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Short Communication

Interaction between the respiratory syncytial virus G glycoprotein cytoplasmic domain and the matrix protein

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Paramyxovirus assembly at the cell membrane requires the movement of viral components to budding sites and envelopment of nucleocapsids by cellular membranes containing viral glycoproteins, facilitated by interactions with the matrix protein. The specific protein interactions during assembly of respiratory syncytial virus (RSV) are unknown. Here, the postulated interaction between the RSV matrix protein (M) and G glycoprotein (G) was investigated. Partial co-localization of M with G was demonstrated, but not with a truncated variant lacking the cytoplasmic domain and one-third of the transmembrane domain, in cells infected with recombinant RSV or transfected to express G and M. A series of G mutants was constructed with progressively truncated or modified cytoplasmic domains. Data from co-expression in cells and a cell-free binding assay showed that the N-terminal aa 2–6 of G play a key role in G–M interaction, with serine at position 2 and aspartate at position 6 playing key roles.

Respiratory syncytial virus (RSV) belongs to the genus *Pneumovirus* within the family *Paramyxoviridae*. Assembly of enveloped viruses is thought to be coordinated, at least in part, by the cytoplasmic domains of the envelope glycoproteins, as they have the potential to bind to viral components in the interior of the cell. Accordingly, a role in virus assembly has been established for the cytoplasmic domains of influenza virus haemagglutinin (HA) and neuraminidase proteins (Jin *et al.*, 1997), Newcastle disease virus fusion protein (F) (Schwalbe & Hightower, 1982), Sendai virus F and HN (Takimoto *et al.*, 1998), measles virus F (Naim *et al.*, 2000), vesicular stomatitis virus G glycoprotein (Lyles *et al.*, 1992) and Semliki Forest and Sindbis virus E2 proteins (Garoff *et al.*, 1998). However, the protein–protein interactions necessary for assembly of RSV are unknown.

RSV G glycoprotein (G) is a type II membrane protein and the major attachment protein of RSV (Levine *et al.*, 1987). Recent studies have shown that RSV lacking the G gene can replicate efficiently in cell culture, implying that RSV has an auxiliary attachment function, probably involving the F protein (Karron *et al.*, 1997; Techaarpornkul *et al.*, 2001; Teng *et al.*, 2001). Genetically engineered RSV lacking the entire G gene replicates efficiently in some cell lines but not in others, and is attenuated in BALB/c mice (Teng *et al.*, 2001). Thus, although G is not essential for replication in cell lines, it is required for full infectivity and *in vivo* pathogenicity, and a largely intact G gene has been found to be present in all RSV field isolates analysed to date. The RSV matrix protein (M) is postulated to play a major role in virus assembly through its interactions with various components of the virus, as well as with membranes of infected cells (Ghildyal *et al.*, 2002; Henderson *et al.*, 2002).

As observed in other paramyxoviruses, it is proposed that the coalescence of RSV components at budding sites is facilitated by interactions between the cytoplasmic domains of the envelope glycoproteins and M. This is supported by the observation that M associates with cellular membranes by itself, but that the nature of that association is modified in the presence of F (Henderson *et al.*, 2002).

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We investigated the interaction of M with RSV envelope glycoproteins, specifically G. We have provided evidence of an interaction between M and G that involves the G cytoplasmic domain and, in particular, its first 6 aa.

HEp2 cells (Victorian Infectious Disease Reference Laboratory, Melbourne, Australia) were infected with RSV subgroup A, strain A2 (a gift from Paul Young, University of Queensland, Brisbane, Australia) or recombinant RSV lacking the cytoplasmic domain and one-third of the transmembrane domain of G (sG-RSV; Teng *et al.*, 2001) (Fig. 1). In wild-type RSV, G is expressed as a full-length, membrane-associated form that initiates at the first ATG in the 298-codon ORF and as a secreted form that initiates at the second ATG (codon 48), one-third of the way into the transmembrane domain (Roberts *et al.*, 1994). sG-RSV expressed only the secreted form of G due to the engineered deletion of codons 1–47 (Teng *et al.*, 2001).

Infected cells were fixed at 18 h post-infection with either 4 % paraformaldehyde (for visualization of surface proteins; Fig. 1a, c) or 4 % formaldehyde followed by permeabilization with Triton X-100 (for visualization of cytoplasmic proteins; Fig. 1b, d) (Ghildyal *et al.*, 2002). Fixed cells were incubated with a mixture of C781 (anti-M mAb; a gift from Erling Norrby, Karolinska Institute, Sweden; Örvell *et al.*, 1987) and RαG (anti-G polyclonal antibody; a gift from Steve Feldman, Food and Drug Administration, USA). Bound antibodies were detected with a mixture of fluorochrome-conjugated species-specific secondary antibodies. Cells were analysed by confocal laser-scanning microscopy (CLSM) (Ghildyal *et al.*, 2002) and co-localization was scored by a group of investigators who were blinded with respect to the experimental details. In all CLSM, the pinhole was fixed at 1·0, optical slices of 0·2 μm were analysed and an image accumulation and smoothing operation was performed by using Leica TCS software. Images shown are representative of at least 30 cells from two different experiments.

As shown in Fig. 1(a, b), G, but not M, was detected on the surface of infected cells as expected [Fig. 1a(i) and (ii)]. In infected, permeabilized cells, G was observed throughout the cytoplasm with specific localization in patches, suggesting its association with intracellular membranes [Fig. 1b(i)]. M was observed within the cytoplasm, in patches similar to G, and in characteristic cytoplasmic inclusions as reported previously [Fig. 1b(ii)] (Ghildyal *et al.*, 2002). The computer-generated merge showed yellow patches, indicative of co-localization of M and G [Fig. 1b(iii)], which we suggest is occurring at intracellular membranes such as the Golgi. However, not all areas with cytoplasmic G had demonstrable M.

Fig. 1(c, d) shows the surface and intracellular localization of M and sG in sG-RSV-infected HEp2 cells. There was apparent surface localization of sG in cells infected with sG-RSV [Fig. 1c(i)]. Additionally, we routinely detected sG in the culture supernatant, indicating that it was transported

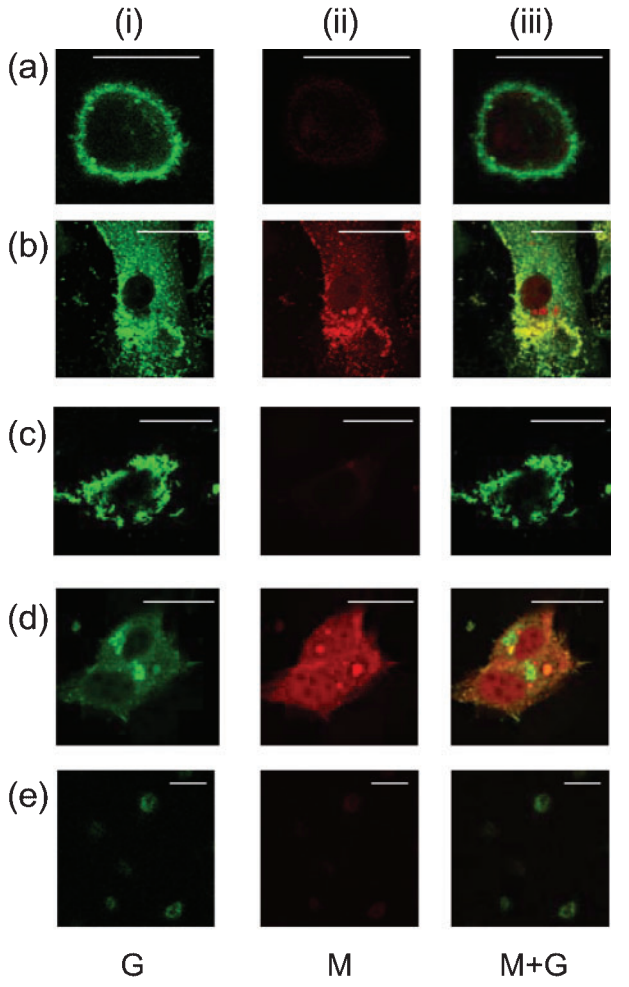

Fig. 1. Localization of RSV M and G in infected cells. Cells infected with wild-type RSV (a, b) or recombinant sG-RSV (c, d) were fixed either for surface (a, c) or for intracellular (b, d) staining and probed for G and M with RαG and C781 antibodies, followed by detection with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse antibodies. Mock-infected HEp-2 cells (e) were fixed for intracellular staining and treated similarly. Column (i), green fluorescence showing G localization; column (ii), red fluorescence showing M localization; column (iii), computer-generated merge at the same optical plane, with a yellow colour denoting co-localization. Bars, 10 μm.

to and across the plasma membrane, as has been reported previously (Teng *et al.*, 2001). Detection of sG at the cell surface was unexpected, but may indicate that it accumulates at least transiently at that site prior to secretion or that secreted sG remains associated with the cell via interaction with cell-surface components (Shields *et al.*, 2003). Interestingly, its cellular distribution was subtly different from that of wild-type G, as it was concentrated in what appeared to be surface projections. The significance of this is unknown, although it may be that the greater spread of wild-type G

along the cell surface is due to its longer residence time. As expected, M was not detected on the surface of infected cells [Fig. 1c(ii)]. sG was observed throughout the cytoplasm, with specific localization in patches [Fig. 1d(i)]. There was also some low-level staining in cytoplasmic inclusions, which may or may not be specific, as we did not observe this localization with full-length G. M was observed in the cytoplasm in patches (similar to sG), as well as in cytoplasmic inclusions [Fig. 1d(ii)]. However, the computer-generated merge showed no distinct regions of yellow colour [Fig. 1d(iii)]. Thus, M and sG did not co-localize in cells infected with sG-RSV. The observation that wild-type G, but not sG, co-localized with M in the context of an authentic RSV infection suggests that the two proteins interact and that this interaction requires the cytoplasmic and/or transmembrane domains of G. Both antibodies (C781 and RαG) were specific for their respective proteins and did not bind to any proteins in mock-infected cells (Fig. 1e).

To define further the region of G that is required for interaction with M, we co-expressed M with full-length G or truncated derivatives thereof (sG, GΔ30, GΔ13 and GΔ6; Fig. 2a). G constructs were cloned into the Semliki Forest virus replicon system (Gibco-BRL) as described previously (JMPpSFV; Ghildyal *et al.*, 1999; Peroulis *et al.*, 1999). Cloning of the full-length G and a variant lacking the first

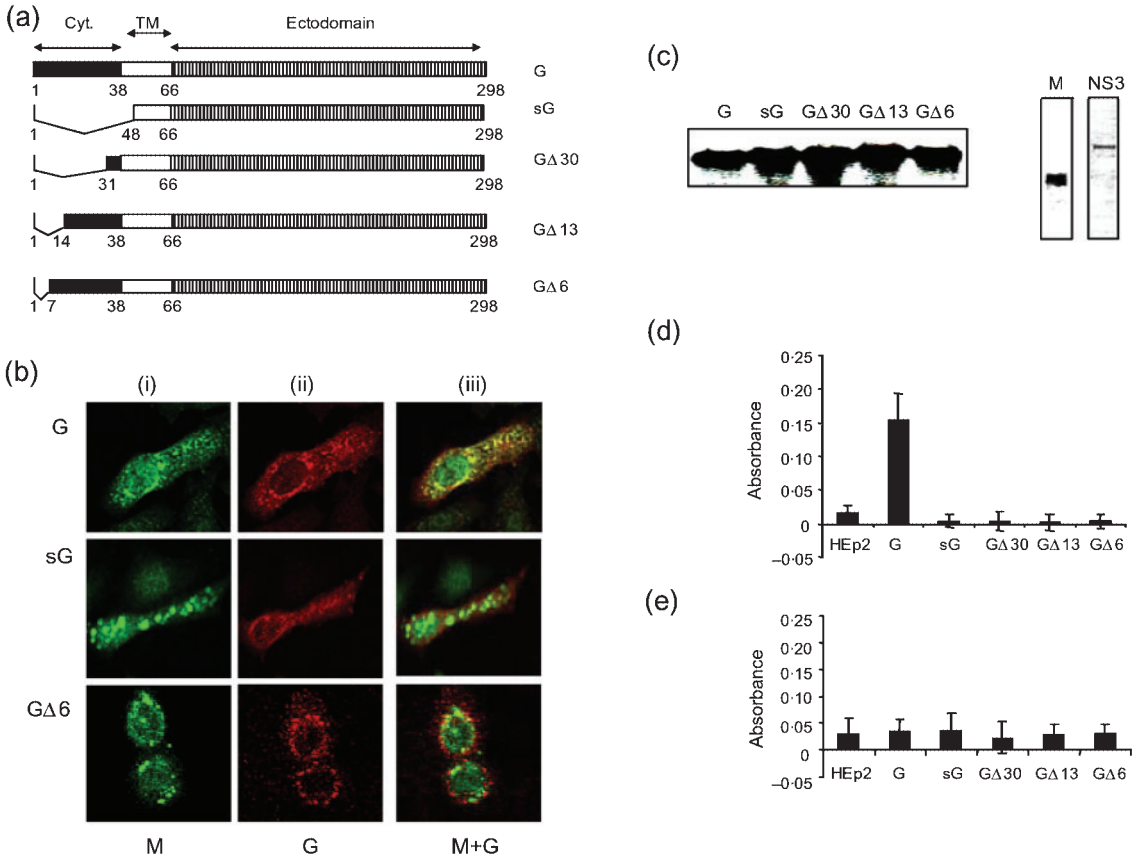

Fig. 2. Ability of N-terminally truncated G variants to bind to M. (a) Schematic representation of the various N-terminally truncated G constructs. Numbers denote amino acid residues. (b) HEp2 cells were co-transfected with M mRNA and mRNA from the indicated G variants. Expression of M was detected by using an anti-HA antibody followed by FITC-conjugated secondary antibody [green, column (i)] and expression of G variants was detected by mAb30 followed by rhodamine-conjugated secondary antibody [red, column (ii)]. Column (iii) denotes a computer-generated merge as described in Fig. 1. (c) The level of expression of G variants in HEp2 cells was determined by Western blotting as described in the text. The purity of bacterially expressed M and NS3 proteins was assessed by Coomassie blue staining following SDS-PAGE. (d) Binding of equal amounts of the indicated cell lysates with M. HEp2, mock-transfected cell lysate. (e) NS3 protein was examined by ELISA as described in the text. Bound G was detected by mAb30 followed by horseradish peroxidase-conjugated goat anti-mouse antibody and TMB. Absorbance values were corrected by subtraction of background values obtained when mock-transfected cell lysates were incubated without M or NS3 proteins (background absorbance values ranged from 0·05 to 0·12). The data shown are the means ± SEM of three separate experiments.

47 aa (G and sG, respectively) within this vector has been described previously (Peroulis *et al.*, 1999). The G construct was used as template for generating the various mutants by PCR, using the same C-terminal primer combined with specific N-terminal primers. The M gene was subcloned from the pET30(a) construct (described below) into the pSD4.2 expression vector (a gift from Douglas S. Lyles, Wake Forest University, Winston-Salem, NC, USA) (Desforges *et al.*, 2002). We and others have found that the paramyxovirus M is difficult to observe by immunological means, especially in transfected cells, as it is not very immunogenic and is usually expressed at low levels (Faaberg & Peeples, 1988). To facilitate the detection of low levels of M, we added an influenza HA-epitope tag to the C terminus of M (M-HA). Recombinant JMPpSFV or M-HA plasmid DNA was linearized, followed by transcription using an mMessage mMachine SP6 transcription kit (Ambion). Freshly prepared mRNA [1 (24-well plates) or 5 (six-well plates) μg per well] was transfected into HEp2 cells using Lipofectamine 2000 (Invitrogen). Transfected cells were fixed at 15 h post-transfection as described above. Immunofluorescence assays were performed as described above, except that C781 was replaced with anti-HA (rabbit anti-influenza HA antibody; Sigma); co-localization of G variants and M was analysed by CLSM.

As shown in Fig. 2(b), M was present in both the nucleus and the cytoplasm, whereas G was found only in the cytoplasm [Fig. 2b, G(i) and G(ii)]. Co-localization of G and M in the cytoplasm (yellow, column 3) was only detected when full-length G and M were co-expressed [Fig. 2b, G(iii)]. M did not co-localize with any of the G derivatives (data for sG and GΔ6 derivatives only are shown). All CLSM micrographs were reviewed by a panel of five investigators who were blinded with respect to the experimental details; only full-length G was found to co-localize with M. Taken together, the CLSM data showed that M and G interact in the absence of other viral proteins and that the N-terminal portion of G is important for interaction with M.

To validate further the CLSM data, we assessed the binding of G variants to M in a cell-free ELISA. G variants were expressed in HEp2 cells by transfection and the level of expression of each variant was assessed by Western blotting (Fig. 2c) prior to use in the ELISA. Transfected cells were lysed 20 h later in lysis buffer as described previously (Marty *et al.*, 2004). Proteins were separated by 10 % SDS-PAGE (Laemmli, 1970) and transferred on to Hybond-C membrane (Amersham Biosciences). Membranes were blocked with BSA, probed with an anti-G-specific mAb (mAb30; a gift from Geraldine Taylor, Institute for Animal Health, UK) and bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse antibody (Dako) followed by ECL (Amersham Biosciences). Full-length G and its derivatives were expressed at similar levels (Fig. 2c). M was expressed in bacteria and purified prior to use. The M gene was PCR-amplified from total RNA extracted from RSV-infected cells and cloned into the pET30(a) vector (Novagen), which introduces a 6-His fusion tag. M was expressed in *Escherichia coli* and purified under denaturing conditions by using Ni^+ affinity chromatography (Qiagen). We also used a recombinant hepatitis C virus NS3 protein, likewise produced in bacteria and purified with a 6-His tag, as an irrelevant control protein. The purity of both M and NS3 proteins was determined by Coomassie blue staining (Fig. 2c).

Approximately 100 ng of either M or NS3 protein was immobilized onto microtitre plates, followed by overnight incubation with cell lysates. Bound G was detected with mAb30 followed by horseradish peroxidase-conjugated goat anti-mouse antibody (Dako) and tetramethylbenzidine hydrochloride (TMB; Sigma Chemicals). As shown in Fig. 2(d), full-length G bound to M, whereas mock-transfected cell lysate and cell lysates containing each of the G derivatives did not bind to M. None of the lysates bound to the unrelated NS3 protein (Fig. 2e). These results further substantiated our CLSM data showing that the N-terminal 6 aa of G are important for its interaction with M. At present, we cannot explain the low absorbance values recorded in the ELISA, but they may be a consequence of the low avidity of the antibodies used, low levels of G in the whole-cell lysate or the presence of G as multimers, making epitopes inaccessible to mAb30.

We next assessed whether a peptide homologous to the N-terminal 36 aa of G (with a C-terminal biotin; 92 % pure peptide; Mimotopes) would bind to M and inhibit binding of G. Recombinant M was immobilized onto microtitre plates, followed by overnight incubation with peptide or with a mix of peptide and full-length purified G (a gift from Dan Speelman, Lederle Praxis Biochemicals, USA) (Hancock *et al.*, 2000). Bound peptide was detected with streptavidin–peroxidase (Dako) followed by TMB. The peptide bound to M in a dose-dependent fashion and 80 ng peptide was able to inhibit 50–70 % of the binding of 20 ng purified G to 100 ng M (data not shown).

The effect of alanine substitution of individual amino acids at positions 2–7 of the G cytoplasmic domain was then examined (Fig. 3a). All of the alanine mutants were expressed in BHK-21 cells and cell lysates were used in a G–M binding ELISA, as shown in Fig. 2(d). The mutants and full-length G were expressed at similar levels, as shown by Western blotting with mAb30 (Fig. 3b). sG bound to M at levels equal to or less than the mock cell lysate, as did the two mutants GS2A and GD6A, whereas the other mutants bound to M at various levels above that of mock cell lysate (Fig. 3c). Thus, loss of either serine at position 2 or aspartate at position 6 in G largely abrogated its interaction with M. These results were also confirmed by CLSM studies of M and G co-expression (data not shown).

We have demonstrated that RSV M and G interact in the cytoplasm of infected cells. This interaction occurred in the absence of any other viral proteins, as shown by co-expression both in cells and in a cell-free system. The N

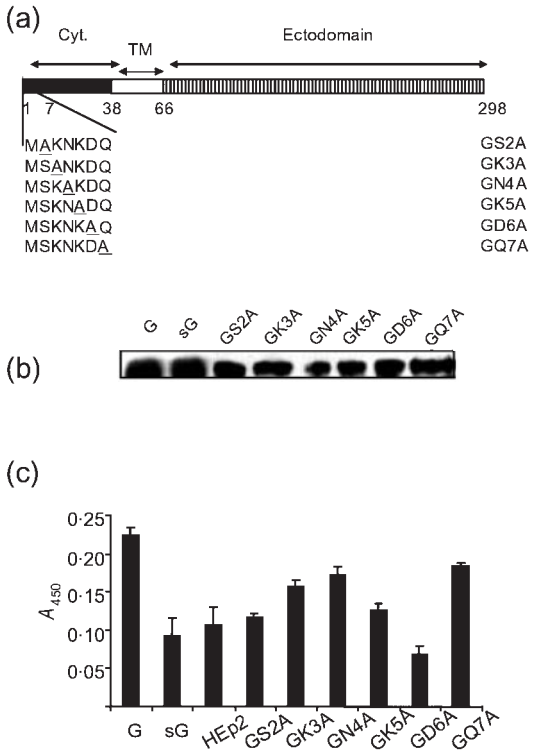

Fig. 3. Binding of G mutants to M. (a) Alanine-scanning mutants of the N terminus of G were constructed and expressed in BHK-21 cells. (b) Cell lysates were analysed by Western blotting as described in the text. (c) Cell lysates were used in an M–G binding ELISA as described in the text. The data shown are the means ± SEM of three separate experiments.

terminus of G was found to be critical for binding to M, and there was a key role for serine at position 2 and aspartate at position 6. Further investigation of the M–G interaction is needed to elucidate whether these two residues are involved directly in the interaction with M or whether they form part of a conformational interacting domain, involving other residues further downstream in the cytoplasmic domain.

As mentioned above, G is not essential for the formation of infectious virus (Karron *et al.*, 1997; Techaarpornkul *et al.*, 2001; Teng *et al.*, 2001) or for the formation of virus-like particles (Teng & Collins, 1998). It is possible that M interacts independently with both G and F and that, whilst interaction with a glycoprotein is essential for assembly, either glycoprotein alone will suffice. In support of this hypothesis, we have observed co-localization of M and F in cells infected with sG-RSV or with a recombinant RSV lacking the entire G gene (data not shown).

In conclusion, we have demonstrated for the first time that RSV M interacts with G, and that this interaction is mediated through the cytoplasmic tail of G. How this interaction relates to other aspects of RSV assembly remains to be determined.

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