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Monoclonal Antibodies to the M Protein of Vesicular Stomatitis Virus (Indiana Serotype) and to a cDNA *M* Gene Expression Product

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Twenty-nine independent hybridomas producing monoclonal antibodies to the matrix (M) protein of vesicular stomatitis virus (Indiana serotype) were prepared by fusion of SP2/0 myeloma cells with spleen lymphocytes obtained from BALB/c mice which had been immunized with the purified M protein. The specific reactivity of each monoclonal antibody was determined by an enzyme-linked immunosorbent assay and a competitive binding assay. Most of the antibodies were of the immunoglobulin G2a and G2b isotypes, although some were immunoglobulin M. By measuring the competitive binding of ^{125}I-antibody, we identified four antigenic determinants in the M protein of the virus; two of these determinants, however, exhibited a large degree of overlap. Western blot analysis revealed little or no cross-reactivity of the antibodies with other viral proteins or with the M protein of the New Jersey serotype. Prolonged trypsin proteolysis removed the first 43 amino acids from the amino-terminal region of the M protein, but it retained its reactivity with monoclonal antibodies to each epitope, except for diminished reactivity with one. To aid in future mapping of these epitopes, we inserted a cDNA clone of the mRNA encoding the M protein of vesicular stomatitis virus into an inducible *lac* expression vector; the M protein produced in the JM103 strain of *Escherichia coli* under induced conditions was found to be approximately the same size as native M protein and was recognized by the monoclonal antibodies. These monoclonal antibodies and the cDNA clone should be useful for studying the role of M protein in virus maturation and the regulation of viral transcription.

The virion of vesicular stomatitis virus (VSV) consists of a ribonucleocapsid core and a limiting membrane derived from the plasma membrane of infected host cells (36). The virion contains five virus-encoded proteins, three of which (N, NS, and L) are associated with the single-stranded RNA genome to form the enzymatically active ribonucleocapsid core. The glycoprotein (G protein) and the matrix (M) protein are intimately associated with the lipid envelope (24, 36). The G protein is an externally oriented transmembrane protein which completely spans the lipid bilayer by a sequence of 20 hydrophobic amino acids, and the viral M protein appears to line the interior surface of the membrane in close association with the nucleocapsid core (29, 43). The M protein has also been implicated in the control of viral transcription (4, 7, 9, 25, 40).

Monoclonal antibodies have been extensively used in recent years to probe the antigenic properties of viral proteins (41). Eleven different non-cross-reacting antigenic determinants have been reported for the G protein of VSV (35); all of the antibodies reacting with four such determinants resulted in virus neutralization. Recent monoclonal antibody analyses of surface G proteins of other enveloped viruses, such as HN of Sendai and Newcastle disease paramyxoviruses and HA (hemagglutinin) of influenza virus, have revealed the presence of multiple antigenic sites in the proteins (17, 18, 23, 39, 42). Only three distinct epitopes were detected on the membrane-associated M protein of influenza A virus; two of these had undergone antigenic variations in a naturally occurring field strain (33).

The M proteins of rhabdoviruses and paramyxoviruses play a critical role in the assembly of virus particles. During the budding of virions from the plasma membrane of host cells, the M protein undergoes two types of interactions. The M protein must recognize the site on the plasma membrane where insertion of the external G protein has occurred and must also bind to the nucleocapsid core. The study of various antigenic domains of the M protein should be useful in understanding the process of virus assembly and its regulation of viral transcription. In this communication we describe the preparation and characterization of monoclonal antibodies to the M protein of VSV. In addition, a cDNA clone of the mRNA encoding the M protein of VSV was inserted into an inducible *lac* expression vector, and the expression of the M protein in *Escherichia coli* was studied with the monoclonal antibodies described here.

MATERIALS AND METHODS

Cells and virus. Baby hamster kidney (BHK-21) cells were grown to 95% confluency at 37°C in Dulbecco modified Eagle medium supplemented with 10% tryptose-phosphate broth, 10% calf serum, and antibiotics as described elsewhere (1). Plaque-purified VSV of the Indiana serotype (San Juan strain) was used to infect the cell monolayer at a multiplicity of 0.1 PFU per cell. Bullet-shaped virions were harvested at 21 h postinfection and purified by differential, rate zonal, and equilibrium centrifugations (1). Purified virions were stored in phosphate-buffered saline (pH 7.4) at a concentration of 2 to 4 mg/ml at −70°C until further use.

Preparation of monoclonal antibodies to the VSV M protein. The VSV M protein was isolated from purified virions

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exposed to 1% Triton X-100–0.25 M NaCl in 10 mM Tris-hydrochloride buffer (pH 7.5) and was purified by column chromatography on Whatman P11 phosphocellulose as described elsewhere (38). M protein isolated by this procedure was found to be >97% pure, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The purified M protein solubilized in 0.55 M NaCl–10% glycerol was dialyzed for 3 h against phosphate buffered saline (pH 7.4) to bring the salt concentration to a physiological level. BALB/c mice were inoculated subcutaneously at 3-week intervals with 100 μg of M protein suspended in 0.2 ml of complete or incomplete Freund adjuvant. After three such injections, each mouse was inoculated intravenously with 50 μg of M protein in phosphate-buffered saline and was sacrificed after 4 days for hybridoma fusion. Fusion of spleen and SP2/0 myeloma cells with 37% polyethylene glycol was performed as described elsewhere (35) with minor modifications. The cells in RPMI medium containing 15% horse serum were plated in 96-well Costar flat-bottomed plates (150 μl per well), and after incubation at 37°C for 24 h, 100 μl of 2.5× hypoxanthine-aminopterin-thymidine medium (22) was added to each well. Clones producing antibodies to the M protein were detected by an enzyme-linked immunosorbent assay (ELISA) and subcloned twice, and single-cell clones were again selected for the final hybridoma (35). Large amounts of each monoclonal antibody were obtained by injecting 10^7 hybridoma cells intraperitoneally into BALB/c mice which had been primed 4 weeks before with an intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane). Ascites fluids were collected from the mice after 5 to 7 days, and immunoglobulins were purified by chromatography on *Staphylococcus* protein A-Sepharose CL-4B (Sigma Chemical Co., St. Louis, Mo.) as described previously (11, 35). Immunoglobulins of the immunoglobulin M (IgM) isotype were not purified further. Protein concentration was determined by absorption at 280 nm with an extinction coefficient of 1.4 per mg of protein. Iodination of immunoglobulins was accomplished with iodogen (Pierce Chemical Co., Rockford, Ill.) and $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.) as described previously (35).

Western blot analysis. Virions (1 μg) or viral M protein (1 μg) was subjected to 12.5% polyacrylamide-SDS slab gel electrophoresis (4) and transferred by electroblotting onto nitrocellulose sheets (0.1 μm; Schleicher & Schuell Inc., Keene, N.H.) as described by Towbin et al. (32). The nitrocellulose sheets were then treated successively with buffered bovine serum albumin (3% bovine serum albumin, 0.9% NaCl, 10 mM Tris-hydrochloride [pH 7.4]), monoclonal antibodies from ascites fluids, and ^{125}I-labeled *Staphylococcus* protein A (specific activity, 33 mCi/mg; Amersham Corp., Arlington Heights, Ill.) in 50 mM Tris (pH 7.4)–10 mM NaCl–5 mM EDTA–0.25% gelatin–0.05% Nonidet P-40 as described elsewhere (33). After being extensively washed with buffer containing 50 mM Tris-hydrochloride (pH 7.4), 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.5% Triton X-100, and 0.1% SDS, the nitrocellulose sheets were air dried and exposed to Kodak X-omat film at −70°C for several hours with an intensifying screen.

Bacterial strains and plasmids. *E. coli* HB101 was obtained from C. S. Schnaitman, and *E. coli* JM103 was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. Plasmid pM309 containing the VSV *M* gene was a generous gift of John K. Rose (Salk Institute, San Diego, Calif.), and plasmid pUC8 was obtained from P-L Biochemicals.

Cloning of the *M* gene insert into the β-galactosidase gene of pUC8. Restriction endonucleases were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Unless otherwise stated, the amount of enzyme used in all these experiments was twice the commercial unit necessary to completely digest 0.5 μg of DNA. Reactions were carried out under the conditions specified by the manufacturers for each specific enzyme. Restriction fragments were separated on 1.5% agarose gels (Sigma Chemical Co.) containing 0.5 μg of ethidium bromide per ml. Electrophoresis buffer consisted of 10 mM Tris acetate (pH 7.8), 5 mM sodium acetate, 1 mM EDTA, and 0.5 μg of ethidium bromide per ml. Plasmid pM309 was digested to completion with *Pst*I, and the fragment containing the *M* gene sequences was isolated by agarose gel electrophoresis. The DNA was excised from the gel and electrophoretically eluted with a buffer consisting of 10 mM Tris acetate (pH 7.9), 5 mM sodium acetate, and 1 mM EDTA. The eluted DNA was extracted with phenol-chloroform and then ethanol precipitated. NACS Prepack (Bethesda Research Laboratories) column chromatography was used to remove agarose contaminants from the purified DNA fragment under the conditions specified by the manufacturer. The DNA fragment eluted from the column was recovered by ethanol precipitation.

Plasmid pUC8 (34) was digested to completion with *Pst*I to produce a linear molecule. The digested sample was extracted with phenol-chloroform, and the DNA was recovered by ethanol precipitation. To reduce self-ligation of linear plasmid DNA, the 5′-phosphates were removed with calf alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The phosphatase-treated DNA was extracted with phenol-chloroform and recovered by ethanol precipitation. The *M* gene and the phosphatase-treated pUC8 DNA molecules were then ligated at 16°C for 12 h by the addition of T4 DNA ligase in buffer containing 50 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP, 0.2 mM EDTA, and 50 μg of bovine serum albumin per ml. The ligated DNA preparation was used to transform competent JM103 cells (8), and ampicillin-resistant colonies were selected. Plasmid DNA was prepared from the isolated colonies by a rapid isolation procedure and was screened for the *M* gene insert by digestion with *Pst*I followed by gel electrophoresis. Recombinant plasmid DNA was analyzed for the correct orientation of the *M* gene insert by combined digestion with *Acc*I and *Bgl*II, which produced a ca. 300-base-pair fragment when the insert was in the right orientation. To place the *M* gene-coding sequence in the right reading frame relative to the β-galactosidase initiation codon, we subjected plasmids with the *M* gene sequences in the correct orientation to digestion with *Acc*I or *Bam*HI and filled them in with the Klenow fragment; the blunt ends were then ligated with T4 DNA ligase as described above. In several experiments, the plasmids, after digestion with *Acc*I or *Bam*HI, were treated with S1 nuclease before being filled in with the Klenow fragment. The ligated mixture was then used to transform JM103 cells, and ampicillin-resistant colonies were selected.

Screening of bacterial lysates for the expression of the M protein. The colonies obtained above were grown in cultures at 37°C overnight in the presence of ampicillin (0.1 mg/ml). JM103 cells were then diluted 50-fold in medium containing ampicillin (0.1 mg/ml), and 2-ml cultures were incubated for 30 min at 37°C. Isopropyl-β-D-thiogalactoside (IPTG; Bethesda Research Laboratories) was then added to the culture at a concentration of 0.24 mg/ml to induce the expression of the M protein, and the incubation was contin-

ued for 6 h. The cells were then pelleted and treated with 0.5 ml of a solution containing 15% sucrose (wt/vol), 50 mM Tris-hydrochloride (pH 8.0), 50 mM EDTA, and 0.5 mg of lysozyme. The samples were incubated in ice for 10 min, and 25 μl of a 10% SDS solution was added to the cell suspension. The solubilized extract was then subjected to 12.5% polyacrylamide gel electrophoresis, and the proteins were blotted onto nitrocellulose and detected with monoclonal antibodies to the M protein and ^{125}I-protein A as described above. In a few experiments, the M protein in an SDS-solubilized bacterial extract was immunoprecipitated with antibodies.

RESULTS

Production of monoclonal antibodies. The spleen cells from two immunized BALB/c mice were fused to SP2/0 myeloma cells. Twenty-nine hybridomas producing monoclonal antibodies to the M protein were recloned and used to produce ascites fluids in BALB/c mice. All the monoclonal antibodies showed strong reactivity with the purified M protein, but no cross-reactivity above background with the G protein was noted. Immunoglobulin isotypes of each antibody were identified by a solid-phase ELISA with an immunoglobulin identification kit supplied by Boehringer Mannheim Biochemicals. Eleven of the immunoglobulins secreted by the 29 hybridoma clones were of the IgM isotype, and the rest of the immunoglobulins were either IgG2a or IgG2b.

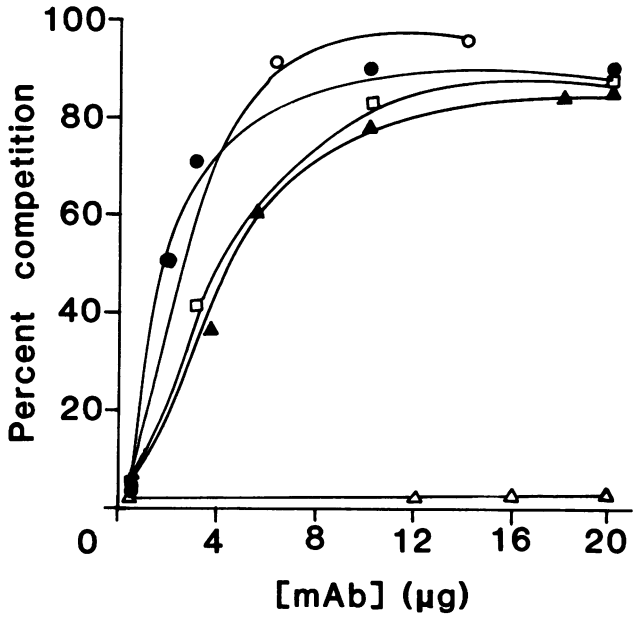

FIG. 1. Competitive binding to the VSV M protein of unlabeled monoclonal antibodies and ^{125}I-labeled monoclonal antibodies mAb2 and mAb13. Microtiter wells coated with 1 μg of M protein were exposed to increasing concentrations of competing unlabeled monoclonal antibody and then to ^{125}I-labeled monoclonal antibody (30×10^3 to 40×10^3 cpm/ml). After 3 h, the wells were thoroughly washed with phosphate-buffered saline containing 0.1% gamma globulin-free horse serum, excised with a hot wire, and counted in a Beckman II gamma counter. Symbols: □, ^{125}I-mAb2 competing with unlabeled mAb2; △, ^{125}I-mAb2 competing with unlabeled mAb13; ▲, ^{125}I-mAb2 competing with unlabeled mAb1; ○, ^{125}I-mAb13 competing with unlabeled mAb13; ●, ^{125}I-mAb13 competing with unlabeled mAb25.

TABLE 1. Antigenic determinants of the M protein of VSV-Indiana identified by competitive binding of monoclonal antibodies (mAb) from 29 hybridoma clones[a]

Determinant no.	mAb clone no.
1	1, 2, 17, 18, 21, 22, 23, 29
2	3, 4, 5, 6, 7, 14, 15, 16, 19, 20, 24, 26
3	8, 9, 10, 11, 12, 13, 25, 27
4	28

[a] Antigenic determinants (arbitrarily numbered) were identified by competitive inhibition of binding of ^{125}I-labeled monoclonal antibody to purified M protein by each of the 29 unlabeled monoclonal antibodies as described elsewhere (35).

Antigenic determinants for each monoclonal antibody. All monoclonal antibodies were classified on the basis of shared versus independent antigenic determinants by means of competitive binding of paired monoclonal antibodies to the purified M protein. To this end, an ammonium sulfate-precipitated immunoglobulin from each ascites fluid was purified through protein A-Sepharose CL-4B and iodinated with Na^{125}I (specific activity, 16.5 mCi/μg) as described above. IgM antibodies were used only as $(NH_4)_2SO_4$-fractionated immunoglobulin. In each test, various amounts of one unlabeled antibody were assayed for the capacity of that antibody to inhibit binding to the M protein of another antibody labeled with ^{125}I. Figure 1 shows such a competition experiment, in which increasing concentrations of unlabeled monoclonal antibodies mAb1, mAb2, mAb13, and mAb25 were tested for their capacity to inhibit binding to the M protein of ^{125}I-labeled mAb2 or mAb13. It is clear from Fig. 1 that mAb2 and mAb1 competed for binding to the M protein and thus shared the same or closely adjacent antigenic determinants. A similar case was observed between mAb13 and mAb25. However, no competition was observed between mAb2 and mAb13, suggesting that these two antibodies bind to two distinct antigenic determinants. Based on these types of competition experiments, four epitopes were identified on the M protein of VSV.

The clones corresponding to all these epitopes are shown in Table 1. Monoclonal antibodies mAb1, mAb2, and mAb22, for example, were assigned to epitope 1 because competitive binding studies revealed >90% competition with each other and with all the other antibodies assigned to epitope 1, whereas the degree of competitive binding to the M protein was <5% for antibodies in all the other epitopes. Similarly, mAb28, assigned to epitope 4, showed very little competition for binding to the M protein with all the antibodies assigned to the other three epitopes. Therefore, epitopes 1 and 4 are unique and do not overlap with epitopes 2 and 3. In sharp contrast, monoclonal antibodies assigned to epitopes 2 and 3 showed various degrees of cross-competition for binding to the M protein. For example, although mAb25 showed >90% competition for binding to the M protein with all the antibodies assigned to epitope 3, it exhibited almost 50% competition with mAb14, mAb15, and mAb16 and almost 80% competition with the other antibodies assigned to epitope 2. Similar results were obtained in cross-competition studies between mAb13 of epitope 3 and the antibodies assigned to epitope 2. By the same token, monoclonal antibodies assigned to epitope 2, such as mAb16, which exhibited >90% competition with all the other epitope 2 antibodies, showed ~50% competition with mAb10 and mAb25 and almost 80% competition with all the other antibodies assigned to epitope 3. It is apparent from

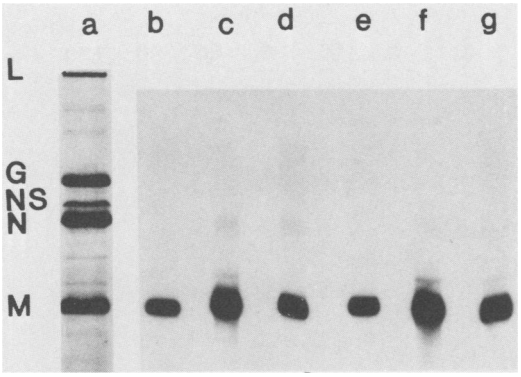

FIG. 2. Western blot analysis of the binding of monoclonal antibodies to the M protein. VSV virions (1 μg) or pure M protein (1 μg) was subjected to 12.5% polyacrylamide gel electrophoresis. The proteins were transferred from the gel to nitrocellulose filters and detected by various monoclonal antibodies as described in the text. Lanes: a, Viral proteins analyzed on a 12.5% polyacrylamide gel and stained with Coomassie brilliant blue; b, viral proteins tested with mAb2 to epitope 1; c, viral proteins tested with mAb3 to epitope 2; d, viral proteins tested with mAb25 to epitope 3; e, pure M protein tested with mAb2 to epitope 1; f, pure M protein tested with mAb3 to epitope 2; and g, pure M protein tested with mAb25 to epitope 3.

these competitive binding studies that the M protein of VSV-Indiana has at least four antigenic determinants; epitopes 1 and 4 appear to be unique, but epitopes 2 and 3 overlap considerably among their monoclonal antibodies and are presumably located in close proximity to each other on the M protein. In experiments in progress in our laboratory, we are attempting to map all antigenic domains to specific peptide regions of the M protein. It should also be noted that epitope 4 is represented by only a single IgM antibody, and because this antibody (mAb28) could not be purified by protein A-Sepharose CL-4B chromatography, it was not suitable for study. Affinity constants for a few of the IgG isotypes were determined by a solid-phase assay (14), and most values were in the range of 10^8 to 10^9 M^{-1} (data not shown).

Specificity of antibodies. The specificities of the antibodies made against the M protein of VSV were determined by both Western blotting and immunoprecipitation reactions. Whole VSV and pure M protein were subjected to gel electrophoresis on a 12.5% polyacrylamide gel, and the viral proteins were transferred by electroblotting from the polyacrylamide gel to nitrocellulose filters. mAb2, mAb3, and mAb25, representative antibodies for epitopes 1, 2, and 3, respectively, showed specific and equivalent binding to the M protein (Fig. 2). Some minor cross-reactivity of mAb3 and mAb25 with the nucleocapsid protein was noted, but no such binding was observed with mAb2. Attempts to use antibody 28 representing epitope 4 were unsuccessful, as the partially purified IgM gave a very high background. The specificities of the monoclonal antibodies made against the M protein were further demonstrated by their capacity to selectively immunoprecipitate intracellular M protein and not other viral proteins present in VSV-infected BHK-21 cells (data not shown).

Cross-reactivity with the VSV-New Jersey M protein. The two major serotypes of VSV, Indiana and New Jersey, have been classically distinguished by type-specific neutralizing antibodies which are directed toward the major antigen, the surface G protein (5, 19). The antibodies prepared here to the M protein of the Indiana serotype were tested for their ability to bind to the M protein of the New Jersey serotype. For this purpose, VSV-New Jersey (Ogden strain) was subjected to gel electrophoresis on a 12.5% polyacrylamide gel, and the proteins were blotted from the gel to nitrocellulose filters as described above. After blocking the nonspecific binding sites with 3% bovine serum albumin in 10 mM Tris–150 mM NaCl (pH 7.6), we exposed the nitrocellulose filters to mAb2, mAb3, and mAb25, representing epitopes 1, 2, and 3, respectively, and then to ^{125}I-protein A. Figure 3 shows an autoradiogram of such a nitrocellulose filter along with the stained proteins of VSV-Indiana and VSV-New Jersey virions separated on a 12.5% polyacrylamide gel. As a positive control, those nitrocellulose filters containing viral proteins from VSV-New Jersey were also exposed to a monoclonal antibody to the M protein of the New Jersey serotype (Ogden strain). The relative electrophoretic mobility of the New Jersey M protein was found to be different from that of the Indiana M protein (Fig. 3). Such a difference in mobility could be due to the differences in the molecular weights of the two proteins or to the degree of phosphorylation. A monoclonal antibody to the M protein of VSV-New Jersey showed strong reactivity with the M protein of VSV-New Jersey, but monoclonal antibodies mAb2, mAb3, and mAb25 to the Indiana M protein cross-reacted only very weakly with the New Jersey M protein. This weak cross-reactivity of the monoclonal antibodies to the New Jersey M protein was also confirmed by a solid-phase ELISA (data not shown).

Peptide maps of M proteins of the Indiana and New Jersey serotypes obtained by proteolytic cleavage were prepared by the method of Cleveland et al. (6) to examine the low degree of cross-reactivity of monoclonal antibodies to the M protein of the Indiana serotype with the M protein of the New Jersey serotype. To this end, VSV proteins from both serotypes were subjected to 12.5% polyacrylamide-SDS gel electrophoresis, and the M protein band was excised. The M

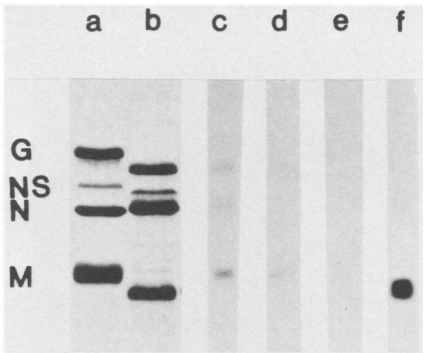

FIG. 3. Western blot analysis of the binding of the VSV-Indiana M protein monoclonal antibodies to the M proteins of VSV-Indiana and VSV-New Jersey (Ogden strain). Proteins extracted from virions of each strain (1 μg per lane) were subjected to 12.5% polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and detected by monoclonal antibodies as described in the text. The L protein is not shown in the gel. Lanes: a, Unblotted VSV-Indiana virion proteins stained with Coomassie blue; b, unblotted VSV-New Jersey virion proteins stained with Coomassie blue; c through e, VSV-New Jersey proteins blotted with mAb2 to VSV-Indiana epitope 1, mAb3 to VSV-Indiana epitope 2, and mAb25 to VSV-Indiana epitope 3, respectively; and f, VSV-New Jersey proteins exposed to a monoclonal antibody made against the New Jersey M protein.

protein from the New Jersey serotype of both Ogden and Hazelhurst strains had a slightly higher mobility in the gel than did that from the Indiana serotype (Fig. 3). The gel slices containing the M protein were equilibrated in 120 mM Tris buffer (pH 6.8), digested with *Staphlococcus* V8 protease, and separated on a 17.5% polyacrylamide gel containing 7 M urea. A number of peptides that were produced from the Indiana M protein after proteolysis with V8 protease were not seen with the New Jersey M protein (Fig. 4). Indeed, similar partial proteolytic cleavages of M proteins by *Staphylococcus* V8 protease, chymotrypsin, and trypsin had already revealed differences in the peptide digestion patterns of the M proteins from the Indiana and New Jersey serotypes (2, 3). This may explain the differences in the antigenic behavior of the M proteins from the two different serotypes.

Trypsin cleavage of the M protein and recognition of the uncleaved fragment by monoclonal antibodies. In an initial attempt to map the antigenic determinants of the VSV-Indiana M protein, the purified M protein was subjected to proteolysis with trypsin. Earlier studies by Morrison and McQuain (20) revealed that the M protein is highly resistant to trypsin, resulting in a 22-kilodalton uncleaved product. Purified M protein (1 mg) in 10 mM Tricine (pH 7.5) (Sigma Chemical Co.) containing 10% glycerol and 0.55 M NaCl was added to 20 μg of acetylated trypsin in the same buffer, and the mixture was incubated for 2 h at 37°C with intermittent shaking. At various times after the initiation of proteolysis, samples were removed and analyzed by gel electrophoresis on a 12.5% polyacrylamide-SDS gel. Figure 5 shows a typical gel electrophoretic pattern of proteolytic cleavage of the M protein at different times. At 5 min after the initiation of proteolytic cleavage, there was no detectable whole M protein remaining, but two bands representing the cleavage products of the M protein appeared. With increasing incubation times, the larger of the two cleavage products gradually disappeared, whereas the smaller product, corresponding to the final fragment, increased in quantity. Proteolytic cleavage was apparently complete by 90 min, as no further cleavage products were apparent at 120 min. Trypsin-resistant fragments were prepared for further study by incubation of the M protein with trypsin for 120 min, followed by chromatography on a soybean trypsin inhibitor-linked Sepharose column to remove trypsin from the mixture. The eluate was dialyzed against 10 mM Tris-hydrochloride (pH 7.6) and assayed for binding to the antibodies by a solid-phase ELISA.

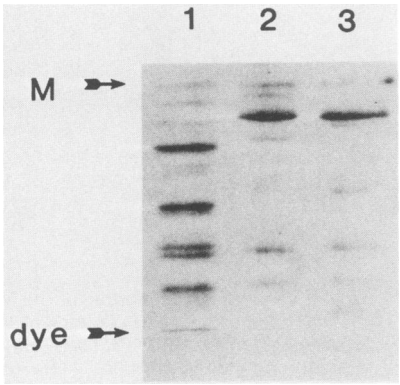

FIG. 4. Cleveland digests of M proteins of VSV-Indiana and VSV-New Jersey. VSV proteins from both serotypes were subjected to 12.5% polyacrylamide gel electrophoresis, and the M protein band was excised. The gel slices containing the M protein were digested with *Staphylococcus* V8 protease as described in the text and separated on a 17.5% polyacrylamide gel containing 7 M urea. Lanes: 1, VSV-Indiana M protein plus 20 μg of V8 protease; 2, VSV-New Jersey (Ogden strain) M protein plus 20 μg of V8 protease; and 3, VSV-New Jersey (Hazelhurst strain) M protein plus 20 μg of V8 protease.

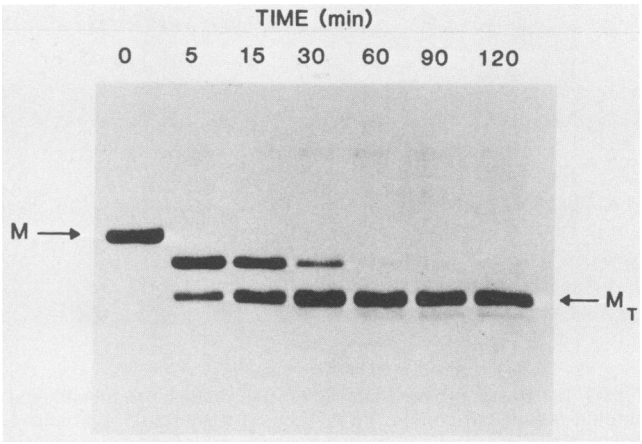

FIG. 5. Progressive proteolysis of VSV-Indiana M protein by trypsin. M protein (1 mg) in 10 mM Tricine (pH 7.5) containing 10% glycerol and 0.55 M NaCl was added to 20 μg of acetylated trypsin, and the mixture was incubated for 2 h at 37°C. At various times, samples were removed and subjected to 12.5% polyacrylamide gel electrophoresis. The figure shows the digestion product formed at 0, 5, 15, 30, 60, 90, and 120 min after exposure to trypsin. M_T, Trypsin-resistant fragment.

It is clear from Table 2 that all the monoclonal antibodies to different epitopes showed some degree of reactivity with the trypsin-resistant fragment. However, antibodies mAb1 and mAb2, reactive with epitope 1, showed considerably less binding to the trypsin-resistant fragment than to the intact M protein. On the other hand, antibodies to other epitopes bound as strongly to the trypsin-resistant fragment as they did to the intact M protein. The sequence of the first eight amino acids from the N-terminal region of the trypsin-resistant fragment was determined by Jay Fox at the Uni-

TABLE 2. Binding to the M protein and to the trypsin-cleaved fragment of monoclonal antibodies (mAb) from various hybridoma clones representing different epitopes[a]

mAb		Optical density at 415 nm of:	
Clone no.	Epitope no.	M protein	Trypsin-cleaved fragment[b]
1	1	1,027	0.594
2	1	1.036	0.613
3	2	1.611	2.000
4	2	1.543	1.829
9	3	2.000	2.000
12	3	1.300	1.850
13	3	1.490	1.800
25	3	1.297	1.890
28	4	1.059	0.889

[a] Binding of antibodies to purified antigens was assayed by an ELISA on microtiter plates with $(NH_4)_2SO_4$-precipitated ascites fluid (1:300 dilution of a 0.4-mg/ml stock) and 400 ng of antigen.

[b] Purified M protein was cleaved with acetylated trypsin as described in the text.

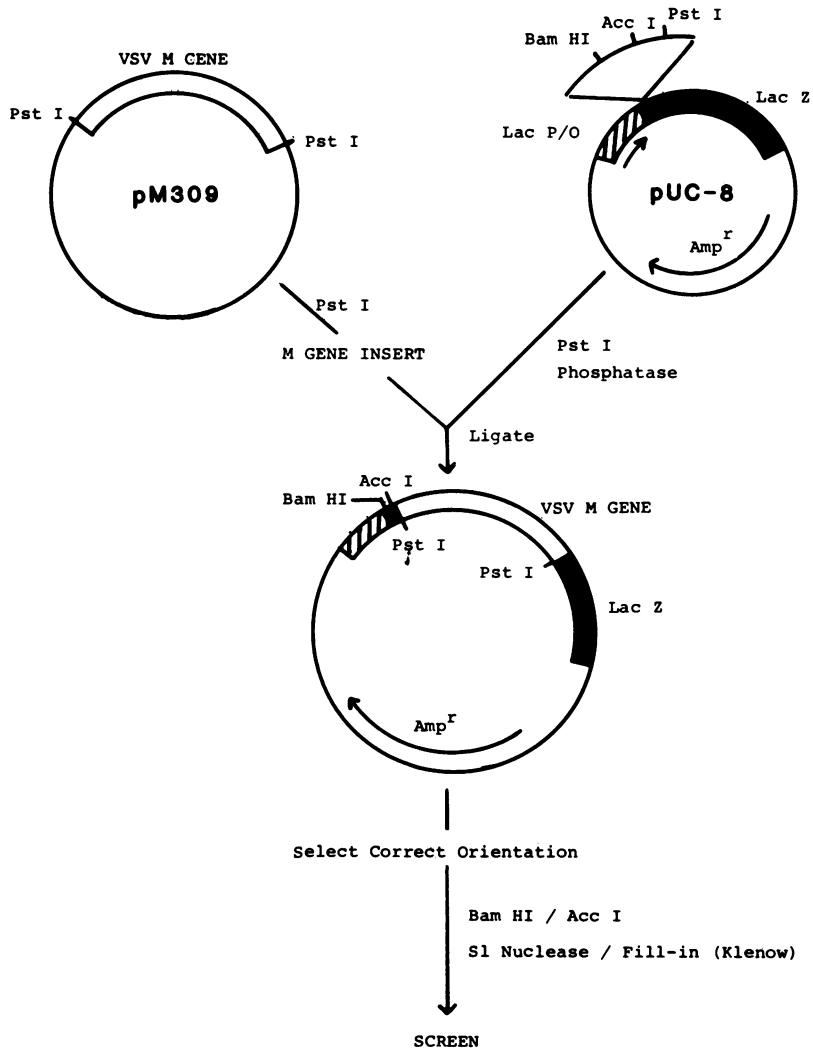

FIG. 6. Construction of plasmid pUC8 containing the *M* gene insert (pUC8-M).

versity of Virginia Sequencing Center and was as follows: Ser-Tyr-Phe-Gly-Val-Asp-Glu-Met. Comparison of this sequence with that derived from the cDNA clone of the intact M protein (27) indicated that the trypsin-resistant fragment had lost the first 43 amino acids from its amino terminus. It also seems likely that the first 43 amino acids from the amino-terminal segment of the M protein contribute to epitope 1 reactive with mAb1 and mAb2.

Expression of the M protein of VSV in *E. coli* and recognition of the expressed protein by monoclonal antibodies. Epitopes recognized by specific monoclonal antibodies are often useful in defining the structural and immunological domains of proteins. Most of the methods used to define sites of antibody binding involve low-resolution mapping of specific chemical and proteolytic fragments of the protein (10). The expression of molecularly cloned DNA fragments and the recognition of the expressed protein fragment by monoclonal antibodies have been used to obtain a precise map of antigenic determinants in a protein (21). The complete nucleotide sequences of VSV mRNAs for the G protein and the M protein were determined from cDNA clones that contained the complete coding sequence from each mRNA (27). Stable expression of the G protein in cGI cells has been established with a hybrid expression vector (26), and the G protein expressed in mammalian cells was found to be processed, glycosylated, and transported to the cell surface in a normal fashion. Further, it has been demonstrated that the G protein expressed on mammalian cell surfaces induces cell fusion at a low pH (12, 13).

It was of interest to determine whether the M protein could be expressed in a stable form in *E. coli* and whether such an expressed protein could be recognized by the monoclonal antibodies to the M protein. To this end, a cDNA recombinant of the *M* gene, containing both coding and noncoding sequences, was inserted into the β-galactosidase-coding sequences at the *PstI* site in pUC8 (Fig. 6). The *M* gene insert retained its own translation stop codon, so the carboxy-terminal end of the protein would not be fused to β-galactosidase. The *PstI* site in pUC8 is 41 base pairs downstream from the translational initiation codon of β-galactosidase, and the *PstI M* gene insert contained the normally noncoding sequences as well as the GC tails from the cDNA cloning upstream of the first ATG (27). Thus, the fusion protein was expected to contain additional amino-terminal amino acids, adding ca. 3 kilodaltons to the M protein. The expression of the M protein was assessed after

growing the bacteria in cultures for 6 h in the presence of IPTG. Total solubilized extract from the bacterial cells was prepared and subjected to gel electrophoresis. The proteins were then blotted onto nitrocellulose and detected by monoclonal antibodies to the M protein and by ^{125}I-protein A as described above.

Figure 7 shows the autoradiogram of Western blots in which a mixture of antibodies to epitopes 1 and 2 was used to detect the expressed protein. As expected, no M protein was detected in the solubilized cell extract from the control bacteria containing only the pUC8 plasmid. In the absence of IPTG, there was very little M protein expression in bacteria containing the pUC8-M plasmid, but the amount increased markedly when the cells were induced by IPTG. It should be noted that a protein larger in molecular weight than the M protein was found to cross-react slightly with the antibodies in cells containing both the pUC8 and pUC8-M plasmids. To examine whether the M protein expressed in *E. coli* was recognized by individual monoclonal antibodies to epitopes 1 and 2, we performed similar experiments, but the antibodies to epitopes 1 and 2 were used separately to detect the protein. Figure 7 also shows blots in which pure VSV M protein was subjected to gel electrophoresis along with the bacterial cell lysate. Both of the antibodies reacting with epitopes 1 and 2 also reacted with the expressed M protein, which appeared to migrate like native M protein in polyacrylamide gels. Although the antibody to epitope 1 was found to cross-react nonspecifically with the higher-molecular-weight protein, no such binding reaction occurred with the antibody to epitope 2.

Immunoprecipitation of the expressed M protein in a bacterial lysate was done with a mixture of antibodies to epitopes 1 and 2, and the results are shown in Fig. 7. As expected, no M protein was immunoprecipitated from cells containing plasmid pUC8 alone. However, a significant amount of M protein was immunoprecipitated from the bacterial lysate containing plasmid pUC8-M. The recombinant M protein appeared to have a molecular weight on SDS-polyacrylamide gels similar to that of the native M protein, even though it was expected to be ~3 kilodaltons larger. However, the native M protein migrated to a position ~3 kilodaltons larger than its actual size, presumably because of its phosphorylation. The hybrid recombinant M protein may not be phosphorylated correctly, which may explain its migrating at its correct molecular weight.

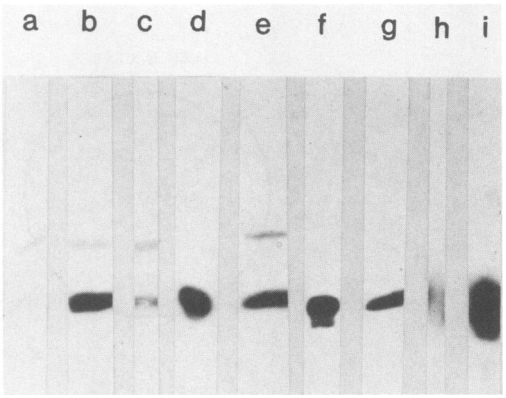

FIG. 7. Detection of the M protein expressed in *E. coli* JM103 cells by Western blot analysis with monoclonal antibodies. JM103 cells containing plasmid pUC8 or pUC8-M were grown in the presence of ampicillin (0.1 mg/ml) and without or with IPTG (0.1 mg/ml) for 6 h. The cells were pelleted, solubilized, and subjected to 12.5% polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose filters and tested for antibody binding by the Western blot method. Lanes: a, Extract from cells containing plasmid pUC8 (devoid of the *M* gene) tested with a mixture of mAb2 and mAb3 antibodies to epitopes 1 and 2; b, extract from cells containing plasmid pUC8-M induced with IPTG and tested with a mixture of mAb2 and mAb3 antibodies to epitopes 1 and 2; c, extract from cells containing plasmid pUC8-M not induced with IPTG and tested with a mixture of mAb2 and mAb3 antibodies to epitopes 1 and 2; d, native VSV M protein (1 μg) reacted with mAb2 antibody to epitope 1; e, extract from IPTG-induced cells containing plasmid pUC8-M tested with mAb2 antibody to epitope 1; f, native VSV M protein (1 μg) reacted with mAb3 antibody to epitope 2; g, extract from IPTG-induced cells containing plasmid pUC8-M tested with mAb3 antibody to epitope 2; h, extract from IPTG-induced cells containing plasmid pUC8 immunoprecipitated by mAb2 and mAb3 antibodies to epitopes 1 and 2; and i, extract from IPTG-induced cells containing plasmid pUC8-M immunoprecipitated by mAb2 and mAb3 antibodies to epitopes 1 and 2.

DISCUSSION

Monoclonal antibodies reactive with the M protein of VSV-Indiana were isolated and characterized. Of the 29 monoclonal antibodies isolated here, 11 were of the IgM isotype. The formation of IgM antibodies against the M protein may represent a unique antigenic property of the VSV M protein, because none of the 19 monoclonal antibodies formed against the VSV G protein were of the IgM isotype (35). Although two of the antibodies reported for the G protein were found to cross-react with the M protein (35), none of the antibodies isolated here showed any significant cross-reactivity with the G protein in either the ELISA or the Western blot analysis. However, in the Western blot analysis, the antibody to epitope 2 (clone mAb3) showed very weak but reproducible cross-reactivity with the nucleocapsid protein. Four epitopes were recognized by the monoclonal antibodies to the M protein, two of which were found to overlap quite extensively. The fourth epitope was represented by only a single antibody of the IgM isotype which did not compete with any other antibodies for binding to the M protein.

The internal M protein of influenza virus has been shown to be quite similar in size and functions to the VSV M protein (15, 44). Both of them have a molecular weight of ~25,000 and are involved in interactions with the membrane and ribonucleocapsid core of the virions (33). Three distinct epitopes on the M protein of influenza virus were also identified with monoclonal antibodies. At least two of these three epitopes were shown to be located in a nonoverlapping domain on the M protein molecule (33).

Indiana and New Jersey are the two major serotypes identified for VSV virions. These serotypes have been classically distinguished by serotype-specific neutralizing antibodies which are directed towards the major surface G protein (5, 19). The monoclonal antibodies to the M protein of the Indiana serotype were shown to be only very weakly reactive with the M protein of the New Jersey serotype. This was not surprising, as antigenic variations have been observed in the M protein of various strains of viruses. For example, monoclonal antibodies against five structural proteins of measles virus were used to determine the degree of antigenic variation within these proteins in nine different strains of measles virus (31). Limited variations were observed in the epitopes on surface, polymerase, and nucleocapsid proteins of various strains, but the epitopes on the M protein of different measles virus strains exhibited extensive

variation in their reactivity with the nine anti-M protein monoclonal antibodies. It was also observed that in influenza A virus, two of the three epitopes on the M protein underwent antigenic variation in a number of virus strains, and the third epitope appeared to be an invariant type-specific determinant for influenza A virus (33).

It has been postulated that regions of a protein rich in hydrophilic amino acids serve as possible antigenic sites (16). The amino-terminal region of the M protein, rich in lysine, is highly hydrophilic (27) and should be an ideal region for an antibody-binding site. The trypsin-resistant fragment does not contain the highly hydrophilic region from the amino terminus; however, it still shows binding by the monoclonal antibody to epitope 1, but at a significantly lower level than that of the monoclonal antibodies directed against the other epitopes. The failure to obtain antibodies specific for peptides residing in the first 43 amino acids suggests that this lysine-containing amino-terminal region may be very protease sensitive and might have been cleaved when the protein was injected into mice. A degradation product of a similar size was found in VSV-infected Chinese hamster ovary cells within 4 to 5 h after infection, also supporting the view that this region of the M protein is particularly sensitive to proteases in vivo (28).

Insertion of the *M* gene into the *lac* expression vector pUC8 resulted in the synthesis of the M protein in *E. coli*. The protein was found to be stable in bacteria, had mobility on polyacrylamide gels similar to that of the native M protein, and was found to be recognized by monoclonal antibodies to three epitopes on the M protein. Experiments in progress in this laboratory are designed to express various segments of the M protein in *E. coli* by molecular cloning of different regions of the *M* gene in the pUC8 expression vector. Recognition of these expressed protein segments by monoclonal antibodies should enable us to map the antibody-binding sites in the protein.

The M protein of VSV plays an important role in virus maturation and the regulation of viral transcription. Recent studies have elucidated two functions of the M protein, one structural and one regulatory. Experiments with VSV temperature-sensitive mutants and viral pseudotypes have shown that the M protein is essential for the budding of virions from the plasma membrane (30, 37). It has also been shown that the M protein has a regulatory role in virus-directed RNA synthesis in infected cells and acts as an inhibitor of VSV transcription (4, 7, 9, 25, 40). For fulfillment of these roles, the M protein must interact with both the viral nucleocapsid and the plasma membrane. Models of such interactions have been described recently for M protein reconstituted with lipid vesicles and in viral transcription systems with ribonucleocapsid cores (9, 25, 38). Monoclonal antibodies made against the M protein could be useful in determining the mode of interaction of the M protein with ribonucleocapsid cores and with lipids as well as the sites of the M protein involved in such binding. Research described in a future communication will show the use of monoclonal antibodies as probes to study the regulation of viral transcription by the M protein of VSV.

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