Functional and Antigenic Domains of the Matrix (M_1) Protein of Influenza A Virus

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The membrane- and ribonucleocapsid (RNP)-binding domains of the matrix (M_1) protein of influenza A virus (WSN strain) were partially mapped and characterized by reactivity with monoclonal antibodies (MAb) as well as by proteolytic cleavages and amino acid sequencing of the resulting peptides. Of two peptides formed by formic acid hydrolysis, a 9-kilodalton fragment at the amino-terminal third of the M_1 protein was recognized by MAb M2-1C6 (to epitope 1), and a 15-kilodalton fragment at the carboxy-terminal two-thirds was recognized by MAb 289/4 (to epitope 2). Partial cleavage by staphylococcal V8 protease gave rise to a 16-kilodalton peptide, mapping to amino acid 8, which was recognized by MAbs to all three epitopes but rather weakly by MAb 904/6 to epitope 3. These studies suggest that epitope 1 of the M_1 protein resides between amino acids 8 and 89, whereas epitopes 2 and possibly 3 are located between amino acids 89 and 141 or somewhat more carboxy distal. The intact M_1 protein and its N-terminal 9- and 10-kilodalton peptides generated by formic acid or V8 protease cleavage, respectively, reconstituted with dipalmitoylphosphatidylcholine vesicles, but these N-terminal peptides had little effect on *in vitro* transcription of the RNP core. In sharp contrast, both intact M_1 protein and the C-terminal 15-kilodalton formic acid fragment were able to inhibit viral transcription markedly. Moreover, MAb 289/4 (to epitope 2) reversed this inhibited transcription significantly. These studies suggest that the lipid-binding domain of the M_1 protein is located within the amino-terminal third, whereas the site involved in the interaction of the M_1 protein with RNP cores is located within the carboxy-terminal two-thirds.

Influenza virus is a negative-strand RNA virus which is composed of eight single-stranded genomic segments coding for 10 or more polypeptides (16, 17). The matrix (M_1) protein encoded by RNA segment 7 is the most abundant protein found within the virion and has been shown to play a central role in virus assembly (1, 16–19). This protein is located at the inner surface of the lipid bilayer of the virion envelope in close proximity to the ribonucleocapsid protein (RNP) complex (2, 7, 8, 28). Strong interaction of M_1 protein with membranes was demonstrated by reconstituting the isolated protein *in vitro* with lipid vesicles, with planar lipid dispersions, and by lipid-protein cross-linking studies (3, 11, 13–15, 24). In addition to its interaction with membrane lipids, the M_1 protein influences the RNP-associated transcriptase activity of influenza virus; addition of M_1 protein *in vitro* to transcribing RNP cores significantly inhibits their transcriptase activity (37).

Monoclonal antibodies have been used in recent years to probe the structural and functional domains of viral polypeptides (35). Three distinct epitopes have been identified on the M_1 protein of influenza A virus (WSN/33 strain), two of which undergo antigenic variation in some virus strains (29). Competitive binding assays and Western blot analyses revealed that at least two of these epitopes are located on nonoverlapping domains of the M_1 protein (29). These antibodies (kindly supplied by Kathleen van Wyke), as well as chemically and enzymatically cleaved fragments of the M_1 protein, were used in these studies to characterize the domains on the WSN matrix protein involved in its interaction with liposomes and with the RNP complex of the virus.

MATERIALS AND METHODS

Virus and cells. All experiments were performed with influenza virus A/WSN/33 kindly supplied by Anastasia Gregoriades. Unlabeled virus was produced by infecting 10-day-old chicken embryos with seed virus and harvesting allantoic fluids 48 h after inoculation. Viral proteins were radiolabeled with [35 S]methionine (1,022 Ci/mmol; New England Nuclear Corp., Boston, Mass.) by infecting confluent monolayers of MDCK cells with influenza virus at a multiplicity of 1.5 PFU per cell in the presence of [35 S]methionine (50 μ Ci/ml) in Eagle minimal essential medium. Radiolabeled virus released from MDCK cells 48 h postinfection or unlabeled virus from allantoic fluids were concentrated by centrifugation and purified by banding in 15 to 60% sucrose gradients as described by Gregoriades (12).

Purification of M_1 protein from WSN virions. The matrix (M_1) protein of influenza virus was purified by acidic chloroform-methanol extraction as described by Gregoriades (12). The virions (1 mg/ml) were delipidated by the addition of 2 volumes of chloroform-methanol (2:1, vol/vol). The viral proteins were collected from the interphase and extracted with 2 ml of chloroform-methanol (1.8:1, vol/vol) containing 0.04 N HCl. The organic phase was then mixed with 1 ml of buffer containing 0.01 M Tris (pH 7.4), 0.1 M NaCl, 1 mM EDTA, evaporated under nitrogen for 30 min, and lyophilized. M_1 protein obtained by this procedure was more than 90% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentration was determined by the method of Lowry et al. (21).

Cleavage of M_1 protein. The M_1 protein of WSN virus was cleaved into oligopeptide fragments by enzymatic digestion with *Staphylococcus aureus* V8 protease (Cooper Biomedical, Inc., West Chester, Pa.), by hydrolysis with formic acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.), or by treat-

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ment with cyanogen bromide (Aldrich). Purified M₁ protein (125 µg) in 125 mM Tris hydrochloride buffer (pH 6.8)–0.5% sodium dodecyl sulfate was digested with V8 protease for 2 h or overnight. The proteolytic fragments were then purified by high-pressure liquid chromatography (HPLC)-C8 column chromatography. In separate experiments, the M₁ protein (400 µg) was dissolved in 200 µl of 75% formic acid, and the sample was incubated at 37°C for 48 h. The solution was then lyophilized to remove formic acid, and the fragments were purified by HPLC-C8 column chromatography.

Amino acid sequence analysis of peptide fragments. The N-terminal amino acid sequences of peptide fragments generated from M₁ protein by chemical or enzymatic cleavages were determined with the aid of an Applied Biosystems gas-phase sequencer. For determining the site of cleavage on the protein molecule, these sequences of the WSN M₁ protein were then compared with the amino acid sequence of the M₁ protein of the closely related A/PR8/34 strain inferred from the nucleotide sequence of cloned cDNA prepared from the PR8 virus RNA genome segment coding for the matrix protein (34).

Monoclonal antibodies to M₁ protein. Hybridoma cell lines producing monoclonal antibodies to M₁ protein of influenza virus A/WSN/33 (29) were a generous gift of Kathleen van Syke of the National Institute of Allergy and Infectious Diseases. Purified monoclonal antibodies were prepared from mouse ascites fluid by protein A column chromatography as described previously (30). The specificity of each monoclonal antibody was determined by Western blot analysis as described recently (22). Fab fragments from monoclonal antibodies were prepared as described by Einck and Bustin (10). Briefly, purified monoclonal antibodies (1 mg) were treated with 0.01 mg of papain (Worthington Diagnostics, Freehold, N.J.) in 0.2 M sodium phosphate buffer (pH 6.5) for 6 h at room temperature. Samples were then dialyzed against 0.1 M Tris hydrochloride (pH 7.4) and passed over a Sepharose staphylococcal protein A column (Sigma Chemical Co., St. Louis, Mo.) to remove Fc fragments and undigested molecules.

Reconstitution of M₁ protein or its cleaved fragments with sonicated vesicles. Viral M₁ protein or its cleaved polypeptides were reconstituted with sonicated vesicles by the method of Wiener et al. (32). In short, 500 nmol of dipalmitoylphosphatidylcholine (DPPC) alone or mixed with dipalmitoylphosphatidylglycerol (DPPC-DPPG, 1:1 molar ratio) containing 0.05 µCi of [³H]DPPC in chloroform was dried under nitrogen and lyophilized overnight. The lipid samples were then suspended in 2 ml of 10 mM Tricine (pH 7.4) (Sigma) containing 0.55 M NaCl and sonicated at 42°C for 3 to 5 min under nitrogen with a Heat-Systems W-350 sonicator. Multilamellar vesicles were removed by centrifuging the sonicated lipid sample at 150,000 × g for 3 h. The resulting suspension of small unilamellar vesicles was then mixed with the M₁ protein or its fragmented peptides at the desired protein-to-lipid ratio and then dialyzed for 24 h against 10 mM Tricine (pH 7.4) at 42°C. The reconstituted samples were next analyzed for protein and lipid composition after density gradient centrifugation in 0 to 30% sucrose gradients as previously described (32).

RNP core preparations. Enzymatically active RNP cores of influenza virus were obtained by disrupting virions (0.5 mg/ml) in 10 mM Tricine (pH 8.0)–1% Nonidet P-40 (NP-40)–0.25 M NaCl–1.25 mM dithiothreitol. After disruption of virions at room temperature for 30 min, RNP cores were then pelleted by centrifugation in an SW50.1 rotor at 150,000 × g for 2 h through a 50% glycerol pad. The

supernatant fluid containing lipids and membrane proteins was discarded, and the pellet containing RNP cores was suspended in 10 mM Tris (pH 8.0). RNP cores prepared by this method had no membrane-bound HA and NA proteins but contained trace amounts of M₁ protein.

Influenza virus transcription assays. Transcription of whole virions or RNP cores was assayed as described by Zvonarjev and Ghendon F(37) with minor modifications. Briefly, RNP cores or whole virions were suspended in transcription buffer containing 10 mM Tris (pH 8.0), 1 mM dithiothreitol, 80 mM NaCl, 7.5 mM MgCl₂, 0.12 mM MnCl₂, 0.04 mM ApG, 1 mM each ATP, GTP, and CTP, and 0.1 mM UTP containing 10 µCi of [α-³²P]UTP and 0.25% NP-40. Transcription mixtures were incubated for 2 h at 31°C, and the reactions were terminated by the addition of 0.6 ml of sodium PP_i (67 µM). Carrier yeast RNA (50 µg) was then added to the reaction mixture, and the total RNA was precipitated with an equal volume of 10% trichloroacetic acid. The mixture was kept on ice for 20 min, and the acid-insoluble RNA was collected by filtration through glass fiber filters (GSA; Whatman, Inc., Clifton, N.J.). The radioactive content in the filter paper was measured by scintillation spectrometry.

RESULTS

Chemical cleavage of M₁ protein and recognition of fragments by monoclonal antibodies. The matrix (M₁) protein of influenza virus undergoes two types of interactions which appear to be critical for maturation of the virion and budding from the infected-cell plasma membrane: it binds to the lipid bilayer and interacts strongly with the RNP cores (7, 8). These two types of interactions have been demonstrated in vitro by reconstituting M₁ protein with phospholipid vesicles simulating the virus membrane (3, 13) and by inhibiting transcription of viral RNP cores by the M₁ protein (37). Three distinct antigenic domains have been identified in the M₁ protein of WSN influenza virus (29). Three monoclonal antibodies designated by van Wyke et al. (29) as M2-1C6, 289/4, and 904/6, corresponding to epitopes 1, 2, and 3, respectively, seemed good candidates to serve as probes to characterize the antigenic and functional domains of the M₁ protein. In addition to performing these experiments, fragmentation of the protein by chemical and enzymatic methods would also appear to be useful means to localize the domains on the M₁ protein that are involved in its interaction with lipid bilayers and RNP complexes.

The M₁ protein was cleaved by both chemical and enzymatic methods, and the binding of monoclonal antibodies to these fragments was assayed by Western blot analysis (22). The peptide bonds linking aspartyl to prolyl residues have long been recognized to be labile to mild acids such as formic acid (20), resulting in partial cleavage. The incomplete cleavage products of M₁ protein generated by treatment with formic acid are shown in Fig. 1. In addition to uncleaved M₁ protein, exposure of the M₁ protein to 75% formic acid for 48 h resulted in partial hydrolysis into two major fragments; based on the molecular weights of coelectrophoresed standard proteins, these peptides were assigned approximate molecular size values of 15 and 9 kilodaltons. Comparison of the N-terminal amino acid sequences of these two fragments with that of the M₁ protein of the closely related A/PR8/34 strain (34) revealed that the A/WSN/33 M₁ protein was cleaved at amino acid residue 89 by formic acid hydrolysis. Therefore, the 9-kilodalton fragment was derived from the amino-terminal region, whereas the 15-kilodalton fragment comprised the carboxy-terminal region.

FIG. 1. Western blot analysis showing the selective binding to influenza virus M₁ protein or to its formic acid-cleaved fragments (FA) of monoclonal antibodies that react with three separate epitopes (Ep1, Ep2, Ep3) of the M₁ protein. Intact M₁ protein (1 µg) or M₁ protein exposed to 75% formic acid for 48 h at 37°C (5 µg) was subjected to electrophoresis on 15% polyacrylamide gels. The intact protein or the fragments were then transferred by electroblotting to nitrocellulose paper and reacted with monoclonal antibodies followed by ¹²⁵I-labeled *S. aureus* protein A for autoradiography. The first three lanes illustrate unblotted proteins stained with Coomassie brilliant blue (DYE) showing molecular weight standards (S), intact M₁ protein (M₁), and M₁ protein partially cleaved with formic acid (FA). The next six lanes labeled Ep1, Ep2, and Ep3 illustrate electroblotted intact M₁ protein (labeled M₁ in lanes 4, 6, and 8) or M₁ protein cleaved with formic acid (labeled FA in lanes 5, 7, and 9) exposed to monoclonal antibodies M2-1C6 (epitope 1; lanes 4 and 5), 289/4 (epitope 2; lanes 6 and 7), and 904/6 (epitope 3; lanes 8 and 9). Arrows indicate positions of migration of intact M₁ protein and the 15- and 9-kilodalton (K) formic acid cleavage products.

The binding of three monoclonal antibodies to these two fragments was assayed by Western blot analysis with ¹²⁵I-labeled staphylococcal protein A and autoradiography to identify bound monoclonal antibodies. The results presented in Fig. 1 show the expected strong reactivity of all three monoclonal antibodies with the intact M₁ protein. Monoclonal antibody M2-1C6 (to epitope 1) also reacted strongly with the N-terminal 9-kilodalton fragment, whereas monoclonal antibody 289/4 (to epitope 2) bound only to the C-terminal 15-kilodalton fragment. However, also present with or without formic acid treatment was a spontaneous 15-kilodalton protein shown in Fig. 1, which reacted with monoclonal antibody M2-1C6 directed to epitope 1 (Fig. 1, lane 4) but not with monoclonal antibody 289/4 directed to epitope 2. Strikingly, monoclonal antibody 904/6 (to epitope 3) showed no significant binding to either of the two formic acid fragments. The formic acid cleavage data reveal that epitope 1 of the M₁ protein resides in a 9-kilodalton N-terminal peptide and that epitope 2 is a determinant within the 15-kilodalton C-terminal peptide.

Enzymatic cleavage of M₁ protein and recognition of its fragments by monoclonal antibodies. Purified M₁ protein was subjected to enzymatic cleavage with staphylococcal V8 protease as described in Materials and Methods. Digestion of M₁ protein resulted in the formation of three distinct peptides of approximate molecular weights of 16,000, 10,000, and 4,000. Of the three peptides generated by V8 protease, the 16-kilodalton fragment reacted strongly with monoclonal antibody M2-1C6 (epitope 1) and with monoclonal antibody 289/4 (epitope 2) and weakly with monoclonal antibody 904/6 (epitope 3). The 16-kilodalton fragment appears to be a partial digestion product of reaction with V8

protease because prolonged exposure (16 h) resulted in its disappearance and the formation of only 10- and 4-kilodalton peptides (data not shown). The 10-kilodalton peptide reacted only with monoclonal antibody M2-C16 (to epitope 1), whereas the 4-kilodalton peptide failed to bind any of the three monoclonal antibodies.

The sequences of the N-terminal amino acids of the 16-, 10-, and 4-kilodalton V8 peptides of the M₁ protein were determined and compared with the known amino acid sequence of the closely related PR/8/34 influenza A M₁ protein (34). The 16- and 10-kilodalton peptide fragments had exactly the same N-terminal amino acid sequences, showing that they were both derived by proteolytic cleavage at residue 8; quite clearly, the 10-kilodalton peptide emanates from further cleavage of the 16-kilodalton peptide, which is a partial V8 protease cleavage product. The 4-kilodalton peptide was formed by V8 cleavage at amino acid residue 141 and has no determinants for binding any of the three monoclonal antibodies. These studies indicate that the antigenic determinants for all three monoclonal antibodies are located on the WSN M₁ protein between amino acids 8 and 141, or possibly extending through amino acid sequences further downstream.

Fragments of the M₁ protein obtained by V8 protease or by chemical cleavage and their reactivity with the three monoclonal antibodies are summarized in Fig. 2. It is clear from both types of digestions that epitope 1, which is the binding site of monoclonal antibody M2-1C6, is located near the amino-terminal region of the protein molecule. Thus, the 9- or 10-kilodalton peptide derived from formic acid or V8 protease cleavage, respectively, representing ~35% of the protein from the N terminus, contains the antigenic determinant for epitope 1 recognized by monoclonal antibody M2-1C6. On the other hand, epitope 2 representing the binding site of monoclonal antibody 289/4 is located further downstream toward the carboxy-terminal portion of the M₁ protein; the 15-kilodalton formic acid fragment, which makes up ~65% of the protein at the carboxy terminus, was recognized only by monoclonal antibody 289/4 (to epitope 2). Of potential significance is the finding that smaller peptides representing portions of the amino-terminal 9-kilodalton and carboxy-terminal 15-kilodalton fragments obtained by V8 protease or cyanogen bromide cleavage (25) were not recognized by any of the three monoclonal antibodies (Fig. 2). Furthermore, monoclonal antibody 904/6 (to epitope 3) showed low but significant binding potential only to the 16-kilodalton cleaved product, which is presumably the same as the 14.8-kilodalton V8 cleavage product identified but not further characterized by van Wyke et al. (29). These data collectively suggest a tentative map for all three antigenic determinants on the M₁ protein in which epitope 1 is located somewhere between amino acid residues 8 and 43, whereas both epitopes 2 and 3 are likely to be located between amino acid residues 89 and 141.

Binding of M₁ protein and its cleaved fragments to lipid vesicles. Reconstitution of the M₁ protein of influenza virus with preformed DPPC small unilamellar vesicles was readily demonstrated by dialyzing a mixture of DPPC small unilamellar vesicles and M₁ protein in 10 mM Tricine (pH 7.4)–0.55 M NaCl against salt-free 10 mM Tricine (pH 7.4). Figure 3 illustrates the buoyant density profiles obtained by sucrose gradient centrifugation of DPPC vesicles alone (Fig. 3A) or DPPC vesicles reconstituted with M₁ protein at a protein/lipid molar ratio of 1:100 (Fig. 3B). Vesicles reconstituted with M₁ protein exhibited a single homogeneous peak and displayed an increase in buoyant density of ~0.028

FIG. 2. Staphylococcus V8 protease, cyanogen bromide (CNBr), and formic acid (FA) cleavage sites depicted on a linear map of influenza M₁ protein and the resulting peptides specifically interacting with monoclonal antibodies (Ab) M2-1C6 (directed to epitope 1), 289/4 (to epitope 2), and 904/6 (to epitope 3). The location of each peptide generated by V8 protease or chemical cleavage was determined by sequencing N-terminal amino acids after HPLC purification and aligning the peptide generated with the known amino acid sequence of the M₁ protein of the very closely related influenza virus A/PR/8/34 (34). Binding of monoclonal antibody to each peptide was determined by Western blots (see Fig. 1) and scored as strong reactivity (+) or no detectable binding (-). Protein cleavage methods are described in Materials and Methods. K, Kilodaltons.

g/cm³. Density gradient centrifugation of M₁ protein alone in the absence of lipid vesicles resulted in pelleting of the protein in the centrifuge tube (data not shown). Unlike the M protein of vesicular stomatitis virus (VSV) (32, 36), acidic phospholipids did not enhance liposome binding of the influenza virus M₁ protein but actually resulted in moderate inhibition of vesicle binding (data not shown).

The availability of formic acid and V8 protease peptides provided a means for identifying those domains of M₁ protein involved in its binding to lipid bilayers. To this end, the degree of reconstitution with DPPC vesicles was compared for intact or fragmented M₁ protein. The percent reconstitution calculated from the output-to-input ratio of protein and lipid in the bilayer with intact M₁ protein or its cleaved peptides (purified by HPLC) is presented in Table 1. Intact M₁ protein exhibited nearly 75% binding to the DPPC vesicles under the conditions used in these experiments. Both the formic acid-cleaved 9-kilodalton fragment and the V8 protease-cleaved 10-kilodalton peptide, each derived from the amino-terminal third of M₁ protein, exhibited an ability to bind DPPC vesicles equivalent to that of intact M₁ protein (Table 1). In contrast, the 15-kilodalton carboxy-terminal fragment derived by formic acid hydrolysis of M₁ protein showed only 30% binding with DPPC vesicles. The 4-kilodalton V8 protease-cleaved fragment, located in the same region as the carboxy-terminal 15-kilodalton fragment, also showed ~35% binding to lipid vesicles (data not shown). This level of 30 to 35% binding may represent the basic level of nonspecific protein binding to DPPC vesicles. These results indicate that the N-terminal portion of the M₁ protein exhibits considerable affinity for membrane binding, whereas that portion of the protein corresponding to the carboxy-terminal two-thirds showed significantly less binding affinity to the DPPC lipid bilayer.

We also tested the effect of Fab fragments derived from monoclonal antibodies 289/4 and M2-1C6 on the capacity of the V8 protease-cleaved 10-kilodalton peptide to reconstitute with DPPC vesicles. The Fab fragment from monoclonal

FIG. 3. Comparative buoyant densities of sonicated DPPC vesicles alone (A) or reconstituted in the presence of 1 mol% of M₁ protein of WSN influenza virus (B). All vesicle preparations contained [³H]DPPC in trace amounts to label the lipid, and the M₁ protein was metabolically labeled with [³⁵S]methionine. Buoyant density was determined by flotation upward through 0 to 30% sucrose gradients by centrifugation at 200,000 × g for 16 h at 42°C. Gradients were fractionated, and the samples were counted for ³H and ³⁵S radioactivity by scintillation spectrometry. Densities were determined by refractometry. Symbols: ●, [³H]DPPC; ○, [³⁵S]methionine-labeled M₁ protein; ρ = density (grams per cubic centimeter).

TABLE 1. Comparative binding of M₁ protein or its cleaved peptides to DPPC small unilamellar vesicles and effect of monoclonal antibodies

Protein or peptide ^a	MAB (Fab) present ^b	% Reconstitution with SUV ^c
Intact M ₁ protein	None	73
9 kilodalton (formic acid)	None	78
15 kilodalton (formic acid)	None	30
10 kilodalton (V8 protease)	None	76
10 kilodalton (V8 protease)	289/4 (epitope 2)	69
10 kilodalton (V8 protease)	M2-1C6 (epitope 1)	41

^a M₁ protein was purified from whole virus by acidic chloroform-methanol extraction as described in Materials and Methods. Preparations of formic acid-cleaved 9- and 15-kilodalton fragments were obtained by treating M₁ protein (400 µg) with 200 µl of 75% formic acid at 37°C for 48 h and purified by HPLC. Preparations of V8 protease-cleaved 10-kilodalton fragments were obtained by digesting M₁ protein (50 µg) with *S. aureus* V8 protease (1 µg) at 37°C overnight as described in the text and purified by HPLC.

^b Monoclonal antibodies (MAB) were added as Fab fragments prepared by treating immunoglobulin G monoclonal antibodies M2-1C6 and 289/4 with papain as described in Materials and Methods. The 10-kilodalton peptide and Fab fragments were reacted at a 1:1 molar ratio.

^c M₁ protein or its peptides were reconstituted with DPPC small unilamellar vesicles (SUV) as described in Materials and Methods. The percent reconstitution of M₁ protein or the peptides was measured from input-to-output protein/lipid ratios. The output of ratio of protein to lipid was measured in protein-reconstituted vesicles collected after 0 to 30% sucrose density gradient centrifugation as described in the text.

antibody M2-1C6 (directed to epitope 1), which reacts strongly with this peptide fragment, inhibited the reconstitution process significantly but far from completely (residual 41% binding) (Table 1). On the other hand, the Fab fragment from monoclonal antibody 289/4 (directed to epitope 2) had relatively little effect, if any, on the reconstitution with DPPC vesicles of this 10-kilodalton V8 protease fragment, which does not react with this antibody. To complicate the interpretation of these data, it must be mentioned that none of the Fab fragments from any of the three monoclonal antibodies had any significant effect on the reconstitution of intact M₁ protein with DPPC vesicles (data not shown).

Effect of M₁ protein and its fragments on viral transcription. Zvonarjev and Ghendon (37) demonstrated that the in vitro transcriptase activity of influenza virus was inhibited by ~50% in the presence of the matrix protein. We confirmed this observation by comparing the transcriptase activity of RNP cores rendered largely free of M₁ protein by salt wash with that of whole detergent-disrupted virions at various concentrations. In these experiments, preparations of WSN influenza virus (0.5 mg/ml) were disrupted in 10 mM Tricine (pH 7.4)–0.25 M NaCl–1% NP-40; the released RNP cores were collected by centrifugation through a glycerol pad. The transcriptase activity of RNP cores was measured as a function of equivalent virion protein concentration and compared with the transcriptase activity displayed by whole detergent-disrupted virions of equivalent concentration. The RNP cores lacking most of the M₁ protein exhibited a linear increase in transcriptase activity over a considerable range of concentration, from 0.2 to 1.2 mg/ml (data not shown). In sharp contrast, the transcriptase activity of whole virions, the RNPs of which contained a full complement of M₁ protein, exhibited diminishing levels of RNA synthesis at increasing protein concentrations, reaching a saturating level of transcription around 0.8-mg/ml concentration (data not shown). This inhibition of transcription by whole virions containing M₁ protein, as compared with that of RNP cores essentially free of M₁ protein, clearly suggests that the M₁ protein inhibits the in vitro transcription of influenza virus.

This inhibitory effect of the M₁ protein on viral transcription was further demonstrated in vitro by use of a reconstituted system in which isolated M₁ protein was added to fully transcribable RNP cores. In these experiments, the level of transcription by RNP cores decreased linearly with increasing concentrations of M₁ protein added to the transcription reaction mixture; at an M₁ protein concentration of 0.08 mg/ml the level of RNP transcription was reduced ~75% (data not shown).

The antigenic domain(s) of the M₁ protein potentially involved in its interaction with RNP cores was examined by comparing the transcription of naked RNP cores in the absence or presence of M₁ protein and the three monoclonal antibodies for each distinct epitope (Fig. 4). In these experiments, M₁ protein was incubated without or with increasing concentrations of each of the three separate monoclonal antibodies and then added to transcribing RNP cores in a

FIG. 4. Differential effects of monoclonal antibodies (mAb) on in vitro transcription by RNP cores in the presence of M₁ protein preincubated with increasing concentrations of monoclonal antibodies M2-1C6 (to epitope 1), 289/4 (to epitope 2), or 904/6 (to epitope 3) (A) and in the presence of the formic acid-cleaved 15-kilodalton peptide preincubated with increasing concentrations of monoclonal antibody 289/4 (to epitope 2) (B). RNP cores were prepared by solubilizing whole virions (2.0 mg/ml) in 10 mM Tricine (pH 8.0) containing 0.25 M NaCl, 1% NP-40, and 1.25 mM dithiothreitol as described in Materials and Methods. The 15-kilodalton peptide was obtained by exposing M₁ protein (400 µg) to 200 µl of 75% formic acid for 48 h at 37°C and purified by HPLC as described in Materials and Methods. Transcription reaction mixtures (100 µl) in duplicate contained 10 mM Tris (pH 8.0), 80 mM NaCl, 7.5 mM MgCl₂, 0.12 mM MnCl₂, 0.04 mM ApG, 0.25% NP-40, M₁ protein (40 µg) or the 15-kilodalton peptide (25 µg), 1 mM each ATP, CTP, and GTP, 0.1 mM $[\alpha\text{-}^{32}\text{P}] \text{UTP}$, and various concentrations of monoclonal antibodies. Transcription reaction mixtures (100 µl) in duplicate were incubated for 2 h at 31°C after which the incorporation of $[^{32}\text{P}] \text{UMP}$ into trichloroacetic acid-precipitable materials was measured by scintillation spectrometry. (A) Transcription of RNP cores in the presence of M₁ protein prebound to monoclonal antibody M2-1C6 (●), prebound to monoclonal antibody 289/4 (○), or prebound to monoclonal antibody 904/6 (□); (B) transcription of RNP cores in the presence of the formic acid-cleaved 15-kilodalton fragment (FA-15K) prebound to monoclonal antibody 289/4.

transcription reaction buffer containing 0.08 M NaCl. Addition of M_1 protein alone to the transcription reaction mixture inhibited the incorporation of [32 P]UMP into trichloroacetic acid-precipitable RNA transcripts by 40% (compare RNP alone with RNP plus M_1 protein in the absence of monoclonal antibody) (Fig. 4A). If M_1 protein was first incubated with monoclonal antibody M2-1C6 (to epitope 1) or with monoclonal antibody 904/6 (to epitope 3) and then added to the transcribing RNP cores, the M_1 protein inhibition of transcription activity was not significantly affected. In sharp contrast, increasing concentrations of monoclonal antibody 289/4 (to epitope 2) preincubated with M_1 protein gradually restored RNP transcription and at 0.5 mg/ml almost completely reversed the M_1 protein-inhibited transcription of the RNP cores. This result suggests that the site on the M_1 protein at which epitope 2 is located plays a significant role in the regulation of *in vitro* transcription by influenza virus RNP cores.

Since monoclonal antibody 289/4 (to epitope 2) was the only monoclonal antibody that reacted with the 15-kilodalton fragment generated by formic acid hydrolysis of the M_1 protein (Fig. 2), it was of interest to test the effect of this peptide on the transcriptase activity of RNP cores. This 15-kilodalton oligopeptide, representing the entire carboxy-terminal two-thirds of the M_1 protein, was able to inhibit the transcription of RNP cores by nearly 70% (compare in Fig. 4B RNP alone with RNP plus FA-15K in the absence of monoclonal antibody). Moreover, increasing concentrations of monoclonal antibody 289/4 prebound to this 15-kilodalton fragment were able to reverse the transcription inhibitory activity of this peptide by ~60% (see Fig. 4B). It should also be noted that the 9-kilodalton peptide obtained by formic acid hydrolysis of M_1 protein failed to have any significant effect on the transcription activity of RNP cores (data not shown). These results indicate that the portion of the M_1 protein encompassing the carboxy-terminal two-thirds, where epitope 2 is located, apparently contains the amino acid sequences that mediate interaction of M_1 protein with the RNP complex and regulate its transcription.

DISCUSSION

The matrix (M) proteins of enveloped viruses play a critical role in the assembly process of virions budding from the infected-cell plasma membrane at sites where the integral viral glycoproteins are inserted (7, 8, 31). These M proteins also appear to interact specifically with RNP cores to form a compact infectious unit. Characterization of the domains of M proteins involved in such interactions with membrane and RNP cores could lead to better understanding of the maturation process of enveloped virions. Furthermore, monoclonal antibodies could provide useful probes for recognizing those regions of the matrix proteins responsible for membrane and RNP binding. Three distinct epitopes have been identified thus far in the M_1 protein of influenza virus A/WSN/33 (29). In the present study, these monoclonal antibody-binding sites were partially mapped by chemical and enzymatic cleavages of the M_1 protein. It must also be noted that van Wyke et al. (29) reported somewhat different results in immunoblotting studies with the same three monoclonal antibodies tested with V8 protease-cleaved fragments of M_1 protein, which were not mapped by amino acid sequencing. We can only attribute the discrepancies in our data with those of van Wyke et al. (29) to the different methods used for extraction of M_1 protein and its peptides; they electroeluted their protein and peptides from polyacryl-

amide gels, whereas we extracted the M_1 protein with acidic chloroform-methanol and purified the peptides by HPLC. It seems quite conceivable that altered antigenicity owing to conformational changes resulted from different extraction procedures.

The M_1 protein of influenza virus has been shown to interact with lipid bilayers in a manner similar to that of an integral membrane protein as judged by the fact that a protease-resistant peptide of ≈ 5 kilodaltons was isolated after exposure to trypsin of vesicles reconstituted with M_1 protein (14). This mode of interaction of influenza M_1 protein with lipid bilayers differs from that of the M protein of VSV, which binds to membranes primarily by electrostatic forces and only secondarily by hydrophobic interaction (32, 36). The highly basic M protein of VSV ($pI \approx 9.1$) requires the presence of negatively charged phospholipids in the vesicle for any reconstitution (32, 36). As shown here, the M_1 protein of influenza virus is able to reconstitute with neutral DPPC vesicles better than with mixed lipid vesicles consisting of equimolar amounts of DPPC and acidic DPPG. This striking difference in the mode of reconstitution of the M_1 protein of influenza virus from that of the M protein of VSV is probably attributable to the presence of several long stretches of neutral and hydrophobic amino acids in the influenza M_1 protein sequence (1, 14, 34) compared with no significant stretches of hydrophobic amino acids present in the M protein of VSV (27). However, Rose et al. (26) have also detected a certain degree of homology in the sequences of these two matrix proteins which leads to a prediction of evolutionary conservation in the structure of matrix proteins in general that enables them to perform similar crucial functions leading to virus assembly.

Georgiades and Frangione (14) suggested that the major site of the influenza virus M_1 protein for insertion into the lipid bilayer is located in the middle region containing a large cluster of 20 hydrophobic and neutral amino acids. A second potential membrane insertion site was postulated to be present approximately one-fourth of the way downstream from the amino terminus of the M_1 protein, where a smaller stretch of 13 consecutive uncharged amino acids is located (14). In the present study we showed that both a V8 protease-cleaved 10-kilodalton peptide and a formic acid-cleaved 9-kilodalton peptide, each containing lipophilic amino acid stretches, interact strongly with DPPC vesicles. Each of these fragments represents about 35% of the N-terminal region of the protein and is probably the binding site for reconstitution of the M_1 protein with the lipid bilayer. Of considerable interest is the finding that one of the monoclonal antibodies (M2-1C6 directed to epitope 1) recognized and bound to this fragment and inhibited its membrane reconstitution partially but significantly.

Matrix proteins of rhabdoviruses also interact strongly with RNP cores and regulate their *in vitro* transcription. In the VSV model system, Carroll and Wagner (4) have demonstrated that the M protein of VSV serves as an endogenous inhibitor of *in vitro* viral transcription. This observation was subsequently confirmed and extended in various laboratories (5, 6, 9, 23, 33). In a separate study with influenza virus, Zvonarjev and Ghendon (37) demonstrated that the transcriptase activity of RNP cores was significantly inhibited by purified M_1 protein. This observation was confirmed here by studying the *in vitro* transcription of whole virions and RNP cores at various protein concentrations.

The possible site of interaction of M_1 protein with the RNP complex was examined by studying the effect of monoclonal antibodies and chemically cleaved fragments of M_1 protein

on RNP transcription. The transcriptase activity of RNP cores was inhibited by ~40% in the presence of M₁ protein, but prebinding of monoclonal antibody 289/4 (to epitope 2) to the M₁ protein reversed the transcription inhibition significantly. This suggests that the domain of the M₁ protein corresponding to epitope 2 is involved in its interaction with RNP cores. This was further supported by the fact that monoclonal antibody 289/4 bound strongly to the 15-kilodalton peptide obtained from M₁ protein by formic acid hydrolysis and that this peptide alone also markedly inhibited the *in vitro* transcription of RNP cores. Furthermore, prebinding of monoclonal antibody 289/4 to this 15-kilodalton peptide reversed the inhibited transcription of the RNP complex quite significantly. Since the 15-kilodalton fragment is derived from the carboxy-terminal two-thirds of the M₁ protein, it is quite likely that amino acid sequences within the carboxy-terminal portion of the M₁ protein mediate its interaction with RNP cores. This M₁ protein site appears to be distinct and well separated from the membrane-binding domain localized in the amino-terminal portion of the protein. Further characterization of these domains is required by precise mapping of monoclonal antibody sites in the M₁ polypeptide, as well as the use of deletion mutants.

ACKNOWLEDGMENTS

We thank Anastasia Gregoriades for supplying the virus and for helpful advice and Kathleen van Wyke for hybridoma cells.

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

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