antibodies. It is worth noting in this regard that the in vitro product of the control G-gene plasmid pKG97 was not immunoprecipitated by MAb 9 directed to disulfide bond-dependent discontinuous epitope IV present in this region of the native glycoprotein (data not shown). This prompted us to determine whether expression of the VSV-G/FMDV-ASA plasmids in vivo would result in presentation of antigenic determinants as is the case for posttranslational modification required for proper folding of the VSV-NJ G protein (13, 29); proper folding of G protein occurs as it migrates through cellular endoplasmic reticulum and Golgi compartments of the infected cell (23).

Figure 2B shows that vTF1-6.2-infected CV1 cells transfected with pKG97 and the plasmids expressing chimeric VSV-G/FMDV-ASA proteins all synthesize a product immunoprecipitated by polyclonal anti-VSV G serum that migrates by SDS-PAGE like native VSV-NJ G protein, as did the chimeric protein expressed by pGF3(Δ gly₁₄₃) with a glycine residue in ASA deleted at amino acid 143 (lane 6). All the chimeric VSV-G/FMDV-ASA proteins expressed by plasmids pGF1, pGF2m, pGF2d, and pGF3 were readily immunoprecipitated by polyclonal anti-FMDV serum (lanes 8 to 11). As expected, the wt G protein expressed by pKG97 was not immunoprecipitated by anti-FMDV serum, nor was the chimeric protein expressed by pGF3(Δ gly₁₄₃) with a glycine deleted from the RGD motif of the ASA sequence (lane 12). These results confirm the requirement for proper folding of the chimeric VSV-G/FMDV-ASA protein in the endoplasmic reticulum-Golgi complex to assume the correct conformation to be recognizable by anti-FMDV serum; conversely, the failure of anti-FMDV antibody to recognize the ASA undecapeptide in the in vitro-synthesized VSV-G/FMDV-ASA chimeric protein (Fig. 2A) is probably due to failure of proper conformational folding. Laser density scanning of the gel lanes shown in Fig. 2B demonstrated that chimeric protein GF2d (dimeric ASA insert) has almost twice the anti-FMDV binding activity of the monomeric ASA GF2m (data not shown), suggesting that all of the duplicated antigenic sites are exposed and contribute equivalently to the overall GF2d reactivity with FMDV-specific antibodies. These experiments were performed with excess

antigen as determined by residual GF2m and GF2d in supernatant fluids after anti-FMDV immunoprecipitation.

Epitope IV of the VSV-NJ G protein is probably located in a disulfide-bonded region between cysteine residues 108 and 169 (13). Therefore, insertion of the FMDV ASA sequence at the *Cla*I site between amino acids 160 and 161 (position 2 of G protein; Fig. 1A) might be expected to result in loss of epitope IV. As shown in Fig. 2C, MAb 9, directed to epitope IV, failed to immunoprecipitate chimeric proteins synthesized by plasmids pGF2m and pGF2d transfecting CV1 cells (lanes 8 and 9). In contrast, chimeric proteins synthesized in CV1 cells transfected by pGF1 and pGF3, as well as that synthesized by pKG97, were readily precipitated by MAb 9 (lanes 6, 7, and 10). These data indicate that insertion of the small unidecapeptide ASA into the nonlinear epitope IV results in loss of this antigenic determinant despite the fact that the inserted ASA peptides are readily recognized by anti-FMDV serum (lanes 3 and 4). As alternative explanations, the ASA sequence inserted at position 2 of the VSV-NJ G protein could directly interfere with binding of MAb 9 without altering conformation, or the inserted ASA could operate at long range by disrupting loop conformation.

Baculovirus vectors constructed to express chimeric VSV-G/FMDV-ASA proteins. A vector providing more efficient expression was required to raise sufficient antigen to immunize mice with chimeric VSV-G/FMDV-ASA proteins. For this purpose, we chose the baculovirus vector. Crystallographic studies (1) provide evidence for coexistence within the ASA unidecapeptide of both linear and conformational epitopes highly dependent on spatial distribution of their component sequences (27). Such studies are highly relevant to the question of whether conformation-dependent epitopes present in the ASA loop of FMDV protein VP₁ are essential for the B-cell response required for induction of antibodies that neutralize the infectivity of FMDV.

To create the baculovirus vector, we used the vaccinia virus-based chimeric plasmid pGF2d, which expresses the FMDV ASA dimer, because in CV1 mammalian cells it appears to produce chimeric protein twice as reactive with FMDV antibody as the vectors containing the ASA monomer (Fig. 2). Figure 3 shows stick models of plasmids capable of expressing chimeric VSV-G/FMDV-ASA proteins when inserted into baculovirus DNA (Fig. 3A) and electropherograms of the immunoreactive proteins after expression by baculovirus vectors (Fig. 3B). As described in Materials and Methods, the coding region of the vaccinia virus-based plasmid pGF2d was moved into the baculovirus plasmid pAcSG2 to create the chimeric VSV-G/FMDV-ASA plasmid pGF2d-bac shown in Fig. 3A. This plasmid was recombined with linearized baculovirus DNA by cotransfection of Sf9 cells to create the recombinant baculovirus vGF2d-bac expressing chimeric protein G/ASA dimer GF2d-bac. A second plasmid, designated pΔGF2d-bac (Fig. 3A), which contained the coding region for the first 213 amino acids of GF2d-bac, was created by truncating the chimera with the restriction enzyme *Pst*I. This truncated plasmid pΔGF2d-bac was also cotransfected into Sf9 cells with linearized baculovirus DNA to form the recombinant baculovirus vΔGF2d-bac.

Figure 3B shows Western blot analyses of chimeric proteins present in lysates of Sf9 cells infected with recombinant baculovirus vGF2d-bac or vΔGF2d-bac reacted on nitrocellulose filters with polyclonal anti-G serum or polyclonal anti-FMDV serum. As noted, anti-VSV G antibody reacted strongly with both the truncated chimeric protein ΔGF2d-bac (lane 2) and the complete chimeric protein GF2d-bac (lane 3) as well as the native G protein (lane 1) but not with FMDV-infected cell

FIG. 3. Baculovirus vectors pGF2d-bac and deletion mutant pΔGF2d-bac showing stick models of the FMDV ASA (black boxes) insertion site (A) and Western blots (B) with anti-VSV-G or anti-FMDV polyclonal antibody reactive with purified VSV-NJ G protein (lanes 1 and 5), protein synthesized in vΔGF2d-bac-infected Sf9 cells (lanes 2 and 6), protein synthesized in Sf9 cells infected with vGF2d-bac (lanes 3 and 7), and proteins synthesized in BHK-21 cells infected with FMDV (lanes 4 and 8). Shown in the stick models are the polyhedrin promoter (PHp), the *Cla*I site for insertion of ASA in position 2 of the VSV-NJ G gene, and the *Pst*I site for truncation of the G protein by deleting C-terminal amino acids beyond amino acid 193. Sf9 cells in spinner cultures were infected at a multiplicity of 10 PFU per cell with vGF2d-bac or vΔGF2d-bac and, as described in Materials and Methods, incubated for 5 days at 29°C. Cell extracts were boiled and subjected to electrophoresis in SDS-15% polyacrylamide minigels, blotted onto nitrocellulose paper, and exposed either to anti-G or anti-FMDV polyclonal antibody. The signal was developed by a chemiluminescence protocol and exposed to X-ray film for the requisite period of time.

lysates (lane 4). Similarly, the anti-FMDV serum reacted strongly with the truncated and the complete chimeric proteins, ΔGF2d-bac and GF2d-bac (lanes 6 and 7, respectively), as well as the VP₀ and VP₁₋₃ proteins of FMDV (lane 8) but not the VSV-NJ G protein (lane 5). The rapid migration of ΔGF2d-bac protein shown in lanes 2 and 6 is consistent with the extensive deletion of the carboxyl terminus of the G protein in this chimera, compared with the similar gel migration of the intact chimeric protein GF2d-bac and *wt* G protein. Despite loss of its transmembrane anchor, no evidence was found for secretion of ΔGF2d-bac from transfected Sf9 cells (data not shown).

Immunogenicity of the VSV-G/FMDV-ASA baculovirus chimeric proteins. To test the immunogenicity of GF2d-bac and ΔGF2d-bac proteins synthesized in Sf9 cells, groups of three mice were injected i.p. with either 10 or 30 μg of the partially purified chimeric proteins in complete Freund's adjuvant and boosted by i.p. injections with the same amounts of each chimeric protein in incomplete Freund's adjuvant 10 and 20 days after the initial injection. As positive controls, other mice were injected on the same regimen with three doses of 1 μg each of inactivated FMDV virions. Sera obtained 35 days after the final injection of antigen were tested for specific anti-FMDV activity in an ELISA using captured whole inactivated FMDV C3,Arg₈₅ as the source of antigen, as described in Materials and Methods. Sera from mock-infected mice and anti-VSV G sera were used as controls.

Figure 4 illustrates the complete ELISA optical density readings (Fig. 4A) and compares the average anti-FMDV titers (Fig. 4B) in the sera of three mice injected with GF2d-bac

FIG. 4. Comparative anti-FMDV ELISA titers in serum of mice serially inoculated with 30- μ g doses of GF2d-bac protein or Δ GF2d-bac protein or 1- μ g doses of FMDV virions (A) and average reciprocal ELISA titers in serum of mice injected serially with 10 or 30 μ g of GF2d-bac protein, 10 or 30 μ g of Δ GF2d-bac protein, or 1 μ g of FMDV virions (B). Mice injected i.p. with either of the two chimeric VSV-G/FMDV-ASA proteins or FMDV virions in complete Freund's adjuvant were boosted by i.p. injection of the same doses of each antigen in incomplete Freund's adjuvant 10 and 20 days later. Sera collected 35 days after the initial injection were tested by serial dilution for the ability to bind excess amounts of native FMDV virions captured in microtiter wells. ELISA optical density (O.D.) titers shown in panel A are values averaged for groups of three mice corrected by subtracting background values of sera from control mice mock injected in parallel. The bar graphs shown in panel B represent the average log₁₀ ELISA titers in sera of mice inoculated with various amounts of chimeric proteins or FMDV antigens.

protein, Δ GF2d-bac protein, or inactivated FMDV. Sera of mice inoculated with 30- μ g doses of the chimeric protein GF2d-bac exhibited anti-FMDV titers of 2.5 log₁₀ ELISA units, a response clearly inferior to that elicited by inoculation with FMDV virions but somewhat greater than that present in the sera of mice inoculated with the ASA synthetic unidecapeptide at a dose about 40 times greater than that of ASA present in 30 μ g of GF2d-bac protein (data not shown). Injections of mice with 10- μ g doses of GF2d-bac or Δ GF2d-bac proteins elicited anti-FMDV responses significantly lower than that elicited by 30 μ g of the same proteins (Fig. 4B).

Quite remarkably, the deletion mutant chimeric protein Δ GF2d-bac elicited a much higher serum titer of anti-FMDV antibody than did the complete chimeric protein GF2d-bac

(Fig. 4). Anti-FMDV titers in response to inoculation with 30 μ g of Δ GF2d-bac protein were about 4 log₁₀ ELISA units, a titer only 10-fold less than that elicited by FMDV virions. Titers in the sera of mice inoculated with 10 μ g of Δ GF2d-bac protein were roughly a log lower than that in mice inoculated with 30 μ g doses of Δ GF2d-bac protein. The 2.5-fold greater concentration of ASA peptide in Δ GF2d-bac protein than in GF2d-bac protein does not account for the much greater efficiency of Δ GF2d-bac protein in eliciting anti-FMDV antibody. The additional fact that anti-FMDV titers induced by 30- μ g doses of GF2d-bac were consistently lower than those induced by 10 μ g of Δ GF2d-bac suggests that the C-terminal amino acid sequence present in GF2d-bac protein exerts a negative effect on its capacity to elicit anti-FMDV antibodies, at least under the experimental conditions used. Alternatively, better recognition of helper T cells or B-cell receptors could explain the augmented antigenicity of Δ GF2d-bac.

Comparative capacity of ASA 11 to reverse the FMDV reactivity with anti- Δ GF2d-bac serum and anti-ASA serum. To further characterize the nature of the anti-FMDV antibody induced by chimeric VSV-G/FMDV-ASA protein, we tested the capacity of the synthetic ASA unidecapeptide (ASA 11) and its RGD→KAE mutant to compete with anti- Δ GF2d-bac and anti-ASA 11 sera for the ability to react by ELISA with FMDV virion antigen. Concentrations of synthetic ASA 11 oligopeptide ranging from 0 to 200 μ g/ml, or 200 μ g of its RGD→KAE mutant unidecapeptide per ml, were incubated at room temperature for 30 min with anti- Δ GF2d-bac serum or anti-ASA serum prior to ELISA titration of each antibody to react with captured FMDV antigen.

Figure 5 shows that the *wt* ASA 11 synthetic unidecapeptide had limited capacity to inhibit the FMDV reactivity of anti- Δ GF2d-bac serum even at a concentration of 200 μ g/ml and that the mutant ASA unidecapeptide had a minimal effect at a concentration of 200 μ g/ml. In contrast, 50 μ g of the ASA 11 *wt* unidecapeptide per ml completely reversed the capacity of anti-ASA serum to react by ELISA with FMDV antigen, whereas the mutant (RGD→KAE) ASA unidecapeptide exhibited little or no effect on the FMDV-reactivity of anti-ASA serum (Fig. 5).

The concentrations of the competing synthetic ASA 11 unidecapeptide used in these experiments are 3 to 4 orders of magnitude greater than the ASA concentrations present in the captured FMDV ELISA antigen tested for reactivity with anti- Δ GF2d-bac serum. Therefore, these competition data strongly suggest that a major fraction of anti-FMDV antibody present in the anti- Δ GF2d-bac serum, but not in the anti-ASA 11 serum, is directed to virion VP₁ ASA conformation(s) poorly represented in the isolated pool of the synthetic ASA 11 unidecapeptide. ASA 11 at concentrations of 200 μ g/ml reduced anti-FMDV serum ELISA titers by 0.5 units or less (data not shown). Moreover, the negligible effectiveness in competing for FMDV antibody in anti- Δ GF2d-bac or anti-ASA 11 sera of the mutant ASA unidecapeptide, in which the RGD tripeptide is replaced by KAE, indicates the significance of the RGD motif in the overall immunogenicity of the ASA unidecapeptide in VP₁ antigenicity.

When this experiment was repeated with antisera of comparable ELISA titer (2.5 to 2.9 units), synthetic oligopeptide ASA 11 at concentrations up to 200 μ g/ml showed limited capacity to compete with anti- Δ GF2d-bac serum for binding to immobilized FMDV, whereas even 1 to 2 μ g of ASA 11 per ml markedly reduced the anti-ASA serum ELISA titer (data not shown). These studies clearly show that a VSV-G/FMDV-ASA chimeric protein gives rise to a preponderance of antibodies that recognize non-linear ASA epitopes.

FIG. 5. Comparative capacity of the synthetic unidecapeptide ASA 11 and its RGD→KAE mutant to compete by ELISA with FMDV antigen for antibodies present in anti-ΔGF2d-bac and anti-ASA 11 sera. Anti-ΔGF2d-bac serum was prepared as described in the legend to Fig. 4, and anti-ASA 11 serum was prepared similarly by serial inoculation of mice with 50-μg doses of synthetic unidecapeptide ASA 11 in Freund's adjuvant. Serial dilutions of anti-ΔGF2d-bac or anti-ASA 11 serum were each incubated for 30 min at room temperature with 0, 2, 50, or 200 μg of synthetic ASA 11 unidecapeptide per ml or with 200 μg of the mutant (MUT) unidecapeptide in which amino acids KAE were substituted for the RGD motif per ml. Each of these preincubated serum dilutions was tested by ELISA for its capacity to react with excess FMDV captured in microtiter plate wells. The residual ELISA titers of anti-ΔGF2d-bac and anti-ASA 11 sera preincubated with or without ASA 11 or its mutant are expressed as the percentage of the maximum (100%) ELISA titers of sera preincubated in the absence of ASA 11 (0 μg/ml). ELISA titers in the presence of 0, 2, 50, and 200 μg of competing ASA 11 were 4.5, 4.35, 3.2, and 2.6 log₁₀ units, respectively, for the anti-ΔGF2d-bac serum and 2.7, 2.15, <0.1, and <0.1 units, respectively, for the anti-ASA 11 serum. In the presence of 200 μg of the RGD→KAE mutant ASA per ml, the ELISA titers for anti-ΔGF2d-bac and anti-ASA 11 sera were 4.4 and 2.4 log₁₀ units, respectively.

Comparative reactivities of anti-ΔGF2d-bac and anti-FMDV sera with native and denatured FMDV particles. The preceding synthetic ASA unidecapeptide competition experiments suggested that most of the affinity of anti-ΔGF2d-bac antibody for FMDV virions is dependent on a restricted set of conformation-dependent ASA epitopes present only in native FMDV particles. To explore this hypothesis further, we compared the reactivities of anti-FMDV serum and anti-ΔGF2d-bac serum with those of native and SDS-denatured FMDV particles by both ELISA and Western blot techniques (Fig. 6).

Shown in Fig. 6A are ELISA plots of the affinity of anti-FMDV and anti-ΔGF2d-bac sera for binding to FMDV antigen, either undenatured or denatured by exposure to 3% SDS at 100°C for 3 min prior to their capture in 96-well plates. The results show that SDS denaturation of FMDV antigens resulted in loss of binding affinity by anti-ΔGF2d-bac antibody by almost 3 log units, declining to negligible reactivity of denatured FMDV. By comparison, exposure of FMDV to SDS resulted in only a minimal decline in its capacity to recognize anti-FMDV antibody, only a 0.5-log₁₀-unit loss. Clearly, FMDV denaturation produces only minimal effects on its reactivity with polyclonal anti-FMDV antibodies, compared with the virtually complete loss in reactivity with anti-ΔGF2d-bac antibody directed to the ASA sequence of FMDV VP₁ (Fig. 6A).

Figure 6B compares by Western blot analysis the reactivity of a mixture of anti-VSV/G and anti-FMDV antibodies with that of anti-ΔGF2d-bac serum with VSV-NJ G protein or SDS-denatured FMDV proteins separated by SDS-PAGE and electroblotted onto nitrocellulose paper. As noted in lanes 1, 3, and

FIG. 6. Comparative binding of antibodies in anti-ΔGF2d-bac serum and anti-FMDV serum to native and SDS-denatured FMDV as determined by ELISA (A) and Western blot (B) analyses. Clarified lysates obtained from FMDV-infected BHK-21 cells (containing ~5 μg of 140S virus particles per ml) were used either directly (native antigen) or after treatment with 3% SDS for 3 min at 100°C (denatured antigen). After dilution to appropriate concentrations, the native and denatured antigens were added to microtiter plate wells coated with 1:5,000 dilutions of anti-FMDV rabbit polyclonal capture antibody. After blocking with buffered BSA, serially diluted anti-ΔGF2d-bac and anti-FMDV sera were tested for the capacity to bind native or denatured FMDV antigens as tested by ELISA. The native and SDS-denatured FMDV proteins were separated by SDS-PAGE (15% gel), electroblotted onto nitrocellulose paper, reacted with anti-ΔGF2d-bac serum (1:100 dilution), and after SDS stripping and reblotting exposed to a mixture of anti-VSV G and anti-FMDV polyclonal rabbit sera (anti-FMDV/G). Reactivity with the respective antibodies was monitored by anti-mouse or anti-rabbit immunoglobulin G conjugated with peroxidase in the chemiluminescence signal development system. O.D., optical density. In panel B, lanes 1, 3, and 3' contain purified VSV G protein, and lanes 2, 4, and 4' contain SDS-denatured FMDV proteins. Lanes 3' and 4' represent computer-contrasted images (Adobe Photoshop) of lanes 3 and 4, respectively, showing the positive, but weak, reactive VP₁ protein with anti-ΔGF2d-bac serum (lane 4'). The FMDV samples contain BSA as a contaminant reactive with anti-BSA antibodies present in the anti-ΔGF2d-bac serum. Coomassie blue staining confirmed that this band is BSA (data not shown).

3', the VSV-NJ G protein was recognized equally well by the anti-FMDV/anti-G serum mixture and the anti-ΔGF2d-bac serum. The anti-FMDV/anti-G antibody mixture reacted quite well with denatured FMDV proteins VP₀ and VP₁₋₃ (lane 2), whereas the anti-ΔGF2d-bac serum reacted extremely weakly with denatured FMDV capsid proteins (lane 4), exhibiting only minimal reactivity with SDS-denatured VP₁ by computer-contrasted imaging using the Adobe Photoshop enhancement method (lane 4').

Taken together, the results of these experiments support the hypothesis that a major fraction of the antibodies induced in

FIG. 7. Comparative neutralization of FMDV infectivity by anti-GF2d-bac, anti-ΔGF2d-bac, and anti-FMDV sera. Serial dilutions of each antiserum were incubated for 60 min at room temperature with ~100 PFU of infectious FMDV. These mixtures were plated on monolayers of BHK-21 cells, overlaid with agar, incubated for 48 h at 37°C, and stained with crystal violet for plaque assay. Neutralization titers of each antiserum are recorded as the reciprocal of the antiserum dilution that reduced the FMDV plaque titer by 50%. The control represents the composite titer of sera obtained from uninoculated mice or mice inoculated serially with 1 μg of inactivated FMDV. Anti-GF2d-bac and anti-ΔGF2d-bac sera were from the same mice immunized with 30-μg doses of the respective antigens and anti-FMDV serum from the same mice immunized with 1-μg doses of FMDV shown in Fig. 4.

mice by inoculation of ΔGF2d-bac protein targets a restricted set of VP₁ ASA conformations exclusively present in native FMDV virions. It is also worth noting in this regard that anti-ΔGF2d-bac serum was not able to react by ELISA with FMDV antigens degraded by the action of trypsin-like proteases present in infected-cell lysates known to be targeted mainly to the ASA region of VP₁ but not other FMDV structural proteins (data not shown).

Neutralization of FMDV infectivity by antibodies directed to VSV-G/FMDV-ASA chimeric proteins. The ASA site of FMDV protein VP₁ has been postulated to be responsible for induction of antibodies that will neutralize the infectivity of FMDV (2, 10, 26). The efficient binding to FMDV proteins of antibodies raised against VSV-G/FMDV-ASA chimeras suggests that these antibodies might be capable of neutralizing FMDV infectivity. This postulate was tested by a standard FMDV neutralization assay in which dilutions of anti-GF2d-bac or anti-ΔGF2d-bac serum were mixed with 50 to 100 PFU of FMDV and incubated at room temperature for 1 h. Polyclonal anti-FMDV serum was also used as a positive control for neutralizing activity for FMDV. After plating on monolayer cultures of BHK-21 cells overlaid with agar and incubation for 48 h at 37°C, plaques were counted after staining with crystal violet and the serum neutralization titer was calculated as the reciprocal of the serum dilutions capable of reducing PFU by 50%, using the average of two independent assays.

The bar graphs in Fig. 7 compare the neutralization titers of the three sera. The results show that the anti-GF2d-bac antibody neutralized the 50% plaque-forming activity of FMDV at a titer of 200, compared with that of 2,000 for the FMDV-neutralizing activity of anti-ΔGF2d-bac serum. The neutralization titer of anti-FMDV serum was almost 3,000. These results closely parallel the ELISA titers of the same antisera shown in Fig. 4. These data indicate that the immunogenicity of chimeric VSV-G/FMDV-ASA proteins, particularly ΔGF2d-bac, approaches that of FMDV ASA itself, including the capacity to

induce the formation of neutralizing antibodies in the serum of vaccinated mice.

DISCUSSION

The data presented here show that VSV-NJ G-gene vectors with insertions of in-frame sequences coding for ASA of the FMDV VP₁ protein express in eukaryotic cells chimeric proteins that react with and give rise to FMDV antibodies. Quite striking was the fact that the chimeric G genes with ASA sequences inserted at three different sites expressed in vitro chimeric proteins that failed to recognize FMDV antibodies, albeit they were strongly reactive with VSV-NJ G antibodies. These results strongly indicate that antibody recognition of ASA sequences in chimeric G protein requires specific conformations of the G protein, probably related to folding and proper disulfide bond formation in endoplasmic reticulum (13, 29).

Critical to an understanding of its antigenicity is the fact that the ASA sequence of FMDV (C3 serotype) is composed of several overlapping epitopes located on the G-H loop of the VP₁ protein at the exterior surface of the FMDV virion (1, 27, 28). Crystallographic and immunological data obtained with the O1 serotype of FMDV indicate that these short amino acid sequence arrays display considerable structural flexibility (1, 20, 22), suggesting that these short peptides could function, at least partially, as conformational epitopes. In the specific case of the FMDV C3 serotype, it has been proposed that the ASA oligopeptide in solution has no unique conformation but rather can assume multiple conformations, each of which displays a particular immunogenicity (15, 28). The available data seem to indicate that only a restricted number of the many possible ASA conformations are able to raise or react with FMDV neutralizing antibody. The fact that the VSV-G/FMDV-ASA chimeric proteins reported here can react strongly with whole FMDV antiserum, and can elicit high ELISA titers of anti-FMDV antibodies in mice, clearly indicates that the ASA sequences in these hybrid proteins are able to mimic one or several of the alternative conformations of ASA presented in FMDV virions. Moreover, the relatively high titers of neutralizing antibody elicited in serum of mice, particularly by ΔGF2d-bac protein, support this assumption that a significant fraction of these chimeric proteins display in mice conformations of ASA identical to those on the surface of FMDV virions that elicit high titers of neutralizing antibody.

It is of considerable interest that newly synthesized chimeric protein ΔGF2d-bac extracted from host Sf9 cells with SDS retains its conformation-dependent capacity to elicit an immune response in mice. This ability of chimeric protein ΔGF2d-bac to retain or regain full ASA antigenic capacity after SDS denaturation is probably a reflection of the capacity of the VSV-NJ G protein to refold and recover its original topography after denaturation (29). The fact that the ASA sequence is inserted in a region of the G protein believed to overlap with epitope IV, an epitope that exhibits greater stability than the other nonlinear epitopes in the nonglycosylated G protein (14), supports this hypothesis. It is tempting to speculate that the superior capacity of truncated ΔGF2d-bac protein to elicit neutralizing antibody in mice, compared with that of the complete chimera GF2d-bac protein, is due to removal of the labile region of the VSV-NJ G protein distal to amino acid 213. The capacity of GF2d-bac protein and especially ΔGF2d-bac protein to elicit formation of FMDV neutralizing antibodies in mice seems to be superior to that of the ASA-containing isolated VP₁ protein of the FMDV O1 sero-

type and matches that of intact FMDV virions at doses higher than those used in the current experiments (39).

Although different vaccination protocols are difficult to compare, particularly because of variability in antigenic dose, schedule, and animal species, the immunogenic capabilities of the VSV-G/FMDV-ASA chimeric proteins tested here appear to match or exceed those reported previously for other recombinant chimeric proteins containing FMDV antigenic determinants (3, 8, 18, 21). For example, the FMDV neutralization titer in mice of $3 \log_{10}$ units obtained here compares favorably with the FMDV neutralization titers of 2.5 to $2.7 \log_{10}$ units reported for a single inoculation of hepatitis B core antigen-FMDV chimeric constructs (4); these constructs approach the specific immunogenicity of FMDV itself when tested in guinea pigs (3).

In considering hybrid proteins as subunit vaccines, one should stress the importance of aligning T-helper epitopes because of their often critical roles in enhancement and targeting of the immune response for protection against specific pathogens, such as FMDV (11, 30, 33, 38). The facts that the isolated VSV G protein strongly elicits the formation of neutralizing antibodies (17) and protects cattle against challenge with live VSV (24) strongly suggest the presence in G protein of T-helper cell epitopes that could enhance the FMDV vaccination potential of VSV-G/FMDV chimeras, such as those examined here. Such VSV G-protein chimeras containing the ASA sequence and/or other relevant FMDV sequences (9, 31, 37) could play a significant role as virus-free alternatives to FMDV vaccines in cattle and pigs, the main targets of foot-and-mouth disease as well as vesicular stomatitis. Quite obviously, critical factors to be considered in the future are dose response to single injections, heterotypic immunity, and the degree of resistance to challenge infection.

Finally, the data presented here open up the possibility of using the VSV G protein as a multipurpose carrier of short amino acid sequences with potential immunogenic properties as vaccines for a variety of pathogens.

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