The M1 and M2 Proteins of Influenza A Virus Are Important Determinants in Filamentous Particle Formation

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Influenza A virus is highly pleomorphic with particles exhibiting either spherical or filamentous morphology. The mechanisms behind this pleomorphism and its importance in viral pathogenesis are not clearly understood. We have observed that budding of filamentous influenza A/Udorn virus particles can be readily visualized by immunofluorescence microscopy. Filamentous particle formation was inhibited by treatment of cells with the anti-M2 14C2 antibody, but was not inhibited with the isotype identical SC7 anti-M2 antibody or by anti-neuraminidase antibody. To further explore the viral determinants of filamentous particle formation, we investigated the morphology and growth characteristics of three variants of A/Udorn/72 virus, which had previously been selected for their resistance to growth inhibition by the 14C2 anti-M2 monoclonal antibody. Two of the variant viruses, 5A and 10A, contain single amino acid substitutions in the cytoplasmic domain of the M2 protein, whereas the 1A variant contains a single amino acid substitution in the viral matrix protein, M1. Variants 5A and 10A both were found to retain the filamentous particle phenotype found in the parental strain A/Udorn/72, and the production of filamentous virions by both variants was resistant to inhibition by the 14C2 antibody. However, immunofluorescence and electron microscopy revealed that the variant 1A was composed almost exclusively of spherical particles. The 1A variant displayed higher viral yields and a larger plaque size than the filamentous viruses. In addition, we separated distinct populations highly enriched in spherical or filamentous particles by velocity gradient centrifugation. Analysis of the protein compositions of these particles revealed that the NP:M1 or NP:HA ratios in filamentous particles were significantly lower than in spherical particles, but the filaments have higher levels of NP per particle. The spherical and filamentous particles were found to have similar specific infectivity. These results indicate that the filamentous morphology of the A/Udorn virus depends upon the matrix (M1) and/or M2 proteins.

INTRODUCTION

Influenza A virus morphology ranges from spherical particles with a mean diameter of approximately 120 nm, which are commonly found in laboratory-adapted strains, to greatly elongated filamentous particles with lengths of greater than several micrometers, which are commonly found in original human isolates and early egg passages of virus (Choppin et al., 1960; Chu et al., 1949). It has been reported that egg adaptation of original human isolates results in loss of the filamentous phenotype (Burnet and Lind, 1957; Kilbourne and Murphy, 1960). Although the genetic elements responsible for filament formation have not been identified conclusively, recent reports suggest that alterations in several viral structural gene products may modulate or influence virus morphology (Enami and Enami, 1996; Hughey et al., 1995; Jin et al., 1997; Mita et al., 1996; Smirnov et al., 1991). In addition, the properties of the host cell itself and the host cell microfilament actin array have also been implicated in modulation of virus morphology (Roberts and Compan, 1997). Thus, it appears that viral morphology is determined in part by interactions between viral structural proteins and cytoskeletal components of the host cell.

A monoclonal antibody to the M2 protein designated 14C2 has been shown to restrict growth of influenza A/Udorn virus, both in vivo by passively transferred antibody (Treanor et al., 1990) and in vitro by plaque size reduction (Zebbedee and Lamb, 1988, 1989). Treatment of infected cells with this antibody was also shown to inhibit viral assembly and release (Hughey et al., 1995). Several A/Udorn virus variants that are resistant to the inhibitory effects of this anti-M2 antibody have mutations within viral RNA segment 7, which codes for the M1 and M2 proteins. Reassortment analysis of antibody-resistant variants revealed that the resistance to antibody-induced effects on virus growth can be attributed to mutations in segment 7 of the viral genome (Zebbedee and Lamb, 1989).

In the present study, we have further investigated the effect of the inhibitory 14C2 antibody directed against the N-terminal extracellular domain of the M2 protein on viral morphology, assembly, and release. We have utilized immunofluorescence microscopy in conjunction with biochemical characterization to further investigate the
requirements for filamentous particle production. M2 antibody-resistant variants of A/Udorn/72 virus were investigated for their ability to retain the filamentous phenotype of the parental virus in cell culture. Three variants with single amino acid exchanges in the segment 7 gene products were evaluated for growth properties, morphological phenotype, antibody-induced inhibition of filament formation, and virion protein compositional differences. The results provide new insights into viral and cellular protein interactions involved in determining influenza virus morphology.

MATERIALS AND METHODS

Cells and viruses

Madin-Darby canine kidney cells (MDCK) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone). Influenza virus A/Udorn/301/72 and antibody-resistant variants 1A, 5A, and 10A (Zebedee and Lamb, 1989) were propagated either in MDCK cells in the presence of 1.5 g/ml TPCK-trypsin (Sigma) or in the allantoic sac as described previously (Zebedee and Lamb, 1989) and stored as stock virus in the presence of 1% bovine serum albumin at −70°C until use. Infectivity titers were determined by plaque assay in MDCK cells in an agar overlay system containing 15 g/ml of TPCK-treated trypsin (Tobita et al., 1975). An allantoic stock of influenza A/FW/1/50 was a kind gift from Drs. J. Katz and N. Cox (Influenza Branch, Centers for Disease Control, Atlanta, GA).

Antibodies

Guinea pig anti-Udorn antisera, goat anti-HA (H3 serotype), anti-NA (N2 serotype), anti-M1, and anti-RNP specific antisera were obtained from the Influenza Virus Repository, National Institute of Allergy and Infectious Diseases (Bethesda, MD). Anti-NP monoclonal antibody was kindly provided by Dr. J. Katz (CDC, Atlanta, GA). Anti-14C2 and anti-5C4 monoclonal antibodies specific for the influenza virus M2 protein have been described previously (Zebedee and Lamb, 1988; Holsinger and Lamb, 1991).

Virus infection in the presence of antibodies

MDCK cells were infected with A/Udorn/72 or variant viruses at a multiplicity of infection (m.o.i.) of 3 plaque-forming units (PFU) per cell. Following an adsorption period of 1 h at 37°C, the cells were further incubated in DMEM supplemented with 2% FBS for an additional 1.5 h prior to the addition of antibody to the incubation media. At 2.5 h pi, specific viral antisera (goat anti-N2, mouse 14C2 anti-M2, or mouse 5C4 anti-M2 antibody) or non-specific control antibody (goat serum or mouse anti-isotype IgG1 antibody) were diluted 1:100 in DMEM/2% FBS and the infections were continued in the presence of antisera until 9 h pi. Cells were subsequently processed for indirect immunofluorescence as described below.

Immunofluorescence

 Cultures of MDCK cells grown on glass coverslips were infected with influenza virus at a m.o.i. of 3 PFU per cell. After an 8-h incubation in DMEM/2% FBS, the cells were washed three times with PBS and stained for cell surface viral antigen by sequential incubations with antisera specific for M2, HA, or NA at 4°C, followed by fluorescein isothiocyanate (FITC)-conjugated or rhodamine tetramethylisothiocyanate (TRITC)-conjugated rabbit anti-goat or goat anti-mouse immunoglobulin Ig. Alternatively, cells were incubated with guinea pig anti-Udorn antisera followed by FITC- or TRITC-conjugated goat anti-guinea pig Ig. Following cell surface staining, cells were fixed with 3% freshly made paraformaldehyde in PBS for 15 min at RT. Internal viral structural proteins were visualized by fluorescence staining of fixed cells permeabilized either by treatment with 0.2% Triton X-100 in PBS for 15 min or by a 2-min treatment with acid: alcohol (95% ethanol:5% glacial acetic acid, at −20°C). Cells were mounted in Mowiol (Calbiochem) and photographed with a Zeiss Axiophot microscope equipped with epifluorescence or dual fluorescence filters (Omega Optical).

Metabolic labeling of viral proteins and virus particles

Influenza virus-infected cells (m.o.i. = 3) were incubated in DMEM/2% FBS. Prior to labeling, cells were incubated for 15 min in Eagle’s medium deficient in methionine and cysteine. Cells were subsequently labeled for 20 min at 37°C in Eagle’s deficient medium supplemented with 50 μCi 35S cell labeling mix (American Radiolabeled Chemicals, Inc.) followed by washing twice with PBS and lysed in 200 μl of lysis buffer containing 1% Triton X-100 and 0.5% sodium deoxycholic acid in MNT [20 mM 2-(N-morpholino)ethanesulfonic acid (MES, Sigma), 100 mM NaCl, 30 mM Tris·HCl (pH 8.0)], 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Chymostatin, pepstatin, leupeptin, and antipain were routinely included as protease inhibitors (10 μg/ml). Aliquots of cell extracts were analyzed by SDS±13.5% polyacrylamide gel electrophoresis (SDS±PAGE) and fluorography.

For labeling of virus particles, influenza virus-infected cells (six-well plates) were metabolically labeled from 5 to 16 h pi with 100 μCi/ml 35S cell labeling mix in Eagle’s deficient medium at 37°C. Supernatants were collected and precleared of cellular debris by centrifugation for 5 min at 500 g at 4°C prior to analysis of virus by sucrose gradient centrifugation.
Sucrose gradient analysis of virus particles

After a 16-h infection in MDCK cells (m.o.i. = 3), the supernatants of infected cells were harvested and precleared by centrifugation at 300g for 10 min at 4°C. Virus was pelleted through 25% sucrose cushion at 100,000g for 1 h at 4°C. Pelleted virus was resuspended by velocity centrifugation at 100,000g for 2.5 h at 4°C. Virus was recovered by fractionation into 0.5-ml fractions. When radiolabeled virus was analyzed, the cpm per fraction was determined by scintillation counting of 50-μl aliquots of each fraction. Hemagglutination assay was performed on fractions in parallel as described previously (Compans et al., 1969). Virus was concentrated from pooled or individual fractions by ultracentrifugation at 100,000g for 1 h at 4°C.

Electron microscopy of viral particles

For negative staining, virions in culture media or from sucrose gradient fractions were allowed to adhere to carbon/formvar grids and stained for 15 s with 1% ammonium phosphotungstate, pH 7.4. Specimens were viewed with a Philips CM10 electron microscope. The percentage of filamentous and spherical virus particles was determined by counting a minimum of 600 particles in low-magnification micrographs of several different preparations. Filamentous particles were defined as particles with a length of greater than 400 nm, which is roughly 3× the observed diameter of spherical particles.

RESULTS

The 14C2 anti-M2 antibody inhibits A/Udorn filament formation

The influenza A/Udorn/301/72 virus represents a strain which has retained the capacity for viral filamentous particle production despite passage in cell culture. Previous studies have shown that antibody to the M2 protein can restrict growth and assembly of the A/Udorn virus (Zebedee and Lamb, 1988, 1989; Hughey et al., 1995). To further explore this inhibitory effect, we investigated the assembly of filamentous particles at the surface of influenza A/Udorn infected MDCK cells by surface immunofluorescence.

As shown in Fig. 1a, numerous long filamentous viral particles are observed in the process of budding at cell surfaces when A/Udorn virus-infected cells were examined by surface immunofluorescence in the absence of antibody treatment. The filamentous particles seen by immunofluorescence correspond to the long filamentous virus particles observed budding at the cell surface in thin sections of A/Udorn infected cells (Hughey et al., 1995; data not shown). In contrast, cells incubated in the presence of the 14C2 antibody to the M2 protein (Fig. 1b) were completely devoid of budding filaments, but showed intense fluorescence, demonstrating the presence of viral antigens distributed over the cell surface. The specificity of the effect of this antibody is shown by the fact that a different monoclonal antibody (5C4) to the M2 protein, which does not inhibit virus spread and is of the same isotype, was found to have no inhibitory effect on filament formation (Fig. 1c). To demonstrate further the specificity of the inhibitory effect of the 14C2 antibody, we also examined the effect of antibody to another viral surface protein, the NA protein. As seen in Fig. 1d, cells incubated in the presence of antibody to NA continued to produce numerous long filamentous particles. These results demonstrate that the inhibition of formation of filamentous virus particles is a specific effect of the 14C2 anti-M2 antibody, which was previously shown to inhibit the release of the A/Udorn virus. In the presence of 14C2 antibody, the morphology of released particles was found to be almost exclusively spherical (less than 1% filamentous) when examined by negative staining and electron microscopy (Fig. 2). Thus, there appears to be a correlation between antibody-induced inhibition of virus growth and the inhibition of production of filamentous particles.

In agreement with previous electron microscopy results (Hughey et al., 1995), we also observed that treatment with the 14C2 antibody resulted in clustering of the M2 antigen at the cell surface (Fig. 3b). However, we found that similar clustering of cell surface M2 was also observed with the M2-specific 5C4 monoclonal antibody (Fig. 3c), but not in untreated cells (Fig. 3a). Both monoclonal antibodies recognize the N-terminal, extracellular domain of the M2 protein. These results indicate that the 14C2 antibody-induced clustering of the M2 protein at the cell surface is not responsible per se for the antibody-induced inhibition of virus release or filament formation.

Antibody-resistant A/Udorn variants differ in morphology

In order to examine further the role of the M2 protein in virus assembly, we analyzed the assembly of three plaque-purified variants of A/Udorn/301/72 virus, which had been previously selected for resistance to the growth inhibitory effects of the anti-M2 14C2 antibody (Zebedee and Lamb, 1989). Antibody resistance in these variants has been mapped to mutations in viral RNA segment 7 and was shown to result from single amino acid substitutions in either the M2 or the M1 proteins (Zebedee and Lamb, 1989). The A/Udorn 5A variant has a Lys78Gln amino acid substitution in the cytoplasmic tail domain of the M2 protein, and the 10A variant has a Ser71Tyr substitution, whereas the 1A variant has an Ala41Val substitution in the M1 protein.
At 9 h pi, MDCK cells infected with either the 5A or the 10A variants displayed intense staining of filamentous particles emanating from the cell surface. Furthermore, with both of these variants, no inhibition of filament formation was observed in the presence of the inhibitory 14C2 anti-M2 antibody (not shown). Using antibodies to specific viral structural proteins, these filamentous particles were found to contain HA, NA, NP, and M1 viral antigens (Figs. 4b and 4c, and data not shown). Some staining of the filamentous particles was also detected with anti-M2 monoclonal antibodies (not shown). However, no F-actin could be detected in the viral filaments, thus clearly distinguishing them from cellular microvilli. In contrast to the results with the 5A and 10A variants, no F-actin could be detected in the viral filaments, thus clearly distinguishing them from cellular microvilli.

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To further investigate the structure of released virions, we examined the morphology of negatively stained virus particles released into the media (Fig. 4, right). Both the 5A and the 10A variants were found to produce numerous filamentous particles (Figs. 4e and 4f, respectively), whereas exclusively spherical particles were observed with the 1A variant (Fig. 4d). Thus, the mutation in the 1A variant has resulted in the production of spherical rather than filamentous particles.

We also observed that the percentage of filamentous

FIG. 1. Inhibition of filament formation by 14C2 anti-M2 antibody. MDCK cells were infected with A/Udorn virus at an m.o.i. of 3 plaque-forming units per cell. Media containing a 1:100 dilution of normal goat serum (a), mouse anti-M2 14C2 ascites fluid (b), mouse anti-M2 5C4 ascites fluid (c), or goat anti-N2 polyclonal antiserum (d) was added at 2.5 h pi and the infection was continued for an additional 6.5 h. At 9 h pi the cells were sequentially incubated with guinea pig anti-Udorn antiserum and FITC-conjugated rabbit anti-guinea pig IgG. Immunofluorescent staining was performed at 4°C and the cells were fixed in 3% paraformaldehyde. Viral filaments are numerous in a, c, and d, but are absent in cells treated with anti-M2 14C2 ascites fluid.
particles released from infected cells varied considerably with the different variants. As shown in Table 1, the 1A variant produces exclusively spherical particles. The 5A variant virus population is composed of approximately 18% filamentous particles, which is similar to the level observed for the parental A/Udorn virus. The 10A variant yield, however, was found to be composed of greater than 40% filamentous particles. These results demonstrate that single amino acid substitutions in the M1 or M2 proteins can either enhance or prevent filamentous particle production.

A/Udorn variants differ in growth properties

It has been reported that serial passage of filamentous strains in eggs results in variants which display
faster growth rates and yield higher titers (Burnet and Lind, 1957; Kilbourne and Murphy, 1960). In at least some cases this has been attributed to loss of the filamentous phenotype (Burnet and Lind, 1957). Thus, we expected that there might be significant differences in the growth properties of the A/Udorn variants. When we compared the yields of the variant viruses after infection of MDCK cells, we observed that the 1A variant consistently yielded viral infectivity titers approximately 3.3- to 8.6-fold higher than those of either the 5A or the 10A variants, respectively (Table 1). The physical particle production as measured by hemagglutination assay also correlated with the infectivity titers of the variants. These results indicate that the spherical particles in the 1A variant are produced at higher levels than the filamentous particles produced by the other variants. No major differences were observed in the levels of viral protein expression among the variants (data not shown), indicating that differential protein expression is not the cause of the observed growth differences. Although we can not rule out that mutations in other genes may have occurred, it seems likely that the specific amino acid substitutions in M1 and/or M2 are responsible for the altered phenotypes and growth characteristics.

Variant virus particles differ in physical and biochemical characteristics

Little information has been obtained on the protein composition of influenza virus filamentous particles. Additionally, strains producing filaments also produce spherical particles, which can complicate the interpretation of the results. We investigated the protein composition of the A/Udorn variant viruses after separation of spherical and filamentous particles by velocity density centrifugation.

After radiolabeling of virus from MDCK cells infected with A/Udorn or variant viruses, the 35S-radiolabeled particles were harvested and layered onto a 20–60% sucrose gradient and separated by centrifugation. As can be seen in Fig. 5, after fractionation of the gradients the distribution of radioactivity was largely confined to fractions 8 to 13, but the distribution differed for the variant viruses. The parental A/Udorn strain, and the 5A and 10A variants, exhibited a heterogeneous profile as evidenced by the broad distribution of radioactivity in fractions 8±13. In contrast, the 1A virus sedimented much more homogeneously, with a major peak in fractions 12 and 13. As shown in Fig. 6 (left), pooled fractions 11±13 were found to be almost exclusively composed of spherical particles. In contrast, the faster sedimenting virus population (fractions 7–9) is highly enriched in filamentous particles. We did not observe any major differences in the specific infectivity ratios between the two virus populations (Table 2), indicating that both filamentous virions and spherical particles are similar in infectivity.

The virion protein profiles of the distinct particle populations from fractions 8±13 were also compared by polyacrylamide gel electrophoresis (Fig. 7). Interestingly, two forms of viral NP appear to be present in virus particles, which may represent different phosphorylated forms (Almond and Felsenreich, 1982; Kistner et al., 1985, 1989). Quantitative analysis revealed that lower NP levels were associated with filamentous particles (fractions 8±9) than with spherical particles (fractions 11±13), whereas the M1 to HA ratios were similar in both populations of virions (Table 3). In spherical particles the NP to M1 or HA ratio was approximately 1:2. The NP to M1 or HA ratio in filamentous particle fractions 8±9 ranged from 1:2.5 to 1:11 depending on the virus. The decrease in the

FIG. 3. Cell surface localization of viral M2 antigen after antibody treatment. MDCK cells were infected and treated with antibody during infection as described in Fig. 1, and were sequentially incubated with anti-14C2 monoclonal antibody and FITC-conjugated goat anti-mouse Ig at 8 h p.i. Depicted are: (a) cells not treated with antibody during infection; (b) cells treated with 1:100 dilution of 14C2 anti-M2 monoclonal antibody from 2±8 h p.i.; and (c) cells treated with a 1:100 dilution of 5C4 anti-M2 from 2±8 h p.i. Immunofluorescent staining was performed at 4°C prior to fixation with 3% paraformaldehyde. The M2 antigen is evenly distributed across the cell surface in untreated cells (a), but clusters in 14C2 or 5C4 anti-M2 antibody-treated cells.
NP:M1 ratio does not indicate a reduction in the total amount of NP per filamentous particle, however, since the mass of a filamentous particle is much greater than that of a spherical particle.

**DISCUSSION**

The filamentous phenotype is a genetic trait, which can be transferred by reassortment between different
influenza A viruses (Choppin, 1963; Choppin et al., 1960; Kilbourne and Murphy, 1960; Smirnov et al., 1991). The filamentous phenotype is often lost upon low-dilution serial passage of virus in eggs (Burnet and Lind, 1957). In this report, we present evidence that the viral M1 and M2 proteins can modulate viral morphology. Genetic reas-
sortment studies between filamentous and spherical virus strains have also suggested that the gene products of segment 7 play a major role in regulation of the morphological phenotype of influenza A virus (Smirnov et al., 1991). We observed loss of the filamentous phenotype in a variant with an alanine to valine substitution in the M1 protein at position 41. This Ala41Val substitution in the M1 protein is also found in other strains, including A/WSN/33 and A/PR8/34, both of which produce largely spherical particles (Winter and Fields, 1980; Zebedee and Lamb, 1989). However, these strains have undergone extensive laboratory adaptation, which may have led to additional mutations affecting morphology. Comparison of the M1 proteins of various avian and human strains of influenza did not reveal a direct correlation between the presence of valine at position 41 and spherical morphology. However, there is very little information regarding the passage history of viruses with known sequences, and other mutations resulting in spherical morphology may have arisen during passage.

Recently, Mitnaul and co-workers (1996) reported that deletion of the cytoplasmic tail of the influenza virus NA protein led to a shift from a spherical to a filamentous phenotype. A much greater shift toward filamentous particle production was observed when the cytoplasmic tail

<table>
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<th>Virus</th>
<th>PFU/ml</th>
<th>HAU/ml</th>
<th>PFU/HAU (Log10)</th>
<th>% F-virions (n)</th>
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<td>Udorn</td>
<td>$4.3 \times 10^6$ (±0.5)</td>
<td>512</td>
<td>3.92</td>
<td>15 (n= 753)</td>
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<tr>
<td>1A</td>
<td>$2.4 \times 10^6$ (±0.2)</td>
<td>512</td>
<td>3.69</td>
<td>&lt;2 (n= 622)</td>
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<tr>
<td>5A</td>
<td>$7.3 \times 10^5$ (±0.04)</td>
<td>256</td>
<td>3.44</td>
<td>18 (n= 624)</td>
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<tr>
<td>10A</td>
<td>$2.8 \times 10^5$ (±0.02)</td>
<td>128</td>
<td>3.34</td>
<td>46 (n=1214)</td>
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The plaque-forming units per milliliter were determined by plaque assay in MDCK cells in the presence of trypsin (1.5 µg/ml). The standard error of the mean is shown in parentheses. The Student t test analysis reveals significant differences between all variant viruses compared to the A/Udorn virus ($P < 0.05$). The values obtained for the 1A variant were highly significant when compared to the variants 5A and 10A ($P < 0.01$ and $P < 0.001$, respectively.)

The percentage of filamentous particles was determined as described under Materials and Methods. A minimum of 600 particles per variant were counted (n).

TABLE 1
Growth Characteristics of A/Udorn Variant Viruses

FIG. 5. Sedimentation profiles of variant viruses. $^{35}$S-radiolabeled virus (A/Udorn or variant viruses 1A, 5A, or 10A) was analyzed by velocity gradient centrifugation in 20–60% sucrose gradients. Gradients were fractionated and the cpm per fraction was determined by scintillation counting of fraction aliquots.
domains of both the NA and the HA were deleted (Jin et al., 1997). However, the particles produced with these mutants differ from the filamentous A/Udorn particles, in that particles of abnormal diameter and greatly distended structures were frequently observed. Interactions between the cytoplasmic tails of the viral glycoproteins and the matrix (M1) protein are likely to be essential for accurate assembly of virions with either the spherical or the normal filamentous morphology.

The present results indicate that the viral M2 protein also plays a role in regulating viral morphology. We observed previously that the 14C2 M2-specific antibody can restrict virus growth by inhibiting virus assembly and release (Hughey et al., 1995). Treatment of cells with this M2 antibody dramatically suppresses filamentous particle production, but does not prevent the assembly and release of spherical particles. The mechanism by which anti-M2 antibody restricts virus growth and filamentous particle formation is still not clear. Several lines of evidence indicate that the cytoplasmic tail domain of the M2 protein and not the ion channel activity is involved in modulating virus morphology: (i) the ion channel activity of the M2 protein is not influenced by 14C2 M2-specific antibody binding (Holsinger, Pinto, and Lamb, unpublished observations); (ii) amantadine treatment failed to inhibit filamentous particle formation (data not shown); and (iii) antibody-resistant filamentous variants have mutations in the M2 cytoplasmic tail domain. These results suggest that the cytoplasmic domain of the M2 protein may undergo a conformational change due to antibody

<table>
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<th>Virus</th>
<th>F-fractions</th>
<th>S-fractions</th>
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<td></td>
<td>PFU/ml ($\times 10^8$)</td>
<td>PFU/mg of viral protein ($\times 10^8$)</td>
</tr>
<tr>
<td>Udorn</td>
<td>10.5</td>
<td>2.4</td>
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Note. Virus harvested from MDCK cells was pelleted and layered over a 20–60% sucrose gradient. Following velocity centrifugation and fractionation, fractions 7–9 and 11–13 were pooled to represent fractions enriched in filamentous (F) and spherical (S) particles, respectively. Aliquots of pooled fractions were analyzed for infectivity by plaque assay on MDCK cells in the presence of 1.5 μg/ml TPCK-trypsin. Protein concentrations were determined using the Micro BCA Protein Assay (Pierce, Rockford, IL).
we observed that the percentage of NP is reduced in fractions enriched in spherical (S) or filamentous (F) particles. The relative optical units from scanned by a densitometer and the relative optical units of each polypeptide band were determined. The relative optical units from spherical and filamentous particles (Table 3), filamentous A/Udorn particles contain approximately 60% of the relative NP amount found for spherical particles. Based on the relative particle mass and assuming that a filament is composed of 70% protein (Scholtissek et al., 1969), then a filamentous particle would contain approximately 56,000 molecules of NP. Thus, the amount of NP is estimated to be 56-fold higher in a 10-μm filamentous particle than in a 120-μm spherical particle. This is consistent with previous results of Smirnov and co-workers (1991), who found no significant differences in RNA content between spherical and filamentous particle-enriched fractions. In fact, it is likely that the infectivity observed for the fractions enriched in filaments after sucrose gradient separation was reduced due to fragility of the long filamentous particles upon ultracentrifugation (Table 2).

The filamentous variants possess many of the characteristics described for newly isolated human strains of virus (Kilbourne, 1963; Kilbourne and Murphy, 1960). They produce smaller plaques and yield lower titers than the laboratory-adapted strains. Previous work has demonstrated that egg adaptation of newly isolated strains increases viral yields, an important prerequisite for use of a strain in vaccine development (Kilbourne and Murphy, 1960). Segment 7 encoded proteins have also been correlated to high-yielding properties of some viral strains (Klimov et al., 1991). It is interesting to note that segment 7 gene products have also been implicated in limitation of avian influenza viruses to growth in the respiratory tract of primates (Buckler-White et al., 1986; Tian et al., 1985). It will be interesting to determine whether differences in morphology could play a role as a determinant of influenza viral pathogenesis.

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