The M1 and NP proteins of influenza A virus form homo- but not heterooligomeric complexes when coexpressed in BHK-21 cells

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The nucleoprotein (NP) and matrix protein (M1) are the most abundant structural proteins of influenza A virus. M1 forms a protein layer beneath the viral envelope and NP constitutes the protein backbone of the ribonucleoproteins (RNPs). In order to elucidate the functions of these proteins in virus assembly we have expressed NP and M1 in BHK-21 cells using Semliki Forest virus replicons and analysed their molecular interactions. We found that both M1 and NP engaged in extensive homooligomerization reactions soon after synthesis. However, there was no detectable heterooligomerization taking place between the two viral proteins, nor between these and host proteins. One interpretation of these results is that homooligomers, and not monomers, of NP and M1 are used as building blocks during RNP assembly and formation of the submembranous M1 layer, respectively. The complete absence of M1–NP heterooligomers suggests, on the other hand, that these two major viral proteins do not interact directly with each other during virus assembly. We also found that a fraction of M1 associated with cellular membranes. This did not, however, result in membrane budding or vesicularization as was the case with the matrix protein of vesicular stomatitis virus when expressed separately (P.A. Justice and others, Journal of Virology 69, 3156–3160, 1995).

Introduction

The matrix (M1) and nucleoprotein (NP) are the two most abundant structural proteins in the influenza A virion (Lamb, 1989). This implies that they have a central function in the assembly process of the virus. In the virion the M1 proteins form a continuous layer at the internal surface of the envelope thereby enclosing the eight viral ribonucleoproteins (RNPs) (Murti et al., 1992; Fujiiyoshi et al., 1994). The NPs, on the other hand, form the backbone of the RNPs (Pons et al., 1969; Kingsbury et al., 1987; Ruigrok & Baudin, 1995).

In the infected cell newly synthesized NPs are imported into the nucleus where they take part in a RNA elongation/RNP assembly process (Davey et al., 1985; Honda et al., 1988; Krug et al., 1989). According to a favoured assembly model one or a few NPs bind to newly synthesized viral RNA, which will then stimulate further NP associations by cooperative NP–NP and NP–RNA interactions (Yamanaka et al., 1990). In contrast to NP, the bulk of the newly synthesized M1 protein binds to cellular membranes, in particular the plasma membrane (PM) (Meier-Ewert & Compans, 1974; Hay, 1974; Hay & Skehel, 1975). Here the M1 appears to act both as an adapter, bringing the viral spikes and the RNPs together, and as a budding device, that functions through lateral M1 self-association reactions on the PM (Compans & Choppin, 1975; Dubois-Dalcq et al., 1984). A part of M1 is also imported into the nucleus where it inhibits viral RNA transcription and facilitates RNP export into the cytoplasm (Meier-Ewert & Compans, 1974; Hay & Skehel, 1975; Zvonariev & Ghendon, 1980; Ye et al., 1987, 1995; Patterson et al., 1988; Martin & Helenius, 1991).

So far the models of NP- and M1-directed assembly processes are mostly supported by in vitro studies with proteins isolated from virions or produced in E. coli. For instance, NPs have been shown to associate into homopolymers ranging from trimers to large RNP-like structures in vitro (Ruigrok & Baudin, 1995). Furthermore, isolated NPs bind to RNA in a way that is consistent with the involvement of cooperative NP–RNA and NP–NP interactions (Yamanaka et al., 1990). Studies with isolated M1 protein have, on the other hand, clearly demonstrated that it has a lipophilic feature. Most notably, it binds efficiently to artificial membranes (liposomes) (Gregoriades, 1980; Gregoriades & Frangione, 1981; Bucher et
al., 1980). In addition, it has been demonstrated that M1 proteins bind cooperatively to both naked RNA and RNP (Wakefield & Brownlee, 1989; Watanabe et al., 1996). This suggests that M1–RNA interactions might be involved in the assembly reaction between RNP and viral envelope.

Recently, a crystal structure of a major (NH2-terminal) proteolytic fragment of the M1 protein of influenza A/PR/8/34 virus was reported (Sha & Luo, 1997). This revealed that residues 2–158 of the 258-residue-long M1 fold into two domains, the NH2-terminal (N) and the middle (M) ones. Each of them contained four α-helices. Further, the M1 fragment was shown to be oligomerized into a dimer via M domain interaction. The two M domains formed a common positively charged surface-patch with a putative RNA-binding function. A membrane-binding function was suggested to be mediated by a hydrophobic surface region of the N domain.

In order to fully understand the assembly interactions of influenza A virus it is important to compare the in vitro and structural analyses with experiments done in vivo. A very useful in vivo approach is to express the viral components in cells, either individually or in various combinations, and analyze their interactions with each other and with host components. In this work, we have studied NP and M1 protein interactions in baby hamster kidney (BHK)-21 cells that had been transfected with Semliki Forest virus (SFV)-NP and -M1 RNA expression vectors. We were interested in the possible formation of NP–M1 complexes which would help to explain how RNP is incorporated into virions during assembly. However, no such complexes were demonstrated suggesting that RNP incorporation is mainly mediated through M1–RNA interactions. Instead, the M1 and NP proteins were found to undergo extensive homooligomerization reactions soon after synthesis.

Methods

DNA and antibodies. Plasmids pAPR701 and pAPR501, monoclonal antibodies (MAB) against M1 of WSN (39-4) and against NP of PR8 (HT103) were kindly provided by Peter Palese (Mount Sinai School of Medicine, New York, USA). MAB against M1 of influenza A virus (for immunofluorescence staining) was purchased from Serotec. Influenza A/PR/8/34 virus stock was obtained from Mikael Jondal (MTC Karolinska Institute, Sweden). Plasmids pSFV-C and pSFV1 have been described before (Liljestrom & Garoff, 1991; Suomalainen et al., 1992). SFV stock was prepared in BHK-21 cells as described before (Wahlberg et al., 1989).

Plasmid constructions. The DNAs encoding the M1 and NP proteins of influenza A/PR/8/34 virus were obtained from plasmids pAPR701 and pAPR501 by digestion with EcoRI. The ends of the fragments were converted to blunt-ends by Klenow polymerase reaction and then cloned into the unique Smal site of pSFV1 to generate plasmids pSFV1-M and pSFV1-NP. To achieve a higher level of expression, plasmids pSFV-C-M1 and pSFV-C-NP were made. These contain the M1 and the NP gene of influenza A/PR/8/34 virus in fusion with the capsid (C) gene of SFV. A precise fusion of the 3' coding region of the C gene and the 5' coding region of the M1 or NP genes was carried out by PCR using four different primers and plasmids pSFV1-M1 and pGEM1-7Z-SFV (our unpublished data) or pSFV1-NP and pGEM1-7Z-SFV as templates. The primers for making pSFV-C-M1 were:

(i) 5′ TTGGTCCATGTTATTTGGATC 3′ (nucleotides 289–310 in the M1 gene, encompassing a BamHI site)
(ii) 5′ CAACCGAAAACGGCACAGC 3′ (nucleotides 7672–7691 in the capsid gene, encompassing an AsuII site)
(iii) 5′ CCCGAGGATCCTGGAATGAGT GAGTCTTCTAACCAGGG-TGCAG 3′ (M1–C gene fusion primer)
(iv) 5′ TTCGACCTCGGTATAAGAACCTACCTTCGGGACACCCCT-GCGG 3′ (C–M1 gene fusion primer)

The primers for making pSFV-C-NP were:

(i) 5′ GAGACGACATCTCTGGAGTCC 3′ (nucleotides 522–541 in the NP gene, encompassing a BamHI site)
(ii) 5′ CAACGGAAAAACGGACAGAC 3′ (nucleotides 7672–7691 in the capsid gene, encompassing an AsuII site)
(iii) 5′ CCCGAGGATCCTGGAATGAGT GAGTCTTCTAACCAGGG-TGCAG 3′ (NP–C gene fusion primer) and
(iv) 5′ TCATGTTGGTCTTCTGAGACGCCCCATCCTTCGGGACCCCT-CGGG 3′ (C–NP gene fusion primer)

The PCR products were digested with BamHI and AsuII. Finally, pSFV-C-M1 and pSFV-C-NP were constructed by ligating the 701 bp or 913 bp AsuII–BamHI PCR fusion fragment together with the 10553 bp AsuII–BamHI fragment of pSFV-C and the 1611 bp BamHI–SpeI fragment of pSFV1-M1 or 1887 bp BamHI–SpeI fragment of pSFV1-NP. The constructs were confirmed by sequencing.

RNA transcription in vitro and transfection. RNA transcripts were synthesized in vitro by SP6 RNA polymerase using SpeI-linearized plasmid as described previously (Liljestrom & Garoff, 1991). Monolayers of BHK-21 cells (105 cells in a 75 cm² bottle) (ATCC), grown in BHK medium (Gibco) supplemented with 5% foetal calf serum, 10% tryptose phosphate broth, 2 mM glutamine and 20 mM HEPEs, were trypsinized and washed with phosphate-buffered saline without MgCl2 and CaCl2 (PBS). The cells were suspended in 0.6 ml PBS, mixed with 20 μl RNA transcription reaction mixture and then transferred into a 0.4 cm electroporation cuvette (Bio-Rad). Electroporation was done by two consecutive pulses at 0.85 kV and 25 μF, using a Bio-Rad Gene Pulser apparatus. Electroporated cells were suspended in 15 ml complete BHK medium and plated at 35 or 60 mm plates (Nunc) and incubated at 37 °C.

Co-expression of NP and M1 proteins. Equal amounts (20 μl) of SFV-C-M1 and SFV-C-NP RNAs were mixed and transfected into BHK-21 cells as described above. Co-expression of M1 and NP was monitored by indirect immunofluorescence staining (as described later) using anti-M1 or anti-NP MABs. The results showed that over 90% of the cotransfected cells expressed M1 and that over 90% expressed NP, indicating that more than 80% of cells expressed both M1 and NP proteins.

Metabolic labelling of transfected cells

Labelling of viral proteins. At 7 h after transfection, when host protein synthesis is shut off, cells were starved in methionine-free minimal essential medium (MEM) for 30 min and pulsed in the same methionine-free medium containing 100 μCi/ml [35S]methionine (Amersham, > 800 Ci/mM) for 15 min at 37 °C. After washing twice with MEM containing 100-fold excess cold methionine, the cells were incubated for various times in MEM with a 10-fold excess of methionine at 37 °C.

Labelling of host and viral proteins. Monolayers of BHK-21 cells were washed twice with phosphate-buffered saline containing MgCl2 and CaCl2 (PBS) and then incubated in methionine-free MEM for 30 min at 37 °C. The medium was replaced with 7 ml of the same methionine-free medium containing 1 μCi [35S]methionine and the cells were incu-
bated for 2 h at 37 °C. The labelling was then stopped by washing the cells twice with MEM containing a 100-fold excess of cold methionine and then incubated in the same medium for 5 min at 37 °C. The labelled cells were washed twice with PBS* and trypsinized. After washing with PBS, the cells were resuspended in 0.8 ml PBS*, mixed with in vitro-transcribed RNA and transfected as described above. The transfected cells were incubated for 7 h and viral proteins were pulse-labelled and chased as described above.

**Virus infection.** Wild-type SFV infection was carried out as described previously (Zhao & Garoff, 1992). After 3.5 h infection at 37 °C, the cells were starved and pulse-labelled as described above. Wild-type influenza virus infection was performed according to an earlier protocol (Barrett & Inglis, 1985). Briefly, monolayers of BHK-21 cells were grown on 35 mm dishes, rinsed with PBS, and then 10 p.f.u. per cell of virus stock diluted in PBS* was added. After 60 min of adsorption at 37 °C, the medium was replaced with fresh MEM and the incubation continued for 6 h. At this time-point the cells were starved, and pulse-labelled as described above.

**Preparation of cell lysate and immunoprecipitation.** The cells were solubilized with NP40 lysis buffer [1% (w/v) NP40, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 10 µg/ml PMSF and 0.1 mM N-ethylmaleimide (NEM)] and the lysates were clarified by low-speed centrifugation at 3000 g for 5 min in an Eppendorf centrifuge. The lysates were either processed directly for SDS–PAGE or first incubated with anti-M1 or anti-NP MAb in order to precipitate the respective antigen as described previously (Zhao & Garoff, 1992). When indicated, the cells were lysed in SDS lysis buffer [1% SDS, 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 10 µg/ml PMSF and 0.1 mM NEM] prior to SDS–PAGE.

**Fractionation of nuclear and cytoplasmic proteins.** Transfected and radio-labelled BHK-21 cells were lysed in NP40 lysis buffer and clarified as described. The lysate was transferred to a new tube, whereas the pelleted material, which contains the cell nuclei, was suspended in SDS lysis buffer. Samples of both fractions were mixed with SDS–PAGE sample buffer and analysed by SDS–PAGE.

**Membrane flotation.** This was done essentially as described in Chong & Rose (1993). Transfected BHK-21 cells were pulse-labelled for 20 min, chased for 15 min, and then scraped into an ice-cold cell homogenization buffer containing 10% (w/v) sucrose, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1 mM NEM and PMSF (10 µg/ml). Cells were disrupted by passing the mixture 30 times through a 23 gauge needle. Nuclei and other large material debris were removed from the homogenate by centrifugation at 6000 r.p.m. for 5 min at 4 °C in an Eppendorf centrifuge. A sample of the supernatant was adjusted to 50% sucrose in water. A 3 ml sample of this was placed at the bottom of a Beckman SW 41 centrifuge tube, and overlaid with 5.5 ml 65% (w/v) sucrose in water and 2.5 ml 10% (w/v) sucrose in water. The step gradient was centrifuged at 35000 r.p.m. for 18 h at 5 °C. Fractions were collected from the top, 1 ml per fraction, and samples of these were analysed directly on SDS–PAGE.

**Extraction of M1 protein from membranes.** Membranes were isolated from transfected cells by flotation in a sucrose gradient as described above, incubated with (i) 2 M KCl/10 mM Tris–HCl (pH 7.4) for 1 h at 25 °C or (ii) 100 mM Na₂CO₃ for 30 min at 0 °C (Chong & Rose, 1993; Fujiyoshi et al., 1994). Treated membranes were adjusted to 80% (w/v) sucrose, and subjected to a new membrane flotation centrifugation. Fractions were collected from the top, and portions of these were analysed directly by SDS–PAGE.

**Immunofluorescence.** RNA-transfected or influenza virus-infected BHK cells were incubated for 7 h at 37 °C, washed twice with PBS*, fixed in ice-cold methanol for 6 min at —20 °C, and then washed three times with PBS-E (1.8 mM NaH₂PO₄, 12 mM Na₂HPO₄, 50 mM NaCl, pH 7.4) and twice with PBS-E containing 0.2% gelatin to block unspecific antibody binding. The cells were first incubated with either anti-M1 or anti-NP MAb as the primary antibody and later with an FITC-conjugated rabbit anti-mouse antibody as the secondary antibody. All incubations with antibody were in PBS-E containing 0.2% gelatin, for 30 min at room temperature. After each incubation, cells were washed three times with PBS-E and twice with PBS-E containing 0.2% gelatin. Finally, the cells were rinsed with water, and mounted in Mowiol 4-88 containing 2.5% DABCO (1,4-diazabicyclo [2.2.2] octane). Fluorescence staining was observed and photographed on a Zeiss Axiophot photomicroscope with Kodak Tₘₐₓ p3200 film.

**Sedimentation analysis.** Transfected cells were incubated for 7 h, pulse-labelled for 10 min and chased for either 5 or 60 min. The cells were then lysed with NP40 lysis buffer and insoluble material was pelleted by low-speed centrifugation. Samples of the supernatant fractions were loaded on to either a 5–30% (w/v) sucrose gradient in TNE [50 mM Tris–HCl (pH 7.4), 100 mM NaCl and 0.5 mM EDTA] containing 0.1% NP40 and centrifuged in an SW41 rotor at 39000 r.p.m. for 20 h at 4 °C, or a 15–30% (w/v) sucrose gradient in TNE and centrifuged in an SW41 rotor at 40000 r.p.m. for 2 h at 4 °C. The gradients were fractionated from the bottom into 21–22 fractions (550 µl per fraction). Aliquots of the fractions were analysed by SDS–PAGE.

**RNase A digestion.** RNA-transfected cells were incubated for 5 h, labelled for 60 min with [³⁵S]methionine and chased for 5 min. The cells were then lysed in NP40 lysis buffer, clarified as described and incubated with 100 µg/ml RNase A (Boehringer Mannheim) for 10 min on ice. The samples were separated on 5–30% (w/v) sucrose gradients and analysed by SDS–PAGE as described.

**Trichloroacetic acid precipitation.** Proteins in the medium of transfected and pulse-labelled cells were precipitated with trichloroacetic acid (TCA) and analysed by SDS–PAGE. Precipitation was done in 10% TCA for 1 h on ice. Precipitates were collected by centrifugation at 16000 g for 10 min at 4 °C in an Eppendorf tabletop centrifuge. The pellets were washed once in cold acetone and then dissolved in SDS–PAGE sample buffer by sonication for 20 min and heating at 95 °C for 5 min.

**SDS–PAGE and quantification.** Aliquots of all lysates or washed immunoprecipitates were mixed with SDS–PAGE sample buffer [0.1 M Tris–HCl (pH 8.8), 0.5 M sucrose, 0.02% bromphenol blue, 5 mM EDTA (pH 8.0), 10 mg/ml methionine and 4% SDS], incubated for 5 min at 70 °C and separated on 12% SDS–PAGE gels (Lobigs et al., 1990). After electrophoresis, the gels were soaked for 30 min in 1 M sodium salicylate, dried and exposed to Fuji medical X-ray film. The amount of [³⁵S]methionine in the protein bands were measured by a Fuji phosphoimager (Sjöberg et al., 1994).

**Results**

**Synthesis and localization of M1 and NP**

Transfection of BHK-21 cells with SFV-C-M1 and SFV-C-NP RNA resulted in expression of the influenza virus NP and M1 proteins. These were seen as 56 and 27 kDa protein bands in the SDS–PAGE analyses (Fig. 1). The identities of the influenza virus proteins were confirmed by immunoprecipitation and gel-mobility comparisons with viral proteins from infected cells. Note the concomitant expression of the SFV C protein. This is released from the initially synthesized C–M1 complex.
Fig. 1. Expression of influenza virus M1 and NP proteins from SFV replicons. BHK-21 cells were transfected with SFV-C-M1 or SFV-C-NP RNA by electroporation and incubated for 7 h. Control cells were infected with SFV and influenza virus. The cells were labelled with $^{[35]S}$methionine for 15 min, chased for 15 min and lysed in NP40 lysis buffer. Aliquots of the lysates were analysed either directly or after immunoprecipitation with anti-M1 or anti-NP MAb by SDS–PAGE (12%). (A) SFV-infected cell lysate which was used as a control (lane 1), SFV-C-M1 RNA-transfected cell lysate (lane 2), and anti-M1 MAb-precipitated material from SFV-C-M1 RNA-transfected cell lysate (lane 3). (B) SFV-infected cell lysate (lane 1), SFV-C-NP RNA-transfected cell lysate (lane 2), and anti-NP MAb-precipitated material from SFV-C-NP RNA-transfected cell lysate (lane 3). (C) Influenza A/PR/8 virus-infected cell lysate (lane 1), SFV-C-NP RNA-transfected cell lysate (lane 2) and SFV-C-M1 RNA-transfected cell lysate (lane 3).

Fig. 2. Stability and release of NP and M1 proteins. Cells were transfected with SFV-C-M1 RNA (A) and SFV-C-NP RNA (B) separately or in combination (C). After incubation for 7 h, cells were pulse-labelled for 15 min and chased for 5, 60 or 180 min. Media (labelled M in the figure) were then collected and subjected to TCA-mediated protein precipitation. Cells (labelled C) were solubilized in SDS-lysis buffer.

and C–NP fusion proteins by a cotranslational, C-mediated autoproteolysis (Sjöberg et al., 1994). The fates of the newly synthesized M1 and NP proteins were initially studied in three kinds of pulse–chase experiments. In the first one we analysed the distribution of the proteins in cells and media. The results showed that both influenza virus proteins remained associated with the cells during a 180 min chase (Fig. 2). There was neither a significant degradation nor release into medium of these proteins during this time. In the second experiment we followed the extractability of M1 and NP with NP40. We found that both proteins accumulated with time in the NP40-insoluble cell fraction, which include cell nuclei and cytoskeleton (Fig. 3). In the samples chased for 5 min about half (51%) of M1 and a minor amount (25%) of NP were found in the NP40-insoluble fraction. After a 180 min chase most M1 (85%) and NP (63%) were found in this fraction. In the third experiment we studied the membrane association of M1 and NP using a cell homogenization/membrane flotation assay. The results showed that about 15% of M1, but virtually no NP, was bound to the floating...
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Fig. 3. NP40 extraction of M1 and NP. Cells were transfected with SFV-C-M1 (A) or SFV-C-NP RNA (B) separately or in combination (C). The cells were pulse-labelled (15 min) and chased as indicated and lysed in NP40 lysis buffer. Solubilized material (labelled S) was separated from particulate material (labelled P) by centrifugation and both fractions were analysed by SDS–PAGE.

Membranes (Fig. 4). Additional studies showed that the membrane binding of M1 occurred within 5 min after synthesis and that it was partially sensitive to treatments with 2 M KCl and 100 mM Na$_2$CO$_3$ (data not shown).

The intracellular localizations of M1 and NP were studied by immunofluorescence analyses of transfected cells. The NP analyses showed strong staining in the nucleus and weaker staining in the cytoplasm (Fig. 5E). In contrast, the M1 analyses showed a strong patchy and reticular staining in the cytoplasm, predominantly in a perinuclear region (Fig. 5B). Influenza virus-infected cells were analysed as controls. These showed, both for M1 and NP, strong staining in the nucleus and weaker staining in the cytoplasm (Fig. 5A, D).

When the M1 and NP proteins were coexpressed in cells they behaved the same in our pulse–chase and immunofluorescence analyses as when expressed separately (Figs 2C, 3C and 5C, F). This was also the case when the M1 and NP proteins were expressed at reduced (1/10–1/20) levels using SFV1-M1 and SFV1-NP RNAs (data not shown). However, there was one notable exception. When expressed at lower level the bulk of M1 was found to be NP40-soluble at all chase times.

M1 and NP oligomerization

The oligomeric state of the M1 and NP proteins was studied, by sedimentation in sucrose gradients, in NP40 extracts of cells transfected with SFV-C-NP and SFV-C-M1 RNA. We used the SFV nucleocapsid (NC, 150S), the SFV membrane-protein complex p62–E1, which matures into an E2–E1 complex (4–5S; molecular mass, with bound detergent, 150 kDa), and the ribosomal subunits (60S and 40S) as markers (Ulmanen et al., 1976; Simons et al., 1973). Rapidly sedimenting
large structures and slowly sedimenting smaller structures were analysed by short (2 h) and long centrifugation times (20 h), respectively. We also chose two different chase times (5 and 60 min) after pulse-labelling of transfected cells to see whether there was a progressive oligomerization reaction for either protein during the first hour after synthesis.

The results of the 20 h sedimentation analyses showed that both NP and M1 proteins oligomerized into a series of complexes (Fig. 6 A, B). In the case of the M1 protein, most migrated slightly slower than the SFV p62–E1 membrane protein complex (compare Fig. 6 A with 6 D). The larger M1 complexes formed a continuous series that decreased in amount as their sizes increased. In the 2 h sedimentation analyses the largest M1 oligomers were shown to migrate somewhat
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Fig. 6. Oligomerization of the NP and M1 proteins. Cells were transfected with SFV-C-M1 RNA (A, E), SFV-C-NP RNA (B, F) or cotransfected with SFV-C-M1 RNA and SFV-C-NP RNA (C, G). Control cells were transfected with wt SFV RNA (D, H). After pulse-labelling for 10 min and chasing for 5 or 60 min, the cells were lysed with NP40 lysis buffer. Clarified lysates were layered on top of a 5–30% (w/v) sucrose gradient in TNE, which was run in an SW41 rotor for 20 h at 4 °C. Gradients were fractionated and analysed by SDS–PAGE. Each panel shows fractions from bottom to top of the gradients, and the original NP40 extract (total). Note that in all analyses of gradients two separate gels were been used. In the figures the autoradiographs of both gels have been combined and this has resulted in some mobility differences of proteins when comparing left- and right-half of each panel.

Fig. 7. Immunoprecipitation of M1 and NP oligomers. Cells were labelled with [35S]methionine for 2 h and then transfected with SFV-C-M1 RNA, SFV-C-NP RNA or a mixture of SFV-C-M1 and SFV-C-NP RNAs. After a 5 h incubation, cells were pulse-labelled (a second time) for 30 min and chased for 5 min. The cells were lysed in NP40 lysis buffer and then processed for immunoprecipitation with anti-M1 or anti-NP MAb. Samples of cell lysates (C) and immunoprecipitates (αM1 and αNP) were analysed by SDS–PAGE.
slower than the ribosomal subunits (data not shown). There was no significant change in the oligomerization pattern of pulse-labelled M1 when comparing samples chased for 5 and 60 min (Fig. 6A, E). Note that in the SFV control sample, which was chased for 60 min, most of the p62–E1 membrane protein complex matured into E2–E1 complex (Fig. 6H). Note also that all SFV C protein is present as NCs, which are pelleted in the 20 h sedimentation analyses.

In the short (2h) sedimentation analyses the NP was found in all fractions of the gradient and in the pellet (data not shown). However, the bulk of the material migrated slower than the ribosomal subunits. In the 20 h sedimentation analyses of the sample chased for 5 min there was a clear peak of NP complexes that migrated slightly faster than the SFV spike complexes (compare Fig. 6B with 6D). In addition, NP complexes were present in all fractions towards the bottom. About 15% of the NP complexes was pelleted under these conditions. Interestingly, the peak of small-sized NP complexes was absent in the cell sample chased for 60 min whereas the large NP oligomers were still present (Fig. 6 F).

The M1 and NP oligomers in lysates from cotransfected cells showed essentially the same sedimentation pattern as when expressed separately (compare Fig. 6A, B and C). Altogether we conclude that the M1 and NP proteins show a strong tendency to oligomerize soon after synthesis.

**Nature of NP and M1 oligomers**

The possibility that the NP and M1 proteins formed complexes with host proteins was investigated by immunoprecipitation. In this experiment, cell cultures were labelled with \[\textsuperscript{35}S\]methionine for 2 h before transfection with SFV-C-NP or SFV-C-M1 RNA, respectively. After a 5 h incubation, when viral protein synthesis was most efficient, the cells were labelled a second time, for 30 min. The cultures were then chased for 5 min, lysed and immunoprecipitated with anti-M1 or anti-NP MAb. The electrophoretic analyses of the NP- and M1-transfected cell lysates showed the presence of a large amount of host proteins in addition to the viral protein (Fig. 7, lanes 1 and 3). However, immunoprecipitation resulted in an
essentially clean recovery of NP or M1 protein (Fig. 7, lanes 2 and 4). This suggests that the oligomeric complexes of the two influenza virus proteins do not contain any significant amounts of host proteins.

Immunoprecipitation analyses were also used to study whether the NP and M1 proteins interacted and formed heterooligomers. In this case, we used prelabelled cells that were cotransfected with SFV-C-NP and SFV-C-M1 RNAs. When an NP40 lysate of such cells was reacted with anti-NP MAb and the precipitate analysed, only NP (no M1 or host proteins) was recovered (Fig. 7, lane 5 and 7). Similar analyses with the anti-M1 MAb showed mostly M1 but also a small amount of NP (Fig. 7, lanes 5 and 6). The latter corresponded to the amount of NP that the anti-M1 MAb precipitated nonspecifically from a lysate of cells expressing only NP (data not shown). Therefore, we conclude that the NP and M1 protein did not form heterooligomers. This conclusion was further supported by the fact that the characteristic sedimentation profiles of the separately expressed NP and M1 oligomers (Fig. 6A, B) did not change when the two proteins were coexpressed (Fig. 6C).

A further possibility was that the viral proteins formed complexes with cellular RNA. In particular, this could be the case with the large NP complexes. We therefore incubated NP40 lysates of prelabelled and transfected cells with RNase and compared the remaining viral protein complexes with those of untreated lysates by sedimentation analyses. The results showed that the large NP protein complexes, which were pelleted in the 20 h sedimentation (Fig. 8C), were digested into smaller units that migrated as a broad band in the
bottom half of the sucrose gradient (fractions 8–12 in Fig. 8D). In contrast, the RNase treatment of the SFV-C-M1 RNA-
transfected cell lysate did not change the migration of the M1 complexes in the sucrose gradient (Fig. 8A, B). We conclude that a significant amount of the NP forms complexes with RNA.

**Discussion**

In this work we have used SFV replicons to express two influenza A virus genes, those of NP and M1. This cytoplasmic gene expression system directed the synthesis of the authentic gene products, which remain stable in the transfected cells for at least 3 h. Using two variants of the SFV vectors, SFV1 and SFV-C, we were able to express different levels of the two influenza virus proteins. However, the two proteins behaved mostly the same in all our assays independent of concentration. In each of the two systems, it was possible to analyse the cell samples without using immunoprecipitation because the recombinant RNA replication results in a very efficient gene expression and concomitant shut-off of host mRNA translation (Kääriäinen & Ranki, 1984; Wengler, 1980; Liljestrom & Garoff, 1991).

The aim of this study was to analyse what kind of interactions newly synthesized NP and M1 proteins can make in the cell. Among others we were interested in whether NP and M1 proteins were able to complex with each other because this could explain the mechanism by which RNP are assembled into viral particles. Our analyses demonstrated, however, that heterooligomeric complexes were not formed between NP and M1 proteins. This was most convincingly shown by our immunoprecipitation analyses using lysates of cells that had been co-transfected with SFV-C-NP and SFV-C-M1 RNAs. Therefore, our result suggests that M1 does not interact with NP during virus assembly. Most likely, the interaction of RNP with M1 is mediated by its RNA component. Indeed, the viral RNA is known to be exposed in the RNP and it has been shown that isolated M1 binds to RNA in vitro (Wakefield & Brownlee, 1989; Yamamoto et al., 1990; Watanabe et al., 1996). A tentative RNA-binding region has also been mapped in the M1 protein structure (Sha & Luo, 1997).

Although we could not find NP and M1 heterooligomers our analyses did show that both proteins efficiently formed homooligomers soon after synthesis. Among the heterogeneously sized NP homooligomers there was a preference for small-sized complexes that migrated slightly faster than the membrane-protein heterodimer of SFV. If one considers that the molecular mass of the latter complex is about 150 kDa one can estimate the NP complexes to be homotrimers (molecular mass 168 kDa) or tetramers (molecular mass 224 kDa). Such NP complexes have been observed before in an in vitro study of NP assembly and recently in influenza virus-infected cells also (Ruigrok & Baudin, 1995; Prokudina-Kantorovich & Semenova, 1996). Most interestingly, our pulse–chase analyses demonstrated that the NP trimers/tetramers were almost completely removed from the NP40 lysate within 1 h of synthesis. This coincided with the increased recovery of NP in the NP40-nonextractable fraction (see below). A likely explanation of this phenomenon is that the NP trimers/tetramers are transported from the cytoplasm into the cell nucleus. These results could be interpreted such that NP homooligomers/tetramers (and not monomers) are used for RNP assembly in the nucleus of influenza virus-infected cells. A significant amount of NP was further present in large complexes that contained RNA. These might be RNP-like assemblies between NP and host or SFV recombinant RNA. We could not observe any M1 binding to these complexes.

The M1 protein formed oligomers which were slightly smaller, of equal size or larger than the SFV membrane-protein complex. In view of the recent structural data on M1 crystals, it is possible that the M1 oligomers we observed in our study represent M1 dimers and multimers thereof (Sha & Luo, 1997). M1 multimerization is thought to be involved both in the process of M1 binding to the RNA of the RNP and during the formation of the submembranous M1 layer during virus budding (Fujiyoshi et al., 1994; Watanabe et al., 1996).

Our cell-fractionation studies showed that both NP and M1 proteins accumulated in the NP40-extraction-resistant fraction with time after synthesis. The behaviour of NP can be explained by nuclear transport of NP but the reason for the increasing resistance of M1 to NP40 extraction is more difficult to understand. It might be related to the characteristic patchy immunofluorescence staining in the perinuclear region of the cell. Recently, a similar extraction behaviour of M1 was observed in influenza virus-infected cells (Zhang & Lamb, 1996; Avalos et al., 1997). In the latter studies, it was proposed that M1 associates with cytoskeletal elements of the cell. This had also been suggested earlier on the basis of immunofluorescence analyses (Bucher et al., 1989). Interestingly, the resistance to NP40 extraction was not observed when M1 was expressed separately or in combination with NP using a vaccinia virus expression system (Zhang & Lamb, 1996; Avalos et al., 1997). The authors’ explanation for this discrepancy was that M1 binds to the cytoskeleton via a viral component, e.g. RNP, which was not present in the experiments with the vaccinia expression system. However, we have here shown that separately expressed M1 also becomes resistant to NP40 extraction. Furthermore, we demonstrated that this effect depends on the concentration of M1. When M1 was expressed at high level using the SFV-C-M1 RNA vector it became resistant to NP40 extraction but not when it was expressed at a considerably lower level using the SFV1-M1 RNA vector. In the earlier studies on the resistance of M1 to NP40 extraction its possible dependence on M1 concentration was not considered. Therefore, we conclude that the reason for the resistance of M1 to NP40 extraction is still unclear as is also the possible significance of this phenomenon for virus maturation.
We also found that a significant fraction of M1 was associated with cellular membranes. This confirms the similar results of earlier studies done in vitro using isolated M1 and other studies where M1 has been expressed in cells (Bucher et al., 1980; Gregoriades, 1980; Gregoriades & Frangione, 1981; Enami & Enami, 1996; Kretzschmar et al., 1996; Zhang & Lamb, 1996). Our analysis of the medium of transfected cells suggested, furthermore, that the membrane-associated M1 protein did not have the capacity to drive membrane budding at the PM, neither when it was expressed separately, nor together with NP. This finding has recently been confirmed by electron-microscopic analyses of thin sections of transfected cells. These did not reveal any budding profiles at the PM or at intracellular membranes (H. Zhao, unpublished). Our results with the influenza virus M1 protein are clearly different form those obtained with matrix (M) protein of the rhabdovirus vesicular stomatitis virus (VSV). Like the M1 protein of influenza virus the M protein of VSV is also an internal protein which binds to membranes in vivo and in vitro (Lenard & Vanderoe, 1990; Chong & Rose, 1993, 1994; Zakowski et al., 1981; Dubovi & Wagner, 1977). When VSV M was expressed separately in insect and animal cells it induced efficient membrane vesicularization at the PM (Li et al., 1993; Justice et al., 1995). This indicates that the activities of the matrix protein of different enveloped negative-stranded RNA viruses are not equivalent. It also points to differences in the budding mechanisms of these viruses, although the M–M and M–membrane interactions probably provide the bases for the budding reaction in all cases. Thus influenza virus budding with M1 might be especially dependent on supporting M–RNP and M–spike interactions. In future, further coexpression experiments involving in particular the viral RNPs might shed more light on the assembly process of influenza virus and also on that of other enveloped negative-stranded RNA viruses.

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