Reverse genetics studies on the filamentous morphology of influenza A virus

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We have investigated the genetic determinants responsible for the filamentous morphology of influenza A viruses, a property characteristic of primary virus isolates. A plasmid-based reverse genetics system was used to transfer the M segment of influenza A/Udorn/72 (H3N2) virus into influenza A/WSN/33 (H1N1) virus. While WSN virions display spherical morphology, recombinant WSN-Mud virus acquired the ability of the parental Udorn strain to form filamentous virus particles. This was determined by immunofluorescence studies in infected MDCK cells and by electron microscopy of purified virus particles. To determine the gene product within the M segment responsible for filamentous virus morphology, we generated four recombinant viruses carrying different sets of M1 and M2 genes from WSN or Udorn strains in a WSN background. These studies revealed that the M1 gene of Udorn, independently of the origin of the M2 gene, conferred filamentous budding properties and filamentous virus morphology to the recombinant viruses. We also constructed two WSN viruses encoding chimeric M1 proteins containing the amino-terminal 1–162 amino acids or the carboxy-terminal 163–252 amino acids of the Udorn M1 protein. Neither of these two viruses acquired filamentous phenotypes, indicating that both amino- and carboxy-terminal domains of the M1 protein contribute to filamentous virus morphology. We next rescued seven mutant WSN-M1ud viruses containing Udorn M1 proteins carrying single amino acid substitutions corresponding to the seven amino acid differences with the M1 protein of WSN virus. Characterization of these recombinant viruses revealed that amino acid residues 95 and 204 are critical in determining filamentous virus particle formation.

INTRODUCTION

Influenza A virus is a member of the Orthomyxoviridae family of segmented negative-strand RNA viruses. Influenza A virions are enveloped particles of pleomorphic morphology, ranging from small spherical particles 80–120 nm in diameter to long filamentous particles up to several micrometres in length. Fresh clinical isolates of influenza viruses are characterized by the presence of a significant proportion of filamentous virions in virus preparations (Chu et al., 1949). However, after multiple passages in eggs or tissue culture, virus preparations often consist mainly of virions with spherical morphology (Choppin et al., 1960; Kilbourne & Murphy, 1960). The filamentous morphology of influenza A virus is genetically determined, as shown by virus gene reassortment experiments (Smirnov et al., 1991). Proteins encoded by the M segment, M1 and M2, appear to be involved in modulating filamentous versus spherical virion morphology (Hughes &expand; et al., 1995). In addition, alterations in some other structural gene products can also affect virus morphology. For instance, virus mutants lacking the cytoplasmic tails of both the NA and the HA viral glycoproteins showed increased lengths and irregular virus particle shape (Jin et al., 1997).

Host cell type and actin cytoskeleton have also been shown to play an important role in influenza virus morphology. Specifically, the formation of filamentous influenza A viruses is best seen in polarized epithelial cells. Moreover, disruption of the actin microfilament network by cytochalasin D treatment leads to a significant reduction in the formation of filamentous virus particles (Roberts & Compans, 1998).

Filamentous virions have been described for many negative-strand RNA viruses. Several paramyxoviruses such as human parainfluenza virus type 2 (HPIV2) and respiratory syncytial virus (RSV) form filaments on the surfaces of infected cells and these are believed to represent filamentous viruses in the process of budding (Roberts et al., 1995; Yao & Compans, 2000). The formation of extremely long cord-like structures with lengths up to 500 μm has been described upon infection of cells with some influenza C virus strains (Nishimura et al., 1990). Marburg and Ebola filoviruses are pleomorphic, appearing as long, sometimes branched, filamentous virions (Klenk et al., 1994). In the present study, we used plasmid-based reverse genetics techniques to study the genetic determinants responsible for the differences in virus particle morphology between two
strains of influenza viruses: influenza A/WSN/33 (WSN) virus, whose virions have a predominantly spherical morphology, and influenza A/Udorn/72 virus, whose virions are predominantly filamentous. Reombinant virions were analysed for their ability to form filaments in infected cells by indirect immunofluorescence. The morphology of virus particles was visualized by electron microscopy. Using these techniques, we have identified amino acid residues in the M1 protein responsible for the difference in virion morphology between WSN and Udorn influenza A viruses.

METHODS

Cells and viruses. 293T human embryonic kidney cells were maintained in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Madin-Darby bovine kidney (MDBK) cells were cultured in reinforced minimal essential medium containing 10% FBS. Madin-Darby canine kidney (MDCK) cells were maintained in MEM with 10% FBS. Influenza A/WSN/33 (H1N1) virus (WSN) and its derivatives were routinely propagated in MDBK cells. Influenza A/Udorn/72 (H3N2) virus (Udorn) was propagated either in 10-day-old embryonated chicken eggs or in MDCK cells [supplemented with 1.5 μg TPCK-treated trypsin ml⁻¹ (Sigma)].

Plasmids. pcAGGS-PB1, -PB2, -PA and -NP express viral proteins required for the replication and transcription of influenza virus RNA (Basler et al., 2001). The eight WSN viral RNA (vRNA) expression plasmids pPOLI-PB1, -PB2, -PA, -HA, -NP, -NA, -M and -NS have been described previously (Fodor et al., 1999). The sequences of the M1 and M2 genes of WSN virus in pPOLI-Mwsn are identical to the GenBank sequence (accession number X08088), except for nucleotide 502 (an A in X08088 and a G in pPOLI-Mwsn, resulting in a substitution of Thr-168 in X08088 by an Ala in our M1 clone) and nucleotide 507 (a T in X08088 and a C in our clone, which is a silent change). These changes most likely represent sequence differences between our tissue culture-passaged WSN virus clone, which is a silent change). These changes most likely represent sequence differences between our tissue culture-passaged WSN virus and the one sequenced by Zebedee & Lamb (1989).

The sequence of the Udorn M cDNA in pPOLI-Mud is identical to the corresponding GenBank sequence accession number J02167. The PCR product was digested with SapI and cloned into SapI-digested pPOLI-SapI-RT (Pleschka et al., 1996). The sequence of the Udorn M cDNA in pPOLI-Mud is identical to the corresponding GenBank sequence accession number J02167.

pPOLI-M1wsn and pPOLI-M1ud plasmids express M vRNA segments encoding only the M1 protein (Fig. 1B). M2 protein expression was abrogated in these plasmids by the insertion of a stop codon and the generation of a small deletion after amino acid 24 of the M2 protein. This was achieved as follows. First, a PCR product was obtained using as template either pPOLI-Mwsn or pPOLI-Mud plasmids and primers 5’-gacctgtgacAGTCTTCTAACCAGGAGGC-3’ (annealing to 3–22 nt position of the M1 ORF) and 5’-ggcatctgatgtaATCCTTGAAT-GTTTGCATCTG-3’ (corresponding to the last 21 nt of the M1 ORF and generating a stop codon in M2 ORF, underlined). PCR products were digested with Stul and NsiI enzymes and inserted into Stul/NsiI-digested pPOLI-Mwsn or pPOLI-Mud plasmid.

The sequence of the Udorn M cDNA in pPOLI-Mud is identical to the corresponding GenBank sequence accession number J02167.

pPOLI-M2wsn-BiP-NA encodes a bicistronic NA segment containing M and NA ORFs flanked by the NA vRNA noncoding sequences and separated by an internal ribosomal entry site derived from the 5’ noncoding region of the human immunoglobulin heavy chain-binding protein (BiP) mRNA (Fig. 1B). This arrangement allows translation of both M2 and NA proteins from the same mRNA. To construct this plasmid, pt3GP2/BiP-NA (Garcia-Sastre et al., 1994) was modified by
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replacing the T3 promoter located at the 5' end of NA vRNA-specific sequence with a truncated human RNA polymerase I (POLI) promoter, and inserting the hepatitis delta virus ribozyme sequence at the 3' end of the NA vRNA sequence. Next, the M2 WSN cDNA was RT-PCR amplified from WSN virus-infected cell extracts using primers N, 5'-ggcgcgtacATGAGTCTGTTAACGAGAGGC-3' (containing an Nhel site followed by the first 19 nt of the M2 ORF) and primer 5'-ggcgcgtacTATCCAGCTTATGTGAC-3' (containing a SalI site followed by the last 21 nt of M2 WSN ORF in negative sense). The PCR product was digested with Nhel and SalI and inserted into Nhel/SalI-digested pPOLI-GP2/Bip-NA. To construct plasmid pPOLI-M2ud-Bip-NA, the Udorn M2 cDNA was RT-PCR amplified using total RNA isolated from Udorn virus-infected cells and primers N and 5'-ggcgcgtacTATCCAGCTTATGTGAC-3' (containing a SalI site followed by the last 21 nt of M2 WSN ORF in negative sense). This PCR product was cloned into Nhel/SalI-digested pPOLI-GP2/Bip-NA.

Generation of recombinant influenza viruses. To generate recombinant viruses, 0.25 µg each of 12 plasmids was transfected into 293T/MDKB cocultures. Each transfection contained four protein expression plasmids, pCAGGS -P1, -P2, -PA and -NP, and eight vRNA expression plasmids, pPOLI -PB1, -PB2, -PA, -HA, -NP, -NA, -M and -NS (Basler et al., 2001; Fodor et al., 1999). pPOLI-M plasmid was replaced with the appropriate construct to generate viruses containing mutations in their M genes. For the generation of viruses containing M1 and M2 non-overlapping ORFs, both pPOLI-M1udM2wsn plasmids were replaced with pPOLI-M2-BiP-NA and pPOLI-M1udM2wsn plasmids were used in transfections to generate recombinant viruses.

Negative staining, electron microscopy and measurements of virus particles. Supernatants from virus-infected MDCK cells were preclarified by low speed centrifugation (6000 r.p.m. for 5 min at 4°C) and viruses were subsequently pelleted through a 25% sucrose cushion in NTE (NaCl 100 mM, Tris 10 mM, EDTA 1 mM, pH 7.4) by centrifugation at 28000 r.p.m. for 90 min in an SW41 rotor at 4°C. Negative staining of viruses was performed using these sucrose-cushion purified virions resuspended in NTE buffer. Virus suspensions were allowed to adsorb to carbon/Formvar grids (Electron Microscopy Sciences) for 3–5 min, washed once with PBS and stained for 15 s with 1% ammonium phosphotungstate (Sigma) pH 7.0. Specimens were viewed using a Hitachi H7000 transmission electron microscope and three representative photographs per virus were taken. Photos were scanned using an AGFA-DuoScan scanner and a coloured line was drawn over the image of every particle along its major axis in Adobe Photoshop 5.0. Lengths of drawn lines were measured using image analysis software IPLab Spectrum.

RESULTS

The M segment of influenza A/Udorn/72 virus confers filamentous morphology to influenza A/WSN/33 virus

In our work we have used two strains of influenza A virus with distinct morphologies of virus particles. WSN virus forms predominantly small spherical virions. By contrast, Udorn virus preparations have a significant number of filamentous particles (Hugh et al., 1995; Roberts et al., 1998). Previous studies (Roberts et al., 1998; Smirnov et al., 1991) suggested that the viral M segment encodes the determinants responsible for the filamentous morphology of virions. Recently established plasmid-based reverse genetics systems allowed us to generate recombinant influenza A viruses by transfection of cells with 12 plasmids (Fodor et al., 1999; Neumann et al., 1999). Using these techniques, from transfected cells at 3 or 4 days post-transfection were used to infect fresh MDBK cells. Three days later, recombinant viruses were plaque-purified in MDBK cells. Plaque-purified viruses were grown in MDBK cells and titrated in MDBK cells by plaque assay. The identity of the recombinant viruses was confirmed by restriction digestion and sequence analysis of RT-PCR products derived from the M and/or NA genes.

Indirect immunostaining of surfaces of infected cells. Confluent monolayers of MDCK cells grown on cover glasses in 24-well plates were infected at an m.o.i. of 3 with influenza A viruses. Sixteen hours post-infection, cells were fixed with 3% formaldehyde in PBS for 10 min, washed three times with PBS and incubated for 1 h at room temperature with a 1:500 dilution of a rabbit anti-serum (in PBS with 3% BSA) raised against WSN virus. Cells were washed three times with PBS and incubated for 1 h at room temperature with a 1:750 dilution of a secondary FITC-conjugated goat anti-rabbit IgG (Boehringer Mannheim). Cells were washed three times with PBS and mounted using ProLong Antifade reagent (Molecular Probes). Surface fluorescence of cells was observed with a Zeiss Axiovert 2 microscope and digital images of cells were taken using a Hamamatsu Orca CCD camera. Udorn virus-infected cells were processed using an identical protocol, except that the primary antibody consisted of rabbit polyclonal antiserum raised against X-31 (H3N2) virus.

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we generated recombinant WSN (Basler et al., 2001) and WSN-Mud viruses. These two viruses differ only in the origin of their M segments, which are derived from WSN or Udorn viruses, respectively. Restriction digestion analysis and sequencing of an RT-PCR product derived from the M gene of WSN-Mud virus confirmed the presence of the M Udorn vRNA segment (data not shown).

In order to investigate the ability of recombinant WSN and WSN-Mud viruses to form filaments on the surface of infected cells, we performed immunofluorescence analysis of virus-infected MDCK cells using polyclonal influenza virus antisera (Fig. 2A–C). As a control we also infected cells with wild-type Udorn virus. As expected, surface staining of recombinant WSN virus-infected cells showed a punctuated pattern of viral glycoprotein staining, typical of cells infected with influenza viruses of spherical morphology (Fig. 2A). By contrast, surface staining of Udorn virus-infected cells revealed the formation of long filamentous protrusions containing viral glycoproteins (Fig. 2B). These filamentous structures were also present in WSN-Mud virus-infected cells (Fig. 2C) and most likely represent filamentous viruses in the process of budding (Cox et al., 1980).

The formation of hair-like filaments in infected cells correlated with the presence of long filamentous virus particles, as seen by electron microscopy of negatively stained virions purified from supernatants of infected cells (Fig. 2D–F). These results demonstrated that the M gene of Udorn virus confers the ability to form filamentous virions to WSN virus.

The M1 gene of influenza A/Udorn/72 virus confers filamentous morphology to influenza A/WSN/33 virus

The M segment of influenza A virus encodes two proteins, M1 and M2, in partially overlapping ORFs. In order to find out which protein is responsible for the filamentous phenotype of WSN-Mud virus, we separated the M1 and M2 ORFs into different segments and constructed four recombinant WSN viruses carrying different sets of M1 and M2 genes derived from WSN or Udorn viruses (Fig. 1B). These viruses have M segments encoding only the M1 protein. M2 protein expression is achieved from a bicistronic NA gene. Four recombinant viruses were made expressing M1 and M2 proteins from WSN and/or Udorn viruses in all possible combinations: M1wsn/M2wsn, M1ud/M2wsn, M1wsn/M2ud and M1ud/M2ud viruses. RT-PCR amplification and restriction digestion analysis of the M1 and M2 ORFs from these recombinant viruses confirmed the correct Udorn or WSN origin of these genes.

The M1/M2 viruses were used to infect MDCK cells and 16 h post-infection we performed immunostaining of viral antigens on cell surfaces. Formation of hair-like filaments were only observed upon infection of cells with M1ud/M2ud and M1ud/M2wsn viruses (Fig. 3A–D). The presence of long filamentous particles in purified M1ud/M2ud and M1ud/M2wsn virions was confirmed by electron microscopy (data are not shown). This clearly demonstrates that the substitution of the M1 gene of WSN virus by the corresponding gene of Udorn virus is sufficient to confer filamentous virus morphology to WSN virus.

Fig. 2. Substitution of the M gene of WSN virus by the M gene of Udorn virus results in a virus with filamentous budding phenotype. MDCK cells were infected with WSN (A), Udorn (B) and WSN-Mud (C) viruses, and the cell surface was stained at 16 h post-infection with polyclonal antisera against WSN (A, C) or X-31 viruses (B). Electron microscopy pictures of negatively stained virus preparations are shown at the bottom (D, E, F). The bar represents a length of 300 nm.
Both amino- and carboxy-terminal parts of M1 Udorn protein are needed for the acquisition of the filamentous phenotype

There are seven amino acid differences between the M1 proteins of our WSN and Udorn viruses at positions 41, 95, 167, 168, 204, 205 and 218. We generated two recombinant viruses, Mchim1 and Mchim2, carrying chimeric M segments (Fig. 1A). The Mchim1 RNA segment encodes a chimeric M1 protein identical to the M1 protein of WSN virus except for amino acids 41 and 95, which are identical to the corresponding amino acids of the M1 protein of Udorn virus. The Mchim2 RNA segment encodes a chimeric M1 protein whose carboxy-terminal part (containing amino acids 167, 168, 204, 205 and 218) is derived from the M1 Udorn protein. Due to our cloning strategy, M2 genes are derived from WSN and Udorn viruses in Mchim1 and Mchim2 viruses, respectively. RT-PCR amplification and sequencing of the M vRNAs from these viruses confirmed the presence of the introduced mutations. M1udA41V, M1udR95K, M1udA167T, M1udT168A, M1udE204D, M1udV205I and M1udA218T, were compared with a recombinant WSN virus, WSN-M1ud, expressing a wild-type Udorn M1 protein. As expected, WSN-M1ud virus showed characteristics typical of filamentous viruses, both by immunostaining of infected cell surfaces and by electron microscopy analysis of purified virus particles (Fig. 3E, F). Indirect immunofluorescence of surface viral antigens in cells infected with the seven viruses carrying single amino acid mutations in the M1 Udorn protein is shown in Fig. 4(A–G). Formation of filaments was not observed upon infection of cells with M1udR95K and M1udE204D viruses. By contrast, the

Mutation of amino acid residues arginine to lysine at position 95 and glutamate to aspartate at position 204 in the Udorn M1 protein impaired filamentous morphology of virus particles

In order to understand the single contribution of the seven Udorn-specific amino acid residues of the M1 protein in filamentous particle formation, we generated a panel of M1 mutant recombinant viruses. These viruses were engineered to express Udorn M1 mutant proteins containing single amino acid changes representing the seven amino acid differences between the M1 proteins of WSN and Udorn virus. All other viral sequences in these recombinant viruses, including the M2 gene sequence, were derived from WSN virus (Fig. 1A). RT-PCR amplification and sequencing of the M vRNAs from these viruses confirmed the presence of the introduced mutations. The corresponding viruses, M1udA41V, M1udR95K, M1udA167T, M1udT168A, M1udE204D, M1udV205I and M1udA218T, were compared with a recombinant WSN virus, WSN-M1ud, expressing a wild-type Udorn M1 protein. As expected, WSN-M1ud virus showed characteristics typical of filamentous viruses, both by immunostaining of infected cell surfaces and by electron microscopy analysis of purified virus particles (Fig. 3E, F). Indirect immunofluorescence of surface viral antigens in cells infected with the seven viruses carrying single amino acid mutations in the M1 Udorn protein is shown in Fig. 4(A–G). Formation of filaments was not observed upon infection of cells with M1udR95K and M1udE204D viruses. By contrast, the
M1udA167T virus formed filaments in infected cells indistinguishable from those in WSN-M1ud virus-infected cells. Immunofluorescence staining revealed filaments in cells infected with the remaining four mutants, M1udA41V, M1udT168A, M1udV205I and M1udA218T viruses, although filament formation seemed reduced either in number or in length when compared with WSN-M1ud virus-infected cells.

To compare virus particle morphology among the different mutant viruses, we examined negatively stained purified virions by electron microscopy. These experiments were performed in a blind fashion, in which samples were coded before the analysis in order to prevent potential bias. Pictures of several fields for each specimen were taken and representative images from virus preparations are shown in Fig. 5A. These pictures were scanned and lines were drawn over each virus particle along its major axis (Fig. 5B). The lengths of approximately 700–1500 particles per virus preparation were measured and the data are summarized in Fig. 5C. We arbitrarily classified virus particles as filamentous if their lengths exceeded 300 nm, which approximately corresponds to an increase of three times in length as compared with spherical particles. Among the single mutant M1 Udorn viruses analysed, M1udA41V, M1udA167T, M1udT168A, M1udV205I and M1udA218T contained a percentage of long filamentous particles either similar (approximately 7%) to that of parental WSN-M1ud virus, or even higher (9.4% and 13.2% for M1udV205I and M1udA218T viruses, respectively). Although all our panel of M1 Udorn mutant viruses retained a higher percentage of slightly elongated particles [with diameters between 120 and 300 nm (Table 1)], only two single-amino-acid mutants, M1udR95K and M1udE204D viruses, showed a low content of long filamentous particles – 1.6% and 1.5% respectively. We repeated the electron microscopy analysis a second time and obtained similar results (data not shown). Based on these results, we conclude that the major viral sequence determinants responsible for the morphological differences seen between WSN (spherical) and Udorn (filamentous) viruses are amino acid residues 95 and 204 in the M1 protein.

## DISCUSSION

In these studies, the use of plasmid-based reverse genetics techniques to generate recombinant influenza A viruses allowed us to investigate viral determinants responsible for the formation of long filamentous viruses after infection of polarized epithelial cells. Primary isolates of influenza A viruses are characterized by the presence of elongated virus particles ('filamentous phenotype'). Egg or tissue culture adaptation often results in the loss of the filamentous
Fig. 5. For legend see page 524.
phenotype, and laboratory strains of influenza A viruses, such as influenza A/WSN/33 virus, usually display a ‘spherical phenotype’. One exception to this norm is influenza A/Udorn/72 virus. Despite tissue culture adaptation, Udorn viruses mirror primary influenza A virus isolates in morphology. Preparations of Udorn virus have been reported where about 15% of virus particles are longer than 400 nm (Roberts et al., 1998). Through the generation of recombinant influenza A viruses using plasmid DNA reverse genetics techniques, we have now demonstrated that the ‘filamentous phenotype’ of Udorn virus is genetically linked to the M1 gene. These results are in agreement with previous virus gene reassortment experiments that pointed to the contribution of the viral M segment to the filamentous morphology of virus particles (Smirnov et al., 1991).

We have used two criteria to determine the filamentous/spherical phenotype of recombinant influenza viruses (Cox et al., 1980; Roberts & Compans, 1998; Roberts et al., 1998). Indirect immunofluorescent staining of viral glycoproteins on the surfaces of infected cells can be used to visualize the formation of long processes that presumably represent filamentous viruses in the course of budding. By contrast, cells infected with viruses with spherical morphology give a punctuated surface staining without the appearance of long processes. The second criterion is electron microscopy analysis of negatively stained virions purified from supernatants of infected cells that gives direct information on the shape and size of virus particles. Based on both criteria, it is clear that WSN and Udorn viruses have spherical and filamentous phenotypes, respectively, and that substitution of the WSN M gene by the Udorn M gene in a recombinant WSN virus results in the acquisition of a filamentous phenotype (Fig. 2).

The M RNA segment of influenza A virus encodes the matrix protein M1 and the small integral membrane protein M2. The M1 protein is translated from a linear unspliced transcript derived from the M gene, while the M2 protein is the product of a spliced M-specific mRNA (Inglis & Brown, 1981; Lamb et al., 1981). Splicing of the M-specific pre-mRNA is controlled by the viral polymerase complex.

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**Table 1.** Length of virus particles of recombinant influenza viruses as determined by electron microscopy

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**Fig. 5.** Effect of mutations in the M1 protein of Udorn virus on virion morphology. (A) Purified WSN, WSN-Mud, WSN-M1ud, Mchim1, Mchim2, M1udA41V, M1udR85K, M1udA167T, M1udT168A, M1udE204D, M1udV205I and M1udA218T viruses were visualized by electron microscopy. Representative pictures taken at 39,900 x magnification are shown. Figs 2(D), 2(F) and 3(F) also show a closer look at the WSN, WSN-Mud and M1ud panels, respectively. (B) In Adobe Photoshop, lines were drawn along the longest diameter of virus particles using electron microscopy pictures as templates. Virus preparations containing less than 1% of particles longer than 300 nm are shown in black. Virus preparations containing between 1–2% of particles longer than 300 nm are shown in blue. Virus preparations containing more than 6% of particles longer than 300 nm are shown in red. (C) Percentage of virus particles longer than 300 nm present in the virus preparations. The same colour code as in (B) is used.
and by the cellular splicing factor SF2/ASF (Shih & Krug, 1996; Shih et al., 1995). Roberts et al. (1998) found single amino acid mutations in M1 and M2 proteins in escape mutants of Udorn virus selected by treatment of infected cells with antibodies specific for the extracellular domain of the M2 protein. These M2-specific antibodies were also shown to inhibit virus replication and filamentous particle formation by A/Udorn/72 virus. According to these studies, we expected that the M1 and/or M2 proteins encoded by the M segment would contain the genetic determinants responsible for the differences between the filamentous and spherical phenotypes of WSN and Udorn viruses. In order to further delineate the contribution to the filamentous phenotype of the M1 and M2 genes, we generated recombinant influenza A viruses in which M1 and M2 protein expression is uncoupled from splicing regulation. We took advantage of a previously described system to express additional ORFs from bicistronic NA segments using an internal ribosomal entry site (Garcia-Sastre et al., 1994). We constructed four M1/M2 viruses carrying different sets of M1 and M2 genes from WSN and Udorn strains in independent viral segments. Our results clearly demonstrate that regulation of M2 expression by premRNA splicing is not required for virus replication, at least in cell culture, nor for the generation of filamentous virions. In addition, the presence of only the M1 gene from Udorn virus conferred filamentous morphology to an otherwise spherical virus. This demonstrates a crucial role of the M1 protein from Udorn virus in determining virus filamentous morphology.

The M1 protein is a major structural component of influenza A viruses that lies underneath the virus envelope, surrounding the ribonucleoprotein (RNP) core (Fujiyoshi et al., 1994; Lamb & Choppin, 1983; Ruigrok et al., 2000). The M1 protein is a multifunctional protein that plays several roles at different stages of virus replication. It participates in the transport of viral RNPs (vRNPs) into and out of the nucleus (Herz et al., 1981; Jackson et al., 1982; Martin & Helenius, 1991; O’Neill et al., 1998; Yasuda et al., 1993). It binds single-stranded RNA (Elster et al., 1997; Watanabe et al., 1996) and vRNPs (Watanabe et al., 1996; Ye et al., 1999) in vitro and it has been shown to inhibit viral RNA polymerase activity, presumably by aggregating onto RNPs (Baudin et al., 2001; Watanabe et al., 1996; Ye et al., 1999). Moreover, the M1 protein has the inherent ability to bind membranes in vitro and in vivo (Ali et al., 2000; Avalos et al., 1997; Bucher et al., 1980; Enami & Enami, 1996; Kretzschmar et al., 1996; Ruigrok et al., 2000; Ye et al., 1987; Zhang & Lamb, 1996). Importantly, it has been suggested that the M1 protein plays a crucial role in virus assembly and budding by interacting with RNPs in the cytoplasm and with the host cell membrane as well as with cytoplasmic tails of viral glycoproteins on the plasma membrane. Our results demonstrate a crucial role for the M1 protein in filamentous virus morphology, and are consistent with studies suggesting that this viral protein provides the major driving force in virus budding (Gomez-Puertas et al., 2000; Latham & Galarza, 2001). Interestingly, a recent study on the VP40 protein of Ebola virus, which is considered to be the matrix protein of this negative-strand RNA virus, showed that expression of VP40 in mammalian cells led to production of filamentous virus-like particles that could be visualized by electron microscopy (Noda et al., 2002).

Our results show that both amino- and carboxy-terminal regions of the M1 protein contain sequence determinants responsible for filamentous morphology of virus particles. There are seven amino acid differences between the M1 proteins of the Udorn and WSN strains used in our studies. We found that the single amino acid mutations Arg-95→Lys and Glu-204→Asp caused a significant reduction in the percentage of long virus particles. All other single mutations did not significantly affect the filamentous phenotype of WSN-M1ud virus as analysed by electron microscopy of purified virions. By contrast, Roberts et al. (1998) reported that an Ala-41→Val mutation in the Udorn M1 protein causes the loss of the filamentous phenotype of Udorn virus. It is possible that changes in genetic background between WSN and Udorn viruses are responsible for differential effects on the Ala-41→Val change. Nevertheless, it should be noted that, with the exception of the Ala-167→Thr change, all seven single mutations affected to some extent the number and/or length of filaments in infected MDCK cells as visualized by immunofluorescence, although a clear drastic reduction was again only observed with the Arg-95→Lys and Glu-204→Asp M1 mutants. Interestingly, mutation of alanine to threonine at position 218 resulted in an even more penetrant filamentous phenotype (twofold increase in long virus particles as compared to the parental WSN-M1ud virus by electron microscopy). Although the significance of these differences remains unknown, our results clearly indicate that amino acids 95 and 204 of the M1 protein play a major role in determining a filamentous versus spherical phenotype. In these studies, our mutational analysis was restricted to positions different between WSN and Udorn M1 proteins. It is likely that several other M1 amino acids are also critical for the filamentous phenotype.

The crystal structure of the amino-terminal part of the M1 protein (Arzt et al., 2001; Harris et al., 2001; Sha & Luo, 1997) revealed that lysine at position 95 is one of the highly positively charged amino acid residues on the surface of the molecule and therefore this amino acid may participate in functionally significant interactions with other viral or cellular components. Other investigators have suggested that this positively charged area binds to membranes (Baudin et al., 2001; Ruigrok et al., 2000). Since the three-dimensional structure of the carboxy-terminal region on the M1 protein remains unsolved, it is unclear whether amino acid residue 204 is surface-exposed or not. Nevertheless, it is likely that changes in amino acids 95 and 204 result in changes in the strength of interactions with other viral proteins, membranes or cellular factors.
important for virus budding. Although these changes may be subtle, they can become amplified due to the oligomeric structure of the M1 protein, and lead to a significant change in virus particle morphology.

The role that filamentous virus morphology may play in virus pathogenesis and disease is not known. Since long filamentous virions could theoretically infect neighbouring cells prior to their release, it has been suggested that filamentous virus morphology may facilitate cell-to-cell transmission of viruses in the respiratory mucosa (Roberts & Compan, 1998). On the other hand, small spherical virions would be expected to be more efficiently incorporated into aerosols and therefore may be easily transmitted from person to person. Future experiments are required to determine the biological role that filamentous viruses play in the influenza virus life-cycle and pathogenicity. The fact that clinical isolates of influenza A viruses invariably show filamentous phenotypes strongly suggests that this property is important for virus survival in nature. In addition, studies on the molecular requirements for filamentous virus formation may give some insights into the mechanism of virus assembly and budding. Our results point to a critical role of both amino- and carboxy-terminal domains of the M1 protein of influenza A virus in modulating the budding of filamentous influenza A viruses.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health (A. G.-S.). Microscopy was performed at the MSSM-Microscopy Shared Resource Facility, supported, in part, with funding from NIH-NCI shared resources grant (1 R24 CA095823-01). We express our great appreciation to Scott Henderson for help with the fluorescence microscopy, and to Valerie Williams and Vladimir Protopopov for assistance with the electron microscopy. We also thank Estanislao Nistal-Villán and Richard Cadagan for excellent technical assistance. We are also grateful to all members of Peter Palese’s and A. G.-S.’s laboratories for critical discussions.

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