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Glycoprotein targeting signals influence the distribution of measles virus envelope proteins and virus spread in lymphocytes

Nicole Runkler, Erik Dietzel, Markus Moll,† Hans-Dieter Klenk and Andrea Maisner

Correspondence
Andrea Maisner
maisner@staff.uni-marburg.de

Institute of Virology, Philipps University Marburg, Hans-Meerwein-Str. 2, D-35043 Marburg, Germany

We previously demonstrated the presence of tyrosine-dependent motifs for specific sorting of two measles virus (MV) glycoproteins, H and F, to the basolateral surface in polarized epithelial cells. Targeted expression of the glycoproteins was found to be required for virus spread in epithelia via cell-to-cell fusion *in vitro* and *in vivo*. In the present study, recombinant MVs (rMVs) with substitutions of the critical tyrosines in the H and F cytoplasmic domains were used to determine whether the sorting signals also play a crucial role for MV replication and spread within lymphocytes, the main target cells of acute MV infection. Immunolocalization revealed that only standard glycoproteins are targeted specifically to the uropod of polarized lymphocytes and cluster on the surface of non-polarized lymphocytes. H and F proteins with tyrosine mutations did not accumulate in uropods, but were distributed homogeneously on the surface and did not colocalize markedly with the matrix (M) protein. Due to the defective interaction with the M protein, all mutant rMVs showed an enhanced fusion capacity, but only rMVs harbouring two mutated glycoproteins showed a marked decrease in virus release from infected lymphocytes. These results demonstrate clearly that the tyrosine-based targeting motifs in the MV glycoproteins are not only important in polarized epithelial cells, but are also active in lymphocytes, thus playing an important role in virus propagation in different key target cells during acute MV infection.

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INTRODUCTION

Measles virus (MV) is still one of the leading causes of death among young children in developing countries, despite the availability of an effective vaccine for 40 years (WHO, 2007). During the course of an acute MV infection, many cell types, including polarized cells, are infected. As MV is transmitted via aerosols or droplets and replicates initially in the respiratory mucosa, polarized nasal or bronchial epithelial cells are among the first MV target cells. MV then enters local lymphatic tissues and spreads systemically through the lymphatic and blood systems. In the systemic phase of infection, monocytes and lymphocytes are the main target cells. Infected blood mononuclear cells are responsible for MV-induced transient immunosuppression (Schneider-Schaulies *et al.*, 2001) and carry MV to various organs, such as skin, intestine, liver, lung and kidney, where different types of polarized cell are infected (Esolen *et al.*, 1993; Osunkoya *et al.*, 1990; Yanagi *et al.*, 2006).

Polarized cells differ from non-polarized cells in their ability to segregate proteins and lipids into distinct surface subdomains accompanied by morphological and functional asymmetry, as occurs with the apical and basolateral surfaces in polarized epithelia and the axonal and dendritic processes in neurons (Rodriguez-Boulan & Powell, 1992). Lymphocytes can also develop a polarized phenotype if they carry out certain functions, such as cell–cell interactions or migration (Bretscher, 1996; Sanchez-Madrid & del Pozo, 1999). In migrating T cells, polarization involves the formation of a leading edge, which is enriched in receptors involved in recognition of chemokines, antigens and substrate-adhesion molecules (Negulescu *et al.*, 1996; Nieto *et al.*, 1997), and a trailing edge, termed the uropod (Campanero *et al.*, 1994). The uropod selectively concentrates molecules involved in intercellular adhesion (del Pozo *et al.*, 1995). Upon contact of T cells with other T cells or antigen-presenting cells, a characteristic polarized arrangement of molecules at cell–cell junctions, known as the immunological synapse, is induced (Grakoui *et al.*, 1999). However, formation of synapse-like structures not only is required to respond effectively to antigenic challenge, but might also be important for the dissemination of lymphotropic retroviruses. For human immunodeficiency virus type 1 (HIV-1) and human T-lymphotropic virus (HTLV), cell-to-cell spread is believed to occur via a stable adhesive junction, the so-called

†Present address: Center for Infectious Medicine, Karolinska Institute, Stockholm, Sweden.

virological synapse (Jolly & Sattentau, 2004). Thus, transient polarization of lymphocytes, similar to the permanent polarized nature of epithelia or neurons, is not only central to their physiological function, but also influences virus replication. Selective transport of viral surface and matrix proteins to a specific domain can critically determine cell-to-cell spread and targeted virus release from polarized cells (Danis *et al.*, 2004; Deschambeault *et al.*, 1999; Fuller *et al.*, 1984; Lodge *et al.*, 1997; Mora *et al.*, 2002; Sanger *et al.*, 2001; Tashiro *et al.*, 1990; Zimmer *et al.*, 2002).

MV, as a member of the family *Paramyxoviridae*, encodes two surface glycoproteins, the receptor-binding H protein and the fusion (F) protein. Both are required for virus entry, spread via cell-to-cell fusion and virus release. Functional virus assembly depends on the presence of the cytoplasmic domains of H and F and their interaction with the matrix (M) protein (Spielhofer *et al.*, 1998). Furthermore, interaction of the F cytoplasmic tail was shown to be required for downregulation of MV-induced syncytium formation and cytopathogenicity (Cathomen *et al.*, 1998a, b; Moll *et al.*, 2002). We found previously that both MV surface glycoproteins F and H contain specific polarized sorting signals within their cytoplasmic domains, which mediate expression on the basolateral surface of epithelial cells upon both stable expression and infection with MV_{Edm}. Targeted F and H expression is dependent on tyrosine residues in the cytoplasmic tails (Y_{549} in the F protein; Y_{12} in the H protein) and is of crucial importance for fusion of polarized epithelial cells. Mutations in the basolateral sorting signals prevent direct cell-to-cell spread in epithelial monolayers and thus compromise the ability of MV to overcome epithelial barriers, restricting virus spread *in vitro* and *in vivo* (Maisner *et al.*, 1998; Moll *et al.*, 2001, 2004).

As it has been shown for HIV that tyrosine-based targeting signals can also be of functional importance for the infection of lymphocytes (Deschambeault *et al.*, 1999), we wanted to determine the impact of the basolateral sorting signals in the MV glycoproteins for propagation in lymphocytes, the main target cells during the systemic phase of infection. To study MV replication and glycoprotein targeting, lymphocytes were infected with recombinant MVs (rMVs) carrying mutations in the cytoplasmic tyrosines Y_{549} in the F protein and/or Y_{12} in the H protein (tyrosine mutants). Immunolocalization analysis in rMV-infected lymphocytes revealed that transport of F and H to the uropod of polarized lymphocytes, as well as clustering of the glycoproteins on the surface of non-polarized lymphocytes, are dependent on the cytoplasmic tyrosines. Interestingly, all tyrosine mutants had an enhanced fusion activity. rMVs carrying mutations in both glycoproteins displayed the most pronounced fusogenic phenotype and were barely released into the supernatant of infected lymphocytes. The finding that mutated glycoproteins have lost their marked colocalization with M on the surface of infected cells indicates that M–glycoprotein binding is disturbed by the tyrosine mutation in either the F or the H protein. As a consequence, M-mediated downregulation of fusion is reduced. In summary, our data indicate clearly that the cytoplasmic tyrosines in the MV glycoproteins, which are responsible for basolateral expression in polarized epithelia, also act as uropod-targeting signals in lymphocytes. Furthermore, they are involved in M–glycoprotein interaction, thereby regulating cell-to-cell fusion and virus propagation in lymphocytes.

METHODS

Cells and viruses. Primary peripheral blood lymphocytes (PBLs) of human donors were isolated as described by Erlenhoefer *et al.* (2001). PBLs and Jurkat cells (a human T-leukaemic cell line) were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS), 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (all from Gibco). B95a cells (an adherent marmoset B-cell line) and Vero cells (African green monkey kidney cells) were maintained in Dulbecco's modified minimal essential medium (DMEM; Gibco) containing 10 % FCS, penicillin and streptomycin. Madin–Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium (MEM; Gibco) supplemented with 10 % FCS and antibiotics.

Recombinant MV Edmonston B (rMV_{Edm}) and all rMV tyrosine mutants ($rMV_{F549Y/A}$, $rMV_{H12Y/A}$ and $rMV_{FHY/A}$) were rescued from cDNA, grown and titrated on Vero cells as described previously (Moll *et al.*, 2004).

Immunostaining. PBLs stimulated with 2.5 μg phytohaemagglutinin (PHA) ml^{-1} for 48 h and Jurkat cells, both grown in suspension, as well as adherent B95a and MDCK cells, were infected with the different rMVs at an m.o.i. of 0.5 for 2 h at 37 °C. After washing with PBS, cells were cultured in medium containing 10 % FCS together with a fusion-inhibitory peptide to prevent disruption of the cells by syncytium formation (Weidmann *et al.*, 2000). As MV infection in Jurkat cells proceeds very fast, infected Jurkat cells were analysed at 1 day post-infection (p.i.), whereas PBLs and B95a and MDCK cells were processed at 2 days p.i. Before immunostaining, PBLs (48 h p.i.) and Jurkat cells (21 h p.i.) were seeded onto fibronectin-coated coverslips (BD BioCoat) and incubated for 1.5 h at 37 °C to allow uropod formation. Then, the cells were fixed with 4 % paraformaldehyde for 10 min at room temperature and subsequently blocked with DMEM containing 10 % FCS for 1 h at 4 °C. To visualize MV glycoproteins on the cell surfaces, cells were incubated with F- or H-specific mAbs (A504 and K83, kindly provided by S. and J. Schneider-Schaulies, Institut für Virologie und Immunbiologie, Universität Würzburg, Germany) for 1 h at 4 °C and rhodamine-conjugated goat anti-mouse IgG (Dianova) for 45 min at 4 °C. For M–H costaining, cells were incubated with an H-specific rabbit anti-MV serum (anti-Hc, kindly provided by R. Cattaneo, Mayo Clinic College of Medicine, Rochester, MN, USA) for 1 h at 4 °C. The primary antibody was detected by incubation with rhodamine-conjugated anti-rabbit IgG (Dako) for 45 min at 4 °C. After treating the cells with methanol : acetone (1 : 1) for 5 min at room temperature, the M protein was labelled with the M-specific mAb 8910 (Chemicon) for 1 h at 4 °C and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Dako) for 45 min at 4 °C. For F–M costaining, cells were incubated with anti-F antibody (A504) and rhodamine-conjugated secondary antibody, followed by permeabilization, blocking with 5 % normal mouse serum and incubation with an FITC-labelled anti-M antibody as described previously (Moll *et al.*, 2002). After immunostaining, cells were mounted in Mowiol (Merck) and 10 % 1.4

diazabicyclo(2.2.2)octane (Sigma), and fluorescence images were recorded by using a Zeiss ApoTome/Axiovert 200M microscope.

Fusion assay. To analyse fusion activity of the different rMVs, 5×10^5 Jurkat cells were infected in suspension with rMV_{Edm}, $rMV_{F549Y/A}$, $rMV_{H12Y/A}$ or $rMV_{FHY/A}$ at an m.o.i. of 0.01. After incubation for 2 h, cells were washed and incubated with RPMI 1640 medium containing 10 % FCS at 37 °C. To study cell-to-cell fusion in adherent B95a cells, the cells were grown to confluence in 24-well plates (2.5×10^5 cells), then infected with the different rMVs at an m.o.i. of 0.05 and maintained in DMEM containing 10 % FCS at 37 °C. rMV-infected cells were monitored regularly for syncytium formation by phase-contrast microscopy.

Growth analysis. Virus growth was analysed by infecting 2.5×10^5 Jurkat cells with rMV_{Edm}, $rMV_{F549Y/A}$, $rMV_{H12Y/A}$ or $rMV_{FHY/A}$ at an m.o.i. of 0.01 for each time point of analysis. After 2 h, cells were washed to remove unbound viruses and were cultured in 1 ml RPMI 1640 medium containing 10 % FCS at 37 °C. Every 12 h, cells were pelleted by low-speed centrifugation, and cell-free rMVs in the supernatant were titrated by plaque assay. Dilutions of the cell supernatant were adsorbed to Vero cells for 2 h, then the inoculum was removed and cells were overlaid with MEM containing 2 % FCS and 0.9 % Bacto Agar (BD). After 4 days, the plaques were stained with 0.0125 % neutral red (Merck) and counted.

***In vitro* proliferation assay.** Human PBLs were stimulated with PHA (2.5 μg ml^{-1}) for 48 h and subsequently infected for 2 h with rMV_{Edm}, $rMV_{F549Y/A}$, $rMV_{H12Y/A}$ or $rMV_{FHY/A}$ at an m.o.i. of 0.5, or were left uninfected (mock). After several washings, 10^5 cells were seeded into a 96-well plate in a volume of 200 μl per well and incubated in the presence of PHA for 72 h. Cells were then labelled for 16 h with [^{3}H]thymidine [18.5 kBq (0.5 μCi) ml^{-1}]. Incorporation rates of ^{3}H were determined by using a β-plate reader. The assay was performed in triplicate.

RESULTS

Mutation of the tyrosine-based targeting signals alters the distribution of the MV glycoproteins on the surface of non-polarized and polarized lymphocytes

To study the role of tyrosine-based signals in the MV glycoproteins for localization in lymphocytes, Jurkat cells (a human T-cell line) and primary PBLs, both growing in suspension, and an adherent marmoset B-cell line (B95a) were infected with standard virus (rMV_{Edm}) and rMV tyrosine mutants. These viruses, previously rescued from cloned cDNA, harbour glycoproteins in which the cytoplasmic tyrosine residues were replaced with alanine residues either in the F ($rMV_{F549Y/A}$) or H ($rMV_{H12Y/A}$) protein only, or in both glycoproteins ($rMV_{FHY/A}$) (Moll *et al.*, 2004). For immunodetection of the MV glycoproteins on the surface of rMV-infected cells, Jurkat cells and PBLs were adsorbed to fibronectin-coated coverslips at 21 and 48 h p.i., respectively. As a polarized phenotype can be induced *in vitro* upon adhesion to extracellular matrix components (Johansson *et al.*, 1997), about 5–10 % of the lymphocytes form uropods under these conditions. After 1.5 h, Jurkat cells and PBLs adsorbed to coverslips were fixed with paraformaldehyde. Infected B95a cells were fixed directly at 48 h p.i. To visualize MV glycoproteins on the plasma membrane, the infected lymphocytes were incubated with anti-F protein- or anti-H-specific mAbs and rhodamine-conjugated secondary antibodies. Fig. 1 depicts the surface distribution of F and H in polarized (Fig. 1a, b) and non-polarized (Fig. 1c, d) lymphocytes. In rMV_{Edm}-infected polarized Jurkat cells (Fig. 1a) and PBLs (Fig. 1b), standard MV glycoproteins were clearly localized at the uropod, whereas the F protein in $rMV_{F549Y/A}$-infected cells, as well as the H protein in $rMV_{H12Y/A}$-infected cells, were no longer concentrated at this trailing edge. In $rMV_{FHY/A}$-infected cells, both MV glycoproteins were distributed more or less homogeneously all over the cell surface. This demonstrates clearly that the tyrosine-based signals in the MV glycoproteins are not only required for polarized transport in epithelia, but also act as uropod-targeting signals in polarized lymphocytes. Interestingly, the cytoplasmic tyrosines not only affect protein localization in polarized cells, but also influence H and F distribution in non-polarized lymphocytes. In both non-polarized, spherical Jurkat cells (Fig. 1c) and adherent B95a cells (Fig. 1d) infected with standard rMV_{Edm}, F and H accumulated in large aggregates on the cell surface. In cells infected with rMV tyrosine mutants, mutated glycoproteins displayed a punctate distribution (F in $rMV_{F549Y/A}$- and H in $rMV_{H12Y/A}$-infected cells), whereas non-mutated glycoproteins were still found in large aggregates. In $rMV_{FHY/A}$-infected cells, neither H nor F accumulated in larger clusters at the cell surface. The finding that glycoprotein distribution in Jurkat and B95a cells was changed similarly indicates that the effect of the tyrosine mutations on glycoprotein clustering at surface membranes of lymphocytes is not restricted to a certain subset of lymphocytes.

rMV tyrosine mutants are more fusogenic in lymphocytes

To test whether changes in F or H distribution in lymphocytes have an effect on virus spread, we compared MV propagation via cell-to-cell fusion. For this, Jurkat and B95a cells were infected with standard and mutant rMVs, and syncytium formation was photographed at 48 h p.i. (Fig. 2). In both cell lines, the fusion capacity of the mutants $rMV_{F549Y/A}$ and $rMV_{H12Y/A}$ was enhanced in comparison with that of standard $rMV_{Edm,}$ and syncytium formation was most pronounced in $rMV_{FHY/A}$-infected cells. To determine the mean size of syncytia induced by the different rMVs in B95a cells, the number of nuclei in 12 randomly chosen syncytia of each sample was counted and averaged. Syncytia induced by rMV_{Edm}, $rMV_{F549Y/A}$, $rMV_{H12Y/A}$ and $rMV_{FHY/A}$ contained on average 88, 219, 227 and 390 nuclei, respectively. Thus, the single tyrosine mutants were 2.5-fold more fusogenic than standard rMV_{Edm}, demonstrating that mutation in only one glycoprotein already increases viral fusogenicity. $rMV_{FHY/A}$ was 4.4-fold more fusogenic than rMV_{Edm}, showing that the combination of two tyrosine-mutated MV glycoproteins has an additive effect on fusion enhancement, generating a highly cytopathic virus.

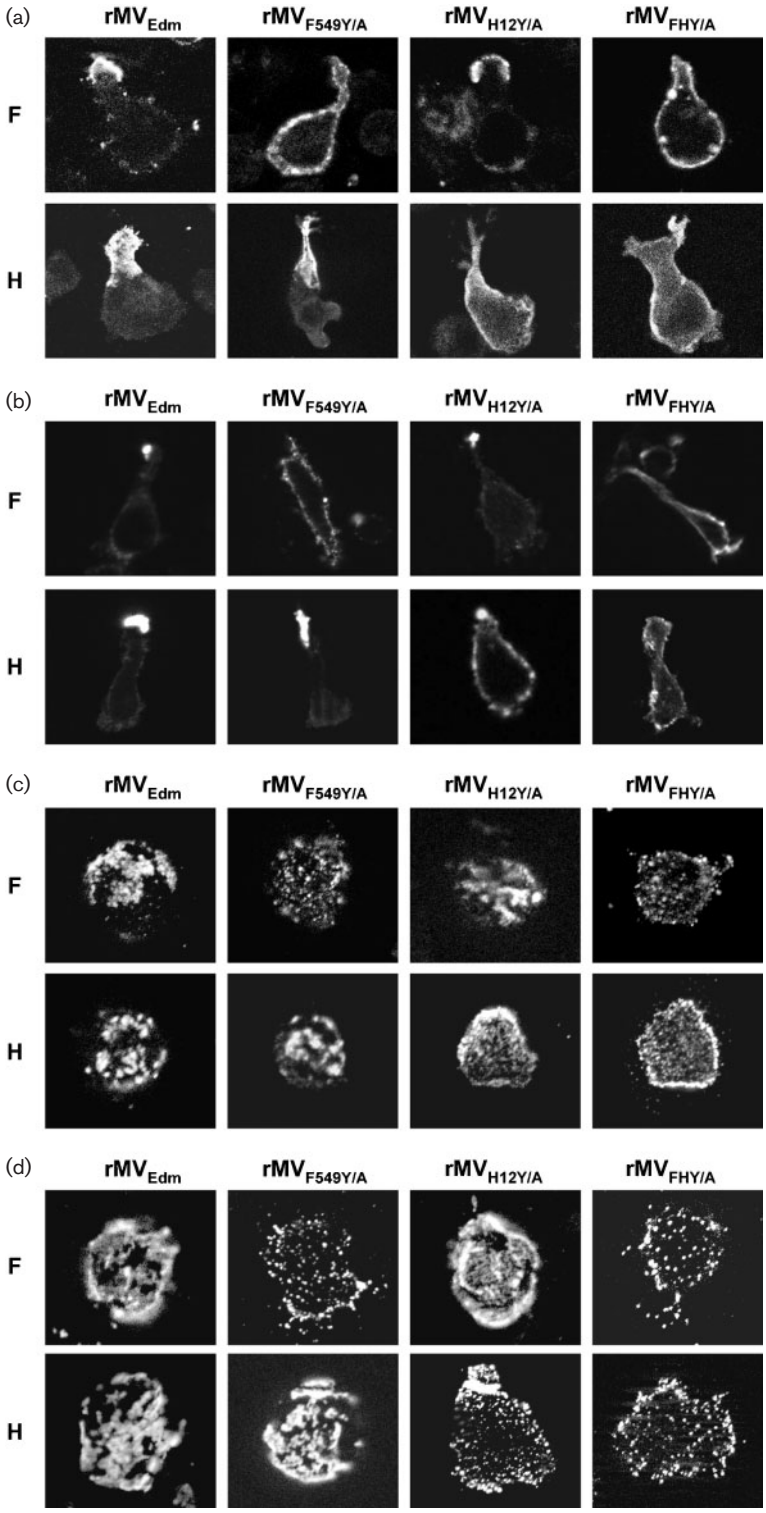

Fig. 1. Surface distribution of MV F and H proteins in infected lymphocytes. (a, c) Jurkat cells were infected with rMV$_{Edm}$, rMV$_{F549Y/A}$, rMV$_{H12Y/A}$ or rMV$_{FHY/A}$ and seeded on fibronectin-coated coverslips at 21 h p.i. After fixation with paraformaldehyde at 22.5 h p.i., surfaces were labelled with anti-F- or -H-specific primary mAbs and rhodamine-conjugated goat anti-mouse immunoglobulins. (b) PHA-stimulated PBLs were infected with the different rMVs for 48 h, seeded on fibronectin-coated coverslips for 1.5 h, fixed and probed for F and H proteins as described above. (d) rMV-infected B95a cells were fixed and stained for F and H expression at 48 h p.i. Analyses were performed with a Zeiss ApoTome/Axiovert 200M microscope. Optical sections through the middle of polarized Jurkat cells (a) and PBLs (b) and cell surfaces of non-polarized Jurkat cells (c) and B95a cells (d) are shown.

Virus release and lymphocyte proliferation are downregulated only if both glycoproteins harbour tyrosine mutations

To assess whether alterations in the glycoproteins of the rMV tyrosine mutants not only affect virus spread via cell-to-cell fusion, but also influence multi-step virus growth, virus release from infected Jurkat cells was analysed. At various time points p.i., cell-free viruses in the culture medium were harvested and titres were determined by plaque assay (Fig. 3a). rMV$_{F549Y/A}$ and rMV$_{H12Y/A}$ did not show a prominent difference in virus

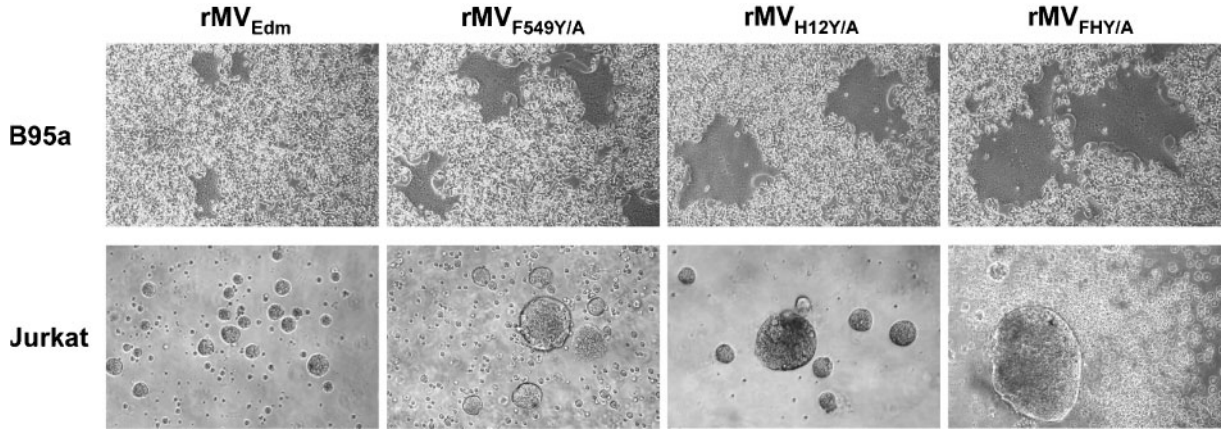

Fig. 2. Cell-to-cell fusion in rMV-infected lymphocytes. B95a and Jurkat cells were infected with rMV$_{Edm}$, rMV$_{F549Y/A}$, rMV$_{H12Y/A}$ or rMV$_{FHY/A}$ and incubated at 37 °C. Syncytium formation was monitored by phase-contrast microscopy at 2 days p.i. Magnification ×100.

release compared with standard rMV$_{Edm}$. Maximal virus titres were reached between 36 and 48 h p.i., and infected Jurkat cells constantly produced high amounts of infectious virus over at least 3 days. In contrast, growth of rMV$_{FHY/A}$ was clearly impaired. Maximal virus release was also observed at 36 h p.i., but the titre was 10-fold lower (8×10^4 p.f.u. ml^{-1}) and dropped rapidly below 10^4 p.f.u. ml^{-1}. The defective virus propagation in rMV$_{FHY/A}$-infected cells is probably due to the extensive syncytium formation and the concomitant, pronounced cytopathic effect in these cells, which presumably interferes with virus protein synthesis and functional virus assembly and budding at late time points of infection. Surprisingly, the enhanced fusion capacity of rMVs with only one mutated glycoprotein (rMV$_{F549Y/A}$, rMV$_{H12Y/A}$) had no measurable effect on multi-step virus propagation in lymphocytes. In PBLs, the growth properties of the different rMVs were essentially the same as in Jurkat cells (Fig. 3b). This indicates that the tyrosine-dependent transport signals are of similar importance for virus propagation in lymphocytic cell lines and in primary lymphocytes.

Proliferative inhibition of PBLs after MV infection is well-documented and is mediated by contact of F–H complexes on the surface of MV-infected cells with uninfected lymphocytes (Schlender *et al.*, 1996). To assess whether rMV tyrosine mutants affect lymphocyte proliferation differently, infected primary human PBLs were subjected to a [^{3}H]thymidine incorporation proliferation assay *in vitro* (Fig. 3c). In agreement with data published by Schlender *et al.* (1996), rMV$_{Edm}$ showed a dramatic proliferation inhibition of 76.7 % (Fig. 3c). The single tyrosine mutants (rMV$_{H12Y/A}$ and rMV$_{F549Y/A}$) induced a similar inhibitory effect (inhibition of 67.7 and 81.5 %, respectively), whereas the double mutant rMV$_{FHY/A}$ caused a much less pronounced proliferation inhibition (38.6 %). This indicates that the less efficient growth of the hyperfusogenic rMV$_{FHY/A}$ is accompanied by a reduced inhibitory effect on contact-mediated proliferation.

Colocalization of M and the MV glycoproteins is affected by the tyrosine mutations

By using rMVs with tail-truncated glycoproteins, it has been shown that MV-induced cell-to-cell fusion is critically dependent on the interaction of F and/or H with the M protein. Binding to the glycoprotein cytoplasmic tails is obviously required for M-mediated downregulation of the glycoprotein-dependent fusion process (Cathomen *et al.*, 1998b; Moll *et al.*, 2002). Thus, increased syncytium formation of our rMV tyrosine mutants might be a result of deficient glycoprotein–M interaction. In order to evaluate this idea, colocalization of M and the mutated glycoproteins on the surfaces of infected cells was analysed. As the protocol for double immunostaining has been established previously for infected MDCK cells (Moll *et al.*, 2002), colocalization studies were performed in MDCK, B95a and Jurkat cells infected with standard rMV$_{Edm}$ or mutants rMV$_{H12Y/A}$, rMV$_{F549Y/A}$ or rMV$_{FHY/A}$. Immunofluorescence analyses of MDCK and B95a cells were performed at 48 h p.i. and Jurkat cells were examined at 22.5 h p.i. For costaining of the F and M proteins, cell surfaces were labelled with an anti-F mAb and a rhodamine-conjugated secondary antibody. To visualize the M protein, cells were permeabilized with methanol : acetone, blocked with normal mouse serum and subsequently incubated with FITC-labelled anti-M antibodies. For H and M costaining, H proteins were labelled on the cell surfaces with a rabbit anti-H serum and M proteins were labelled after permeabilization with an anti-M mAb. Primary antibodies were then detected by rhodamine-conjugated anti-rabbit and FITC-conjugated anti-mouse sera. In Fig. 4, merged pictures of the rhodamine and FITC channels are shown. M–glycoprotein colocalization is thus

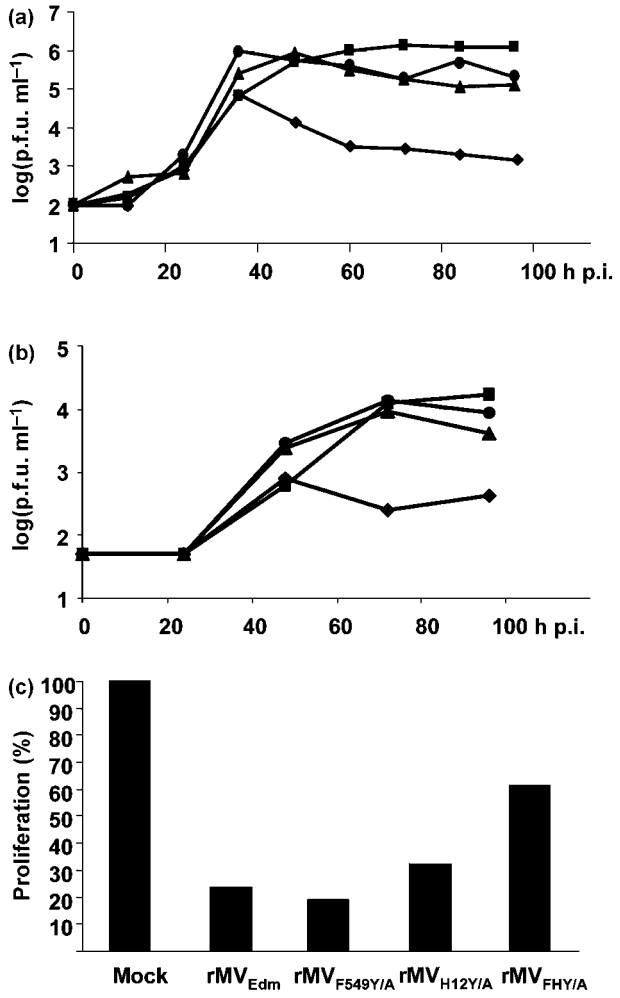

Fig. 3. Growth and proliferation of rMVs in infected lymphocytes. (a) Jurkat cells and (b) PHA-stimulated primary human PBLs were infected with rMV_{Edm}, $rMV_{F549Y/A}$, $rMVH_{12Y/A}$ or $rMV_{FHY/A}$ at an m.o.i. of 0.01 and incubated at 37 °C. Virus titres in the supernatant at different time points p.i. were determined by plaque assay. ●, rMV_{Edm}; ■, $rMV_{F549Y/A}$; ▲, $rMV_{H12Y/A}$; ◆, $rMV_{FHY/A}$. The values plotted represent mean results from two experiments. (c) PHA-stimulated PBLs were infected with rMVs at an m.o.i. of 0.5 and activated with PHA (2.5 μg ml^{-1}). Proliferative activity was determined after 72 h by labelling with [^{3}H]thymidine for 16 h and is indicated in relation to uninfected, PHA-stimulated PBLs (mock).

indicated by a yellow colour. In Fig. 4(a), the overlay of the F + M and the H + M staining in MDCK cells is shown. As the results for the adherent B95a cells were found to be essentially the same, only H + M costaining is shown exemplarily in these cells (Fig. 4b). Fig. 4(c) depicts the double staining of F + M and H + M in Jurkat cells. In all three cell lines infected with standard rMV_{Edm}, both MV glycoproteins accumulated in large aggregates on the cell surface and colocalized completely with the M protein. In contrast, neither F and M in $rMV_{F549Y/A}$-infected cells nor H and M in $rMV_{H12Y/A}$-infected cells showed a marked colocalization. In cells infected with $rMV_{FHY/A}$, neither of the two MV glycoproteins colocalized markedly with the M protein. This result indicates clearly that both MV glycoproteins interact individually with the M protein and that the interaction depends on functional tyrosine residues in the cytoplasmic domains. Thus, the observed differences in the fusion activity of the rMV tyrosine mutants are probably due to defective M–glycoprotein interactions, interfering with fusion downregulation by the M protein.

DISCUSSION

Lymphocytes travel widely throughout the host circulatory system and interact intimately with one another and with other cell types through transient, but nevertheless robust, bonds. As infectious MV is highly cell-associated *in vitro* and *in vivo*, virus spread depends on such direct cell–cell contacts (Ehrengruber *et al.*, 2002; Hyypia *et al.*, 1985; Lawrence *et al.*, 2000; Mrkic *et al.*, 2000; Udem, 1984; van Binnendijk *et al.*, 1994). The results presented in this paper reveal a critical role of the cytoplasmic tyrosines in the MV F and H glycoproteins for virus transmission from infected to uninfected lymphocytes, not only because they are required for localization in uropods, the cell pole involved in cell–cell interactions, but also because they mediate binding to the M protein, which downregulates H- and F-mediated cell-to-cell fusion, thereby preventing rapid syncytium formation and cell damage.

In contrast to epithelial cells, lymphocytes polarize only transiently, for example upon direct cell–cell contact with other lymphocytes or antigen-presenting cells, or in response to soluble factors such as chemokines (Krummel & Macara, 2006; Vicente-Manzanares & Sanchez-Madrid, 2004). If lymphocytes have acquired a polarized or migrating phenotype, proteins and lipids are delivered specifically to one cell domain (del Pozo *et al.*, 1997; Gomez-Mouton *et al.*, 2001; Sanchez-Madrid & del Pozo, 1999). However, whilst protein sorting to the apical or basolateral domain in epithelial cells is well-characterized, not much is known about polarized transport to the leading edge or the uropod of lymphocytes. As lymphocyte migration is accompanied by extensive rearrangements and polarization of microtubules and the actin cytoskeleton (Krummel & Macara, 2006), one determinant for localization at one cell pole is binding to actin-associated proteins, such as proteins of the ezrin–radixin–moesin family, which are located specifically at the uropod (del Pozo *et al.*, 1997; Serrador *et al.*, 1998). In addition, interaction with proteins of the polarity network, e.g. Scrib, Lgl, Dlg and PAR, or specific recruitment into detergent-insoluble, glycolipid-enriched membrane domains (rafts) may account for polarized protein localization. It has been shown that rafts are essential for the generation, maintenance and functionality of T-cell anteroposterior polarity and that acquisition of a migrating phenotype in T lymphocytes results in the asymmetrical redistribution of

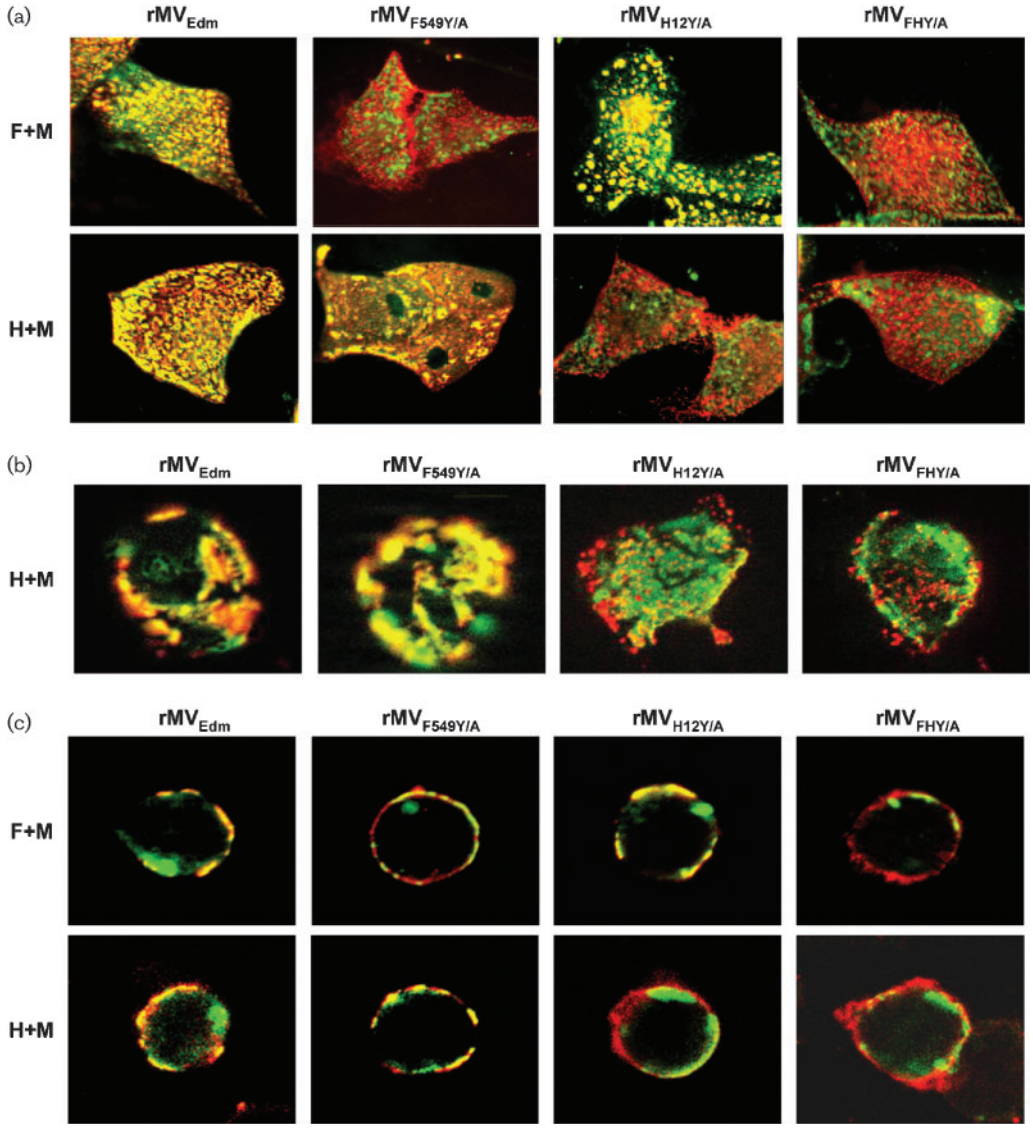

Fig. 4. Colocalization of M with F and H proteins in rMV-infected cells. MDCK (a), B95a (b) and Jurkat (c) cells were infected with rMV$_{Edm}$, rMV$_{F549Y/A}$, rMV$_{H12Y/A}$ or rMV$_{FHY/A}$. For F–M costaining, live MDCK cells (48 h p.i.) or paraformaldehyde-fixed Jurkat cells seeded on fibronectin-coated coverslips (22.5 h p.i.) were labelled with a mAb directed against the F protein (A504) and rhodamine-conjugated goat anti-mouse IgG. After permeabilization and blocking with normal mouse serum, the M protein was stained with an FITC-labelled M-specific mAb. For H and M costaining, surfaces of unfixed MDCK and B95a cells or fixed Jurkat cells were labelled with an H-specific MV serum and rhodamine-conjugated goat anti-rabbit IgG. After permeabilization, cells were stained with an M-specific mAb and FITC-conjugated anti-mouse IgG. Merged images of the rhodamine and FITC channels are shown.

ganglioside GM3- and GM1-enriched raft domains to the leading edge and to the uropod, respectively (Gomez-Mouton *et al.*, 2001; Krummel & Macara, 2006; Millan *et al.*, 2002). Also, viral proteins can be transported selectively to one subdomain in lymphocytes (Danis *et al.*, 2004; Millan *et al.*, 2002). For example, localization of the influenza virus haemagglutinin (HA) at the uropod has been linked to its raft association (Millan *et al.*, 2002). In contrast to HA, the MV F protein has only a weak intrinsic ability to associate with rafts, and MV H is supposed to be recruited into these membrane domains exclusively via its interaction with the F protein. Only 15–40 % of F or H proteins are located within rafts (Manie *et al.*, 2000; Vincent *et al.*, 2000). Thus, raft association is probably not the cause of the uropod localization of MV F and H observed in this study. The fact that mutations in the cytoplasmic tyrosines responsible for basolateral transport in polarized epithelial cells prevented concentration in uropods rather indicates that these residues also serve as transport signals in polarized lymphocytes. A similar

mechanism might account for the localization of the Env protein of HIV-1, a basolateral protein that also localizes in uropods (Lodge *et al.*, 1997; Nguyen & Hildreth, 2000). It can therefore be assumed that lymphocytes possess a pathway of transport to the uropod reminiscent of that used for its specific targeting to the basolateral surface of epithelial cells, probably involving cellular adaptor proteins recognizing tyrosine-containing motifs in the cytoplasmic domain of membrane proteins (Bonifacino & Dell'Angelica, 1999; Rodriguez-Boulan *et al.*, 2005).

As lymphocytes recruit bystander cells through their uropod (del Pozo *et al.*, 1997), the localization of F and H at one cell pole might assure rapid and efficient binding to the receptor on the uninfected neighbouring cell, thereby facilitating directed virus transmission. Similar to MV, spread of HTLV-1 is also dependent on direct cell contacts, because naturally infected lymphocytes produce very few cell-free virions. It has been shown that HTLV-1 transmission among $CD4^+$ T cells occurs via a virological synapse, defined as a cytoskeleton-dependent, stable adhesive junction across which virus is transmitted by directed transfer (Igakura *et al.*, 2003). The same method of propagation has also been described for HIV-1 spread between T cells or between dendritic cells and T cells (Jolly *et al.*, 2004; McDonald *et al.*, 2003; Turville *et al.*, 2004). Like the Env proteins of HIV-1 and HTLV-1, the MV envelope proteins F, H and M concentrate at the contact sides between infected and uninfected Jurkat cells (N. Runkler, unpublished data). Furthermore, we recently observed a relocalization of the microtubule-organizing centre to these cell-contact sides, similar to what has been reported for the virological synapse in HTLV-1-infected cells (Igakura *et al.*, 2003). This suggests strongly that MV is also transmitted via a virological synapse. Directed budding of virus into the synaptic cleft would not only facilitate virus transfer into uninfected target cells, but would also protect against neutralization by antibodies or the complement system.

Lymphopenia, cytokine imbalance and the inability of PBLs to expand in response to polyclonal or antigen-specific stimulation *ex vivo* are hallmarks of generalized immunosuppression caused by MV (Borrow & Oldstone, 1995). Several studies have shown that T cells can no longer proliferate in response to antigenic stimulation after contact with MV particles or MV-infected cells carrying F and H glycoproteins on the surface. Interaction of the MV glycoprotein complexes with uninfected T cells interferes with activation of the PI3/Akt kinase pathway and rearrangements of the cortical actin cytoskeleton, thus perturbing the ability of T cells to adhere, spread and cluster receptors essential for sustained T-cell activation (Avota *et al.*, 2001; Muller *et al.*, 2006; Schlender *et al.*, 1996). As localization of both MV glycoproteins at cell-contact sides is required for T-cell silencing, changes in the H and F surface distribution might influence not only virus dissemination from infected lymphocytes, but also MV-induced immunosuppression. This idea is clearly supported by the finding that $rMV_{FHY/A}$ had a reduced inhibitory effect on PBL proliferation (Fig. 3c).

For efficient MV assembly, all virus components must interact specifically with each other at the plasma membrane. The M protein is known to play the key role during the assembly and budding process (Cathomen *et al.*, 1998a; Peebles, 1991), because it mediates the contact between the outer surface glycoproteins and the inner nucleocapsids. We have shown very recently that M is required for nucleocapsid transport from intracellular inclusions to the plasma membrane (Runkler *et al.*, 2007). At the inner side of surface membranes, M is able to form large aggregates by self-aggregation, and budding is induced after recruiting the glycoproteins via M binding to the F and H cytoplasmic tails. For functional assembly, the glycoproteins must thus colocalize in M clusters at the plasma membrane. Here, we demonstrated that this colocalization depends critically on one amino acid, the cytoplasmic tyrosine in the H and F proteins. The importance of a tyrosine motif in the glycoprotein cytoplasmic tail has also been proposed for Sendai virus. Here, binding of the HN glycoprotein to M depends on an SYWST motif (Takimoto *et al.*, 1998). In agreement with the independent binding of each MV glycoprotein to M, cell-to-cell fusion of infected and uninfected lymphocytes is enhanced if only one MV glycoprotein fails to colocalize with M clusters at the cell surface. As it is generally assumed that interaction of the glycoproteins with large M aggregates lowers the lateral mobility of F and H in the plasma membrane of the infected cell, thereby down-regulating the formation of active fusion complexes, as well as accumulation of these complexes at the sites of cell-to-cell fusion (Henis *et al.*, 1989), defective interaction of one MV glycoprotein with M probably increases lateral mobility. This effect is synergistic if both glycoproteins are mutated, as demonstrated by dramatically enhanced syncytium formation in $rMV_{FHY/A}$-infected cells. As virus release, in contrast to fusogenic properties, is not changed in $rMV_{F549Y/A}$-infected and $rMV_{H12Y/A}$-infected cells, the virus-assembly process is obviously not disturbed markedly if only one glycoprotein has lost its ability to bind stably to M clusters. A negative effect on virus release was only found in $rMV_{FHY/A}$-infected cells, which is probably due to the extensive cytopathic properties of this virus, destroying the infected cell before new virions can be assembled. Supporting this view, we found that the amount of cell-associated virus in $rMV_{FHY/A}$-infected cells was reduced similarly to the virus titres in the supernatant (N. Runkler, unpublished data). Thus, reduced virus release is accompanied by lower intracellular virus production. However, the possibility that the simultaneous lack of M interaction with both glycoproteins has a direct negative effect on virus assembly by affecting the amount of surface glycoproteins incorporated into budding virions, thereby decreasing the infectivity of the cell-free viruses, cannot be excluded completely.

Our data emphasize the critical role of the tyrosine residues in the cytoplasmic tails of both MV glycoproteins for

efficient MV propagation. These residues not only account for basolateral glycoprotein expression in polarized epithelia, thus allowing fusion of infected epithelial cells with neighbouring or underlying cells, helping the virus to overcome epithelial barriers, but they are also responsible for F and H transport to one pole of polarized lymphocytes, the main MV target cells in acute MV infection. Accumulation at cell-contact sides probably allows direct virus transfer to uninfected cells, perhaps via a virological synapse. As the cytoplasmic tyrosine residues also mediate interaction with the M protein, cell-to-cell fusion is limited, and virus assembly and propagation are not prevented by an overshooting cytopathic effect.

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