Measles virus M protein-driven particle production does not involve the endosomal sorting complex required for transport (ESCRT) system

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Assembly and budding of enveloped RNA viruses rely on viral matrix (M) proteins and host proteins involved in sorting and vesiculation of cellular cargoes, such as the endosomal sorting complex required for transport (ESCRT). The measles virus (MV) M protein promotes virus-like particle (VLP) production, and we now show that it shares association with detergent-resistant or tetraspanin-enriched membrane microdomains with ebolavirus VP40 protein, yet accumulates less efficiently at the plasma membrane. Unlike VP40, which recruits ESCRT components via its N-terminal late (L) domain and exploits them for particle production, the M protein does this independently of this pathway, as (i) ablation of motifs bearing similarity to canonical L domains did not affect VLP production, (ii) it did not redistribute Tsg101, AIP-1 or Vps4A to the plasma membrane, and (iii) neither VLP nor infectious virus production was sensitive to inhibition by dominant-negative Vps4A. Importantly, transfer of the VP40 L domain into the MV M protein did not cause recruitment of ESCRT proteins or confer sensitivity of VLP release to Vps4A, indicating that MV particle production occurs independently of and cannot be routed into an ESCRT-dependent pathway.

INTRODUCTION

Budding of enveloped viruses such as measles virus (MV) is a multi-step process involving directional membrane transport of virus components for assembly of core and envelope subunits prior to particle formation and release (Bieniasz, 2009; Chen & Lamb, 2008; Welsch et al., 2007). The MV matrix (M) protein plays a key role in virus maturation steps occurring subsequent to transcription, for which it acts as a negative regulator by as-yet-unknown mechanisms (Iwasaki et al., 2009; Reuter et al., 2006; Suryanarayana et al., 1994). It associates and is co-transported with the nucleocapsid to the plasma membrane (Iwasaki et al., 2009; Reuter et al., 2006), but also with the cytoplasmic domains of the viral F/H glycoproteins, thereby negatively regulating membrane fusion and promoting virus budding (Moll et al., 2001, 2002; Tahara et al., 2007). Its crucial role in MV budding has been revealed by the failure of M protein-deficient MVs to support release of infectious particles in persistent infections and, more directly, by the ability of the M protein to promote release of virus-like particles (VLPs) when expressed transiently in tissue-culture cells (Cathomen et al., 1998a; Cattaneo et al., 1986; Pohl et al., 2007; Runkler et al., 2007).

The MV M protein oligomerizes and associates strongly with membranes, and a limited fraction of M protein is found in detergent-resistant membrane microdomains (DRMs), which serve as platforms for assembly and release of many enveloped viruses, including filoviruses (Bavari et al., 2002; Panchal et al., 2003), although their importance in MV budding has not yet been demonstrated directly (Chazal & Gerlier, 2003; Maneie et al., 2000; Pohl et al., 2007; Vincent et al., 2000). Remarkably, co-expression with the F protein enhanced DRM association, but not the VLP-promoting activity, of the M protein, indicating that recruitment into these domains might be required, but not sufficient, for particle production. Whether the M protein associates with tetraspanin (tspan)-enriched membrane microdomains (TEMs), identified as exit gateways for other enveloped viruses such as human immunodeficiency virus (HIV) and influenza A virus, is as yet

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unknown (Hemler, 2005; Khurana et al., 2007; Nydegger et al., 2006).

How the MV M protein promotes particle formation has not yet been analysed. Some enveloped RNA virus M proteins, including filoviral VP40 and retroviral Gag proteins, contain short cis-acting domains, referred to as late (L) domains, which assist in budding by recruitment of host factors to assembly sites (Demirov & Freed, 2004; Hartlieb & Weissenhorn, 2006; Usami et al., 2009). They are linked to the cellular ESCRT (endosomal sorting complex required for transport) pathway and are redistributed to budding sites upon interaction with well-defined, canonical L domains [PT(S)AP for Tsg101, YxxL for AIP-1/ALIX, and PPxY, which binds various members of the large family of Nedd4 ubiquitin ligases] (reviewed by Chen & Lamb, 2008; Raiborg & Stenmark, 2009; Welsch et al., 2007). Ablation of these domains often results in accumulation of fully assembled particles tethered to the plasma membrane. Thus, recruitment and requirement of a functional ESCRT system for particle formation have been documented for ebolavirus VP40, which harbours two overlapping N-terminal L domains (PTAPPEY) (Irie et al., 2005; Licata et al., 2003), and for simian virus 5 and mumps virus M proteins, which contain a suboptimal functional N-terminal L domain (Li et al., 2009; Schmitt & Lamb, 2004; Schmitt et al., 2005). For other RNA viruses, particle production can, however, be independent of a functional ESCRT system (Chen & Lamb, 2008).

To characterize M protein association with host-cell components, we compared its co-segregation with DRMs, TEMs and ESCRT components with that of filoviral VP40, for which membrane-trafficking pathways (Kolesnikova et al., 2002, 2004, 2007, 2009) are known and the relevance of the ESCRT pathway for budding is established (Dolnik et al., 2008; Gomis-Ruth et al., 2003). Association of the VP40 and M proteins with membrane microdomains was similar, yet, unlike for VP40, the VLP production promoted by the M protein and also MV release did not rely on ESCRT components. In line with this, M protein did not harbour functional canonical L domains. Further stressing the ESCRT independence of M protein-driven particle production, insertion of the overlapping ebolavirus VP40 PTAPPEY L domain did not alter M protein subcellular distribution.

RESULTS

VP40 and M protein associate similarly with TEMs and DRMs

When co-expressed in HeLa cells, VP40 and M proteins co-localized to only a limited extent, and this referred especially to the plasma membrane. In line with previous observations (Pohl et al., 2007; Runkler et al., 2007), only a very limited fraction of the M protein was detected in association with cortical actin, whereas VP40 accumulated there much more efficiently (Fig. 1a). To compare the ability of both proteins to associate with membrane microdomains, we first analysed their established co-\footnote{co-\footnote{fraction with CD55, used as a DRM marker, to comparable levels in transfected or infected cells (Fig. 1d). In agreement with their preferential accumulation at cell boundaries or internal compartments, respectively, VP40 and M proteins co-localized strongly with CD9 [Fig. 1d(i, ix)] and moderately with CD63 and CD81, which was apparently more pronounced for the M protein [Fig. 1d(iv, xii)]. tidal–M protein association in MV-infected cells did not differ from that seen in cells transfected to express the M protein alone [Fig. 1d(v–viii)].}

MV M protein-driven VLP production does not involve canonical L domains

ESCR\footnote{T-dependent VP40-driven particle production involves overlapping canonical L domains (PTAPPEY) (Dolnik et al., 2008; Hartlieb & Weissenhorn, 2006; Schmitt & Lamb, 2004), and we aimed to analyse whether this also applies to particle release promoted by the M protein. The protein harbours several conserved motifs with similarity to canonical L domains [PIQP (20–23), PTYY (23–26), PSVP (311–314), FKVL (332–335) and YMFL (52–55)] (Fig. 2a), which we replaced within the pCG-M(ED) expression plasmid by four-alanine stretches to yield pCG-M/PIQP (PIQP→AAAA, referred to as ‘PIQP’), pCG-M/PTTY (PTTY→AAAA, ‘PTTY’), pCG-M/YMFL (YMFL→AAAA, ‘YMFL’), pCG-M/PSVP (PSVP→AAAA, ‘PSVP’) or pCG-M/FKVL (FKVL→AAAA, ‘FKVL’). When transfected into HeLa cells, all constructs gave rise to proteins that accumulated to comparable levels (as detected by Western blot analysis, not shown; also Fig. 2d). The subcellular distribution of the M protein species did not differ detectably. Except for PTTY, which apparently accumulated in association with cytosolic compartments [Fig. 2b(iii)], a minor fraction of all recombinant M proteins,}}T-dependent VP40-driven particle production involves overlapping canonical L domains (PTAPPEY) (Dolnik et al., 2008; Hartlieb & Weissenhorn, 2006; Schmitt & Lamb, 2004), and we aimed to analyse whether this also applies to particle release promoted by the M protein. The protein harbours several conserved motifs with similarity to canonical L domains [PIQP (20–23), PTYY (23–26), PSVP (311–314), FKVL (332–335) and YMFL (52–55)] (Fig. 2a), which we replaced within the pCG-M(ED) expression plasmid by four-alanine stretches to yield pCG-M/PIQP (PIQP→AAAA, referred to as ‘PIQP’), pCG-M/PTTY (PTTY→AAAA, ‘PTTY’), pCG-M/YMFL (YMFL→AAAA, ‘YMFL’), pCG-M/PSVP (PSVP→AAAA, ‘PSVP’) or pCG-M/FKVL (FKVL→AAAA, ‘FKVL’). When transfected into HeLa cells, all constructs gave rise to proteins that accumulated to comparable levels (as detected by Western blot analysis, not shown; also Fig. 2d). The subcellular distribution of the M protein species did not differ detectably. Except for PTTY, which apparently accumulated in association with cytosolic compartments [Fig. 2b(iii)], a minor fraction of all recombinant M proteins,
although mainly membrane-proximal, co-localized strongly with cortical f-actin (Fig. 2b). The PTTY mutant was also reproducibly overrepresented in DRM fractions, whilst the association of the other mutant M proteins with those was identical to that of the wild-type protein (Fig. 2c).

The ability of the mutant M proteins to promote VLP production from 293 T cells after 48 h was assessed. All expression constructs gave rise to M proteins of the expected size and similar accumulation levels in cell lysates (Fig. 2d, left panel, lane L) and M protein-containing material in the respective supernatant (Fig. 2d, left panel, lane SN). PIQP, YMFL, PSVP and FKVL accumulated to comparable levels to the wild-type protein (Fig. 2d, right panel), whilst the ability of the PTTY mutant to support VLP production was reproducibly compromised slightly.

Fig. 1. Association of VP40 and M proteins with membrane microdomains (a). VP40 and M protein were co-detected in transfected HeLa cells by using specific followed by secondary antibodies conjugated to Alexa 488 (M protein) or Alexa 594 (VP40) (the right panel shows a three-dimensional reconstruction of a z-stack represented in the left panel). (b) Flotation gradient analysis of M proteins in Brij 98 lysates harvested from HeLa cells 24 h following transfection with plasmids encoding M or VP40 proteins. Specific signal intensities were quantified (and their sum set to 100 %) and their representation within the soluble (S, bottom) or non-soluble (NS, top) fractions (as determined by CD55 accumulation) was determined. Blots represent one of three independent experiments, which are summarized (indicated by an asterisk) in the table to the right of the blots (with sd of <3 % for each value). (c) Twenty-four hours following infection of HeLa cells (m.o.i. of 3), membrane cholesterol was depleted by β-MCD (10 μM), and supernatants (SN) and lysates (L) were harvested 8 h after treatment for detection of M protein. Bands were quantified by AIDA software and relative accumulation levels of M protein in supernatants were determined. Table: supernatants from cultures exposed or not to β-MCD were collected for virus titration after 8 h; titres were determined in two independent experiments (#1 and #2). Values are indicated as p.f.u. ml⁻¹. (d) CD9 (i, v, ix), CD63 (ii, vi, x), CD81 (iii, vii, xi) and CD82 (iv, viii, xii) (green) were co-detected with M protein expressed from plasmid (i–iv) or in MV-infected cells (v–viii), or with VP40 protein (ix–xii) (both red), 24 h following transfection or infection in HeLa cells. Co-localization coefficient (m) values determining the efficiency of co-localization are indicated in white (all panels). Bar, 10 μm (representative of all panels).
Altogether, these data suggest that none of the motifs resembling canonical L domains within the MV M protein is important for VLP production.

**MV particle production does not involve recruitment of ESCRT components**

To determine whether recruitment of ESCRT complex components was required for MV particle production, yellow fluorescent protein (YFP)-tagged Tsg101, AIP-1, Vps4A or Vps4A-E/Q (acting as dominant-negative protein) were expressed in HeLa cells along with pCG-M [Fig. 3a(i)] or infected with MV [Fig. 3b]. When plasmid-expressed, the M protein did not co-localize with AIP-1 [Fig. 3b(ii)], yet did co-localize to a moderate extent with Tsg101 and Vps4A, which, however, were not recruited detectably to the plasma membrane [Fig. 3a(i, iii), with Fig. 3a(iii*) showing a three-dimensional reconstruction of the MV M protein.]
a z-stack. Typically, overexpression of Vps4A-E/Q resulted in swollen endosomal compartments where the M protein was partially co-trapped and, seemingly, accumulating less efficiently at the plasma membrane [Fig. 3a(iv, iv*) as a three-dimensional reconstitution]. In agreement with the literature (Silvestri et al., 2007), ebolavirus VP40, when expressed from plasmid and used as a positive control, caused efficient membrane recruitment of Tsg101 [see Fig. 5(c) below] and partially redirected Vps4A and Vps4A-E/Q to the plasma membrane (see Supplementary Fig. S1, available in JGV Online). In MV-infected cells, the subcellular distribution of the M protein clearly segregated at the plasma membrane from that of Tsg101 and AIP-1, but also Vps4A-E/Q [Fig. 3b(i, ii, iv, iv*)], yet there is an apparent partial plasma membrane-proximal overlap with Vps4A clusters [Fig. 3b(iii, iii*)]. Lack of co-segregation between Tsg101 and AIP-1 or their inactive or dominant-negative mutants [Tsg101(1–303) or DN-AIP-1, respectively] and the M protein at the plasma membrane correlated with the inability of these proteins to affect MV release strongly (Fig. 3c; we noted the obvious reduction by Tsg101 by approximately 50 %, yet did not consider this as physiologically relevant). To assess the potential impact of the Vps4A ATPase, we made use of 293 cell clones stably expressing ponasterone-inducible Vps4A or Vps4A-E/Q (Fig. 4a, left panels). The M protein accumulated efficiently 24 h after infection of these and a control 293 T-cell line, with no differences seen with regard to the production of M protein-containing SN material (Fig. 4a, right panel). In line with our previous observations, overexpression of Vps4A-E/Q caused reduction of VP40-driven VLP release by 45 % (Kolesnikova et al., 2009) (Fig. 4a, right panel). Vps4A- and Vps4A-E/Q-expressing cells were comparably susceptible to MV infection, as evidenced by efficient formation of syncytia at 24 h and cell loss at 48 h post-infection (Fig. 4b, left panels), and production of infectious virus, amounts of which did not differ significantly from those released from 293 cells (Fig. 4b, right panel). These findings thus do not support an essential role of ESCRT components for MV particle release.

The VP40 L domain does not route the M protein into an ESCRT-dependent pathway

The similarity of the conserved PIQPTTY motif (aa 20–26) within the MV M protein to the VP40 L domain (PTAPPEY), also with regard to its N-terminal position, prompted us to investigate whether it represents a degenerate canonical L domain that, when restored, could shift M protein-driven particle formation into an ESCRT-dependent mode. We therefore replaced PIQPTTY by PTAPPEY to yield pCG-M/PTAPPEY [Fig. 5a(i)]. The VP40 L domain did not, however, enhance plasma membrane association of the M protein [Fig. 5a(ii, ii*)], nor did it affect VLP production in terms of the overall efficiency or insensitivity
Fig. 4. MV release occurs independently of Vps4A. (a) M protein was detected in SN and L of 293 cells or 293 cell clones stably expressing ponasterone-inducible eGFP-flagged Vps4A or Vps4A-E/Q proteins (left panels) 24 h following pCG-M transfection (right panel). Vps4A- and Vps4A-E/Q-expressing cells released 94 and 95 % of MV M protein, respectively, compared with 293 cells (where M protein release was set as 100 %) (upper panel). In contrast, VLP production driven by transfected VP40 was reduced by 45 % in Vps4A-E/Q cells (bottom panel). (b) Ponasterone A-induced 293–Vps4A (black bars) and 293–Vps4A-E/Q (grey bars) cells (or, for control, 293 cells; white bars) were infected with MV (m.o.i. of 3) and analysed after 24 and 48 h for cytopathic effects (exemplified for Vps4A and Vps4A-E/Q, left panels) and release of infectious virus. Values indicated are derived from three independent experiments.

**DISCUSSION**

The MV M protein is a multifunctional protein that, in addition to acting as a transcriptional regulator, plays a major role in particle assembly and morphogenesis (Naim et al., 2000; Pohl et al., 2007; Reuter et al., 2006; Suryanarayana et al., 1994; Wild & Buckland, 1995). Its interaction with the cytoplasmic tails of the MV glycoproteins essentially favours particle formation at the expense of fusion, as targeted weakening or abolishment of this complex results in enhanced fusogenicity (Cathomen et al., 1998a, b; Tahara et al., 2007). Furthermore, the M protein expressed from plasmids gives rise to production of VLPs morphologically indistinguishable from virus particles (Pohl et al., 2007). In addition, the M protein interacts stably and is co-transported with the nucleocapsid to the plasma membrane for subsequent release (Runkler et al., 2007). In support of the importance of the M protein in assembly and budding, ablation of its reading frame in recombinant MVs did not fully abolish, yet reduced dramatically, the production of infectious material (Cathomen et al., 1998a).

Corroborating their role in MV assembly (Manie et al., 2000; Vincent et al., 2000), DRMs were found to be important in the release of infectious particles (Fig. 1b, c), which also applies to ebolavirus (Bavari et al., 2002) and, in line with these findings, VP40 and M protein co-floated with these fractions to similar extent (Fig. 1b). Differences in their analysed TEM association were subtle (Fig. 1d). Although preferences for CD81 (for VP40) and CD82 (for MV) were noted, it would be difficult to attribute these to interactions with a specific microdomain, as TEMs usually contain several tspan motifs (Hemler, 2005; Khurana et al., 2006; Nydegger et al., 2006) and association with certain tspan motifs may not reflect a functional requirement for the assembly/budding process. This is supported by the finding that CD63, described to co-localize with HIV Gag and to be incorporated into HIV particles, is dispensable for particle production (Deneka et al., 2007; Dong et al., 2005; Khurana et al., 2007; Ruíz-Mateos et al., 2008).

In spite of their similar association with marker and membrane microdomains, the VP40 and M proteins barely co-localized, particularly at the plasma membrane, where substantial amounts of VP40, but not the M protein, were detectable after 24 h (Fig. 1a). This may reflect a higher efficiency of VP40 membrane trafficking to the budding site, which may contribute to the higher efficiency of VP40 to promote VLP production [with >20 % being released from transfected cells into the tissue-culture medium (Timmins et al., 2001) instead of 2 % for MV M protein (Pohl et al., 2007)]. M protein-driven particle production also differs from that promoted by VP40 in its lack of enhancement upon glycoprotein co-expression (Pohl et al., 2007) and, importantly, the independence of MV particle production from the ESCRT complex (Figs 3 and 4).

Because it promotes VLP production, the M protein must harbour an as-yet-unidentified L domain. Clearly, those addressed in this study do not serve this function, and the slightly limited ability of the PTTY mutant to support VLP release may rather link to its preferential accumulation at intracellular compartments (Fig. 2). The similarity in position and sequence with the VP40 PTAPPEY VP40 motif suggest that the M protein 20PIQPTTY27 might represent an ancestral overlapping L domain, having lost the ability to recruit ESCRT components for particle
production. Replacement by PTAPPEY did not, however, alter MV M protein subcellular distribution or budding efficiency, and neither did it confer the ability to recruit Tsg101 to the plasma membrane (Fig. 5). Reasons for the inability to recruit Tsg101 are unclear, yet may not include masking of the N terminus by membrane insertion, as the N terminus is not hydrophobic. Obviously, transfer of a functional L domain does not shift the MV M protein into an ESCRT-dependent budding mode or increase its particle-production efficiency. This is in contrast to observations made upon replacement of the PPPY motif within the vesicular stomatitis virus (VSV) M protein by the VP40 L domain (referred to as M40), where VLP production was increased and, in contrast to that of the VSV M protein, sensitive to dominant-negative Vps4A (Irie et al., 2004, 2005). Suboptimal sequences flanking the transplanted L domain (Silvestri et al., 2007) within the M protein may possibly have contributed to the failure to gain ESCRT dependence, yet it has also been shown that transfer of an L domain alone restored budding activity (Ciancanelli & Basler, 2006). It is thus likely that MV particle production, as that of a growing list of enveloped viruses, follows a completely independent pathway, characterization of which will have to be addressed.

METHODS

Cells, virus and titrations. All media were produced in house. HeLa cells were maintained in minimal essential medium (MEM) containing 5% fetal calf serum (FCS; Biochrom), and HEK 293 cells in Dulbecco’s modified Eagle’s medium/10% FCS, which was, for the ecdysone-responsive (EcR) 293 cells, transfected to stably express inducible Vps4A or Vps4A-E/Q both tagged with enhanced green fluorescent protein (eGFP); kindly provided by C. Crump (Crump et al., 2007) supplemented with zeocin (200 μg ml⁻¹; Invitrogen) and G418 (400 μg ml⁻¹; Roth). When indicated, EcR-293 cells were induced by 1 μM ponasterone A (Invitrogen) for 24 h. The MV Edmonston strain (ED) was grown in Vero cells in MEM/5% FCS and, if not stated differently, used at an m.o.i. of 0.5. Titres were determined on marmoset lymphoblastoid B95a cells (maintained in RPMI 1640/5% FCS). When indicated, cells were treated for 2 h with β-MCD (10 μM; Sigma) 24 h following infection in serum-free MEM, then replenished with MEM/2.5% FCS supplemented with mevalonolactone (25 μM) and mevastine (10 μM) (both from Sigma) (Imhoff et al., 2007; Robinzon et al., 2009). Eight hours later, supernatants were harvested for titrations performed in triplicate. This protocol was used for determination of levels of M protein-containing material in lysates or supernatants upon cholesterol depletion.

Indirect immunofluorescence. Indirect immunofluorescence was performed after fixation of cells in 4% (w/v) paraformaldehyde in PBS. Primary antibodies used included mouse monoclonal antibodies directed against the MV M protein (MAB8910; Chemicon), CD9 (K41, kindly provided by J. Schneider-Schaulies, University of Wuerzburg), CD63, CD81 or CD82 (all from Santa Cruz) or a goat serum raised against VP40. Goat or rabbit anti-mouse antibodies (conjugated to Alexa 488 or 594) or a chicken anti-goat antibody (conjugated to Alexa 594) (all from Molecular Probes) were used as secondary antibodies. For double stainings with mouse monoclonal antibodies, MAB8910 was conjugated directly according to the manufacturer’s protocol (Zenon; Molecular Probes/Invitrogen). Samples were analysed by confocal microscopy. When indicated, co-localization coefficients (m) were determined by measuring co-localization of two different colour signals within an image region.
of interest (ROI) when the number of objects was unequal and therefore independent of signal intensities. The ROI is defined as m = ΣS i(DNA, green) - ΣS i(red); thresholds for the ROI (red) and green signals were determined experimentally by using LSM software (tool-profile). At least 35 cells per co-expression were analysed. m values between 0.5 and 0.7 were classified as minor or transient, and higher as significant, co-localizations.

Plasmid constructions, transfections and detection of proteins. The plasmids used for transfection were pCAGGS-VP40 and pCAG-M(ED) (Pohl et al., 2007) and its derivatives, pCAG-PIQP (‘PIQP’), pCAG-PTTY (‘PTTY’), pCAG-YMEL (‘YMEL’), pCAG-FKVL (‘FKVL’) and pCAG-PMTPAPPEY. These were generated by a site-directed mutagenesis QuickChange kit (Stratagene) (for primers, see Supplementary Table 1A, 2007) and its derivatives, pCG-PIQP (AAAA (‘PSVP’), pCG-FKVL (AAAA (‘FKVL’)) and pCG-PMTPAPPEY. The plasmids, Colin Crump (Department of Pathology, Cambridge University, UK) for the pansterone-inducible 293 cells, Soren Carlsson (Umeå University, Sweden) for the LAMP-1-specific serum, Eric Snijder (Leiden University, The Netherlands) for the LC3 plasmid and serum, and the Deutsche Forschungsgemeinschaft (SPP1175) for funding of the project.

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