Growth of influenza A virus is not impeded by simultaneous removal of the cholesterol-binding and acylation sites in the M2 protein

Bastian Thaa,1 Claudia Tielesch,1 Lars Möller,2 Armin O. Schmitt,3 Thorsten Wolff,2 Norbert Bannert,2 Andreas Herrmann4 and Michael Veit1

1Freie Universität Berlin, Faculty of Veterinary Medicine, Institute of Immunology and Molecular Biology, Philippstraße 13, 10115 Berlin, Germany
2Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany
3Humboldt-Universität zu Berlin, Department for Crop and Animal Sciences, Invalidenstraße 42, 10115 Berlin, Germany
4Humboldt-Universität zu Berlin, Institute of Biology, Molecular Biophysics, Invalidenstraße 42, 10115 Berlin, Germany

Influenza virus assembly and budding occur in the ‘budozone’, a coalesced raft domain in the plasma membrane. The viral transmembrane protein M2 is implicated in virus particle scission, the ultimate step in virus budding, probably by wedge-like insertion of an amphiphilic helix into the membrane. In order to do this, M2 is hypothesized to be targeted to the edge of the budozone, mediated by acylation and cholesterol binding. It was recently shown that acylation and cholesterol binding affect the membrane association of the cytoplasmic tail of M2 and targeting of the protein to coalesced rafts. This study tested whether combined removal of the acylation site (C50) and the cholesterol recognition/interaction amino acid consensus motifs (key residues Y52 and Y57) in the amphiphilic helix of M2 influenced virus formation. Recombinant influenza viruses were generated in the influenza strain A/WSN/33 background with mutations in one or both of these features. In comparison with the wild-type, all mutant viruses showed very similar growth kinetics in various cell types. Wild-type and mutant viruses differed in their relative M2 content but not regarding the major structural proteins. The morphology of the viruses was not affected by mutating M2. Moreover, wild-type and mutant viruses showed comparable competitive fitness in infected cells. Lastly, a global comparison of M2 sequences revealed that there are natural virus strains with M2 devoid of both lipid-association motifs. Taken together, these results indicate that the acylation and cholesterol-binding motifs in M2 are not crucial for the replication of influenza virus in cell culture, indicating that other factors can target M2 to the budding site.

INTRODUCTION

Influenza A viruses are heteromorphous (spherical or filamentous) enveloped viruses in the family Orthomyxoviridae. Their membrane is lined from beneath by a matrix protein layer composed of the protein M1, which in turn envelopes the viral genome, arranged as eight viral ribonucleoprotein particles. The membrane contains three transmembrane proteins, the glycoproteins haemagglutinin (HA) and neuraminidase (NA) and the tetrameric proton channel protein M2. M2 (Fig. 1a) comprises 97 aa per monomer, with the N-terminal 24 aa oriented towards the outside (ectodomain). The next 19 aa form a transmembrane domain assembled into a four-helix bundle that constitutes the proton channel. Aa 44–97 of M2 represent the cytoplasmic tail (M2-CT), and aa 48–62 form a membrane-parallel amphiphilic helix with a hydrophilic (solvent-exposed) and a hydrophobic (partially membrane-inserted) side. In addition, the M2-CT contains a binding site for M1 at aa 71–73 (Chen et al., 2008). In the infected cell, all these components are synthesized and ultimately transported to the apical plasma membrane for assembly and budding of progeny virions (Nayak et al., 2009; Rossman & Lamb, 2011). Assembly is organized in ‘membrane rafts’, cholesterol- and sphingolipid-enriched nanodomains in the apical plasma membrane. These rafts are stabilized to form the ‘viral budozone’ mainly by...
interactions among the viral components and with the raft lipids. The glycoproteins HA and NA have intrinsic raft-association features (Engel et al., 2010; Scheiffele et al., 1997; Scolari et al., 2009) and hence define the site of the bud zone (Hess et al., 2005; Leser & Lamb, 2005; reviewed by Veit & Thaa, 2011). Conversely, the third transmembrane protein, M2, does not associate with typical raft markers (Leser & Lamb, 2005; Schroeder et al., 2005; Thaa et al., 2010; Zhang et al., 2000) and is, accordingly, mostly excluded from virions, although the protein is highly expressed at the surface of the infected cell (Lamb et al., 1985). Nevertheless, some of the M2 molecules need to reach the bud zone for inclusion into virions.

Targeting to the bud zone might be achieved by the amphipathic helix in the M2-CT, which comprises two potential raft-association features: (i) S-acylation (‘palmitoylation’: covalent attachment of a saturated fatty acid, usually palmitic acid, by a thioester linkage) at cysteine residue 50 (C50) (Sugrue et al., 1990; Veit et al., 1991), and (ii) cholesterol binding (Schroeder et al., 2005), probably mediated by cholesterol recognition/interaction amino acid consensus (CRAC) motifs (L/V-X_{1–5}-Y-X_{1–5}-R/K), present up to four times in the amphipathic helix region of the M2-CT, depending on the virus strain. Schroeder et al. (2005) have hypothesized that M2 could accumulate at the edge of raft domains because the transmembrane domain is too short to be completely immersed in the highly ordered raft phase and thus counteracts the raft-targeting features. Positioning of M2 at the raft edge could then entail the scission of virus particles: the amphipathic helix is assumed to induce curvature by wedge-like integration into the membrane. Indeed, there is experimental evidence that M2 plays an active role in scission. M2 accumulates at the neck of budding filamentous virions (the bud zone edge), and M2 as well as a peptide encompassing the amphipathic helix induce the formation of vesicles in giant unilamellar vesicles, an in vitro membrane model system (Rossman et al., 2010a, b).

We recently demonstrated that M2 intrinsically meets the biochemical requirements of the Schroeder model: mutation of tyrosines 52 and 57 (Y52 and Y57, as part of overlapping CRAC motifs) eliminates cholesterol binding to M2-CT as a purified protein. Furthermore, we showed that M2-CT associates with membranes, both in vitro and in cells, and that the membrane-binding properties are modulated by exchange of the acylation site and the CRAC motif tyrosines. Furthermore, M2 has the propensity to partly associate with the coalesced raft phase in cell-derived model membranes, dependent on acylation (Thaa et al., 2011).

Here, we tested whether the same mutations in the amphipathic helix of M2 influence the growth properties of recombinant virus. Others have reported previously that mutations in either the acylation site or in the CRAC motifs of M2 do not significantly affect virus growth, at least in cell culture (Castrucci et al., 1997; Grantham et al., 2009; Rossman et al., 2010a; Stewart et al., 2010). However,

Fig. 1. (a) Topology sketch of M2, showing the orientation of the transmembrane domain and amphipathic helix in the cytoplasmic tail according to nuclear magnetic resonance (NMR) structural analysis (Sharma et al., 2010). The acylation site (C50) and the tyrosine residues (Y52 and Y57) in the CRAC motifs are indicated. (b) A helical wheel plot (axial view) of the amphipathic helix (aa 46–61) of M2 from influenza A/WSN/33 generated using Heliquest (http://heliquest.ipmc.cnrs.fr/), oriented according to the NMR structure (Sharma et al., 2010). Hydrophobic residues are shown in light grey and polar residues in dark grey. Arrows indicate the residues substituted in this study. (c–e) Helical wheel plots of the amphipathic helix of M2 showing the residues changed in the studies by Grantham et al. (2009) (c), Stewart et al. (2010) (d) and Rossman et al. (2010a) (e). The virus strains are WSN (c, d) and Udorn (e, amino acid differences compared with WSN are indicated in italics). See text for details.
the combined replacement of the two raft-association features in the amphiphilic helix of M2 has not been investigated so far in the context of the virus. Our hypothesis was that the combined elimination of both features would disconnect M2 from the budosome, leading to impeded virus budding. However, our results showed that virus replication in cell culture was not markedly compromised by mutating one or both raft-targeting features in M2. This finding is discussed with a focus on molecular interactions and functional redundancy of influenza virus proteins.

RESULTS

Generation of recombinant virus and growth kinetics

In Fig. 1(b), the amphiphilic helix of influenza A virus M2 is displayed as a ‘helical wheel plot’. This view along the helix axis shows the position of the individual amino acid residues: the helix is amphiphilic, with charged/hydrophilic residues (dark grey) clustered at one side of the helix and hydrophobic residues (light grey) at the other, directed towards the membrane. This has also been verified experimentally (Nguyen et al., 2008; Sharma et al., 2010).

In previous studies, the acylation site C50 (Fig. 1c; Castrucci et al., 1997; Grantham et al., 2009) or up to three residues in the CRAC motifs (Fig. 1d; Stewart et al., 2010) were replaced in the background of the influenza virus strains A/WSN/33 (H1N1) and A/Udorn/72 (H3N2), albeit without a noticeable influence on virus replication in cell culture compared with the respective wild-type virus. Note that in Udorn M2, the CRAC motif sequence consensus (L/V-X1–5-Y-X1–5-R/K) is not present. However, the protein associates with cholesterol (Rossman et al., 2010a), and completion of the CRAC motif does not alter viral growth (Stewart et al., 2010). To provoke a marked reduction in virus viability, five residues in the hydrophobic face of the amphiphilic helix had to be replaced in Udorn M2 (Fig. 1e; Rossman et al., 2010a, b). However, none of these investigations examined the effects of a combined mutation of the acylation site and the CRAC motifs on influenza virus growth.

To this end, we produced three different recombinant viruses encoding M2 mutations in the WSN background (Fig. 1b). In the first mutant WSN M2, the mutations Y52S and Y57S were introduced, interrupting the CRAC motifs by replacing the two central tyrosine residues with serines, a mutation that has been shown to eliminate cholesterol binding to purified M2-CT without changing the hydrophobicity pattern of the amphiphilic helix, and not to reduce acylation (Thaa et al., 2011). In the second mutant M2, the acylation site at cysteine 50 was substituted by serine (C50S), and in the third mutant M2, the mutations C50S, Y52S and Y57S were all introduced, thus disrupting the two features simultaneously.

All viruses could be rescued, as evidenced by plaque assay (Fig. 2a). Overall, the plaque size did not differ significantly between the wild-type virus and mutants, indicating that the mutants did not exhibit drastic growth defects. The mutant viruses could be passaged three times in Madin–Darby canine kidney (MDCK) cells without changes in the M2 sequence, showing that the respective mutations were stable (data not shown).

To analyse the replication kinetics of the viruses, MDCK cells were infected with wild-type WSN or one of the mutants at an m.o.i. of 0.01, and the supernatants were collected at various times and assessed for virus titre by plaque assay (Fig. 2b). The mutant with the deleted acylation site reached titles identical to the wild-type virus, confirming data by others for WSN as well as for Udorn (Grantham et al., 2009). The mutant M2: Y52S, Y57S revealed reduced titles at 16 and 24 h post-infection (p.i.), albeit by less than one order of magnitude. However, this virus reached the same titles as the wild-type virus at later times, showing that any possible delay in growth was not long lasting. This is in line with investigations where similar mutations in M2 CRAC motifs did not perturb the growth of WSN (Stewart et al., 2010).

Surprisingly, the mutant lacking both the acylation site and the CRAC motifs (C50S, Y52S, Y57S) did not exhibit any reduction in titre at any time.

We next analysed the viral growth kinetics on cell types derived from the target organ of influenza virus, the respiratory tract. In the human alveolar epithelial adenocarcinoma cell line A549, the virus grew to titres that were generally lower compared with those in MDCK cells, but the growth kinetics were essentially identical for wild-type and mutant WSN (Fig. 2c). Likewise, all M2 mutant viruses also replicated to a similar level to the wild-type virus in non-transformed MRC-5 human fetal lung fibroblasts (Jacobs et al., 1970), which can be regarded as primary cells (Fig. 2d). Similar to the situation in MDCK cells (Fig. 2b), there was a tenfold reduction in virus titre for the M2: Y52S, Y57S mutant at intermediate (48 h p.i.) but not later times. The additional replacement of C50 did not intensify this effect, as the mutant WSN M2: C50S, Y52S, Y57S grew identically to the wild-type virus. Taken together, the mutation of the acylation site in combination with the CRAC motifs in M2 did not perturb the replication of WSN in different cell systems. The mutant viruses characterized in this study may, however, be attenuated in animal hosts, such as mice. Indeed, such findings have been reported both for viruses where the acylated cysteine in M2 was replaced (Grantham et al., 2009) and where the CRAC motifs were disrupted (Stewart et al., 2010).

Electron microscopy

Defects in budding and scission might be reflected by aberrant morphology of the resulting virus particles (Nayak et al., 2009). This was the case when five residues in the hydrophobic face of the amphiphilic helix of M2 were...
replaced in the filamentous Udorn strain (Rossman et al., 2010a). To examine whether acylation and/or the cholesterol-binding moiety of M2 have an influence on virion shape, particles were harvested from the supernatant of infected MDCK cells and visualized by negative-staining transmission electron microscopy. Fig. 3(a) shows that the virus particles were mostly spherical in all cases, yielding no evidence for any perturbation of virus morphology by mutations in M2. In addition, ultrathin sections of infected cells were produced and imaged by transmission electron microscopy. Defects in the last budding step (scission) would be reflected by a ‘beads-on-a-string’ morphology of the budding virions, in which individual virions fail to be separated from each other (Nayak et al., 2009). Despite detailed inspection of samples, no such morphology was observed. The representative images in Fig. 3(b) show MDCK cells with WSN M2: Y52S, Y57S in the process of budding. This was the only mutant that exhibited a slight reduction in titre (see Fig. 2b and d). Thus, WSN virus morphology was not altered by mutating the acylation site and/or the CRAC motifs in the amphiphilic helix of M2.

Viral protein content

We then assessed the content of the individual virus components in virus particles. If there was an influence of the raft-targeting motifs in M2 on the incorporation of viral proteins, there should be a difference in their relative abundance. However, the proportions of the major structural proteins, especially the ratio of HA: M1, was the same in wild-type WSN and the mutants, as illustrated with radioactively labelled virions by SDS-PAGE, fluorography and densitometric quantification (Fig. 4a). This is in line with other reports investigating similar mutations (Grantham et al., 2009; Rossman et al., 2010a; Stewart et al., 2010). M2 could not be discerned in the fluorograms in Fig. 4(a) as only minor amounts are incorporated into virions. Therefore, Western blotting with an anti-M2 antibody was performed (Fig. 4b). The chemiluminescent signal intensity for M2 was related to that of M1 probed in parallel on the same membrane. M2 was detected as a double band, as noted previously (Grantham et al., 2009; Zhirnov et al., 1999). In comparison with wild-type, WSN M2: Y52S,
Y57S showed a significantly reduced M2 : M1 ratio (i.e., reduced M2 incorporation). The mutant WSN M2: C50S, in contrast, contained more than twice the amount of M2 (normalized to M1) than the wild-type virus. Interestingly, the virus in which both the cholesterol-association motifs and the acylation site were disrupted (WSN M2: C50S, Y52S, Y57S) did not display any marked change in the M2 : M1 ratio compared with the wild-type virus. Thus, the opposing effects of CRAC and acylation site mutation on M2 integration into virions appeared to compensate each other.

**Competition of wild-type and mutant viruses**

We next hypothesized that mutating M2 might reduce the competitive fitness of the virus compared with the wild-type virus. If one of the virus strains exhibits a selective advantage, it would rapidly eliminate the other. To test this, we co-infected MDCK cells with wild-type WSN and WSN M2: Y52S, Y57S (the mutant showing slight growth retardation) and analysed the cell-culture supernatant at different times p.i. The viral RNA in the supernatant was isolated, amplified by RT-PCR and sequenced. Fig. 5(a) shows the sequencing chromatograms for the region of interest in the M2 gene in wild-type WSN and WSN M2: Y52S, Y57S (the mutant showing slight growth retardation) and analysed the cell-culture supernatant at different times p.i. The viral RNA in the supernatant was isolated, amplified by RT-PCR and sequenced. Fig. 5(a) shows the sequencing chromatograms for the region of interest in the M2 gene in wild-type WSN and WSN M2: Y52S, Y57S. When the supernatants of cells co-infected with the wild-type and mutant viruses (at a ratio of 1 : 1 or 1 : 5; total m.o.i. 0.005) were analysed by sequencing, both wild-type and mutant nucleotide species were present in all cases, reflected by superimposed peaks for the respective bases (Fig. 5b and c, arrows). Note that differences in the peak heights and areas in the chromatograms should not be interpreted in a precise quantitative manner. Nevertheless, it is obvious that the mutant did not become ‘extinct’ within the time frame of the experiment (96 h).

Competition experiments involving wild-type WSN and the other mutant viruses yielded the same results (data not shown).

This result is in contrast to recombinant viruses where the M2 proton channel is inactivated by the deletion of 3 aa in the transmembrane domain. In this case, an analogous fitness assay revealed that wild-type virus almost completely overgrew the mutant virus within 96 h of co-cultivation, even if the initial amount of wild-type virus was much lower (ratio of wild-type to mutant virus of 1 : 100; Takeda et al., 2002). Thus, our result demonstrated that there was no particular decrease in viral fitness following disruption of the raft-targeting features of M2.

**Conservation of the acylation site and CRAC motifs in influenza A virus M2 protein**

Surprisingly, our combined experimental evidence argued against a synergistic role of the acylation site C50 and the CRAC motif tyrosines of M2 for virus viability in cell culture. However, it has been noted previously that neither C50 (Sugrue et al., 1990; Veit et al., 1991) nor the CRAC motifs (e.g. in the Udorn strain) are strictly conserved in M2, implying that these features are not absolutely essential for virus replication.
Phenylalanine and tyrosine are bulky aromatic residues with relatively high hydrophobicity and membrane-insertion capacity. Thus, they might at least partially compensate for the hydrophobicity of the wild-type S-acylated cysteine, in contrast to serine with its hydrophilic hydroxyl group.

Of the M2 sequences analysed, 1.9% possessed C50 but no CRAC motif. These were mainly sequences from H3N2 viruses of human, porcine or avian origin, including the pandemic strain of the 1968 outbreak (A/Aichi/2/68) and the filamentous laboratory strain Udorn. The M2 of these viruses might, however, still be capable of binding cholesterol, possibly with decreased affinity. The CRAC motif is defined rather loosely (L/V-X1–5-Y-X1–5-R/K) and probably depends on protein conformation rather than just the linear amino acid sequence (Schroeder, 2010). One of the two tyrosines demonstrated to be important for cholesterol association (Thaa et al., 2011) was usually present in these strains.

Lastly, 58 sequences (0.3%) lacked both the acylated cysteine at position 50 and an intact CRAC motif. Of these, 12 sequences were artificial (sequences of M2 fragments for structural analysis), and the M2 sequence of one particular strain [A/Sendai/TU65/2006 (H1N1)] was deposited in the database seven times. Notably, four of these strains carried a cysteine at position 52, which can be assumed to be a potential acylation site instead of the natural C50. Thus, there were 36 different, naturally occurring influenza A virus strains in the database whose M2 lacked both acylation and cholesterol-binding motifs. This proves that such mutants do indeed exist in nature. These strains were mostly human isolates from different geographical regions (Asia, Europe and America), typically isolated in 2000 or later, and exclusively of subtype H1N1. It will be interesting to see whether new strains with these features will emerge; the result from our competition experiment (Fig. 5) is a hint that such mutants are not quickly competed out by wild-type virus.

In summary, this global sequence comparison showed that the ‘standard’ M2 protein contains both a palmitoylated cysteine at position 50 and at least one intact CRAC motif in the amphiphilic helix. However, there are strains in which one or even both of these features are missing.

**DISCUSSION**

Our experimental data collectively show that combined disruption of the acylation site and the CRAC motifs in the amphiphilic helix of M2 had no effect on virus viability in cell culture, although these features determine the membrane-association capacity of the M2-CT and, in the case of acylation, also the partial association of M2 with coalesced rafts in giant plasma membrane vesicles, cell-derived model membranes (Thaa et al., 2011). Nevertheless, an effect on incorporation of mutated M2 into virus particles was observed. Hence, we assume that targeting to the viral
budozone was modified after disruption of CRAC and the acylation site but did not result in diminished virus growth. Although cholesterol binding to purified M2-CT (determined by cross-linking with $[^3H]$photcholesterol) is drastically reduced following mutation of the CRAC motif tyrosines (Thaa et al., 2011), we do not want to exclude the possibility that the mutant M2: Y52S, Y57S expressed during influenza virus infection has residual cholesterol-binding activity that may be critical to virus growth. It can be imagined that the transmembrane region of M2 contributes to cholesterol binding. Moreover, the cholesterol concentration in the plasma membrane, and especially at the viral budozone, might be high enough to allow association with even mutated M2. Technical limitations in labelling M2 and even the established cholesterol-binding protein caveolin (Murata et al., 1995, and data not shown) with $[^3H]$photocholesterol inside cells precluded our attempts to investigate this important issue further. Likewise, the methods we used previously to examine the raft localization of M2, namely fluorescence lifetime imaging/fluorescence resonance energy transfer (Thaa et al., 2010), and the preparation of giant plasma membrane vesicles (Thaa et al., 2011) cannot be performed in the context of virus-infected cells.

Mutating C50 and/or CRAC in M2 did not impede virus replication. However, we observed a reduced incorporation of M2 into virions following CRAC disruption, which corresponded to a slight delay in virus replication. This

<table>
<thead>
<tr>
<th>Presence of CRAC</th>
<th>C50</th>
<th>SS50</th>
<th>F50</th>
<th>Y50</th>
<th>Others</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRAC</td>
<td>15522 (84.0 %)</td>
<td>2035 (11.0 %)</td>
<td>407 (2.2 %)</td>
<td>92 (0.5 %)</td>
<td>6 (0.0 %)</td>
<td>18062 (97.8 %)</td>
</tr>
<tr>
<td>No CRAC</td>
<td>357 (1.9 %)</td>
<td>58 (0.3 %)</td>
<td>0 (0.0 %)</td>
<td>0 (0.0 %)</td>
<td>0 (0.0 %)</td>
<td>415 (2.2 %)</td>
</tr>
<tr>
<td>Total</td>
<td>15879 (85.9 %)</td>
<td>2093 (11.3 %)</td>
<td>407 (2.2 %)</td>
<td>92 (0.5 %)</td>
<td>6 (0.0 %)</td>
<td>18477 (100.0 %)</td>
</tr>
</tbody>
</table>

**Table 1.** Numbers of influenza A M2 sequences retrieved from http://www.ncbi.nlm.nih.gov, grouped according to the identity of residue 50 and the presence or absence of CRAC motifs.
might be due to a weaker interaction of the mutated M2 protein with the cholesterol-rich budozone. In contrast, replacement of C50 led to an increased proportion of M2 in the mature virions. The fatty acyl chains of palmitoylated proteins have been proposed to tilt transmembrane helices relative to the normal bilayer and hence reduce their effective length. Thus, lack of acylation might increase the interaction of M2 with raft domains (and hence the viral budozones), which are considered to be thicker than the bulk phase of the plasma membrane (Charollais & Van Der Goot, 2009; Morozova & Weiss, 2010). The mutant virus in which both the cholesterol-association motifs and the acylation site were disrupted did not display any marked change in the M2:M1 ratio compared with the wild-type virus. Hence, the opposing effects of CRAC and acylation site mutation on the incorporation of M2 into virus particles apparently compensated each other. However, the overall amount of M2 in mature virions is generally very low (14–68 M2 molecules per virion), although the protein is expressed abundantly at the plasma membrane (Zebedee & Lamb, 1988).

So far, only one mutation in the amphiphilic helix of M2 has been described that leads to impairment of virus growth in cells, namely the simultaneous replacement of five amino acids (F47, F48, I51, Y52 and F55) in the hydrophobic face of the helix by less hydrophobic alanines (Rossman et al., 2010a, b; see Fig. 1e). However, this set of mutations severely reduced the hydrophobic moment, $\mu_H$, a measure of the amphiphilicity of the helix (Eisenberg et al., 1984). The $\mu_H$ value was reduced from 0.502 in the wild-type case to 0.244. It should be noted, however, that the additional hydrophobicity of the cysteine-bound palmitate was not considered in this calculation.

In contrast, all other mutations that were engineered in the amphiphilic helix of M2, in this study and those of others, did not markedly change the hydrophobic moment of the helix (Table 2). Concomitantly, viruses carrying these mutations in their M2 showed similar growth properties to the wild-type virus. Thus, virus growth seems to depend on the amphiphilicity of the helix in the M2-CT rather than on the presence or absence of the two potential raft-targeting features, acylation and cholesterol association. We assume that the deepness of membrane insertion exerted by the amphiphilic helix determines whether M2 can fulfil its suggested role as mediator of virus particle scission.

This conclusion is further backed by a new report, published during revision of this manuscript (Stewart & Pekosz, 2011). In this study, alanine scanning mutagenesis in the amphiphilic helix of M2 did not markedly affect virus composition and viability, implying that this region tolerates numerous mutations and that the functionality of M2 for virus formation is based on the overall structure of the amphiphilic helix rather than on its protein sequence.

### Table 2. Amphiphilicity of the helix formed by aa 46–61 in the cytoplasmic tail of M2 and different mutants thereof, studied in the context of the virus in this study and the indicated references

The hydrophobic moment, $\mu_H$, is displayed as calculated using Heliquest. The presence or absence of CRAC motifs is indicated. Mutated amino acids are indicated by bold and underlining.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sequence (aa 46–61)</th>
<th>CRAC</th>
<th>Hydrophobic moment ($\mu_H$)</th>
<th>Impaired virus growth</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WSN strain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>LFFKCIYRFKGYGLKR</td>
<td>+</td>
<td>0.422</td>
<td>No</td>
<td>Stewart et al. (2010)</td>
</tr>
<tr>
<td>R54F</td>
<td>LFFKCIYRFKGYGLKR</td>
<td>+</td>
<td>0.492</td>
<td>No</td>
<td>Stewart et al. (2010)</td>
</tr>
<tr>
<td>L46A, Y52A, R54A</td>
<td>AFFKCIARFFKGYGLKR</td>
<td>–</td>
<td>0.523</td>
<td>No</td>
<td>Stewart et al. (2010)</td>
</tr>
<tr>
<td>C50S</td>
<td>LFFKSIYRFKGYGLKR</td>
<td>+</td>
<td>0.475</td>
<td>No</td>
<td>Grantham et al. (2009), this work</td>
</tr>
<tr>
<td>Y52S, Y57S</td>
<td>LFFKCISSFKSGGLKR</td>
<td>–</td>
<td>0.461</td>
<td>No</td>
<td>This work</td>
</tr>
<tr>
<td>C50S, Y52S, Y57S</td>
<td>LFFKSISSFKSGGLKR</td>
<td>–</td>
<td>0.510</td>
<td>No</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Udorn strain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>LFFKCIYRFEEHGLKR</td>
<td>–</td>
<td>0.502</td>
<td>No</td>
<td>Stewart et al. (2010)</td>
</tr>
<tr>
<td>F54R</td>
<td>LFFKCIYRFEEHGLKR</td>
<td>+</td>
<td>0.451</td>
<td>No</td>
<td>Grantham et al. (2009), Rossman et al. (2010a)</td>
</tr>
<tr>
<td>C50S</td>
<td>LFFKSIYRFEEHGLKR</td>
<td>–</td>
<td>0.533</td>
<td>No</td>
<td>Grantham et al. (2009), Rossman et al. (2010a)</td>
</tr>
<tr>
<td>F47A</td>
<td>LAFKCIYRFEEHGLKR</td>
<td>–</td>
<td>0.439</td>
<td>No</td>
<td>Rossman et al. (2010a)</td>
</tr>
<tr>
<td>F48A</td>
<td>LFAKCIYRFEEHGLKR</td>
<td>–</td>
<td>0.457</td>
<td>No</td>
<td>Rossman et al. (2010a)</td>
</tr>
<tr>
<td>I51A</td>
<td>LFFKCIYRFEEHGLKR</td>
<td>–</td>
<td>0.409</td>
<td>No</td>
<td>Rossman et al. (2010a)</td>
</tr>
<tr>
<td>Y52A</td>
<td>LFFKCIYRFEEHGLKR</td>
<td>–</td>
<td>0.508</td>
<td>No</td>
<td>Rossman et al. (2010a)</td>
</tr>
<tr>
<td>F55A</td>
<td>LFFKCIYRFEEHGLKR</td>
<td>–</td>
<td>0.431</td>
<td>No</td>
<td>Rossman et al. (2010a)</td>
</tr>
<tr>
<td>F47A, F48A, I51A, Y52A, F55A</td>
<td>LAAKCAARFEHGLKR</td>
<td>–</td>
<td>0.244</td>
<td>Yes</td>
<td>Rossman et al. (2010a)</td>
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</table>
The targeting failures of mutant M2 that we have observed for individually expressed M2 (Thaa et al., 2011) are likely compensated for by interactions of M2 in the context of infection. Most importantly, M2 binds to the viral matrix protein M1 via aa 71–73 in the M2-CT, which are not situated within the amphiphilic helix (Chen et al., 2008; McCown & Pekosz, 2006). M1 is assumed to organize virus assembly by interactions with the other viral components, including the cytoplasmic tails of raft-embedded HA and NA (Nayak et al., 2009). M1 might thus drag M2 to the edge of the budozone, even if the intrinsic budozone-targeting features in M2 are disrupted. Accordingly, replacement of the acylation site and the CRAC motifs in M2 does not reduce virus viability in cells but mutating the M1-binding site in M2 does (Chen et al., 2008).

It is the cooperative function of all components that governs budozone organization, virus assembly and budding. Our results, showing that virus growth is not affected by mutation of the two lipid-interaction features in the amphiphilic helix of M2, reinforce the notion that these processes are very robust and partly redundant in their functionalities.

METHODS

Cells. MDCK II (ATCC CRL-2936), human embryonic kidney 293T (ATCC CRL-11268), A549 (ATCC CCL-185) and MRC-5 (ATCC CCL-171) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; PAN Biotech) supplemented with 10% FBS (Perbio) at 37 °C, 5% CO2 and 95% humidity, using standard techniques.

Generation of recombinant viruses. Recombinant influenza A/WSN/33 (H1N1) virus was generated using an eight-plasmid reverse genetics system (Hoffmann et al., 2000), where each plasmid contains the cDNA of one viral RNA segment, flanked by promoters. In the M1-M2 encoding cDNA segment 7, the codons for C50, C51 and C52 of the viral matrix protein M1 via aa 71–73 in the M2-CT, which are not situated within the amphiphilic helix of M2, reinforce the notion that these processes are very robust and partly redundant in their functionalities.

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Radioactive labelling of viruses, SDS-PAGE and Western blotting. Radioactively labelled virus particles were produced by infecting MDCK cells in a 15 cm dish at an m.o.i. of 20. At 3 h p.i., the medium was replaced by DMEM lacking methionine, cysteine and glutamine, supplemented with 0.2% BSA, 0.1% FBS, 5 mM glutamine, 1 μg TPCK-treated trypsin ml−1 and 0.3 μCi (11.1 kBq) [35S]methionine/cysteine (Tran™-S-Label; MP Biomedicals) ml−1. At 24 h after infection, the supernatants were harvested, and virus debris was removed (1500 g, 10 min, 4 °C), and the virus was pelleted from the supernatant by ultracentrifugation (Beckman SW-28 rotor, 28 000 r.p.m., 2 h, 4 °C), resuspended in 100 μl PBS and analysed by SDS-PAGE under reducing conditions and fluorography, as described previously (Veit et al., 2008), or by SDS-PAGE followed by Western blotting using an anti-M2 mAb (clone 14C2; Santa Cruz) and a polyclonal antiserum against a commercial plaque virus, which cross-reacts with WSN M1, and HRP-coupled secondary antibodies for chemiluminescence detection using ECL Plus substrate (GE Healthcare) and a Fusion SL camera system (Promega), which detects photons over a wide linear signal-response range. Densitometric quantifications were carried out using Bio-1D software (Vilmer-Lourmat).

Electron microscopy. For negative staining, virus particles were pelleted from the supernatants of infected MDCK cells as described for radioactive labelling of viruses. In each case, 4 μl of the resulting virus suspension was applied directly onto a carbon-coated, glow-discharged grid. After 10 min of incubation, the grids were washed three times with ddH2O and stained with uranyl acetate (1% in ddH2O).

For ultrathin sections, MDCK cells were infected at an m.o.i. of 100, fixed at 8 h p.i. with glutaraldehyde (2.5% in 50 mM HEPES (pH 7.2)) for 16 h at 4 °C and harvested by scraping. Cells were centrifuged (2000 g, 5 min, 4 °C) and resuspended in 2.5% glutaraldehyde buffer. After washing in 50 mM HEPES (pH 7.2), the pellets were embedded in low melting-point agarose (3% in ddH2O, at a ratio of 1:1). Cells were post-fixed with osmium tetroxide (1% in ddH2O for 1 h), tannic acid (0.1% in 50 mM HEPES (pH 7.2) for 30 min) and uranyl acetate (2% in ddH2O for 2 h), dehydrated stepwise in a graded ethanol series and embedded in epon resin. Thin sections (~60 nm) were prepared with an ultramicrotome (Leica Ultracut UCT) and counterstained with uranyl acetate (2% in ddH2O for 20 min), followed by lead citrate (Reynolds’ solution for 3 min).

Negatively stained samples and ultrathin sections were examined using a JEM-2100 transmission electron microscope (JEOL) at 120 kV (negative staining) and 200 kV (ultrathin sections), respectively. Images were recorded using a Megaview III CCD camera (Olympus SIS).

Bioinformatics. All influenza A virus M2 sequences deposited in the protein database at http://www.ncbi.nlm.nih.gov were downloaded
on 5 May 2011 in FASTA format and stored in one text file. In total, 18,477 sequences with a length of at least 40 aa and including residue 50 were considered for further analysis. The sequences were randomly split up into flat files containing 10,000 sequences each to speed up the generation of multiple sequence alignments using CLUSTAL W (Thompson et al., 2002). The multiple alignments comprising 10,000 sequences were concatenated into one single FASTA sequence file and further processed with custom Perl scripts and Bioperl modules (Stajich et al., 2002). The CRAC motif (LV-X1–5-Y-X1–5-R/K) was extracted using Perl’s regular expression method.

ACKNOWLEDGEMENTS

We thank Robert Webster (St Jude Children’s Research Hospital, Memphis, TN, USA) for influenza virus expression plasmids, Hans-Dieter Klenk (Philips-Universität Marburg, Germany) for fowl plague virus antiserum, Elke Krüger (Charité, Berlin, Germany) for the A549 and MRC-5 cells and Andrea Zoehner (RKI Berlin) for excellent technical assistance. The German Research Foundation (DFG) is acknowledged for funding (SFB 740 TP C3 and SPP 1175).

REFERENCES


