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Respiratory syncytial virus matrix protein associates with nucleocapsids in infected cells

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Little is known about the functions of the matrix (M) protein of respiratory syncytial virus (RSV). By analogy with other negative-strand RNA viruses, the M protein should inhibit the viral polymerase prior to packaging and facilitate virion assembly. In this study, localization of the RSV M protein in infected cells and its association with the RSV nucleocapsid complex was investigated. RSV-infected cells were shown to contain characteristic cytoplasmic inclusions. Further analysis showed that these inclusions were localization sites of the M protein as well as the N, P, L and M2-1 proteins described previously. The M protein co-purified with viral ribonucleoproteins (RNPs) from RSV-infected cells. The transcriptase activity of purified RNPs was enhanced by treatment with antibodies to the M protein in a dose-dependent manner. These data suggest that the M protein is associated with RSV nucleocapsids and, like the matrix proteins of other negative-strand RNA viruses, can inhibit virus transcription.

Human respiratory syncytial virus (RSV) is a non-segmented negative-strand RNA virus belonging to the family *Paramyxoviridae*, genus *Pneumovirus* (Collins *et al.*, 1996). RSV-infected cells contain characteristic cytoplasmic inclusions. Previous studies have shown that these inclusions contain the nucleocapsid (N) protein, the viral polymerase (P and L proteins) (Collins *et al.*, 1996) and the transcription elongation factor protein M2-1 (Garcia *et al.*, 1993). It has been proposed that, as with Sendai virus, the P protein interacts with the newly synthesized N protein to prevent incorrect assembly of the nucleocapsid and to deliver it to the nascent RNA during genome replication (Curran *et al.*, 1995; Garcia-Barreno *et al.*, 1996), whereas the M2-1 protein is required for the synthesis of full-length viral RNA (Collins *et al.*, 1996). These observations suggest that the inclusions are ribonucleoprotein (RNP) aggregates (Garcia-Barreno *et al.*, 1996).

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Matrix proteins of negative-strand RNA viruses have been shown to associate with viral nucleocapsids in infected cells and virions. This association serves two functions: (1) to facilitate assembly and (2) to inhibit the transcriptase activity of the nucleocapsid prior to encapsidation (Coronel *et al.*, 2001; Kaptur *et al.*, 1991; Lenard, 1996). The two functions are genetically unrelated (Kaptur *et al.*, 1991). In this paper, we provide evidence that the M protein of RSV is associated with RNPs and also show that it has transcriptase inhibition activity.

M protein localization in RSV-infected HEp2 cells (Ghildyal *et al.*, 1999) was investigated by immunofluorescence assays at time-points ranging from 12 to 20 h post-infection (p.i.). Cells were fixed with 4% formaldehyde for 10 min and permeabilized with 0·1% Triton X-100 for 5 min (Lyles *et al.*, 1988). We chose formaldehyde as it is a gentle fixative

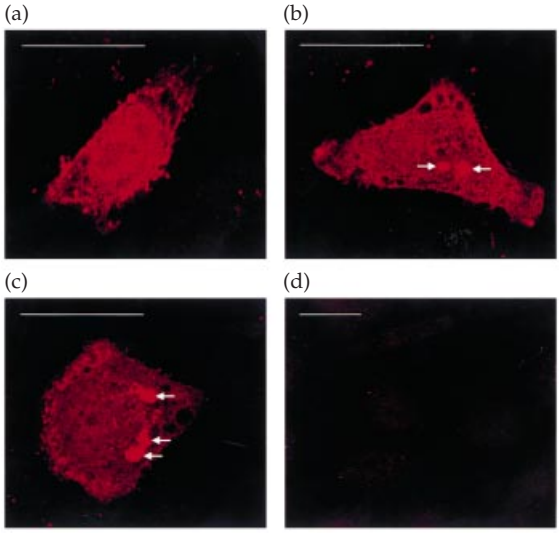

Fig. 1. Localization of the M protein in infected cells. RSV-infected cells were fixed with formaldehyde and Triton X-100 at (a) 16, (b) 18 and (c) 20 h p.i. Mock-infected cells were fixed at 20 h p.i. (d). the M protein was detected by incubating with mAb C781 and TRITC-conjugated goat antibodies to mouse immunoglobulin. Stained cells were observed by confocal microscopy. Cytoplasmic inclusions are indicated by arrows. Bar, 10 μm.

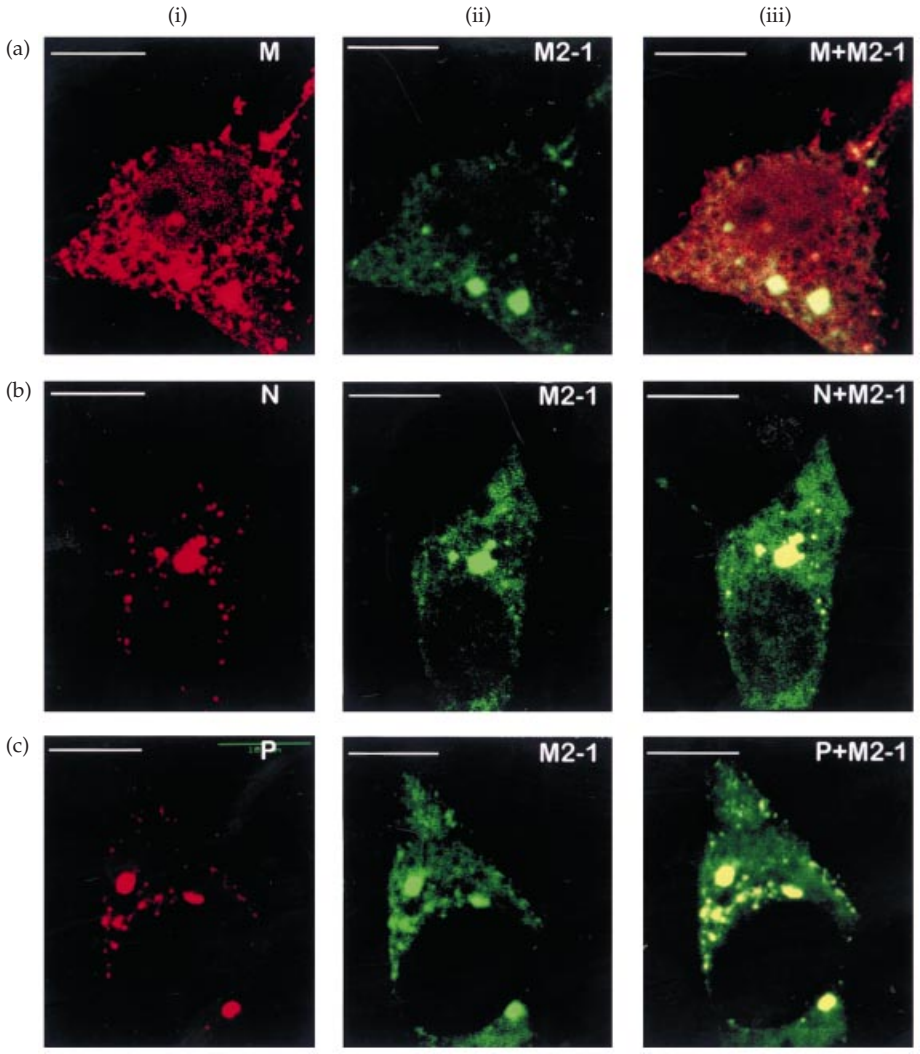

Fig. 2. Co-localization of RSV proteins in cytoplasmic inclusions of infected cells. RSV-infected cells were fixed, as described in Fig. 1, and probed with a mixture of guinea pig antisera to the M2-1 protein and one of mAbs to the M, N or P protein, followed by detection with a mixture of rabbit antibodies to guinea pig immunoglobulin conjugated to FITC and goat antibodies to mouse immunoglobulin conjugated to TRITC. Stained cells were analysed by confocal microscopy. Co-localization of (a) the M and M2-1 proteins, (b) the N and M2-1 proteins and (c) the P and M2-1 proteins is apparent. Localization of (i) the M, N or P protein and (ii) the M2-1 protein is also observed. (iii) A computer-generated image produced by the superimposition of the red and green channel outputs of the same cell at the same optical plane. Yellow areas indicate co-localization of proteins. The three images in each row (a, b and c) represent the same cell, while the images in each column (i, ii or iii) represent three separate experiments. Bar, 10 μm.

compared to acetone or methanol. Fixed cells were incubated with an anti-M monoclonal antibody (mAb C781) (Orvell *et al.*, 1987) diluted 1:100 in PBS (pH 7·2) for 30 min, followed by goat anti-mouse immunoglobulin conjugated to TRITC (Sigma) (diluted 1:40 in PBS). Data for three time-points, 16, 18 and 20 h p.i., are presented in Fig. 1. The M protein was observed throughout the cell at 16 h p.i. (Fig. 1a), whereas at 18 h p.i., the M protein was present in cytoplasmic inclusions as well as diffused throughout the cell (Fig. 1b). At 20 h p.i., the M protein was present predominantly within cytoplasmic inclusions and very little was observed in the rest of the cell (Fig. 1c). The specificity of mAb C781 was demonstrated by the low level of staining in mock-infected cells (Fig. 1d).

To determine if the inclusions to which the M protein localized were the same as those described in RSV-infected cells containing the N, P, L and M2-1 proteins (Garcia-Barreno *et al.*, 1996), we carried out double-labelled immunofluorescent confocal microscopy (Fig. 2). For this experiment, we generated antibodies to M2-1 protein. The M2 gene was amplified by specific RT–PCR from total RNA extracted from RSV-infected cells and cloned, in-frame, into pET-30(a) (Novagen). Recombinant M2-1 was expressed and purified from inclusion bodies by Ni-chelation (Qiagen Ni–NTA spin columns)

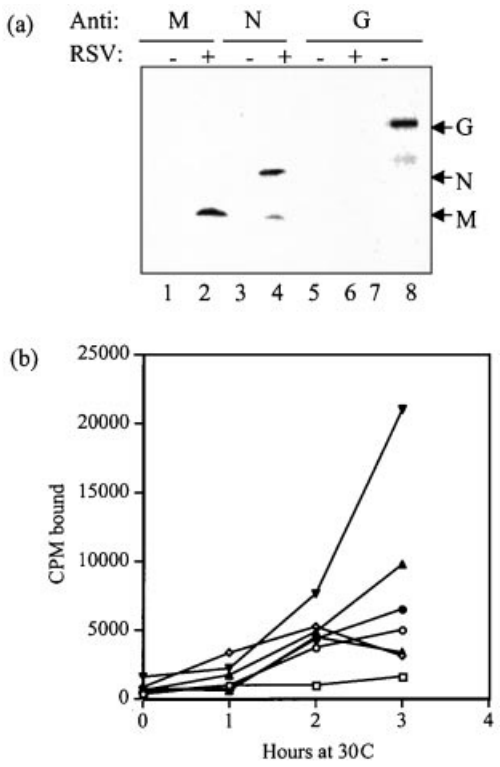

Fig. 3. (a) Immunoblot of purified RNPs. RNPs purified from mock- (−) and RSV-infected cells (+) were electrophoresed on a 10% SDS–polyacrylamide gel and transferred onto a Hybond-C membrane. The same blot was probed sequentially with antibodies to the M (lanes 1 and 2), N (lanes 3 and 4) and G (lanes 5 and 6) proteins. Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibodies, followed by ECL. RSV- and mock-infected cell extracts were electrophoresed in a similar manner and probed with antibody to the G protein (lanes 7 and 8). (b) Inhibition of transcriptional activity of RNPs by the M protein. RNP complexes were prepared and used immediately for transcription assays. Bound c.p.m. are plotted against reaction time. □, RNPs without any soluble proteins; △, soluble proteins without any RNPs. All other reactions were performed with RNPs in the presence of soluble proteins: no mAb C781 (○), mAb C781 diluted 1:1000 (●), mAb C781 diluted 1:100 (▲), mAb C781 diluted 1:10 (▼) and mAb 1420 (a non-specific antibody) diluted 1:10 (◇).

under denaturing conditions, according to the manufacturer's instructions. The purified M2-1 protein was then used to raise polyclonal antibodies in guinea pigs.

RSV-infected cells were fixed at 18 h p.i. and incubated with a mixture of anti-M2-1 guinea pig antisera and any one of the mAbs to N, P or M (mAbs B27, C771 or C781, respectively) (Orvell *et al.*, 1987). All antibodies were used at a dilution of 1:100 in PBS. Bound antibodies were detected by a mixture of goat anti-mouse immunoglobulin conjugated to TRITC and rabbit anti-guinea pig immunoglobulin conjugated to FITC (DAKO). Protein localization was studied by confocal microscopy (Magliano *et al.*, 1998) and co-localization was scored by an observer unaware of the experimental details. The 18 h p.i. time-point was chosen as this was the earliest time when the M protein was detected in cytoplasmic inclusions (Fig. 1). Fig. 2 depicts the results of the double-labelled immunofluorescence assay showing M, N, P (Fig. 2, panel i) and M2-1 (Fig. 2, panel ii) protein localization. Co-localization of the M, N or P proteins with the M2-1 protein (Fig. 2, panel iii) is also apparent. Fig. 2(a) shows that all inclusions containing the M2-1 protein also contain the M protein, whereas Fig. 2(b and c) shows that all inclusions containing the M2-1 protein also contain the N and P proteins, respectively. These data indicate that cytoplasmic inclusions contain aggregates of the N, P, M2-1 and M proteins. Previously, these inclusions have been suggested to be RNP aggregates (Garcia-Barreno *et al.*, 1996); however, no association with the M protein was reported.

To determine if the M protein was indeed associated with RNPs, we purified RNPs from RSV-infected cells and performed immunoblotting assays. RNPs were purified from infected and mock-infected cells, as described previously (Barik, 1992), with some modifications. At 16 h p.i., cells were exposed to actinomycin D (2 μg/ml) for 2 h. At 18 h p.i., cells were washed and lysed (Barik, 1992). The cell lysate was then cleared of nuclei and cell debris and centrifuged at 150 000 ***g*** for 4 h through 40% glycerol in 50 mM Tris–acetate (pH 8) and 1 mM DTT onto a 100% glycerol cushion. The RNPs were collected and diluted in a small volume of 20 mM Tris–acetate (pH 8) containing 1 mM EDTA, re-centrifuged through 40% glycerol and used immediately for immunoblotting or transcription assays (Fig. 3). Mock-infected cells were treated in a similar manner. Soluble proteins were collected from the top of the glycerol gradient as well as the RNP fraction.

Purified RNPs (10 μg) were separated by SDS–PAGE and transferred onto a Hybond-C membrane (Amersham) using a Bio-Rad semi-dry transfer apparatus. The membrane was blocked overnight in 10 mg/ml BSA in 0·1 M Tris–HCl (pH 7·5) containing 0·15 M sodium chloride (TBS) and probed with mAb C781 diluted 1:1000 in 5 mg/ml BSA in TBS containing 0·1% Tween 20. Bound antibody was detected by horseradish peroxidase-conjugated secondary antibodies, followed by ECL (Amersham). Bound antibodies were stripped from the blot by incubation for 30 min at 55 °C in the presence of 0·1 M 2-mercaptoethanol and 2% SDS in 62·5 mM Tris (pH 6·8) and the same blot probed sequentially for the N and G (glycoprotein) proteins (using mAb B27 and rabbit polyclonal antisera, respectively). Finally, the blot was probed once again for the N protein to determine if repeated washing had led to excessive stripping of proteins from the blot. The N and G proteins were used as positive and negative controls, respectively.

The antibodies used in this experiment (Fig. 3a) were specific for RSV proteins, as no bands were observed in the mock lanes (Fig. 3a, lanes 1, 3, 5 and 7). The purified RNP preparation contained the M protein (Fig. 3a, lane 2). When the blot was re-probed, RNPs were found, as expected, to contain the N protein (Fig. 3a, lane 4). The smaller band seen in lane 4 is residual M protein, remaining due to the incomplete

stripping of primary antibodies from the previous experiment. To confirm that the M protein co-purified with the RNPs and was not a contaminant from the membrane or cytosol fractions, we looked for the presence of the RSV G protein in the same samples (Fig. 3a, lanes 5 and 6). The G protein is found in the cytoplasm and membrane of RSV-infected cells and is not associated with RNPs. There was no G protein in the RNP preparation (Fig. 3a, lane 6), indicating that the M protein co-purified with the RNPs. The antibody to G protein recognized a band of 85–90 kDa in RSV-infected cell extracts (Fig. 3a, lane 8). The final probe for the N protein was positive (data not shown).

Next, we studied the role of M protein in association with the RNPs by carrying out transcription reactions with purified RNPs in the presence of increasing concentrations of mAb C781 (Fig. 3b). Standard transcription assays were performed as described previously (Barik, 1992). Briefly, each reaction contained 50 mM Tris–acetate (pH 8), 120 mM K–acetate, 5 mM $MgCl_2$, 5% glycerol, 1 mM DTT, actinomycin D (2 μg/ml), 20 μCi [α-^{32}P]CTP, UTP and GTP, each at 400 μM, 1 mM ATP, 10 μg RNPs and 8 μg soluble protein from mock-infected cells. The indicated amounts of mAb C781 or a non-specific antibody (mAb 1420) (BioDesign) were used in a 20 μl total volume. Reactions containing mAb C781 but without soluble proteins or without RNPs were also included as controls. Soluble proteins from mock-infected cells were added to render the RNPs transcriptionally active, as previous reports have shown that purified RNPs require cellular factors for transcriptase activity (Burke *et al.*, 2000). Reactions were carried out at 30 °C for 3 h. Synthesis of labelled RNA was quantified by applying 5 μl of the reaction mixture to DEAE-embedded glass fibre filtermats. These were processed for scintillation counting in a Wallac Microbeta counter. Incorporation of ^{32}P over a 3 h period was used to measure transcription. Data from one experiment, representative of three separate experiments, are shown in Fig. 3(b).

Results from these assays show that soluble proteins from mock-infected cells were essential for virus transcription, which was enhanced specifically in a dose-dependent manner in the presence of mAb C781, suggesting that the presence of the M protein inhibited virus transcription.

Our studies show that, during RSV infection, the M protein is present in characteristic cytoplasmic inclusions and is associated with RNPs containing the N, P and M2-1 proteins (Collins *et al.*, 1996). This study also provides evidence that the M protein may function as a virus transcription inhibition factor.

An earlier report by Routledge *et al.* (1987) on the immunofluorescent localization of the RSV M protein using mAb C781 did not show the M protein in cytoplasmic inclusions. This discrepancy is attributable to the fixative used (Lyles *et al.*, 1988; Westaway *et al.*, 1997). We found that acetone fixation led to patchy staining around the nucleus (Routledge *et al.*, 1987), while fixation with formaldehyde led to diffuse cytoplasmic staining with intense staining in cytoplasmic inclusions. Another report on RSV M protein localization used formaldehyde–glutaraldehyde fixing followed by immunoelectron microscopy and this report also failed to observe the M protein in cytoplasmic inclusions (Garcia *et al.*, 1993). These results differ from those reported here: this may be due to the different antibodies used in the two studies.

We have also shown that, like many other negative-strand RNA viruses, RSV nucleocapsids and the M protein interact in cytoplasmic inclusions within infected cells. Interactions between the matrix and the nucleocapsid proteins of Sendai virus (Markwell & Fox, 1980; Ryan & Kingsbury, 1988), Newcastle disease virus (Portner & Murti, 1986) and parainfluenza virus type 3 (De *et al.*, 1991) have been documented previously.

The nucleocapsid of negative-strand RNA viruses has to be rendered inactive before it can be packaged into the virus particle; this function has been attributed to the matrix proteins (Collins *et al.*, 1996). Vesicular stomatitis virus, measles virus and influenza virus matrix proteins interact with, and inhibit transcription by, viral nucleocapsids in infected cells (Carroll & Wagner, 1979; Perez & Donis, 1998; Suryanarayana *et al.*, 1994). The data presented here suggest that the RSV M protein is involved in such a function. Depleting the M protein from RNPs in the absence of soluble proteins from mock-infected cells did not lead to the activation of RNPs, confirming that cellular proteins are necessary for transcription by RNPs. Barik (1992) stated that RNPs isolated at 16 h p.i. were activated by the addition of soluble proteins but a later purification (24–30 h p.i.) resulted in RNPs incapable of being activated by the addition of soluble proteins. One explanation for this phenomenon could be that the M protein is associated with RNPs at later times p.i. We observed that, at 16 h p.i., the M protein is not present in cytoplasmic inclusions, whereas at 20 h p.i., it is found mainly within cytoplasmic inclusions.

Our data, taken together with earlier reports of RSV transcription, imply that the M protein associates with RNPs late in the virus life cycle in order to inhibit virus transcription activity, thus facilitating virus assembly. This makes the M protein the third RSV protein to be shown to regulate virus transcription. Previously, the NS1 protein was shown to inhibit virus RNA replication and transcription in a minigenome system and the M2-2 protein was shown to act as a switch from transcription to replication (Atreya *et al.*, 1998; Bermingham & Collins, 1999). In light of our current data, it is obvious that RSV has a very complex mechanism for the modulation of transcription and replication during virion assembly.

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