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# Influenza A Virus $M_2$ Protein: Monoclonal Antibody Restriction of Virus Growth and Detection of $M_2$ in Virions

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**The influenza A virus $M_2$ protein is an integral membrane protein of 97 amino acids that is expressed at the surface of infected cells with an extracellular N-terminal domain of 18 to 23 amino acid residues, an internal hydrophobic domain of approximately 19 residues, and a C-terminal cytoplasmic domain of 54 residues. To gain an understanding of the $M_2$ protein function in the influenza virus replicative pathway, we produced and characterized a monoclonal antibody to $M_2$. The antibody-binding site was located to the extracellular N terminus of $M_2$ as shown by the loss of recognition after proteolysis at the infected-cell surface, which removes 18 N-terminal residues, and by the finding that the antibody recognizes $M_2$ in cell surface fluorescence. The epitope was further defined to involve residues 11 and 14 by comparing the predicted amino acid sequences of $M_2$ from several avian and human strains and the ability of the $M_2$ protein to be recognized by the antibody. The $M_2$-specific monoclonal antibody was used in a sensitive immunoblot assay to show that $M_2$ protein could be detected in virion preparations. Quantitation of the amount of $M_2$ associated with virions by two unrelated methods indicated that in the virion preparations used there are 14 to 68 molecules of $M_2$ per virion. The monoclonal antibody, when included in a plaque assay overlay, considerably slowed the growth of some influenza virus strains. This plaque size reduction is a specific effect for the $M_2$ antibody as determined by an analysis of recombinants with defined genome composition and by the observation that competition by an N-terminal peptide prevents the antibody restriction of virus growth.**

Influenza A virus encodes 10 polypeptides derived from eight negative-strand virion RNA segments (for a review, see reference 22). RNA segment 7 encodes two proteins, the matrix (or membrane) protein $M_1$ and an integral membrane protein of $M_r$ ca. 15,000, designated $M_2$ (21, 26, 43). The $M_1$ protein is encoded by an mRNA that is a colinear transcript derived from RNA segment 7, whereas the $M_2$ protein is synthesized from a spliced mRNA (25). $M_1$ and $M_2$ share the same initiation codon for protein synthesis and the eight subsequent amino acid residues before the 5′ splice junction of the $M_2$ mRNA. The remaining 88 amino acids of $M_2$ are predicted to be encoded in the +1 open reading frame after the mRNA 3′ splice junction, and this has been confirmed by using antisera to synthetic peptides (25, 26).

The $M_2$ protein has been shown to be an integral membrane protein that is anchored in membranes by its single hydrophobic and uncharged region of 19 amino acids (residues 25 to 43 [26]). The orientation of $M_2$ in membranes was determined by using antisera prepared to synthetic peptides predicted from the $M_2$ amino acid sequence (26). Protease treatment of $M_2$ expressed at the infected-cell surface and of $M_2$ in intracellular microsomal vesicles followed by immunoprecipitation with site-specific peptide antibodies demonstrated that a minimum of 18 residues at the N terminus of $M_2$ are exposed extracellularly and that the 54-residue C-terminal domain is cytoplasmic (26, 43).

The finding that $M_2$ is abundantly expressed at the plasma membranes of influenza A virus-infected cells led to the suggestion that a possible function for $M_2$ is in the organization and assembly of virion particles (26). That $M_2$ has a role during the influenza virus replicative cycle was further emphasized when influenza viruses that are resistant to the inhibitory effect of amantadine hydrochloride on replication were examined at the molecular level (10). Amantadine hydrochloride resistance has been shown to be linked to RNA segment 7 from an examination of recombinants (2, 9, 30), and nucleotide sequencing of segment 7 virion RNA (vRNA) from 90 individual resistant mutants indicated that single nucleotide substitutions had occurred in the membrane-spanning domain of the $M_2$ protein (10). To understand the mechanism by which $M_2$ exerts its function, particularly with respect to the inhibitory effect of amantadine hydrochloride and its linkage to $M_2$, it is important to determine whether $M_2$ is packaged in virion particles. Although $M_2$ is readily detected at the cell surface, it has not been detected in purified virions under the assay conditions used. However, it was clear that $M_2$ would be greatly underrepresented in virions compared with its relative abundance in infected cells (26).

We report here properties of a monoclonal antibody specific for $M_2$, show that the antibody binds to the extracellular N-terminal domain, and provide evidence that it is able to restrict the replication of some strains of influenza A virus. A quantitive assay indicates there are 14 to 68 molecules of $M_2$ per virion in the virion preparations used.

## MATERIALS AND METHODS

**Viruses and cells.** Influenza A viruses were grown in 10-day-old embryonated eggs or in MDCK cells. A/Singapore/1/57 was provided by Alan Kendal, Centers for Disease Control, Atlanta, Ga. Other human influenza virus strains used are described below. A recombinant virus which was made by crossing A/mallard/NY/78 × A/Bethesda/NY/1/85 (H2N2) and which contains the A/mallard/78 segment 7 was kindly provided by B. R. Murphy. Other avian strains of influenza virus were kindly provided by V. S. Hinshaw and R. G. Webster and have been described previously (43). Recombinants between A/PR/8/34 and A/HK/8/68 were generously provided by J. Schulman, M. Lubeck, and P. Palese and were those used previously (20, 21). CV1 and MDCK

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cells were maintained and infected as described previously (19, 23).

**Production of monoclonal antibodies.** The $M_2$ protein used as antigen for antibody production was purified from A/WSN/33-infected CV1 cell lysates by separation of the $M_2$ protein on polyacrylamide gels and elution as described previously (26). The protein was emulsified with either Freund complete adjuvant (day 0) or Freund incomplete adjuvant (day 14) and injected (50 μl) into footpads of BALB/c mice. The final injection of $M_2$ protein was administered intraperitoneally after 3 weeks, and mice were sacrificed within 3 to 5 days. Splenocytes of immunized animals were fused to SP2/0 myeloma cells as previously described (32). Selection of hybridoma cell lines, screening of the tissue culture fluids by immunoprecipitation of the $M_2$ protein, and limited dilution cloning were done essentially as described previously (7, 18). Hybridoma and SP2/0 cell lines were injected intraperitoneally into pristane-primed BALB/c mice for the production of ascites fluids.

**Isotopic labeling of infected-cell polypeptides, immunoprecipitation, and polyacrylamide gel electrophoresis.** Influenza virus-infected CV1 or MDCK cells were labeled with [$^{35}$S]cysteine as described previously (43). For immunoprecipitation, labeled infected cells were solubilized in RIPA buffer (23) and incubated with either supernatant fluids from hybridoma cultures (at a 1:3 dilution) or ascites fluids (at a 1:100 dilution) at 4°C for 3 h. Rabbit anti-mouse immunoglobulin G (IgG) (20 μg; Cappel Products, Organon Teknika, West Chester, Pa.) was added, the mixture was incubated at 4°C for 30 min, 30 μl of a 1:1 slurry of protein A-agarose (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added, and the mixture was subjected to rotation at 4°C for 1 h. Immune complexes were washed and prepared for electrophoresis as described previously (23). Polypeptides were immunoprecipitated with the C-terminal peptide antiserum, SP2, as described previously (26). For peptide competition experiments, peptide and antibody were allowed to complex for 1 h before labeled proteins were added. Samples were subjected to electrophoresis on 17.5% polyacrylamide gels containing 4 M urea and prepared for autoradiography as described previously (23).

**Proteolysis of the cell surface and microsomal membranes.** To digest proteins with trypsin at the infected-cell surface, [$^{35}$S]cysteine-labeled, infected CV1 cells were scraped from tissue culture plates with a rubber policeman into phosphate-buffered saline (PBS) and incubated with L- (tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemicals, Organon Teknika, West Chester, Pa.) as described previously (26). Intracellular microsomal vesicles were prepared from infected cells and treated with TPCK-trypsin as described previously (43).

**Immunofluorescence and immunoblotting.** Indirect immunofluorescence of cells fixed with freshly prepared 1% paraformadehyde was done essentially as described previously (43). Ascites fluids specific for the $M_2$ protein were diluted 1:300 with PBS containing 1% bovine serum albumin and used to stain the surfaces of infected cells. Bound antibody was detected with fluorescein isothiocyanate-conjugated goat anti-mouse F(ab')$_2$ (Cappel Products). Wheat germ agglutinin was coupled to tetramethylrhodamine isothiocyanate and used as a marker for the Golgi apparatus (41) as described previously (43).

For immunoblotting, proteins were electrotransferred to nitrocellulose filters at 0.15 A in 50 mM Tris-glycine (pH 9.1)–20% methanol for 10 h. To prevent nonspecific adsorbtion of antibody, the nitrocellulose filter was treated with 5% powdered milk–0.05% Tween 20–0.02% sodium azide in PBS for 12 h at room temperature. Ascites fluids directed against the $M_2$ protein were diluted 1:300 in PBS containing 4% bovine serum albumin and incubated with the nitrocellulose blot for 3 h. Excess antibody was removed by successively washing the filters with PBS and PBS containing 0.05% Nonidet P-40, and the filters were incubated with $^{125}$I-labeled goat anti-mouse F(ab')$_2$, essentially as described previously (4). Radioactivity was detected by autoradiography, using preflashed X-ray film (27) and a Cronex Quanta III intensifying screen (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) at −70°C, and data were quantitated by scanning the bands by using an LKB UltroScan XL laser densitometer (Pharmacia, Inc., Piscataway, N.J.).

**Preparation of influenza virus virions, viral RNA, and dideoxy sequencing.** Influenza virus was harvested at 40 h postinfection (p.i.) from the allantoic fluid of infected embryonated eggs or from the medium of MDCK cell monolayers. [$^{35}$S]cysteine-labeled influenza virus was harvested from the culture medium at 40 h p.i. after the addition of 200 μCi of [$^{35}$S]cysteine per ml in cysteine-deficient medium (5 to 40 h p.i.). Virus was clarified by centrifugation at 5,000 × $g$ for 30 min and then pelleted by centrifugation at 40,000 × $g$. The virus was purified on successive 15 to 60% linear sucrose gradients and 15 to 60% potassium tartrate gradients as described previously (19, 39). Protein concentrations were determined by lysing virions in 0.1% sodium dodecyl sulfate and using the Bio-Rad protein microassay on diluted samples (Bio-Rad Laboratories, Richmond, Calif.).

RNA was isolated from purified influenza virus virions by repeated extraction with phenol and chloroform as described previously (20). Oligonucleotide primers to the $M_2$-encoding regions of RNA segment 7, 5'-d(GCAGGTAGATATT)-3', and 5'-d(GTCTAAAAGATGATCTT)-3' were synthesized by the Northwestern University Biotechnology Facility on a 380B DNA synthesizer (Applied Biosystems, Foster City, Calif.) and purified as described previously (44). The appropriate region of RNA segment 7 was sequenced directly by using the dideoxy nucleotide chain-terminating method with reverse transcriptase and the oligonucleotide primers as described previously (1, 13).

**IgG preparation and purification of the $M_2$ protein by affinity chromatography.** IgG from ascites fluids prepared to the $M_2$-specific antibody-producing cell line (designated 14C2) and the fusion parent SP2/0 cell line were affinity purified by protein A-Sepharose chromatography (35). The IgG fractions were concentrated by using Centricon 30 microconcentrators (Amicon Corp., Danvers, Mass.), and the final protein concentration was determined by Bio-Rad microassay. To prepare IgG-specific columns for affinity chromatography, 2 to 3 mg of IgG was coupled to 1 mg of CNBr-activated Sepharose 4B as specified by the manufacturer (Pharmacia).

To affinity purify the $M_2$ protein, influenza virus-infected cells were fractionated on sucrose gradients as described previously (8, 26). Membrane fractions were pooled, lysed in 2% Triton X-100–0.5 M KCl, and diluted to a final concentration of 0.6% Triton X-100 and 0.15 M KCl.The solubilized membrane fractions were preabsorbed on an SP2/0 IgG-Sepharose column, and the flowthrough was applied to the $M_2$-specific IgG-Sepharose column. Bound protein was eluted with 0.1 M sodium bicarbonate buffer (pH 10.8) containing 0.6% Triton X-100. The elution profile of $M_2$ and its relative purity were assessed by analysis of the fractions on polyacrylamide gels and silver staining (Bio-Rad). The concentration of the purified $M_2$ protein was determined by

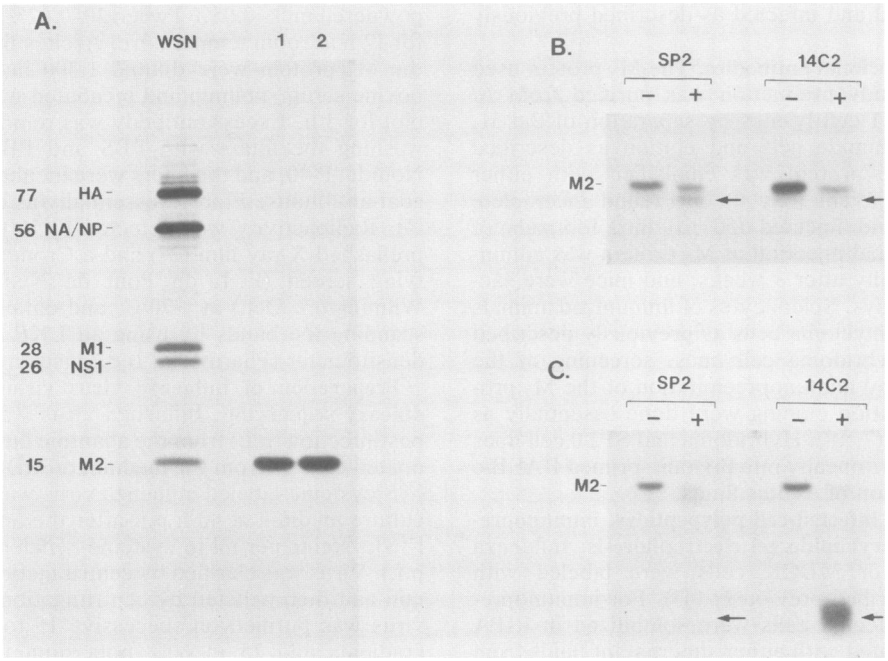


FIG. 1. A monoclonal antibody to the $M_2$ protein recognizes the N-terminal domain. (A) Influenza A/WSN/33 virus-infected CV1 cells were labeled with 100 μCi of [$^{35}$S]cysteine per ml from 6 to 8 h p.i. and immunoprecipitated with 14C2 monoclonal antibody tissue culture supernatant (lane 1) or ascites fluids (lane 2). Lane WSN is a direct lysate of the labeled infected cells. Molecular weights of the infected cell polypeptides are indicated in thousands. (B) Influenza A/WSN/33 virus-infected cells, labeled with [$^{35}$S]cysteine from 6 to 8 h p.i., were removed from the plate and incubated with (+) or without (−) trypsin as described in Materials and Methods. Samples were immunoprecipitated with antisera raised to an $M_2$ C-terminal peptide (residues 69 to 79; lanes SP2) or with tissue culture supernatant from the 14C2 hybridoma line (lanes 14C2). Only the $M_2$ region of the gel is shown, and the trimmed $M_2$ protein ($M_r$ ca. 13,000) is indicated by arrows. (C) Microsomal membrane preparations from influenza A/WSN/33 virus-infected cells labeled at 6 to 7 h p.i. with [$^{35}$S]cysteine and incubated with (+) or without (−) trypsin and immunoprecipitated with SP2 peptide antisera (lanes SP2) or 14C2 hybridoma cell supernatants (lanes 14C2). The $M_r$ ca. 7,000 fragment of $M_2$ is indicated by an arrow.

comparing the silver-stained bands from known quantities of α-lactalbumin ($M_r$ ca. 14,200) and β-lactoglobulin ($M_r$ ca. 18,400) standards (Sigma Chemical Co., St. Louis, Mo.), which were separated on polyacrylamide gels along with $M_2$ protein, dried on sheets of dialysis membrane, and scanned with an LKB UltroScan XL laser densitometer.

**Plaque assays to examine resistance or sensitivity to antibody.** To analyze the resistance or sensitivity of virus replication to the $M_2$-specific antibody in a plaque titration, we diluted virus stocks such that they would yield 50 to 100 plaques on MDCK cells grown in 6-cm dishes. Plaque assays were done essentially as described previously (28). The overlay contained a final concentration of 1% agarose, 1 μg of *N*-acetyl trypsin per ml, and 1× Dulbecco modified Eagle medium. The virus was incubated for 30 min (4°C) with IgG derived from 14C2 and SP2/0 cell line ascites fluids at a final concentration of 5 μg/ml and transferred to the MDCK cell monolayer. The IgG was also included in the agarose-Dulbecco modified Eagle medium overlay at a final concentration of 5 μg/ml. After 2 to 5 days at 37°C, cells were fixed in 2% formaldehyde for 6 h and stained with 0.1% crystal violet in ethanol as described previously (16).

## RESULTS

**A monoclonal antibody recognizes the amino terminus of $M_2$.** To study properties of the influenza A virus $M_2$ protein, including its possible presence in virion particles and its role during virus infection, we required a higher-titer antisera than the $M_2$ antisera to synthetic peptides (SP1 and SP2 [26]). To prepare high-titer monoclonal antibodies, we injected mice with purified $M_2$ protein prepared by elution of the polypeptide from polyacrylamide gels of A/WSN/33-infected cell membrane fractions. The spleens from immunized animals were fused to SP2/0 myeloma cells to produce a hybridoma cell line, designated 14C2, which secretes antibody specific for the $M_2$ protein. The monoclonal antibody derived from the 14C2 culture supernatant (Fig. 1A, lane 1) or ascites fluids (lane 2) immunoprecipitates $M_2$ from [$^{35}$S]cysteine-labeled WSN-infected cells.

To determine biochemically whether the monoclonal antibody recognizes an N-terminal or C-terminal domain of the $M_2$ protein, the surfaces of A/WSN/33-infected cells were treated with trypsin and the polypeptides were immunoprecipitated with the 14C2 monoclonal antibody. As a control for the experiment, the SP2 antipeptide antibody specific for C-terminal residues 69 to 79 was used. Antibody to the SP2 peptide recognizes both untrimmed $M_2$ in transport to the cell surface ($M_r$ ca. 15,000) and trimmed $M_2$ ($M_r$ ca. 13,000) that is digested by trypsin at the cell surface (Fig. 1B). These results confirm our earlier observations (26). The 14C2 antibody recognizes only the untrimmed $M_2$ and not the $M_2$ protein that is proteolytically trimmed at the cell surface (Fig. 1B), suggesting the loss of the antibody-binding epitope. This finding is similar to the lack of recognition of trimmed $M_2$ by the SP1 peptide antibody that is specific for $M_2$ residues 2 to 10 (26).

Direct biochemical evidence that the monoclonal antibody recognizes the N-terminal domain of $M_2$ was provided by

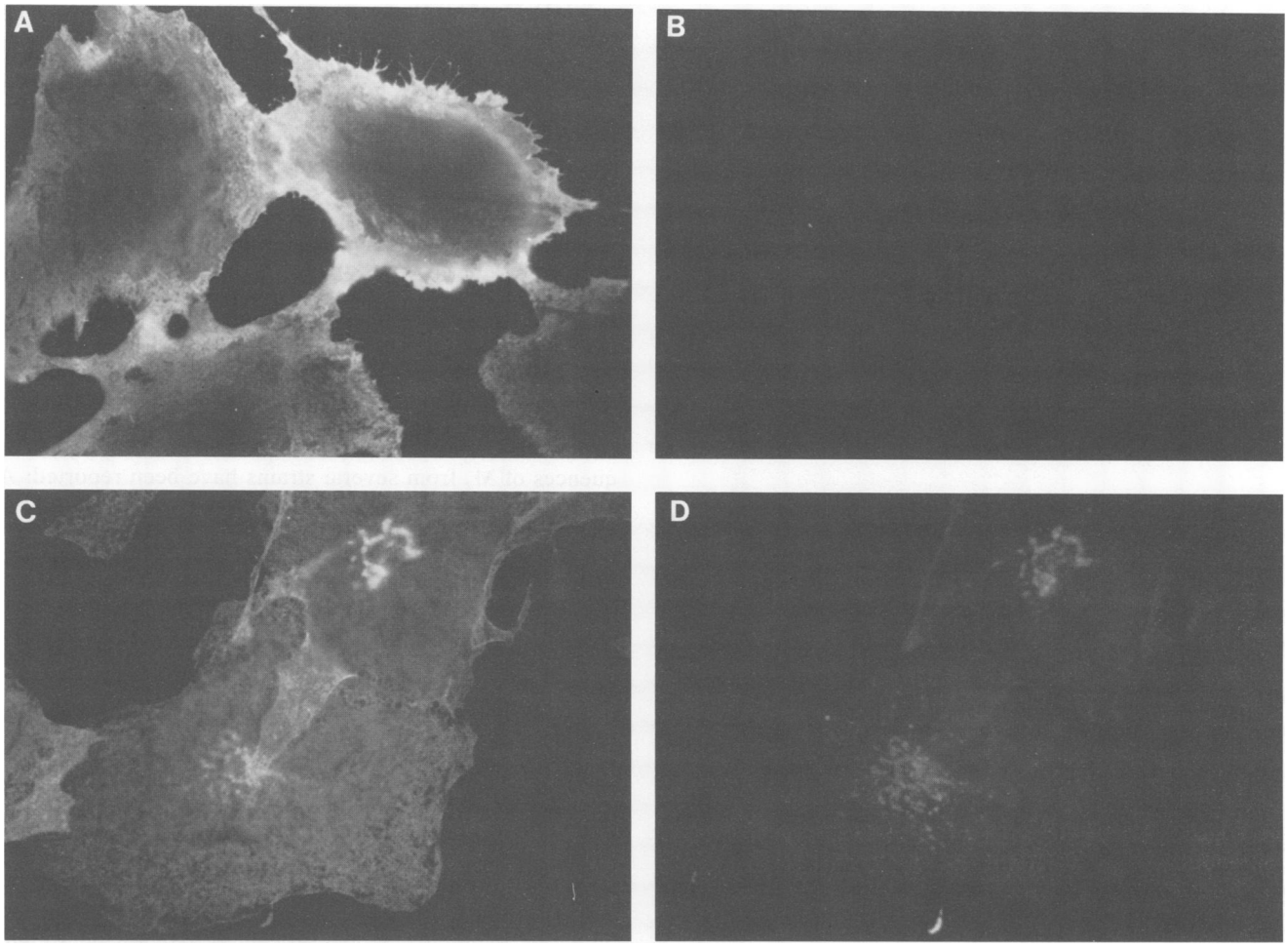


FIG. 2. Immunofluorescent staining of $M_2$ at the cell surface and in the Golgi complex by using the 14C2 monoclonal antibody. Influenza A/WSN/33 virus-infected (A) or mock-infected (B) CV1 cells were fixed with formaldehyde and incubated with 14C2 ascites fluids followed by goat anti-mouse IgG conjugated with fluorescein isothiocyanate as a second antibody. For intracellular staining of $M_2$, cells were fixed and permeabilized with acetone at −20°C for 5 min and stained sequentially with 14C2 ascites fluids, fluorescein isothiocyanate-conjugated goat anti-mouse IgG, and rhodamine-conjugated wheat germ agglutinin. (C) Fluorescein staining of the $M_2$ protein. (D) Rhodamine staining. The exposure time of panel B was manually adjusted to be the same as that of panel A.

immunoprecipitation of $M_2$ from trypsin-treated intracellular vesicles. Microsomal vesicles, prepared by homogenization of [$^{35}$S]cysteine-labeled A/WSN/33-infected cells, were digested with trypsin and immunoprecipitated by using either the SP2 peptide antiserum as a control or the 14C2 monoclonal antibody. The fragment of $M_2$ protected from protease digestion in intracellular membrane vesicles ($M_r$ ca. 7,000) is not recognized by SP2 C-terminal peptide antiserum (Fig. 1C), but is precipitated by the 14C2 monoclonal antibody. We have shown previously that the SP1 peptide antibody also precipitates the N-terminal $M_r$ ca. 7,000 fragment that is protected from protease digestion in intracellular vesicles (43). These data suggest that the 14C2 monoclonal antibody recognizes an N-terminal extracellular region of $M_2$.

To investigate the binding of the 14C2 antibody to the $M_2$ protein in the absence of detergent, we used the antibody in immunofluorescence staining of infected cells. Influenza A/WSN/33 virus-infected CV1 cells were fixed with formaldehyde and incubated with 14C2 antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG as a secondary antibody. The 14C2 monoclonal antibody shows bright surface fluorescence under these conditions (Fig. 2A) and also shows a similar fluorescence pattern on infected live CV1 cells (data not shown). Figure 2B shows the 14C2 surface staining of mock-infected cells as a control. The intracellular staining pattern of $M_2$ observed by using the 14C2 monoclonal antibody on fixed and permeabilized cells is localized to the Golgi apparatus (Fig. 2C), and this intracellular staining pattern is similar to that observed previously with $M_2$-specific antibody (43). Figure 2D shows rhodamine-conjugated wheat germ agglutinin staining of the same field of cells as shown in Fig. 2C to specifically highlight the Golgi apparatus (41).

**Changes in the $M_2$ protein sequence among influenza A virus strains allow for further characterization of the antibody-binding site.** To determine whether the epitope on $M_2$ that is recognized by the 14C2 antibody is conserved in human and avian influenza A virus strains, we examined the antibody recognition of the $M_2$ protein by immunoprecipitation of protein solubilized in RIPA buffer and immunoblotting of sodium dodecyl sulfate-denatured protein transferred to nitrocellulose. MDCK cells were infected with human influenza virus strains (A/WSN/33, A/PR/8/34, A/Singapore/1/57, A/HK/8/68, or A/Udorn/72), the avian influenza virus

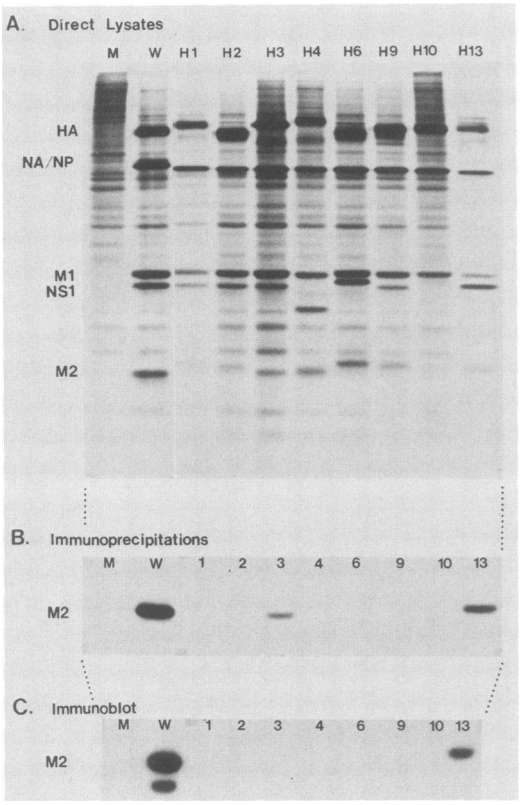


FIG. 3. Immunoprecipitation and immunoblotting of avian strains of influenza A virus by using the 14C2 $M_2$ monoclonal antibody. (A) MDCK cells were infected with avian strains of influenza A virus and labeled with [$^{35}$S]cysteine from 8 to 10 h p.i. Cells were lysed in RIPA buffer, and a sample was analyzed directly on polyacrylamide gels. Lanes: M, mock-infected cells; W, A/WSN/33-infected cells used as a marker for the influenza virus-specific polypeptides; H1, A/duck/Alberta/35/76 (H1N1); H2, A/duck/Germany/12/5/73 (H2N3); H3, A/duck/Ukraine/1/63 (H3N8); H4, A/duck/Czechoslovakia/56 (H4N6); H6, A/turkey/Massachusetts/3740/65 (H6N2); H9, A/turkey/Wisconsin/1/66 (H9N2); H10, A/chick/Germany/N/49 (H10N7); H13, A/gull/Maryland/704/77 (H13N6). (B) The lysates shown in panel A were immunoprecipitated with 14C2 ascites fluids as described in Materials and Methods. Only the $M_2$ region of the gel is shown. (C) Immunoblot of $M_2$ polypeptides detected with 14C2 ascites fluids. MDCK cells were infected with the various subtypes of avian influenza viruses, and at 8 h p.i. cells were lysed and immunoprecipitated with the $M_2$-specific SP2 C-terminal region peptide antiserum to increase the amount of $M_2$ protein on the immunoblot. The $M_2$ polypeptide of all avian strains used can be immunoprecipitated with the SP2 antiserum (43). The immunoprecipitated polypeptides were separated on a polyacrylamide gel and electrophoretically transferred to nitrocellulose filters, and the filters were probed with 14C2 ascites fluids followed by $^{125}$I-labeled goat anti-mouse F(ab')$_2$ as described in Materials and Methods. Only the $M_2$ region of the autoradiogram is shown.

strains as indicated in Fig. 3, or a recombinant virus containing A/mallard/NY/78 segment 7 vRNA, and labeled with [$^{35}$S]cysteine. Lysates were either analyzed directly to show the relative amounts of $M_2$ synthesized with the different strains (Fig. 3A) or immunoprecipitated with the 14C2 monoclonal antibody (Fig. 3B). The antibody immunoprecipitates $M_2$ from all human influenza virus strains tested including A/WSN/33 (Fig. 3B), A/PR/8/34, A/Singapore/1/57, A/HK/8/68, A/Udorn/72, A/FW/1/50, and A/USSR/90/77 (data not shown) and the avian strains A/duck/Ukraine/63 (H3) and A/gull/MD/77 (H13) (Fig. 3B). The 14C2 antibody also recognizes the $M_2$ protein immobilized on nitrocellulose of all human strains tested including A/WSN/33 (Fig. 3C), A/PR/8/34 (see Fig. 5, lane 6), A/Singapore/1/57, A/HK/8/68, and A/Udorn/72 (data not shown), and the avian strain A/gull/MD/77 (H13) (Fig. 3C). Interestingly, the A/duck/Ukraine/63 (H3) $M_2$ protein was not detected by the antibody in the immunoblotting assay. These data for the influenza A viruses tested are summarized in Fig. 4. The observed strain-specific differences in migration of the $M_2$ polypeptide have been observed previously (21, 43). The lower band in the WSN lane of the immunoblot lane (Fig. 3C, lane W) is presumed to be an $M_2$-specific breakdown product which is occasionally seen on overexposed autoradiograms.

To examine for changes in the $M_2$ which could account for some of the differences in 14C2 recognition, we compared the amino acid sequences of $M_2$ from the influenza virus strains described above. The predicted amino acid sequences of $M_2$ from several strains have been reported: A/Udorn/72 (24), A/PR/8/34 (42), A/Singapore/1/57 (10), and A/mallard/NY/6750/78 (3). For the 10 other virus strains tested, the predicted amino acid sequence of the $M_2$ N terminus and hydrophobic domain (resides 1 to 43 [Fig. 4]) was obtained by determining the nucleotide sequence of the appropriate region of their segment 7 vRNAs. The strain-specific differences in the N-terminal amino acid sequence and the ability of the 14C2 antibody to recognize $M_2$ in immunoprecipitation and immunoblot assays correlates with changes in residues 11 and 14. The 14C2 antibody was prepared to $M_2$ purified from A/WSN/33-infected cells, and it can be seen in Fig. 4 that only strains with isoleucine at position 11 can be recognized by the 14C2 antibody in immunoprecipitation assays. In the immunoblot assay, only strains that have isoleucine at residue 11 and glutamic acid at residue 14 can be recognized by the 14C2 antibody. The conformation of the $M_2$ protein that is recognized by the antibody in RIPA buffer or immobilized on nitrocellulose following sodium dodecyl sulfate-gel electrophoresis is not known, but these data suggest that these conformations are not identical.

**Identification of $M_2$ in virions.** To examine for the presence of the $M_2$ protein in purified influenza virus virions by using a highly sensitive assay, we isolated A/WSN/33 and A/PR/8/34 virions from the allantoic fluid of infected embryonated eggs and A/WSN/33 virions from infected MDCK cells and purified them as described in Materials and Methods. Viral proteins (10 μg) were separated on polyacrylamide gels and electrophoretically transferred to nitrocellulose, and the blots were probed with 14C2 antibody followed by $^{125}$I-labeled goat anti-mouse F(ab')$_2$. Influenza virus-infected MDCK cell lysates, unlabeled and [$^{35}$S]cysteine labeled, were used as controls for antibody recognition and as appropriate size markers on the immunoblot. $M_2$ could be detected in preparations of A/WSN/33 virions grown in eggs (Fig. 5, lane 3) or MDCK cells (lane 4) and in virions of A/PR/8/34 grown in eggs (lane 7). By using this immunoblot assay, the $M_2$ protein can also be detected in A/Singapore/1/57, A/HK/8/68, and A/Udorn/72 influenza virions purified from embryonated eggs (data not shown). The presumed proteolytic cleavage product seen in A/WSN/33-infected MDCK cells (Fig. 3C) was also observed with A/WSN/33 virions grown in MDCK cells (Fig. 5, lane 4). The amount of $M_2$ detected in the infected-cell lanes (Fig. 5, lanes 1, 2, 5, and 6) was adjusted to give similar exposure times to those for the virion lanes, and the amount of radioactivity cannot be compared.

**Quantitation of $M_2$ in virions.** To estimate the number of

| | 1 M S L L T E V E T P I R N E W G C R C N D S S D P L V V A A S I I G I L H L I L W I L D | Immuno-precip | Immuno-blot |
|---|---|---|---|
| A/Udorn/72 | M S L L T E V E T P I R N E W G C R C N D S S D P L V V A A S I I G I L H L I L W I L D | + | + |
| A/WSN/33 | * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * | + | + |
| A/PR/8/34 | * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * | + | + |
| A/Sing/1/57 | * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * | + | + |
| A/HK/8/68 | * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * | + | + |
| • A/mallard/NY/6750/78 | T * G E K * S * * I * * * * * * * * * * * * * * * * * * * * * * * | - | - |
| (H1)-A/duck/Alberta/35/76 | T * G E K * S * * I * * * * * * * * * * * * * * * * * * * * * * * | - | - |
| (H2)-A/duck/Germany/1215/73 | T * G E K Y S * * I * * * * * * * * * * * * * * * * * * * * * * * | - | - |
| (H3)-A/duck/Ukraine/1/63 | * * G E * * S * * I * * * * * * * * * * * * * * * * * * * * * * * | + | - |
| (H4)-A/duck/Czech/56 | T * G E * Y S G * I * * * * * * * * * * * * * * * * * * * * * * * | - | - |
| (H6)-A/turkey/MA/3740/65 | T S G E K * S * I F T * V * * * * * * * * * * * * * * * * * * * * | - | - |
| (H9)-A/turkey/WI/1/66 | T * G E K * S * * I * * * * * * * * * * * * * * * * * * * * * * * | - | - |
| (H10)-A/chick/Germany/N/49 | T * G E * * S * * I * * * * * * * * * * * * * * * * * * * * * * * | - | - |
| (H13)-A/gull/MD/704/77 | * * * E * * S * * I * * * * * * * * * * * * * * * * * * * * * * * | + | + |

FIG. 4. Correlation of the $M_2$ N-terminal region predicted amino acid sequences with the reactivity of the 14C2 antibody for $M_2$ in immunoprecipitation and immunoblot assays. The predicted amino acid sequences of the N-terminal 44 residues of the $M_2$ protein of influenza A/WSN/33 virus and avian influenza virus H1 to H13 were determined by dideoxy primer extension sequencing of RNA segment 7 with oligonucleotides complementary to nucleotides 8 to 20 and 703 to 724 of the A/Udorn/72 vRNA segment 7 sequence. Nucleotide sequences reported previously are A/Udorn/72 (24), A/PR/8/34 (42), A/Singapore/1/57 (10), and A/mallard/NY/6750/78 (3). The A/Udorn/72 sequence is shown in the single-letter code, and a blank or an asterisk denotes identity with the A/Udorn/72 sequence. The virus indicated as ●A/mallard/NY/6750/78 is a recombinant containing RNA segment 7 from the avian virus, and the hemagglutinin subtype is omitted. A summary of the results obtained from immunoprecipitation and immunoblotting experiments for the avian strains (data from Fig. 3) and for the human strains (data not shown) with the 14C2 monoclonal antibody are listed, with + and − denoting the ability or lack of ability, respectively, to recognize the $M_2$ protein. The arrows indicate the positions of $M_2$ residues 11 and 14, which are discussed in the text. Other RNA segment 7 nucleotide sequences reported previously include A/FPV/Weybridge/27 (10), A/FPV/Rostock/34 (31), and A/Bangkok/1/79 (33), but these virus strains could not be tested in the assays.

$M_2$ molecules found per influenza virion, we used two independent methods: (i) a quantitative immunoblot procedure and (ii) a direct analysis of the amount of $M_2$ found in radioactively labeled virions. To quantitate the amount of $M_2$ identified in virions by an immunoblot assay, we required a source of pure $M_2$ as a concentration standard. The $M_2$ protein was purified from membrane fractions of A/WSN/33-infected cells by affinity chromatography with the 14C2 antibody covalently coupled to Sepharose. The eluted protein was examined for purity on silver-stained gels, and the specific concentration of the $M_2$ protein was calculated from densitometer scans of the $M_2$ band with known amounts of α-lactalbumin ($M_r$ ca. 14,200) and β-lactoglobulin ($M_r$ ca. 18,400) as standards (data not shown). Figure 6A shows an example of an immunoblot used to compare known concentrations of purified $M_2$ with varied amounts of total virion protein. The amount of secondary $^{125}I$ signal bound to purified $M_2$ protein was quantitated by densitometric scanning of the autoradiogram to create a standard plot of $^{125}I$ signal versus protein concentration. The $M_2$-specific $^{125}I$ signals from total virion protein lanes were compared with the $M_2$ standards to establish a ratio of $M_2$ protein to total virion protein (Table 1). The number of $M_2$ molecules and virion molecules was calculated from these ratios by using the molecular weight of A/WSN/33 $M_2$ as 11,392 (unpublished data) and an assumed average virion molecular weight of $2.1 \times 10^8$ (15). The results from three separate immunoblot experiments indicate that 14 to 68 molecules of $M_2$ are associated with an influenza virus virion particle (Table 1). Data obtained from direct $^{125}I$ counting of the $M_2$ regions of the nitrocellulose blot, rather than densitometer scanning, yielded a similar number of $M_2$ molecules per virion (data not shown).

The amount of $M_2$ per virion was also calculated by a direct analysis with virions labeled to high specific activity

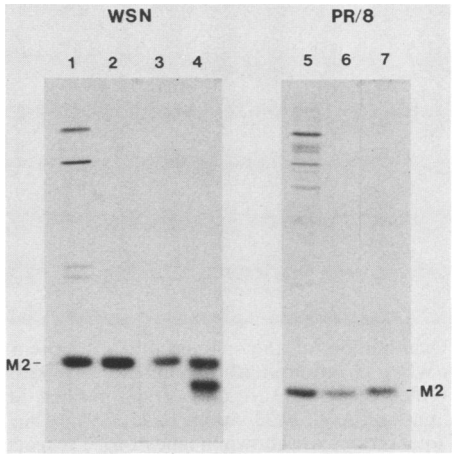


FIG. 5. Detection of $M_2$ protein by immunoblotting in preparations of influenza A/WSN/33 and A/PR/8/34 virions. Influenza A/WSN/33 and A/PR/8/34 viruses were grown in embryonated eggs, and A/WSN/33 virions were also grown in MDCK cells; the virions were purified as described in Materials and Methods. Total virion protein (10 μg) was separated on polyacrylamide gels. As markers, influenza A/WSN/33 or A/PR/8/34 virus-infected [$^{35}S$]cysteine-labeled or unlabeled CV1 lysates were run on the same polyacrylamide gel. The polypeptides were electrophoretically transferred to nitrocellulose filters and incubated with the 14C2 monoclonal antibody (ascites fluids) followed by $^{125}I$-labeled goat anti-mouse F(ab')$_2$ and autoradiography. Lanes: 1, [$^{35}S$]cysteine-labeled A/WSN/33-infected CV1 cells; 2, unlabeled A/WSN/33-infected CV1 cells; 3, A/WSN/33 virions grown in eggs; 4, A/WSN/33 virions grown in MDCK cells; 5, [$^{35}S$]cysteine-labeled A/PR/8/34-infected CV1 cells; 6, unlabeled A/PR/8/34-infected CV1 cells; 7, A/PR/8/34 virions grown in eggs. The infected-cell marker lanes were adjusted to produce a similar exposure period as the virion protein lanes.

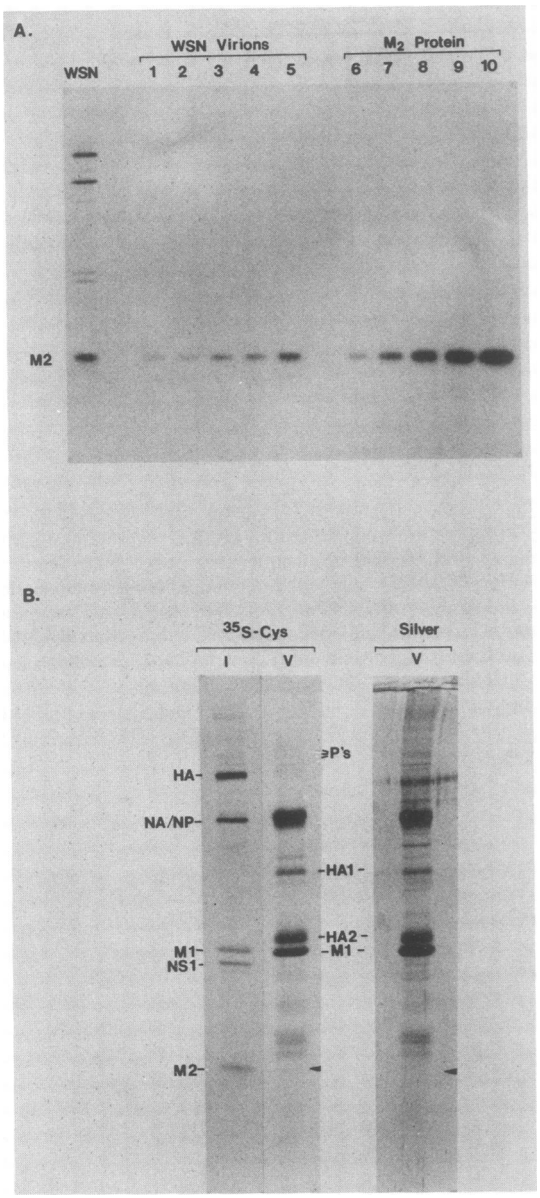


FIG. 6. Quantitation of the amount of $M_2$ protein found in influenza A/WSN/33 virions. (A) Immunoblot comparison of $M_2$ found in purified A/WSN/33 virions versus purified $M_2$ protein. Dilutions of purified A/WSN/33 virions (6.25, 7.95, 9.28, 10.60, and 13.25 μg of total protein are shown in lanes 1 to 5, respectively) and affinity-purified $M_2$ protein (6.8, 13.6, 20.4, 27.2, and 34.0 ng are shown in lanes 6 to 10 respectively) were separated by electrophoresis and transferred to nitrocellulose. The nitrocellulose blot was incubated with 14C2 ascites fluids, and the $M_2$ protein was visualized by incubation with $^{125}$I-labeled goat anti-mouse F(ab')$_2$ and autoradiography. A [$^{35}$S]cysteine A/WSN/33-labeled infected-cell lysate was included as a marker. The $^{125}$I signals from the individual lanes were quantitated by densitometric scanning. The results of this quantitation are shown in Table 1. (B) Direct analysis of [$^{35}$S]cysteine-labeled virion particles. Influenza A/WSN/33 virus-infected MDCK cells were labeled with [$^{35}$S]cysteine (200 μCi/ml) from 5 to 40 h p.i., and the virions were purified as described in Materials and Methods. The virion polypeptides were separated by polyacrylamide gel electrophoresis, the gel was silver stained, and autoradiography was performed. [$^{35}$S]cysteine-labeled influenza A/WSN/33 virus-infected MDCK cell lysates were used as markers for the $M_2$ polypeptide. Left panels, [$^{35}$S]cysteine-labeled samples. Right panel, silver-stained virion polypeptides. Lanes V, purified A/WSN/33 virion preparations. Lane I, A/WSN/33-infected MDCK cell lysate. Arrowheads indicate the $M_2$ polypeptide. The amount of radioactivity contained in the [$^{35}$S]cysteine-labeled $M_2$, $M_1$, and $HA_2$ polypeptides was determined by scintillation counting. Tabulation of the data is shown in Table 2.

TABLE 1. $M_2$ molecules per WSN virion determined by immunoblotting

| Expt[a] | Ratio of virion $M_2$ protein to total protein[b] | | Molecules of $M_2$/virion[c] |
|---|---|---|---|
| | Amt of $M_2$ protein (ng) | Amt of WSN virions (μg) | |
| I. i | 8.12 | 10.60 | 14.2 |
| I. ii | 15.60 | 13.25 | 21.8 |
| II. i | 4.08 | 1.86 | 40.6 |
| II. ii | 8.16 | 2.23 | 67.7 |
| III. i | 4.08 | 2.29 | 26.4 |
| III. ii | 8.16 | 4.44 | 34.0 |
| III. iii | 12.24 | 5.91 | 38.4 |

[a] Data are calculated from three immunoblots (I, II, and III) such as that shown in Fig. 6A. The ratio of virion-associated $M_2$ to total virion protein was compared for separate virion lanes (i, ii, and iii) on each immunoblot that matched the standard $M_2$ plot as described below.

[b] The $M_2$ region of immunoblot autoradiograms was scanned by densitometry to develop a standard plot of nanograms of $M_2$ protein versus bound $^{125}$I. From this plot, the amount of $^{125}$I associated with $M_2$ in a known concentration of purified virions was determined.

[c] Determination of the number of molecules was based on an assumed influenza virion molecular weight of $2.1 \times 10^8$ (15) and an $M_2$ protein $M_r$ of 11,392 for WSN (unpublished results).

with [$^{35}$S]cysteine. Influenza A/WSN/33 virions were purified from infected MDCK cells after a 40-h incubation in the presence of [$^{35}$S]cysteine. Viral proteins were separated on polyacrylamide gels, and the gels were silver stained and prepared for autoradiography. The amount of $M_2$ detected in virions by either silver stain or autoradiography was much lower than that of the $M_1$, NP, $HA_1$, or $HA_2$ viral proteins (Fig. 6B). The silver-stained gel is shown so that an assessment of the relative purity of the virion preparations can be made. To quantitate the amount of $M_2$ protein contained in the [$^{35}$S]cysteine-labeled virions, the $M_2$, $HA_2$, and $M_1$ protein bands were excised from the gel by using the autoradiogram as a template and the amount of radioactivity associated with each band was determined by scintillation counting. The results from three separate experiments are shown in Table 2. From the known sequences of the WSN $M_1$, $M_2$, and $HA_2$ proteins (14; unpublished observations), the cysteine content was determined and the relative number of moles of each polypeptide was calculated. From the number of known molecules of $M_1$ and $HA_2$ calculated previously to be in each virion (6, 15, 39) and presented in Table 2, it was determined that there are 16 to 67 molecules of $M_2$ per virion in the A/WSN/33 preparations used. The relative level of $M_2$ detected by autoradiography is similar to the 15 to 34 molecules of the polymerase (P) proteins (Fig. 6B) determined to be associated with each virion particle (15). However, the P proteins were not used in the quantitation of $M_2$ in virions, since the low levels of radioactivity could lead to significant errors in the calculations.

**The $M_2$ antibody restricts influenza virus replication.** Since the above data indicate that the 14C2 antibody recognizes an extracellular region of $M_2$, we investigated whether the

TABLE 2. $M_2$ molecules per WSN virion estimated from [$^{35}$S]cysteine labeling of virions

| Polypeptide | [$^{35}$S]cysteine cpm[a] in expt: | | | Known molecules per virion[b] | $M_2$ molecules/virion relative to $M_1$ or $HA_2$[c] in expt: | | |
|---|---|---|---|---|---|---|---|
| | I | II | III | | I | II | III |
| $M_2$ | 1,013 | 405 | 729 | | | | |
| $M_1$ | 45,206 | 29,047 | 53,618 | 2,300–3,000 | 51–67 | 32–42 | 31–41 |
| $HA_2$ | 23,287 | 12,362 | 22,633 | 500–1,200 | 22–52 | 16–39 | 16–39 |

[a] The [$^{35}$S]cysteine counts per minute obtained per isolated polypeptide band as shown in Fig. 6B were corrected for the number of cysteine residues predicted from the WSN amino acid sequences of $M_1$ and $M_2$ (unpublished observations) and $HA_2$ (14).

[b] The values shown for molecules of $M_1$ and $HA_2$ per influenza virion are in the range previously reported (6, 14, 39).

[c] The range of $M_2$ molecules associated with WSN virions was calculated by using the upper and lower values for the number of $M_1$ and $HA_2$ molecules contained in virions.

monoclonal antibody can affect the growth of influenza virus in a plaque assay. The IgG purified from either the SP2/0 or 14C2 ascites fluids was included in the overlay of an influenza virus plaque assay in MDCK cells. The effect of the presence of 5 μg of 14C2 IgG per ml on the growth of influenza virus strains A/WSN/33 and A/Udorn/72 is shown in Fig. 7. The size and number of plaques grown in the presence of IgG isolated from the ascites fluids of the fusion parent SP2/0 cell line are identical to those of the control (without IgG; top panel). No effect was observed on the size or number of plaques for the A/WSN/33 strain. However, the size of plaque, but not the number of plaques, for the A/Udorn/72 virus after an equal incubation time in the presence of 5 μg of 14C2 IgG per ml was considerably smaller than in the control plaque assays (without IgG; top panel). Addition of the antibody to the virus dilutions before infection had no effect on plaque size. Other virus strains tested in a similar experiment with and without 14C2 antibody, including A/FW/1/50, A/Singapore/1/57, A/HK/8/68, A/USSR/90/77, and A/PR/8/34, were found to have greatly reduced plaque size in the presence of 14C2 antibody, except A/PR/8/34, which, like WSN, had a normal plaque size. The A/gull/MD/77 (H13) strain of influenza virus, which also synthesizes an $M_2$ protein recognized by the 14C2 antibody in immunoprecipitation assays, did not form plaques in the MDCK cell line used for this multiple cycle replication experiment. As would be expected, influenza A virus strains for which the $M_2$ protein is not recognized by the monoclonal antibody did not show a reduced plaque size in the presence of the antibody.

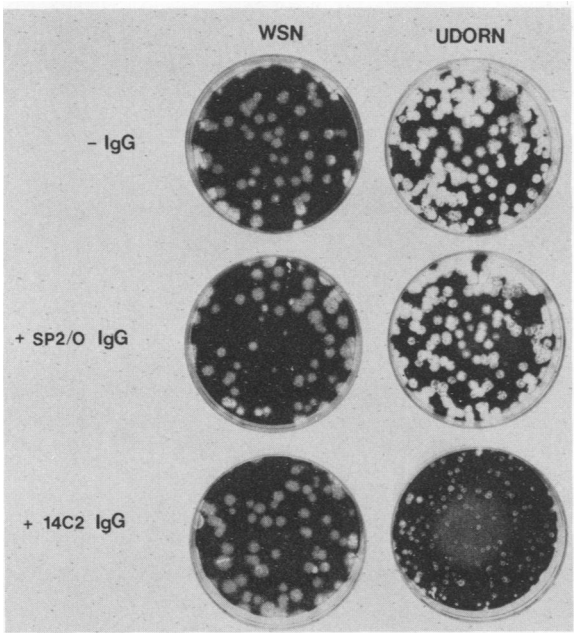


FIG. 7. The 14C2 monoclonal antibody can inhibit plaque growth of influenza A virus. MDCK cells were infected with influenza A/WSN/33 or A/Udorn/72 virus at appropriate dilutions and overlaid with agarose-DME containing 1 μg of *N*-acetyl trypsin per ml and IgG as indicated. After 3 days (A/WSN/33-infected cells) or 4 days (A/Udorn/72-infected cells) of incubation at 37°C, plaques were fixed with formaldehyde and stained with crystal violet. Plates −IgG, no antibody addition to virus dilution and overlay; plates +SP2/0 IgG, addition of 5 μg of IgG per ml from SP2/0 ascites fluid to virus dilution and overlay; plates +14C2 IgG, addition of 5 μg of IgG per ml from 14C2 ascites fluid to overlay.

To investigate the effect of the 14C2 antibody concentration on the plaque size of the A/WSN/33 and A/Udorn/72 influenza virus strains, we included the 14C2 IgG in a plaque assay overlay at concentrations ranging from 0.001 to 25 μg/ml. The plaque size of the A/WSN/33 strain was not reduced at any of the concentrations tested. However, the plaque growth of the A/Udorn/72 strain was inhibited at antibody dilutions down to 0.1 μg/ml. The specificity of the inhibitory effect of the 14C2 antibody on the growth of the A/Udorn/72 virus was also monitored by using antibodies at the same concentration which should have no effect at the cell surface. The replication of A/Udorn/72 was not affected by antibody to the influenza virus membrane protein ($M_1$) (antibody 904/6, generously made available by Kathleen van Wyke Coelingh [40]) or to antibody which recognizes the C proteins of the cellular heterogeneous nuclear ribonucleoprotein complex (antibody 4F4, generously made available by Gideon Dreyfuss [5]).

**Examination of the specificity of the antibody effect on virus growth.** As described above, the 14C2 antibody restricts the growth of all influenza virus strains tested including A/Udorn/72 and A/HK/8/68, yet it does not affect A/WSN/33 or A/PR/8/34. Since the antibody binds to the $M_2$ protein of these four strains as tested by immunofluorescence, immunoprecipitation, and immunoblotting, it was important to rule out a nonspecific effect of the antibody in reducing plaque size. We used 11 recombinants between strains A/PR/8/34 (antibody insensitive) and A/HK/8/68 (antibody sensitive) with gene segments of defined parental origin (characterized by J. L. Schulman, M. D. Lubeck, and P. Palese) to test for segregation of the plaque size phenotype with RNA segment 7. Acquisition of antibody resistance from the A/PR/8/34 parent or antibody sensitivity from the A/HK/8/68 parent (Table 3) segregates only with RNA segment 7 which encodes both the $M_1$ and $M_2$ proteins.

To further investigate the specificity of virus-restricted growth by the 14C2 antibody, we synthesized a peptide to the $M_2$ N terminus for use in competitive binding studies.

TABLE 3. Analysis of A/PR/8/34 and A/Hong Kong/8/68 recombinants

| Recombinant | Segment derived from PR/8 or HK[a] | | | | | | | | Sensitivity to 14C2 antibody[b] |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
| 1 | P | P | P | H | P | H | P | P | R |
| 2 | H | H | H | H | P | H | P | P | R |
| 3 | H | H | H | H | H | H | P | H | R |
| 4 | H | P | P | H | P | H | P | P | R |
| 5 | P | P | P | H | P | P | H | P | S |
| 6 | P | P | P | H | P | P | P | H | R |
| 7 | H | H | P | P | H | H | P | P | R |
| 8 | H | P | H | P | P | H | P | P | R |
| 9 | H | H | H | P | H | P | H | H | S |
| 10 | H | H | H | P | H | P | H | P | S |
| 11 | H | H | H | H | P | H | P | H | R |

[a] P=A/PR/8/34; H=A/HK/8/68. Segments 1, 2 and 3 encode $PB_2$, $PB_1$, and PA; 4 encodes HA; 5 encodes NP; 6 encodes NA; 7 encodes $M_1$ and $M_2$; 8 encodes $NS_1$ and $NS_2$. Data obtained from J. L. Schulman, M. D. Lubeck, and P. Palese (30).

[b] Sensitivity (S) or resistance (R) to the 14C2 monoclonal antibody was determined by plaque size reduction in the presence of 5 μg of IgG per ml.

The synthetic peptide contains residues 2 to 24 of the N-terminal region of the A/Udorn/72 $M_2$ protein (26) and was generously made available by G. Vande Woude. Incubation of the 14C2 antibody with various concentrations of the peptide (0.2 to 10 μg/ml) prior to immunoprecipitation of influenza A/Udorn/72 virus-infected cell lysates was able to block the antibody recognition of the $M_2$ protein (data not shown). In a plaque assay, when the N-terminal peptide (2.5 μg/ml) was incubated with the 14C2 antibody (5 μg/ml) for 1 h before being mixed with the overlay, no reduction in plaque size of influenza A/Udorn/72 virus was observed (Fig. 8). These data strongly suggest that the antibody-mediated reduction in plaque size is a specific effect of the antibody binding to the extracellular N-terminal domain of $M_2$ at the infected-cell surface.

## DISCUSSION

The 14C2 monoclonal antibody recognizes the extracellular N-terminal domain of the influenza A virus $M_2$ protein as shown by proteolysis of whole cells and intracellular microsomes and by immunofluorescence at the infected-cell surface. The ability of the monoclonal antibody to recognize the $M_2$ protein synthesized from avian and human strains in immunoprecipitation and immunoblot assays was correlated with the predicted $M_2$ N-terminal amino acid sequences of these strains. These data indicate that isoleucine at residue 11 and glutamic acid at residue 14 are important for antibody binding to the $M_2$ protein. Other amino acids at the $M_2$ N terminus may also be involved, yet conservation of the residues which surround positions 11 and 14 does not allow for further characterization of antibody binding. Although this region of $M_2$ probably constitutes the antibody-binding site, the observed changes could cause a conformational change in the protein, which leads to an inability of the antibody to bind at another site (29). The predicted amino acid sequences of the human and avian strains obtained here also indicate that the $M_2$ N-terminal residues 1 to 10 are conserved in all strains examined. The significance of this conservation in $M_2$ is not known, because $M_1$ and $M_2$ share N-terminal residues 1 to 9 (owing to their mRNA structures [25]), and conservation of this region could be an important property for the $M_1$ protein. It can also be observed that the potential N-linked glycosylation site (residues 20 to 22) found in $M_2$ of human strains and shown not be used (43) is lost in the avian strains. This provides further evidence that the site for N-linked glycosylation in the human strains is fortuitous.

Our previous attempts to search for $M_2$ as a component of purified virions had been unsuccessful. To increase the sensitivity of the procedure, we used the 14C2 monoclonal antibody to detect the $M_2$ polypeptide with $^{125}I$-labeled secondary antibody in an immunoblot assay of purified virions. This assay indicated that the $M_2$ protein can be detected in influenza A/WSN/33 or A/PR/8/34 virions grown in eggs (Fig. 3) and in influenza A/WSN/33 virions grown in MDCK cells. The $M_2$ protein was also detected in MDCK cell-grown A/WSN/33 virions by [$^{35}S$]cysteine direct labeling. The quantitative methods used for this study indicate that there are 14 to 68 molecules of $M_2$ on average per A/WSN/33 virion particle grown in either eggs or MDCK cells. The amount of $M_2$ found in virions is small, and the calculations would probably be overestimated if virion preparations were contaminated by copurification from the infected cells of plasma membrane or cytoplasmic vesicles containing $M_2$. The finding that there are 14 to 68 molecules of $M_2$ per virion with the known 500 to 1,200 molecules per virion of HA (6, 15) suggests that the $M_2$ protein is selectively excluded from virion particles, particularly when compared with an accumulation of $M_2$ to HA molecules in infected cells in the ratio of 1:1.5.

The $M_2$-specific antibody is not able to inhibit virus adsorption or penetration of virions, but the rate of plaque growth for many influenza virus strains is reduced in the presence of the $M_2$ antibody. The antibody-sensitive strains include A/Udorn/72, A/HK/8/68, A/Singapore/1/57, A/FW/1/50, and A/USSR/90/77. Influenza viruses A/WSN/33 and A/PR/8/34 showed no growth restriction in the presence of the

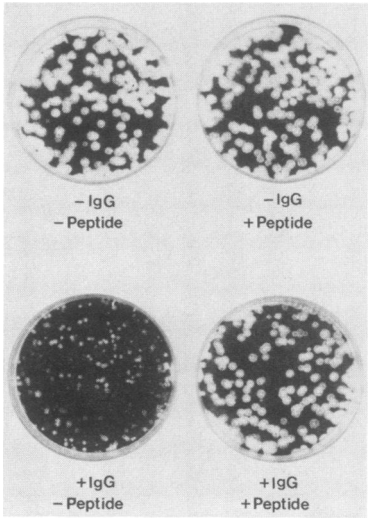


FIG. 8. Restriction of plaque growth by the 14C2 antibody is blocked by preincubation of the antibody with an N-terminal $M_2$ peptide. MDCK cells were infected with influenza A/Udorn/72 virus at a dilution that yielded 50 to 100 plaques per 6-cm plate of cells. Where indicated, agarose overlays included 5 μg of 14C2 IgG per ml or 2.5 μg of an N-terminal peptide synthesized to residues 2 to 24 inclusive of the predicted amino acid sequence of $M_2$ per ml (24). Peptide-antibody complexes were incubated at 25°C for 1 h before being added to the plaque overlay. Cells were fixed and stained with crystal violet after 4 days of incubation at 37°C.

$M_2$-specific antibody, yet, like the sensitive strains, $M_2$ proteins synthesized by these strains are recognized by the antibody in immunofluorescence, immunoprecipitation, and immunoblot assays. A comparison of the $M_2$ amino acid sequences of A/WSN/33 and A/PR/8/34 with those of the antibody-sensitive A/Udorn/72, A/HK/8/68, and A/Singapore/1/57 strains (Fig. 4) indicates that there are no amino acid differences in the extracellular N terminus of $M_2$. One possibility that stems from these findings is that changes elsewhere in the $M_2$ molecule govern antibody-restricted growth. The A/WSN/33 and A/PR/8/34 $M_2$ amino acid sequences are different in the membrane-spanning domain at residues 28 and 31 from the antibody-sensitive strains with known sequences (A/Udorn/72, A/HK/8/68, and A/Singapore/1/57). From the complete sequences of the $M_2$ protein of A/WSN/33, A/PR/8/34, A/Singapore/1/57, and A/Udorn/72, it can be determined that there are three amino acid changes in the cytoplasmic tail that correlate to antibody sensitivity ($M_2$ residues 54, 57, and 89 [10, 24, 42; unpublished results]). Thus, the antibody binding could interfere with a function of the extracellular N-terminal domain of $M_2$ or, alternatively, cause a conformational change in the $M_2$ protein such that there is an alteration in either an activity or a protein-protein interaction of the membrane-spanning domain or cytoplasmic tail.

The phenotype of plaque size reduction of influenza virus growth at a postadsorptive stage observed with the $M_2$ antibody is similar to the effect of antibodies to neuraminidase, which limit plaque size but do not neutralize infection (16, 17, 36). Although the mechanism of neuraminidase antibody inhibition is not entirely clear, it is thought that the antibody blocks a late stage of replication, most probably the release of virus particles budding from the cell surface (34, 37, 38).

The plaque size reduction phenotype is also an effect of the antiviral drug amantadine hydrochloride on the replication of many strains of influenza virus (2, 9, 10). With some strains of influenza virus, the effect of amantadine hydrochloride is a late effect, and there is an alteration in the assembly process of virion particles (11, 12). Interestingly, it has been found that the growth of all human influenza A virus strains tested, except A/WSN/33 and A/PR/8/34, is restricted by the addition of amantadine hydrochloride at micromolar concentrations (2, 10, 30; unpublished observations). Mutations in influenza virus which confer resistance to amantadine hydrochloride map to the hydrophobic domain of $M_2$, and it has been suggested that the drug is targeted against the membrane-spanning domain of $M_2$ (10). It will be interesting to determine whether the phenotype of plaque size reduction observed with the addition of either amantadine hydrochloride or $M_2$ antibody share a common molecular basis. Defining these possible relationships may allow further understanding of the function of the $M_2$ protein and its putative interactions with other viral proteins, the lateral mobility of viral integral membrane proteins in the plane of the membrane, and the overall process of assembly and budding at the plasma membrane.

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