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Molecular requirements of influenza virus hemagglutinin for site-specific S-acylation and virus replication

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von

Diplom-Biologin Katharina Brett

Präsident der Humboldt-Universität zu Berlin
Prof. Dr. Jan-Hendrik Olbertz

Dekan der Lebenswissenschaftlichen Fakultät
Prof. Dr. Richard Lucius
Gutachter/Innen: 1. PD Dr. Michael Veit
                 2. Prof. Dr. Alexander Herrmann
                 3. Prof. Alexey Zaikin

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**Abbreviations**

- **aa**: Amino acid
- **Ac**: Acylation site
- **BSA**: Bovine albumin serum
- **C**: Carboxyl terminus
- **CRD**: Cysteine rich domain
- **CPE**: Cytopathic effect
- **Cys**: Cysteine
- **cDNA**: complementary DNA
- **CT**: cytoplasmic tail
- **Ct**: Cycle threshold
- **CoA**: Coenzyme A
- **CV1**: African green monkey kidney cells
- **DNA**: Deoxyribonucleic acid
- **DHHC**: Asp-His-His-Cys
- **DMEM**: Dulbecco’s modified Eagle medium
- **CHO-K1**: Chinese hamster ovary cells
- **EM**: electron microscopy
- **eNOS**: endothelial nitric oxide synthase
- **ER**: Endoplasmatic reticulum
- **FBS**: fetal bovine serum
- **FP**: fusion peptide
- **FPV**: Fowl plaque virus
- **FRET**: Förster resonance energy transfer
- **GPI**: Glycosylphosphatidylinositol
- **DMSO**: Dimethylsulfoxide
- **HA**: Hemagglutinin
- **HCMV**: Human cytomegalovirus
- **HCV**: Hepatitis C virus
- **HEK 293**: Human embryonic kidney cells
- **HEF**: hemagglutinin-esterase-fusion
- **HPAI**: Highly pathogenic avian influenza
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>HR</td>
<td>hydrophobic region</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LDA</td>
<td>ligation-during-amplification</td>
</tr>
<tr>
<td>LPAI</td>
<td>Low pathogenic avian influenza</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionization-time of flight</td>
</tr>
<tr>
<td>MDCKII</td>
<td>Madin-Darby canine kidney cells</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>M (1 or 2)</td>
<td>Matrix protein (1 or 2)</td>
</tr>
<tr>
<td>m.o.i.</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NEP</td>
<td>Nuclear export protein</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signals</td>
</tr>
<tr>
<td>NP</td>
<td>Nuclear protein</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NS (1 or 2)</td>
<td>Non-structural protein (1 or 2)</td>
</tr>
<tr>
<td>nsP1</td>
<td>non-structural protein 1</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PA</td>
<td>polymerase acidic protein</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post infection</td>
</tr>
<tr>
<td>PATs</td>
<td>Palmitoyl acyltransferases</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PB1(-F2)</td>
<td>Polymerase basic protein 1 (fragment 2)</td>
</tr>
<tr>
<td>PR8</td>
<td>A/Puerto Rico/8/1934 strain</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>ribonucleoprotein</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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</tbody>
</table>
SNARE  soluble N-ethylmaleimide-sensitive-factor attachment receptor
SP   signal peptide
siRNA Small interfering RNA
TMD  transmembrane domain
TMR  transmembrane region
Ud   A/Udorn/1972 strain
vRNA Viral RNA
VLP  Virus-like particle
WB   Western blot
WSN  A/Wilson-Smith/1933 strain
WT   Wild-type

**Amino acid abbreviations**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>1-letter code</th>
<th>3-letter code</th>
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<tr>
<td>Alanine</td>
<td>A</td>
<td>Ala</td>
</tr>
<tr>
<td>Cysteine</td>
<td>C</td>
<td>Cys</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>D</td>
<td>Asp</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>E</td>
<td>Glu</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>F</td>
<td>Phe</td>
</tr>
<tr>
<td>Glycine</td>
<td>G</td>
<td>Gly</td>
</tr>
<tr>
<td>Histidine</td>
<td>H</td>
<td>His</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>I</td>
<td>Ile</td>
</tr>
<tr>
<td>Lysine</td>
<td>K</td>
<td>Lys</td>
</tr>
<tr>
<td>Leucine</td>
<td>L</td>
<td>Leu</td>
</tr>
<tr>
<td>Methionine</td>
<td>M</td>
<td>Met</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
<td>Asn</td>
</tr>
<tr>
<td>Proline</td>
<td>P</td>
<td>Pro</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Q</td>
<td>Gln</td>
</tr>
<tr>
<td>Arginine</td>
<td>R</td>
<td>Arg</td>
</tr>
<tr>
<td>Serine</td>
<td>S</td>
<td>Ser</td>
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<tr>
<td>Threonine</td>
<td>T</td>
<td>Thr</td>
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<tr>
<td>Valine</td>
<td>V</td>
<td>Val</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>W</td>
<td>Trp</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Y</td>
<td>Tyr</td>
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Zusammenfassung


Es wurde vermutet, dass die CD des HA direkt mit dem Virusmatrixprotein M1 interagiert, um es zur Assemblierungsort zu rekrutieren. Wie schon für verschiedene Influenzastämme gezeigt, sind die Acylierungsorten der CD essentiell für die Virusreplikation. Ob auch andere Aminosäuren der CD für die Virusreplikation erforderlich sind, die an der Wechselwirkung mit M1 teilhaben, ist nicht bekannt.


Um das Acylierungsmuster zu bestimmen, wurden rekombinante Viren in MDCK-Zellen und embryonierten Hühnereiern vermehrt und mittels Massenspektrometrie
Summary

The major spike protein hemagglutinin (HA) of Influenza virus is post-translationally modified by S-acylation of three cysteines. Two are located in its cytoplasmic tail (CT) and contain exclusively palmitate and one at the cytosol-facing end of the transmembrane region (TMR) and is acylated primarily with the longer fatty acid stearate. The signals responsible for this differential acylation are not understood. As hypothesized, either the acylation site’s amino acid environment or its location relative to the membrane determines which type of fatty acid is attached.

It was proposed that the CT of HA directly interacts with the viral matrix protein M1 to recruit it to the viral assembly site. Accordingly, the acylation sites in the CT are essential for virus replication, as shown for several influenza virus strains. Whether other amino acids in the tail are required for virus replication, as one would assume if the CT interacts with M1, is not known.

Based on a comprehensive sequence comparison to identify conserved amino acids, I used reverse genetics to create recombinant viruses with amino acid substitutions in the vicinity of the three acylation sites of HA. These substitutions included point mutations, shifting of a transmembrane cysteine to the CT and the deletion of the entire tail. Viruses with a truncated tail and one mutant where the isoleucine (located adjacent to a palmitoylated cysteine) was exchanged by a glutamine could not be rescued indicating that the mutations disabled virus replication. In contrast, a conservative substitution of this isoleucine, other non-conservative exchanges in TMR and CT and moving the TMR cysteine to the CT had only subtle effects on virus growth and on the incorporation of M1 and RNPs into virions and no influence on virus morphology. Yet, several times some of the mutated codons reverted to the original one or other amino acids suggesting that the sequence of the TMR and the tail of HA confer a fitness advantage for the wild-type virus. The implications of the results for the proposed role of the CT of HA for recruitment of M1 are discussed.

To assess the acylation pattern, recombinant viruses were propagated in MDCK cells and embryonated chicken eggs and analyzed by mass spectrometry. No under-acylated peptides were detected, and even the two lethal mutations did not abolish acylation as demonstrated by metabolic labeling of expressed HA with 3H-palmitate. Point mutations in the vicinity of an acylation site only moderately affected the
stearate content. In contrast, relocating the TMR cysteine to the CT virtually eliminated attachment of stearate. Furthermore, the cell type influenced HA’s acylation pattern: more stearate was attached if human viruses were grown in mammalian compared to avian cells. Hence, the location of an acylation site relative to the transmembrane span represents the principal signal for stearate attachment, while the sequence context and the cell type modulate the fatty acid pattern.
1. Introduction

1. INTRODUCTION

1.1 Influenza virus

The influenza virus is a membrane-enveloped RNA virus and belongs to the family of Orthomyxoviridae. It possesses a negative-sense single-stranded RNA genome that is divided into eight separate segments. There are three types of influenza virus, A, B and C, which can be distinguished from each other serologically. The most significant immunogens of the pathogen are the envelope glycoprotein antigens, hemagglutinin (HA) and neuraminidase (NA) which both contribute to the development of an anti-influenza response (Bosch et al., 2010). In influenza C virus, the functions of the two major surface glycoproteins (HA and NA) are combined in the HEF (hemagglutinin-esterase-fusion) protein. Thus, the respective virions contain seven instead of eight RNA segments (Palese et al., 1980; Nakada et al., 1984).

The symptoms resulting from influenza infections can range from mild (feverish chills) to severe illness (pneumonia) and at times lead to death (Thompson et al., 2003; Morens et al., 2008). Typically, after an incubation time of 1-4 days, a tracheobronchitis is developed with some involvement of small airways (Eccles, 2005). The onset of illness starts suddenly and involves headache, sore throat, sneezing, cough, fever and body aches. After 2-6 days the fever declines, while the respiratory signs become more intense, including mucoid cough, nasal congestion, and weakness, that can last for weeks (Taubenberger and Morens, 2008).

In addition to annual winter outbreaks, pandemic influenza viruses occasionally emerge. Among these, the 1918 pandemic (Spanish flu) caused most severe symptoms including massive acute pulmonary hemorrhage and edema and estimated worldwide casualties of 25 million people (Taubenberger et al., 2001; Song, 2014). Furthermore, influenza viruses can also cause some devastating animal diseases in poultry, horses and pigs (Taubenberger and Morens, 2010). Influenza A viruses can infect many different animals, including humans, pigs, horses, mustelidae, whales, seals, dogs, some cats and various birds (Pantin-Jackwood and Swayne, 2009; Yassine et al., 2010). Influenza B viruses can infect humans, ferrets and seals (Jakeman et al., 1994; Osterhaus et al., 2000), but cannot give rise to pandemics (Matsuzaki et al., 2004). Influenza C viruses infect humans, dogs and pigs, and can cause both severe illness and local epidemics (Matsuzaki et al., 2002).
1. Introduction

1.1.1 Structure and composition of virus particles

This work is aiming on the most common and clinically relevant representative of influenza, the influenza A virus and therefore the following sections are focused exclusively on this genus.

The virus particles are approximately 100 nm in diameter, surrounded by a lipid envelope, and have a pleomorphic shape, forming both filamentous and spherical virions (Chu et al., 1949). Laboratory-adapted strains, such as A/Puerto Rico/8/1934 (H1N1, PR8) and A/WSN/1933 (H1N1, WSN), are exclusively made up of spherical particles (Levine et al., 1953). The viral lipid envelope is covered with about 500 projecting spikes, which can be seen clearly under the electron microscope (Figure 1.1).

FIGURE 1.1
Envelope of an influenza virus visualized by transmission electron microscopy. This figure was created in accordance with the Figure 5.5 in the results. It represents the magnification of the mutant G547S. Visible are the viral spikes on the surface of two virions in the photograph. The bar indicates 100 nm.

These spikes are membrane-embedded glycoproteins. About 80% of them are HA, while the remainder is mostly NA (Compans et al., 1974; Harris et al., 2006; Nayak et al., 2009). Additionally, small amounts of the M2 protein are penetrating the lipid membrane of the virus. They form ion channels, permitting protons to enter the inside of the virus during virus entry (Pinto et al., 1992). The interior of the membrane is lined by the matrix protein M1. Internal to the M1 matrix the nuclear export protein (NEP) and the ribonucleoprotein (RNP) complex are found. The RNP complex consists of the viral RNA segments coated with nuclear protein (NP) and the RNA polymerase. The polymerase is composed of two
“polymerase basic” subunits (PB1, PB2) and one “polymerase acidic” subunit (PA) (Zebedee and Lamb, 1988).

The genome consisting of the eight fragments of negative-sense ssRNA adds up to approximately 13 kb in size (Ghedin et al., 2005). The RNA strands encode the following eleven proteins: PB1, PB1-F2, PB2, PA, HA, NP, NA, matrix protein 1 and 2 (M1 and M2), nonstructural protein 1 (NS1) and nuclear export protein/nonstructural protein 2 (NEP/NS2). The ends of each vRNA segment form a helical hairpin, which is bound by the RNA polymerase complex; the remainder of the segment is coated with arginine-rich NP (Baudin et al., 1994). At both ends of each vRNA segment there are non-coding regions, of varying lengths. Nonetheless, these regions are highly conserved among all influenza virus segments. These partially complementary ends base-pair to function as the promoter for vRNA replication and transcription by the viral polymerase complex. The non-coding regions also include the mRNA polyadenylation signal and part of the packaging signals for virus assembly (Compans et al., 1972; Murti et al., 1988).

1.1.2 Genetic variation between of influenza strains

The viral RNA polymerase permits high replication rates but has a poor proofreading ability (Liu et al., 2009) and thus many mutated progeny virions are generated. Variability in HA, generated by such a mechanism is primarily responsible for the continual evolution of new influenza strains and subsequent seasonal outbreaks and occasional epidemics. The antigenic drift occurs due to the accumulation of point mutations in the gene and results in amino acid alterations in the protein, in particular in the five antigenic domains located on the surface of the HA molecule (Wiley et al., 1981). The strains are designated on the basis of the antigenic relationships of the external spike HA and NA proteins: H1-H17 and N1-N9 (Webster et al., 1992; Sun et al., 2013). If at least two mutations have occurred in one HA antigenic site, a new antigenically different strain can emerge (Wiley and Skehel, 1987). Similar to HA, antigenic drift was also found in NA (Paniker, 1968), resulting in similar alterations of the two antigenic sites of the molecule (Colman and Ward, 1985). The two surface antigens HA and NA undergo antigenic variation independently of each other (Laver, 2002). The antigenic drift causes the changes to the seasonal influenza and requires the annual renewal of the vaccine.
1. Introduction

Additionally, major antigenic changes in HA or NA are involved in the so-called antigenic shift. This is due to the segmented genome, in which an influenza A virus strain can reassort by acquiring the HA segment, and possibly the NA segment and others as well, from a different subtype virus. This may happen in cells infected with different human strains or with different avian strains or between human, porcine and avian viruses (Webster et al., 1992). The result may be the appearance of a new subtype that encodes completely novel antigenic proteins to which the human population has no preexisting immunity and is most likely to initiate an epidemic. Furthermore, when a non-human influenza strain acquired the ability to infect humans, this was also due to antigenic shift. The 1918 pandemic, for example, arose when an avian H1N1 strain mutated to enable its rapid and efficient transmission among humans (Basler et al., 2001). A recent example for the antigenic shift is the 2009 novel H1N1 swine influenza strain, which contained genes originated from avian, porcine and human influenza viruses (Smith et al., 2009; Garten et al., 2009).

1.2 The Influenza spike protein Hemagglutinin

1.2.1 Molecular structure and biosynthesis

Hemagglutinin was first identified and named by its ability to agglutinate erythrocytes (Hirst, 1941). It is a typical type I transmembrane glycoprotein with a N-terminal signal peptide (17 amino acids), a large ectodomain, a single transmembrane region (TMR, 27 amino acids) and a short cytoplasmic tail (CT, 11 amino acids) (Figure 1.2) (Skehel and Wiley, 2000). The unprocessed HA (strain A/33/WSN, H1N1) contains 565 amino acids and is composed of the signal peptide, the HA1 subunit (325 amino acids), a single arginine connector residue, and of the HA2 subunit (222 amino acids) (Hiti et al., 1981).
1. Introduction

HA is synthesized as a single polypeptide chain at membrane-bound ribosomes. The molecule forms a non-covalent homo-trimer, the precursor protein HA0 (Gething et al., 1986). The signal peptide is cleaved off and N-linked glycosylation occurs during the co-translational process across the rough endoplasmic reticulum (ER) membrane (Braakman et al., 1991). After trimerization in the ER, transport proceeds through the Golgi to the plasma membrane. HA0 is cleaved by cellular proteases into HA1 and HA2, but these two subunits remain tightly associated by a disulfide bridge (Figure 1.2) (Copeland et al., 1986). Most influenza strains carry a single basic amino acid residue (arginine, sometimes lysine) at the cleavage site (Laver, 1971). Host trypsin-like proteolytic enzymes, found in the respiratory and gastrointestinal tract, cleave HA0 (Lazarowitz and Choppin, 1975). Some avian strains of H5 and H7 subtypes carry multiple basic amino acids at the cleavage site of HA0: Here cleavage can occur by ubiquitous proteases (such as furin) being the reason for systemic viral spread and high mortality.

1.2.2 Three-dimensional structure of HA

The rod-shaped HA forms trimers, with the carboxy-terminus inserting into the viral membrane and the hydrophilic end projecting as a spike away from the viral surface. HA’s crystal structure exhibits two structurally distinct regions for each monomer: a stem, comprising a triple-stranded coiled-coil of α-helices, and a globular head of antiparallel β-sheets, positioned on top of the stem (Wiley and Skehel, 1987).

FIGURE 1.2
Schematic representation of HA. HA0 is cleaved into HA1 and HA2 at the proteolytic activation cleavage (*). SP= signal peptide, FP= fusion peptide, TMR= transmembrane region, CT= cytoplasmic tail.
The sialic acid receptor-binding site is situated within the globular head domain, a part of HA1 (Figure 1.2 and 1.3). A slight cavity between the β-sheets acts as a receptor-binding site and is encircled by the five antigenic sites (Figure 1.3) (Isin et al., 2002), designated Sa, Sb, Ca1, Ca2, and Cb in the H1 subtype (Stevens et al., 2004). The membrane proximal stalk is in contrast to the head domain, conserved among all subtypes (Krammer and Palese, 2013). The stalk is composed of the N- and C-terminus of HA1 and the N-terminus of the HA2 subunit; two cysteine residues, which form a disulfide bond, form the demarcation line between stalk and head domain (Hai et al., 2012; Steel et al., 2010). Each HA2 subunit is anchored at its carboxy-terminus in the viral envelope. The HA2 subunit mediates the fusion of viral and endosomal membranes once the virus is taken up into the cell (Steinhauer, 1999). It also inhabits the TMR, consisting of a long helical chain anchored in the membrane and the CT (Dumard et al., 2013) (Figs. 1.2 and 1.3).

1.2.3 The infection pathway and HA’s role in it

HA’s major functions include receptor binding and fusion of the viral and cellular membranes, but it also plays a crucial role in assembling and releasing newly formed virus particles.
1. Introduction

1.2.3.1 Cell entry

Influenza virus binds to neuraminic (sialic) acid on the cell surface via its HA to initiate an infection. Sialic acids are nine-carbon acidic monosaccharides at the termini of many glycoconjugates, which are ubiquitously distributed. The carbon-2 of the terminal sialic acid is either linked to carbon-3 or carbon-6 of galactose, forming α-2,3- or α-2,6-linkages (Couceiro et al., 1993). Dependent on the kind of sialic acid the virus binds; the virus’s species tropism can be determined to a certain extent. Human influenza virus strains predominantly make use of a glycoprotein where the sialic acid is covalently connected by a α-2,6-linkage, which is mainly present in human respiratory epithelial cells. Influenza viruses infecting birds prefer α-2,3-linkages, the major sialic acid on avian epithelial cells (Matrosovich et al., 2004). Sialic acids with terminal α-2,3-linkages are also present in the lower human respiratory epithelium (bronchioles and alveoli). Thus, avian influenza viruses can also infect humans albeit with a lower efficiency compared to human strains (Tian et al., 1985).

After attachment to the cell surface, the virus particles are mainly internalized by clathrin-dependent endocytosis (Marsh and Helenius, 2006; Matlin et al., 1981) and transported into the endosomes. Here, the fusion of viral and endosomal membranes as well as the uncoating of virus particles and release of vRNPs into the cytoplasm takes place (Stegmann, 2000). The acid pH inside the endosome causes a conformational change in the HA which brings specific catalytic amino acids (which are part of the fusion peptide) into contact with the lipid membrane (Figure 1.4 A) (Skehel and Wiley, 2000). The hydrophobic fusion peptide (20 nonpolar amino acids) at the N-terminus of HA2 (Figs. 1.2 and 1.3) is buried in the interfaces of the coiled coils of the trimers. This sequestering of the fusion peptide prevents the aggregation of virus particles, as well as premature interaction of HA with host cell membranes. The first step in the fusion process is the formation of a hemifusion intermediate, in which the outer leaflets of the two interacting membranes fuse (Figure 1.4 B). The hemifusion concept was initially based on theoretical considerations and is supported by experiments using membrane models, including experimental systems involving HA (Melikyan et al., 1995; Nüssler et al., 1997).
The conformational pH-triggered transition, which generates the long trimeric coiled coil, thrusts the fusion peptide up towards the head of the molecule and into the target membrane (Chen et al., 1999). As a result, HA trimers are connected to the hydrophobic interiors of both the viral envelope via their TMR, and to the target membrane via their fusion peptides. The second rearrangement of HA allows the C-terminal end of the molecule to fold 180° up against the coiled coil and pulls the two membranes together. Moreover, it stresses and destabilizes the membranes, thereby perhaps initiating mixing of the outer leaflets of the membranes (Skehel et al., 1995). The final result is that this process brings the endosomal membrane into juxtaposition with the viral membrane. The presence of more than one HA leads to the formation of a fusion pore (Figure 1.4 C) (Tamm et al., 2003). After the opening of this pore the viral RNPs are released into the host cell cytoplasm (Sieczkarski and Whittaker, 2005). At the same time, protons pass along the M2 ion channel into the interior of the virion. This proton influx disrupts internal protein-protein interactions and enables that the M1 protein is released from the RNP complex (Martin and Helenius, 1991).
1. Introduction

1.2.3.2 Assembly and release of viral particles

The liberated RNP complex can now be trafficked by means of the viral proteins’ nuclear localization signals (NLSs), directing cellular proteins to import the RNPs and associated viral proteins into the nucleus (Cros and Palese, 2003). Once in the nucleus, the viral RNA polymerase uses the negative-sense vRNA as a template for synthesis of mRNA (Resa-Infante et al., 2011).

At later times of infection, the structural proteins are synthesized at high rates. Translation of the envelope proteins HA, NA, and M2 is conducted on membrane-bound ribosomes. All three proteins contain apical sorting signals, which allow them to traffic via the secretory pathway to the plasma membrane for assembly of virus particles. The correct packaging of the eight segments can be explained by two models. One model relies on the random packaging of eight or more segments into one virus particle (Bancroft and Parslow, 2002). As a consequence of this assumption, the great majority of virions are not infectious, since they exhibit the wrong combination of genome segments (Enami et al., 1991; Duhaut and Dimmock, 2002). However, newer evidence suggests that each segment possesses its own unique packaging signal, which ensures the correct adjustment of eight genome segments (Fujii et al., 2003).

The release process of newly assembled virions is called budding. The formation of new virus particles involves bending of the cellular membrane and subsequent scission of the nascent virion from the cell surface to form individual virus particles. So far, several factors are known that contribute to, or are essential for the budding of influenza virus. First of all, it has been shown that influenza virions bud from cellular lipid rafts. Rafts are functionalized, sphingomyelin- and cholesterol-rich microdomains in the plasma membrane. These domains allow viruses to enrich their components and exclude to a certain extent cellular proteins (Suomalainen et al., 2002; Chazal and Gerlier, 2003). In recent years, raft lipids (sphingolipids, cholesterol) were found in high concentrations in the viral membrane (Scheiffele et al., 1999) and HA was demonstrated to be directly associated with the same membrane fractions other viral components were partitioning into (Simons and Ikonen, 1997). HA was not only found in detergent resistant membranes (Scheiffele et al., 1997), but furthermore it has been shown by electron microscopy (Takeda et al., 2003; Hess et al., 2005) as well as Förster resonance energy transfer (FRET) experiments (Scolari et al., 2009; Engel et al., 2010) that HA is located in cholesterol-sensitive clusters or rafts. Secondly, it has been discussed that the line tension of the cellular membrane at the domain boundary might
facilitate the Influenza budding (Lipowsky, 1993; Schuck and Simons, 2004; Reynwar et al., 2007). Another important factor in Influenza assembly might be the intracellular sorting. Since the budding occurs in polarized cells on the apical membrane, it is encompassed by a directed transport of all components (Rodriguez et al., 1980) and both HA (Roth et al., 1983; Barman et al., 2003) and NA (Kundu et al., 1996; Barman and Nayak, 2000) possess signals for the intracellular transport to the apical membrane site. Moreover, the proton channel protein M2 is targeted to the edge of the viral budding site, a large, merged raft phase (see Figure 1.7 A and B). This puts M2 in an ideal position to mediate the scission of newly forming virions. It has been argued that the scission process is triggered by an amphiphilic helix, in M2’s cytoplasmic tail. This helix acts like a wedge into the cellular membrane, thus inducing it to curve (Rossman et al, 2010). Finally, the interaction of the viral proteins M1, NA and HA and RNPs is crucial for the budding to take place. In order to assess which proteins are essential for the budding of the virus, they have been expressed individually in cells. These cells can then release non-infectious, membrane-enveloped vesicles, so-called virus-like particles (VLP). In influenza virus, VLP production is induced by HA and NA (Chen et al., 2007) or if M1 is transported to the plasma membrane by means of an artificially inserted lipid modification (Wang et al., 2010). HA might be the driving force for this process (Chen and Lamb, 2008).

1.3 S-Acylation

S-acylation, also referred to as palmitoylation, is the post-translational attachment of fatty acids to cysteine residues by thioester linkage (Kordyukova et al., 2010; Linder and Deschenes, 2007; Dietrich and Ungermann, 2004). In integral and peripheral membrane proteins it is a quite common modification.
1. Introduction

Originally, protein palmitoylation was discovered in viruses (Schmidt and Schlesinger, 1979). These S-acylated viral proteins can be subdivided into three groups: two types of transmembrane proteins with different membrane topologies and functions, and peripheral membrane proteins.

The first type of transmembrane proteins are viral spike glycoproteins. Usually, they contain an N-terminal cleavable signal peptide, a long luminal (extraviral) ectodomain, one or more membrane-spanning regions and a cytoplasmic tail, which is exposed to the interior of the virus. HA of influenza virus, the fusion (F) protein of measles virus, the glycoproteins of filoviruses and retroviruses (e.g. HIV), the S-protein of severe acute respiratory syndrome (SARS)-coronavirus (CoV) and of other CoVs are the best characterized S-acylated proteins among human pathogenic viruses. Furthermore, spike proteins of widely used model viruses, such as E1 and E2 of togaviruses (Semliki Forest and Sindbis virus) as well as G of vesicular stomatitis virus (VSV) are palmitoylated. Being the main protein component incorporated in the viral membrane, palmitoylated virus spike proteins play major roles in virus entry, receptor binding and membrane fusion (Veit, 2012).

Viroporins represent the second group of palmitoylated viral proteins. In the infected cell membrane they oligomerize to from hydrophilic pores that disrupt numerous cellular physiological properties (Nieva et al., 2012), but are also involved in diverse other steps of the viral replication cycle (Gonzalez and Carrasco, 2003; Wang et al., 2011). However, very few copies are integrated into virus particles. An important example of this group is the M2 protein of influenza A virus, serving as a hydrophilic pore in the viral membrane (Nieva et al., 2012).

Representatives of the third group of palmitoylated proteins of viruses are peripheral membrane proteins. They include the non-structural protein nsP1 of togavirus, UL11 and

![Chemical structure of a S-palmitoylated protein](image)

**FIGURE 1.5**

*Chemical structure of a S-palmitoylated protein.* The C16-carbon saturated fatty acyl chain is attached to the peptide chain via a thioester linkage.

1.3.1 **In viral proteins**

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The first type of transmembrane proteins are viral spike glycoproteins. Usually, they contain an N-terminal cleavable signal peptide, a long luminal (extraviral) ectodomain, one or more membrane-spanning regions and a cytoplasmic tail, which is exposed to the interior of the virus. HA of influenza virus, the fusion (F) protein of measles virus, the glycoproteins of filoviruses and retroviruses (e.g. HIV), the S-protein of severe acute respiratory syndrome (SARS)-coronavirus (CoV) and of other CoVs are the best characterized S-acylated proteins among human pathogenic viruses. Furthermore, spike proteins of widely used model viruses, such as E1 and E2 of togaviruses (Semliki Forest and Sindbis virus) as well as G of vesicular stomatitis virus (VSV) are palmitoylated. Being the main protein component incorporated in the viral membrane, palmitoylated virus spike proteins play major roles in virus entry, receptor binding and membrane fusion (Veit, 2012).

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Representatives of the third group of palmitoylated proteins of viruses are peripheral membrane proteins. They include the non-structural protein nsP1 of togavirus, UL11 and
UL51 of herpesvirus and the core protein of hepatitis C virus (HCV). With fatty acids anchoring the modified protein to membranes, they lack a hydrophobic amino acid sequence (Veit, 2012).

1.3.2 **In cellular proteins**

After their initial discovery, palmitoylated proteins have been identified in every eukaryotic cell type examined so far (Fukata and Fukata, 2010; Linder and Deschenes, 2007) and are also divided into peripheral membrane and transmembrane proteins. Peripheral membrane proteins that are palmitoylated include members of the Ras and Src families, the endothelial nitric oxide synthase (eNOS) and SNARE vesicle fusion proteins (Wedegaertner et al., 1995; Hancock et al., 1989; Koegl et al., 1994; Liu J et al., 1996; Veit et al., 1996). In peripheral membrane proteins no clear consensus sequence was reported and a rather diverse amino acid composition was found in the vicinity/environment of the palmitoylation. Thus, it is assumed that structural similarities rather than strict amino acid sequences are prerequisites to specify palmitoylation (Bijlmakers and Marsh, 2003). It was observed that in many proteins the palmitoylation promotes their association with lipid rafts (Charollais and Van Der Goot, 2009) and supports their transportation between cytosol and membranes (Rocks et al., 2005).

Cellular transmembrane proteins that are palmitoylated include G-protein coupled receptors, T cell co-receptors CD4 and CD8 and ion channels. Transmembrane proteins are palmitoylated on cysteines located inside or close to the transmembrane domain (TMD), usually within ten residues from the TMD boundary (Charollais and Van Der Goot, 2009). If the TMD of a transmembrane protein is too short the surrounding lipids will be compressed, while if it is too long the lipids will be stretched. In case of large mismatches, the protein will be tilted with respect to the lipid bilayer (Schmidt et al., 2008). This in turn leads to an altered trafficking of transmembrane proteins along the secretory pathway (Munro, 1995). It is reported that acylation significantly reduces hydrophobic mismatches in transmembrane proteins with dependence on the length of their TMD (Morozova et al., 2010) and targets its proteins to membrane rafts and other specific membrane domains (Lynes et al., 2012). Since in transmembrane proteins palmitoylation is not required to anchor the protein in the bilayer, the role of the palmitoylation remains unclear (Linder and Deschenes, 2007). However, it has been suggested that the palmitoylation is a protection of transmembrane proteins from
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degradation by the cell (Abrami et al., 2008). Moreover, by associating membrane proteins with specific domains, the acylation might be a regulatory mechanism for sorting signals within the cytoplasmic domains (Stöckli and Rohrer, 2004).

1.3.3 In hemagglutinin

Apart from the receptor binding site and the fusion peptide, HA’s third major structural element is the cytoplasmic tail. HA from Influenza A virus is typically S-acylated at three cysteine residues, two are located in the CT and one at the end of the TMR (Naeve and Williams, 1990; Steinhauer et al., 1991; Veit et al., 1991).

The hydrophobic modification of HA is essential for virus replication: Depending on the virus strain, either virus mutants with more than one acylation site deleted showed drastically impaired growth or could not be created at all by reverse genetics (Zurcher et al., 1994; Chen et al., 2005; Wagner et al., 2005).

1.3.3.1 Functions of HA’s S-Acylation

Being such an essential modification to the virus, the function of S-Acylation has been studied with focus on various aspects (Figure 1.7):

First, acylation facilitates raft-association of HA (Melkonian et al., 1999; Levental et al., 2010) and thus enrichment of the protein in small nanodomains of the plasma membrane (Simons and Gerl, 2010). By means of FRET experiments it could be shown that HA clusters with rafts in the membrane (Engel et al., 2010; Zacharias et al., 2002; Scolari et al., 2009).
Removing HA’s palmitoylation sites increased its’ mobility within the membrane and released it from association with rafts (Engel et al., 2010). In addition, it could be demonstrated in other HA subtypes that the deletion of S-acylation sites abolishes HA’s partition into rafts (Chen et al., 2005; Wagner et al., 2005; Zhang et al., 2000; Takeda et al., 2003).

Another observation endorses the raft association function. As depicted in Figure 1.7 C, HA’s transmembrane α-helix surface has been mapped onto a cylinder to show hydrophobic and hydrophilic residues. In-between hydrophobic amino acids, there are three slightly hydrophilic residues (glycine, alanine and serine) above the acylated cysteine. Possibly, an acyl chain could fit into this hydrophilic groove and it was assumed that rather a stearate than a palmitate would fill it (Veit, 2012). The insertion of a stearate chain could render this surface smooth and support raft association. As already mentioned earlier, an acylation alters the orientation of a TMD towards the membrane and thus avoids hydrophobic mismatches (Charollais and Van Der Goot, 2009). This in turn has impact on trafficking and raft association of the molecule. Importantly, partitioning into rafts not only is the prerequisite for assembly and budding of viruses, but also supports the density of spike molecules (of which

---

**FIGURE 1.7**

**Proposed functions of S-Acylation in HA.** (A) Fatty acids target HA to membrane rafts, (B) Interaction of HA’s acylated cytoplasmic tails with the M1 matrix protein, (C) Surface map of the amino acids in the TMR helix of HA with hydrophilic (blue) to hydrophobic (orange) properties. S-acylation might interact with amino acids in the TMR. The hydrophilic groove composed of Ala 20, Gly 16 and Ser 9 could accommodate the acyl chain of a fatty acid attached to Cys 26. (D) Role during fusion pore opening. The red arrows indicate the location of the fatty acids during membrane fusion. The conformational change of HA shifts the fatty acids. This shifting possibly perturbs the order of membrane lipids and lead to completion of membrane fusion. (Figure modified with permission from Veit 2012, Biol Cell 104(9): 493-515.)
80% are HA) on the surface of the virion (Veit and Thaa, 2011). The density is an important factor for the infection, since the fusion activity is increased with a higher concentration of clustered spike proteins (Bentz and Mittal, 2003).

Lastly, as described earlier, during the fusion process there is an intermediate stage called hemifusion. Here, the lipids of the two membranes are mixed, but the solutes are not exchanged. In cells expressing non-palmitoylated HA, only lipid mixing was observed. Additionally, if only the HA ectodomain (without TMD and CT) was attached in the outer membrane leaflet by a glycolipid anchor, it could only catalyze hemifusion (Kemble et al., 1994). Consequently, TMD and CT are a necessity for full fusion. So far, controversial evidence concerning this hypothesis exists. Accordingly, it was reported that non-palmitoylated HA from the H2 and H3 subtype can mediate full fusion (between cells) (Chen et al., 2007; Naim et al., 1992; Steinhauer et al., 1991). On the other hand, S-acylation of HA from the H1, H7 and a different H2 subtype was required for the opening of the fusion pore (Naeve and Williams, 1990; Sakai et al., 2002; Wagner et al., 2005).

Of note, although the protein is a highly variable molecule with very low amino acid conservation, the acylation sites through all HA subtypes and variants are conserved completely (Veit et al., 2013). This also underlines the necessity of HA palmitoylation for virus replication.

1.3.3.2 Proposed interaction between HA’s S-acylated CT and M1

During an infection, M1 was proposed to interact with either HA or NA in order to initiate the budding process (Figure 1.6 B). The cytoplasmic tail of HA interacts with M1, which remains associated with the RNP. M1 forms a layer at the host cell membrane where it prepares for virus packaging (Nayak et al., 2004) and thus is intended to act as the connection between the virus RNP and HA (and NA and M2).

Such an interaction was indirectly shown for the cytoplasmic part of HA and M1 by altered membrane binding (Enami and Enami, 1996; Gomez-Puertas et al., 2000) or detergent insolubility of M1 in the presence of HA (Barman et al., 2001). In other reports however, this effect was not seen (Kretzschmar et al., 1996; Zhang and Lamb, 1996). Lack of the cytoplasmic tail of HA has a significant impact on the virus morphology and binding of M1 to the membrane, which decrease the budding efficiency (Zhang et al., 2000; Jin et al., 1997).
More precisely, it has been proposed that there is a direct link between HA’s S-acylation and M1. Although it has not been proven by biochemical means that the CT of HA alone (without the CT of NA) is required for an interaction with M1 and, hence, for assembly and packaging of the virions (Jin et al., 1994; Zhang et al., 2000), virus morphology and binding of M1 to the membrane have evidently been shown to be controlled by HA’s cytoplasmic tail (which contains two palmitoylation sites) (Jin et al., 1997). In the H3-subtype, rescued virus particles with a deletion of acylated cysteine residues were able to mediate membrane fusion. Yet, newly formed particles could not be released. When either of the two acylated cysteines in the CT was deleted, incorporation of NP and M1 into virions was affected and their release was decreased. Although being excluded from rafts, non-acylated HA could however be assembled into virions, when the M1 protein was exchanged by that of a different influenza virus (Chen et al., 2005). In the same way, non-acylated HA might not be able to cooperate with other viral proteins that are situated in rafts and this could lead to reduced assembly and budding of virions. An attached fatty acid likely alters the conformation of the only 11-amino acid long CT. Thus, it could improve the binding to a protein nearby; supporting the idea that palmitoylation promotes protein-protein interactions (Charollais and Van Der Goot, 2009). However, no study so far shed light onto the function of the amino acids within the CT. The interaction with M1 and hence formation of virus particles could also be due to other amino acids in HA’s CT than the cysteines.

1.3.4 Differential S-Acylation with palmitate and stearate

It has long been known that some “palmitoylated” proteins contain different acyl chains indicating that the responsible enzyme(s) (in contrast to the N-myristoyl transferase) cannot strictly discriminate between long chain fatty acid species (Schmidt, 1984). However, only advancements in mass-spectrometry then allowed to quantify precisely the type of a fatty acid linked to an acylprotein or even to a single acylation site. By that means, the peripheral membrane protein GAP43, for example, was demonstrated to contain both palmitate (C 16:0) and stearate (C 18:0) at two N-terminal cysteine residues, but a preference of a fatty acid species for one of the cysteines was not observed (Liang et al., 2002).
In contrast, studies with HAs of influenza A virus revealed that stearate is exclusively attached to the cysteine positioned at the end of the transmembrane region, whereas the two cytoplasmic cysteines contain only palmitate (Kordyukova et al., 2008). Site-specific acylation was also observed for the glycoproteins of influenza B and C virus (Figure 1.8). HA of Influenza B virus possesses two cytoplasmic cysteines that contain only palmitate, whereas the HEF of influenza C virus having one transmembrane cysteine is stearoylated (Kordyukova et al., 2008). This confirms previous studies performed with less sophisticated methodology (Veit et al., 1990). Glycoproteins of other enveloped viruses were also analyzed and it was found that site-specific attachment of palmitate or stearate is a common feature of viral spike proteins (Kordyukova et al., 2010). Likewise, this might also go for cellular transmembrane receptors, because in all cases acylation is facilitated by the cellular machinery.

Since this initial observation, more than 40 HA variants from 14 subtypes have been analyzed by MS (Kordyukova et al., 2011; Serebryakova et al., 2011). The percentage of stearate in all HAs differs from 35% (suggesting that each of the TMR cysteines of the trimeric HA spike contains stearate) to 12% (indicating that only one of three TMR cysteines is stearoylated) (Table 1.1). Interestingly, HA present in virus strains isolated from humans contain less stearate compared to HA isolated from other mammals and especially from birds. Since previous MS analysis was performed with viruses purified from the allantoic fluid of embryonated chicken eggs, it is not known whether cell type specific differences in the fatty acid pattern might exist.

**FIGURE 1.8**
Differential acylation of influenza virus types. The putative transmembrane regions and cytoplasmic tails of HA of influenza A and B virus and HEF of influenza C virus are attached with palmitate (Pal) and stearate (Stear) at specific cysteine residues. (From: Kordyukova et al., 2008, Journal of Virology 82(18): 9288-9292.)
1. Introduction

Another reason for the different stearate content in the HA of human and avian viruses might be that other viral membrane proteins, especially the matrix protein M1, which is much less variable than HA, but contains host-specific amino acid substitutions, affect acylation of HA. Yet, variability in the stearate content of HA could not be detected if internal proteins were exchanged between viruses (Serebryakova et al., 2013). Thus, the main molecular signal that determines preferential attachment of palmitate or stearate is likely to be located in HA, either in the amino acid sequence around individual acylation sites or in their positioning relative to the TMD.

1.3.5 S-Acylation: An enzymatic process?

Being essential for the function of a variety of virus proteins and thus for the viral life cycle, it is a major aim to reveal the mechanism of protein palmitoylation on the path to develop novel anti-viral drugs.

Palmitoyl acyltransferases (PATs) catalyze the attachment of fatty acids to proteins. Activity of these enzymes was observed in fractions of the plasma membrane, Golgi and mitochondrial membranes (Dunphy et al., 1996). Furthermore, enrichment of PATs has been
found in lipid rafts, which are associated with both the plasma membrane as well as intracellular membrane systems (Dunphy et al., 2001). For the attachment of palmitate, PATs make use of palmitoyl-CoA, which is available in nanomolar concentrations in the cell (Ren et al., 2008).

However, PATs could not be purified yet. Particularly, cellular enzymes that acylate HA (or any other viral protein) have not been identified. Alternatively, S-acylation of at least some proteins can occur by a non-enzymatic or auto-catalytic mechanism (Kummel et al., 2006; Rocks et al., 2010). Some viral and cellular proteins can be palmitoylated in vitro in the absence of an enzyme source with palmitoyl-coenzyme A (CoA) as lipid donor (Berger and Schmidt, 1984; Duncan and Gilman, 1996; Veit, 2000; Kummel et al., 2006). A consensus sequence in the vicinity of the palmitoylated cysteine, which would support substrate recognition, could not be found. Therefore, a cysteine might become palmitoylated when has been exposed and thus enables a transient access to the cytosolic face of the Golgi (Kummel et al., 2006; Rocks et al., 2010).

Based on this controversial evidence, it is not yet clear how HA is acylated: auto-catalytically or by means of a PAT.

Likely candidates for acylation enzymes of HA are members of the DHHC-family. These are polytopic membrane proteins containing an Asp-His-His-Cys (DHHC) motif within a cysteine rich domain (CRD) that is situated on the cytoplasmic face of the membrane between four transmembrane domains (Linder and Deschenes, 2007; Mitchell et al., 2006).

Most of the DHHC-proteins are located in the ER or Golgi-region where acylation of HA takes place (Ohno et al., 2006; Veit and Schmidt, 1993). Originally, the PAT activity of the DHHC proteins was discovered in yeast. The DHHC protein Erf2 together with Erf4 mediates
the acylation of yeast Ras (Lobo et al., 2002). The DHHC’s substrate specificity remains to be fully characterized. It could be shown that a variety of substrate proteins can be palmitoylated by several, but not every of the various DHHC proteins (Jennings and Linder, 2012; Hou et al., 2009), indicating that these enzymes show distinct, yet overlapping substrate specificities (Roth et al., 2006). For instance, DHHC6 appears to be highly specific for the chaperone calnexin (Lakkaraju et al., 2012), whereas DHHC 7 and 21 acylate the androgen, estrogen and progesterone receptor (Pedram et al., 2012) and the SNARE protein SNAP25 can be modified by DHHC3, DHHC7 and DHHC17 (Greaves et al., 2009). However, it is not known if members of the DHHC-family acylate influenza (and other) viral proteins.
2. Objectives

2. OBJECTIVES OF THE STUDY

Influenza virus A hemagglutinin is acylated at three conserved cysteines, two in the cytoplasmic tail (CT) and one in the transmembrane region. The cysteines in the CT were found to be exclusively palmitoylated in 14 subtypes and more than 40 HA variants (Kordyukova et al., 2008; Serebrakova et al., 2011). The transmembrane proximal cysteine is usually acylated with stearate, but has also been found to be palmitoylated occasionally. It has been supposed that either the amino acid context surrounding the cysteines has an impact on differential acylation or the proximity of the cysteine residue to the transmembrane region is responsible. Computational amino acid sequence analysis so far could not reveal any common sequence around acylation sites that determines whether the respective cysteine is palmitoylated or stearoylated, but such a signal might be complex and not detected by sequence comparison.

Furthermore, it has been shown for various influenza strains that the acylation of its CT is a required modification for the virus to replicate, also because of a proposed interaction of the CT with influenza virus’s matrix protein M1. So far it could not be elucidated, whether this interaction exists only with the acylated cysteines or other amino acids in the CT.

In this project, I aim to address the following questions:

1. Do the amino acids in the vicinity of the acylation sites of HA affect the acylation pattern?
2. Does the location of the cysteine relative to the membrane initiate a different acylation?
3. Are other amino acids in the CT besides the cysteines essential for virus replication?
4. Do these amino acids influence the acylation or interaction with M1 at the assembly site?

To answer these questions, I designed several HA constructs with specific mutations in this region of HA and introduced them into an Influenza reverse genetics system. Point mutations were inserted by utilizing site-directed mutagenesis. Virus mutants generated by the well-established eight-plasmid system were rescued and in order to understand their HA acylation pattern propagated and analyzed by mass spectrometry. Furthermore, I aimed to characterize
the viability and biochemical features of these mutants by growth curves, electron microscopy, radioactive labeling and western blot.

The aim was to clarify the molecular background of the differential S-acylation and the importance of amino acids in the CT of HA on virus replication.
3. Materials

3. MATERIALS

3.1 Cells

3.1.1 Eukaryotic cells

- **CV1** African green monkey kidney cells, fibroblast cell line from the kidney of the green monkey (*Cercopithecus aethiops*), ATCC® CCL-70™
- **MDCKII** Madin-Darby canine kidney cells, epithelial-like cell line from the distal tubule of the kidney of the domestic dog (*Canis familiaris*), ATCC® CRL-2936™
- **293T** epithelial cell line from the kidney of a human embryo (derivative of human embryonic kidney 293 cells, and contains the SV40 T-antigen), ATCC® CRL 3216™

3.1.2 Bacteria

- *Escherichia coli* BL21(DE3)
  - Genotype: F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])
  - An *E. coli* B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene and lacIq
- *Escherichia coli* XL-1 blue (Stratagene/Agilent, Waldbronn)
  - Genotype: endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F' [ ::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+)
  - Nalidixic acid resistant, tetracycline resistant (carried on the F plasmid)

3.2 Reagents

- Agar: Gibco/Life technologies, Karlsruhe, Germany
3. Materials

- Agarose for agarose gels: Invitrogen/Life technologies, Karlsruhe, Germany
- Agarose for plaque tests (SeaPlaque): Lonza, Basel, Switzerland
- Coomassie Brilliant Blue G-250: Serva, Heidelberg, Germany
- Size marker „SmartLadder“ (for DNA in agarose gels): Eurogenc, Cologne, Germany; size marker (for proteins, SDS-PAGE): „ColorPlus Prestained Protein Ladder, Broad Range“, New England BioLabs, Frankfurt, Germany and, peqGOLD Prestained Protein Marker“, Peqlab, Erlangen, Germany
- Palmitic acid, [9, 10-3H(N)]: Perkin-Elmer, Rodgau-Jügesheim, Germany
- Penicillin-Streptomycin (10,000 U/mL), Gibco/Life technologies, Karlsruhe, Germany
- Protease inhibitor cocktail tablets „cOmplete“ Roche, Mannheim, Germany
- Tran35S-Label (> 70% [35S]-methionin, 15% [35S]-cystein): MP Biomedicals, Heidelberg, Germany
- Trypsin (TPCK treated): Sigma-Aldrich, Taufkirchen, Germany
- Cell culture reagents/-media (DMEM, L-glutamine, EMEM with EBSS, Dulbecco’s PBS for cell culture with or without calcium and magnesium, Trypsin-EDTA): PAN Biotech, Aidenbach, Germany
- Fetal calf serum (FCS): Perbio, Bonn, Germany
- Bovine serum albumin (BSA) for cell culture (35%): Sigma-Aldrich, Taufkirchen, Germany
- MEM Eagle with Earle's BSS (2X), without L-glutamine and phenol red: Lonza, Basel, Switzerland
- OptiMEM: Gibco/Life technologies, Karlsruhe, Germany

All used solvents, acids, bases, chemicals and salts which are mentioned were purchased at Roth, Karlsruhe.

3.3 Antibodies and enzymes

- AccuTaq LA DNA polymerase: Sigma-Aldrich, Taufkirchen, Germany
- Enzymes for molecular biology (restriction enzymes, T4 DNA ligase): New England BioLabs, Frankfurt am Main, Germany
- Phusion® High-Fidelity DNA Polymerase, New England Biolabs, Frankfurt am Main, Germany
3. Materials

- Anti-influenza H1N1 virus rabbit polyclonal antibody R309 (kindly provided by Y. Kawaoka, University of Wisconsin, USA; Ultracentrifugation deforms unfixed influenza A virions, Y. Sugita et al., 2011), used in immunoprecipitation
- Rabbit antiserum against fowl plague virus (FPV) (gift from Hans-Dieter Klenk, Philipps-Universität Marburg, Germany), used in western blot 1:2,000
- Secondary antibody for western blot: horseradish peroxidase (HRP)-coupled anti-mouse antibody (Sigma-Aldrich, Taufkirchen, Germany), used in western blot 1:5,000
- Secondary antibody for western blot: horseradish peroxidase (HRP)-coupled anti-rabbit (Abcam, Cambridge, United Kingdom), used in western blot 1:5,000

3.4 Solutions and media

- Coomassie solution: 45% (v/v) ethanol, 10% (v/v) acetic acid, 0.25% (w/v) Coomassie Brilliant Blue G-250
- Freeze medium: 60% DMEM + 30% FBS ( deactivated for 20 min at 56 °C) + 10% DMSO
- Developing and fixing solution Kodak GBX (to develop films): Sigma-Aldrich, Taufkirchen
- Fixing-/destaining solution for SDS-PAGE gels: 10% acetic acid, 10% ethanol
- Infection medium: DMEM with 0.2% (w/v) BSA ( from 35% stock solution), 0.1% FCS ( deactivated for 20 min at 56 °C), 2 mM L-glutamine, 1 μg/mL trypsin (TPCK treated) and Penicillin/Streptomycin (100 units/mL)
- Loading buffer (non-reducing, 1×) for SDS-PAGE: 62.5 mM Tris·HCl, 2% (w/v) SDS, 10% (v/v) glycerin, 0.01% (w/v) bromophenol blue, pH 6.8
- Sucrose cushion:
- Loading buffer (reducing, 1×) for SDS-PAGE: loading buffer (non-reducing, 1×) + 5% (v/v) β-Mercaptoethanol
- Loading buffer for agarose gels (5×): 10 mM Tris·HCl, 100 mM EDTA, 50% (v/v) glycerin, 0.1% (w/v) bromophenol blue, pH 8.0
- Lysis buffer: 1 mM Tris·HCl, 0.1 mM MgCl₂, pH 7.4 (+ protease inhibitor)
- Starving medium: MEM with EBSS (without glutamine, methionine, cystein) + 4 mM glutamine
3. Materials

- Milk powder solution (western blot): 5% (w/v) skim milk powder in PBST
- Neutral red, 0.03% (w/v) in PBS (diluted from a 0.3% stock solution, Biochrom, Berlin)
- PBS: 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.02% (w/v) KH2PO4, 0.135% (w/v) Na2HPO4·2 H2O. – For cell culture Dulbecco’s PBS (with and without calcium and magnesium, PAN Biotech, Aidenbach, Germany) was used.
- PBST: PBS + 0.1% (v/v) Tween-20
- RIPA buffer: 20 mM Tris·HCl, 150 mM NaCl, 10 mM EDTA, 10 mM iodoacetamide, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, pH 7.4 (+ protease inhibitor)
- Roti®-Quant, 5x concentrate, for protein determination (Carl Roth, Karlsruhe)
- Stacking gel solution (SDS-PAGE): 5% (w/v) acrylamide/bisacrylamide (37.5:1; from a 30-% stock solution „Rotiphorese 30“, Roth, Karlsruhe); 0.1% SDS, 125 mM Tris·HCl pH 6.8 (from a 4× concentrated stock solution), 0.075% (w/v) APS, 0.15% (v/v) TEMED
- SDS-PAGE loading buffer: 25 mM Tris·HCl, 192 mM Glycin, 0.1% (w/v) SDS (pH 8.3–8.5)
- TAE buffer (Tris-Acetate-EDTA): 40 mM Tris·HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.0 (from 50× concentrated stock solution)
- Transfer buffer (western blot): 25 mM Tris·HCl, 192 mM glycin, 0.5% (w/v) SDS (from 10× concentrated stock solution), 20% (v/v) methanol
- Seperating gel solution (SDS-PAGE): acrylamide/bisacrylamide (37.5:1) from a 30% stock solution (Rotiphorese 30, Roth, Karlsruhe); for a 15% polyacrylamide gel: 0.1% (w/v) SDS, 375 mM Tris·HCl pH 8.8, 0.05% (w/v) APS, 0.1% (v/v) TEMED
- Overlay medium for viral plaque assays: 2× EMEM w/o phenol red with 0.4% (w/v) BSA (from 35% stock solution), 0.2% FCS (inactivated for 20 min at 56 °C), 4 mM L-glutamine, 2 μg/mL trypsin (TPCK-treated) and penicillin/streptomycin (200 units/mL) mixed 1:1 with 1.8% (w/v) SeaPlaque agarose
- YT agar petri dishes: YT with 1.2% (w/v) agar + 50 μg/mL kanamycin or 100 μg/mL ampicillin
- YT medium (Invitrogen, Karlsruhe): 31 g/L in H2O, autoklaved and supplemented with 50 μg/mL kanamycin or 100 μg/mL ampicillin, respectively
- TYM medium: 2% Bacto™ Trypton, 0.5% Bacto™ Yeast Extract, 0.1 M NaCl, 10 mM MgSO4
- TfB I (pH 6.2, sterile filtered by 0.22 μm): 30 mM potassium acetate, 10 mM CaCl2·2H2O, 15% (w/v) glycerine, 100 mM RbCl, 50 mM MnCl2·4H2O (first solve
3. Materials

potassium acetate, CaCl₂ and glycerine and adjust to pH 6.2 with acetic acid, then only add RbCl and MnCl₂ as solid substances)

- TrB II (pH 7.0, sterile filtered by 0.22 µm): 10 mM MOPS, 75 mM CaCl₂·2H₂O, 1% (w/v) glycerine, 10 mM RbCl, 50 mM MnCl₂·4H₂O (adjust to pH 6.2 with 1N KOH)
- Cell culture media: DMEM with 10% (v/v) FCS (inactivated for 20 min at 56 °C), 2 mM L-glutamine and penicillin/streptomycin (100 units/mL)
- TNE: 10 mM Tris/HCl pH 7.4, 100 mM NaCl, 1 mM EDTA

Solutions and buffers were prepared with deionized water from a "Milli-Q" water purification system (Millipore, Schwalbach).

3.5 Equipment and machines

Materials not mentioned were supplied from VWR, Darmstadt, or Roth, Karlsruhe.

Components of the laboratory-standard equipment are not listed.

- Incubator Heracell (to cultivate eukaryotic cells): Heraeus, Hanau; incubator Certomat BS-1 (to cultivate E. coli): Sartorius, Göttingen
- Pierce ECL Plus Western Blotting Substrate, Pierce/Thermo Scientific, Bonn
- Chemiluminescence substrat: SignalFire™ Elite ECL Reagent, Cell Signaling Technology, Leiden, Netherlands
- Films Kodak Biomax XAR (for autoradiography and fluorography): Sigma-Aldrich, Taufkirchen
- Vacuum gel dryer: UniEquip, Martinsried
- OneStep RT-PCR kit, Qiagen, Hilden
- PCR product purification kit from agarose gels: Invisorb Spin DNA Extraction Kit, Stratec, Berlin
- DNA purification kit from PCR and other enzymatic reaction mixtures: Invisorb Fragment CleanUp Kit, Stratec, Berlin and ExoSAP-IT single-step PCR cleanup, Affymetrix, High Wycombe, UK
- Viral RNA isolation kit (Invisorb Spin Virus RNA Mini Kit): Stratec, Berlin
- Small-scale plasmid purification kit: Invisorb Spin Plasmid Mini Two Kit, Stratec, Berlin
3. Materials

- Large-scale plasmid purification kit: PureYield Plasmid Maxiprep System, Promega, Mannheim
- PVDF membrane (for Western Blot): Amersham/GE Healthcare, Freiburg
- SDS-PAGE equipment: Biometra, Göttingen
- Spectrophotometer NanoDrop 1000: PeqLab, Erlangen;
- BioPhotometer: Eppendorf, Hamburg
- Thermocycler „mastercycler gradient“: Eppendorf, Hamburg
- Sonicator Branson 1210: Branson Ultraschall, Dietzenbach
- Ultracentrifuge TL-100 with rotor TLA-100.2 and L7-65 with rotor Ti-45, with matching centrifugation tubes: Beckman Coulter, Krefeld
- Portable UV lamp HL 254 N, bulbs 2 × 4 W: Waldmann, Villingen-Schwenningen
- Blotting chamber (for Western Blot): Peqlab, Erlangen
- Cell culture flasks and dishes: Greiner Bio-One, Frickenhausen
- Centrifuge Avanti J-25 with rotor JLA-16.250 and centrifuge buckets: Beckman Coulter, Krefeld
- Centrifuge Sigma 3K12: Sartorius, Göttingen
- Vacuum centrifuge Univapo 150H: UniEquip, Martinsried
- Benchtop centrifuge 5417R: Eppendorf, Hamburg

3.6 Computer applications

- Image processing: Microsoft Powerpoint, Adobe Photoshop CS2
- Graphs, charts and plots: Microsoft Excel 2010 und GraphPad Prism 5.01
- Intensity quantification: Bio 1D, Vilber-Lourmat, Eberhardzell
3.7  **Plasmids and oligonucleotides**

3.7.1  **Plasmids**

- Plasmids for reverse genetics, Influenza A/WSN/1933: pHW2000-PB2, -PB1, -PA, -HA, -NP, -NA, -M, -NS, obtained from Thorsten Wolff, Robert Koch Institute, Berlin
- Plasmid for protein expression in vaccinia system: pTM1

![Map of the pTM1 plasmid.](image)

**FIGURE 2.1**


3.7.2  **Generated constructs and oligonucleotides**

All in this work generated constructs and the correspondent oligonucleotides were purified by HPLC. They are listed in the following tables.
3. Materials

### TABLE 3.1
Sites that were altered in generated constructs.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Primer for PCR (5’→ 3’) with sites for mutagenesis based on pHW-2000-HA wild-type, modified bases are in bold and underlined:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pHW2000-HA-G557A</td>
<td>GTTCTGGATGTTCTTCTAATGCT TCTTTTCAGTGCAGAATATGC</td>
</tr>
<tr>
<td>2</td>
<td>pHW2000-HA-G557Stop</td>
<td>GGCAATCAGTTCTGGATGTTCTAATTAATCTTTTCAGTGCAGAATATGC</td>
</tr>
<tr>
<td>3</td>
<td>pHW2000-HA-G557E</td>
<td>GTTTCTGGATGTTCTAATTGAAATCTTTTCAGTGCAGAATATGC</td>
</tr>
<tr>
<td>4</td>
<td>pHW2000-HA-Q560E</td>
<td>GATGTGTTCTAATGGGTCTTTGCAATCAGTTTCTGGGAATGCAATCATGACTGAG</td>
</tr>
<tr>
<td>5</td>
<td>pHW2000-HA-I563Q</td>
<td>GTTCTAATGGGTCTTTTCAGTGCAGACTGCGATCTGAGATTAGG</td>
</tr>
<tr>
<td>6</td>
<td>pHW2000-HA-I563L</td>
<td>GGGTCTTTCAGTGCAGATTTGCTGATCTGAG</td>
</tr>
<tr>
<td>7</td>
<td>pHW2000-HA-G547I</td>
<td>GGTCTCCCTGAATTGCAATCAGTTTCTGG</td>
</tr>
<tr>
<td>8</td>
<td>pHW2000-HA-G547C</td>
<td>GGCTCCCCTGTGGAATCATGTTTCTGG</td>
</tr>
<tr>
<td>9</td>
<td>pHW2000-HA-C554S+L559C</td>
<td>GAGCTCTAATGGGTCTTGCGATGCAATCATGACTGAG</td>
</tr>
</tbody>
</table>

All constructs were confirmed by sequencing (GATC Biotech).

### TABLE 3.2
Oligonucleotides used for cloning and sequencing.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Primer sequence (5’→ 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HA sequencing sense</td>
<td>CGGGATACCAAAACAGG</td>
</tr>
<tr>
<td>2</td>
<td>HA sequencing antisense</td>
<td>TTATATTTTCTGAAATCC</td>
</tr>
<tr>
<td>3</td>
<td>Spe I Cloning</td>
<td>GCTCTCAACTGATGTAAGGCCAAAACACTGGTC</td>
</tr>
<tr>
<td>4</td>
<td>Pst I Cloning</td>
<td>TGAGACCTGAGTCAGATGCAATTTCTGC</td>
</tr>
<tr>
<td>5</td>
<td>Pst I Cloning (for construct #4 in Table 3.1)</td>
<td>TGAGACCTGAGTCAGATGCAATTTCTGC</td>
</tr>
</tbody>
</table>

### 3.8 Sequences

#### 3.8.1 HA-sequence from the WSN plasmid pHW2000

Below is the nucleotide sequence beginning with the start codon (ATG) and ending with the stop codon (TGA).
3. Materials

Below is the translated amino acid sequence beginning with the first amino acid (M) and ending with the stop codon (*).

MKAKLLVLLYAFVATDDTICYGHANNSTDTVDTILEKNNVATVHSVNLLEDSHNGKLCNLKGIAPLQ
LCGNNITGWLGNPECDSSLPARSWSYIVETPNSENGACYPGDLYEELREQLSSVSSLEREIFPPK
ESSPNHHTFGVTSCSHRGKSSFYRRNLWLTKKGDSYPKLTSNYNVNKGKEVLWLWVYHHPSSDEQ
QSLSNGNAYVSASSNYNRFTEIARFPKVRQDHGRMNYYWTLLEPGTDIFFAETGNLIAFWYAF
LSRGFESEIGTSNSMHETCTKQTFCQGAINSNLFPQNIHPVTGECPKYVSTKLRMVGTENIPSI
QYRGLFGAIAFIEEGGGTWGMDWYGYYQNEQGSGYAADQKSTQNAINGTNNKSVIEKMTQFTA
VGKEFNNLEKRMLNKKVDDGLDFTYNALVELLLENERLDHFDNVNYLKEVKSQQLKNKAKI
NGCPEFYHKDCNEMESVNRGTYDPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASLVLV
SLGAISFWMCNSNGSLQCRCI*
4. METHODS

4.1 Molecular biology

4.1.1 Molecular cloning

In molecular cloning a desired DNA fragment (a gene) is integrated into a vector (plasmid or viral vector). It is helpful to make use of such vectors in order to economically amplify the gene of interest in high quantities and then express it in cells.

Firstly, the required plasmid is cut with specific restriction enzymes, to produce overhanging, so-called sticky ends. The target DNA is amplified by means of polymerase chain reaction (PCR) from genomic DNA or another plasmid and then serves as an insert for the vector. Prior to ligating it to the vector it has to be cut with the same specific enzymes, so that complementary ends at vector and target DNA are generated. In the following ligation, vector, insert and a DNA ligase are incubated, so that compatible ends from vector and insert will attach to one another and the ligase can catalyze the covalent binding of the ends of the single stranded DNA.

Constructs with an already altered HA gene (in the plasmid pHW2000 with inserted point mutations, see 4.2.2.1) and a vector for high protein expression were generated using this method.

The following conditions were applied:
As a first step, restriction sites were added by mixing the following components in final concentrations: 1 x Phusion HF buffer, 200 µM dNTPs, 200 µM Primers for SpeI and PstI (see Table 3.2), Phusion® High-Fidelity DNA Polymerase (NEB, Massachusetts, USA). The components were mixed gently, spun down shortly and distributed onto 4 PCR tubes (45 µL each), to which a different DNA template each (5 µL of 100 ng/µL, final conc. 10 ng/µL) was added. The reaction was performed under below listed cycler conditions (Table 4.1).
Then the fragment size was analyzed in a 1 % agarose gel by loading 5 µL of PCR product onto the gel (+ 1 µL loading buffer). The fragment of approx. 2 kb was purified from the gel using the Invisorb Spin DNA Extraction Kit by (STRATEC Molecular GmbH, Berlin, Germany). In a next step the vector (pTM1) and insert were cut by the restriction enzymes SpeI and PstI, pipetted on ice:

Prior to the following step, the fragment size of the empty vector and the cut insert was checked in agarose gel and purified (as mentioned above). Then the fragment was ligated into pTM1: 20 µL insert, 1.5 µL cut vector, 2.5 µL 10 x T4 ligase buffer, 1 µL T4 DNA ligase, incubated for 45 min at room temperature. Subsequently, the ligation assay was transformed and left to incubate overnight. In order to do so competent XL-1 cells were thawed on ice. The ligation assay (25 µL) was mixed with 250 µL of competent cells and incubated on ice for 30 min. After a heat shock for 1 min at 42 °C the mix was kept on ice for 2 min and then

### TABLE 4.1
Cycler conditions.

<table>
<thead>
<tr>
<th></th>
<th>98 °C</th>
<th>30 s</th>
<th>Initial denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x</td>
<td>98 °C</td>
<td>10 s</td>
<td>Denaturation</td>
</tr>
<tr>
<td>30 x</td>
<td>55 °C</td>
<td>30 s</td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>38,25 s</td>
<td>Extension</td>
</tr>
<tr>
<td>1 x</td>
<td>72 °C</td>
<td>10 min</td>
<td>Final extension</td>
</tr>
<tr>
<td>1 x</td>
<td>8 °C</td>
<td>∞</td>
<td>Hold</td>
</tr>
</tbody>
</table>

**TABLE 4.2**
Restriction digest.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Insert</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 µL</td>
<td>vector (1 µg/µL)</td>
</tr>
<tr>
<td>2 µL</td>
<td>BSA (10 x)</td>
</tr>
<tr>
<td>2 µL</td>
<td>buffer (4)</td>
</tr>
<tr>
<td>1 µL</td>
<td>SpeI</td>
</tr>
<tr>
<td>1 µL</td>
<td>PstI</td>
</tr>
<tr>
<td>13 µL</td>
<td>H₂O</td>
</tr>
</tbody>
</table>

| 14 µL   | insert |
| 2 µL    | BSA (10 x) |
| 2 µL    | buffer (4) |
| 1 µL    | SpeI |
| 1 µL    | PstI |

Incubated for 2 h at 37 °C
4. Methods

900 µL of YT medium was added. Incubation for 1 h at 37 °C while shaking followed before the cells were pelleted at 2500 x g for 5 min. 850 µL of the supernatant was decanted. The pellet was resolved with the remaining media and this was plated on an Ampicillin-YT-plate, incubated at 37 °C for 16 h. Eventually, single colonies were picked and an overnight bacteria culture was inoculated and kept at 37°C with vigorous shaking. The DNA from preparations was then isolated and sequenced.

4.1.2 Preparation of competent E. coli cells

E. coli cells (strain XL1 blue) were taken from a glycerol stock and plated on an LB plate using a sterile inoculating loop and were incubated at 37 °C overnight. A single colony was inoculated into 10 mL of TYM medium and incubated overnight at 37 °C on a shaker. 50 mL (preheated) TYM medium was inoculated with 0.5 mL of the overnight culture and incubated at 37 °C on a shaker. The current cell density in TYM medium was monitored by measuring the optical density (OD₆₀₀ = 0.2-0.6, cells doubling every 30 minutes). When the desired cell density was reached, the 50 mL were inoculated in 500 mL of TYM medium (pre-heated) in a 2 L Erlenmeyer flask and incubated in a shaker until OD₆₀₀ = 0.4 was reached. The flask was then held for 10 minutes in ice water, and repeatedly rotated to be cooled uniformly. Then, the suspension was centrifuged for 10 min at 4 °C and 1,000 xg. The pellet was resuspended in 45 mL ice-cold TFB I solution (per bucket), kept in an ice bath for 10 min and then transferred to 50 mL pre-chilled Falcon tubes. After 8 min centrifugation at 4 °C and 1500 xg, the pellet was resolved in 10 mL ice-cold TFB II solution. The well-resuspended cells were then dispensed into sterile ice-cold tubes of 350 µL each, shock-frozen in liquid nitrogen and stored at -80 °C.

4.1.3 Isolation of plasmid DNA

The most common method for isolating plasmid DNA relies on the principle of alkaline lysis. Bacteria with the plasmid are centrifuged and the pellet is resolved in buffer. To degrade RNA, the solution contains an RNAse. By adding a solution of SDS and sodium hydroxide, the cells are lysed by the detergent and the alkaline hydrolysis of the lipids. Adding a slightly
4. Methods

Acidic potassium acetate solution (pH 5.2) causes neutralization and due to the presence of SDS that protein and genomic DNA is precipitated. After a following centrifugation the solved plasmid DNA is additionally purified by a phenol chloroform extraction or an adsorption to silica gel. The DNA can then be precipitated with ethanol or isopropanol, which removes the DNA’s aqueous envelope. 70% ethanol washes the precipitated DNA. The DNA then is eluted in ultrapure water or a Tris/EDTA buffer (pH 7.5).

In this work the isolation was performed using the Invisorb Spin Plasmid Mini Two Kit by (STRATEC Molecular GmbH, Berlin, Germany) for small-scale plasmid purification and the PureYield Plasmid Maxiprep System by (Promega, Wisconsin, USA) for large-scale plasmid purification. Both systems provide a rapid method for purification using a silica-membrane column.

4.1.4 Expression of HA with the Vaccinia T7 system

The Vaccinia T7 system is a system to rapidly and highly express a protein of interest. Firstly, suitable mammalian cells (in this case monkey kidney cells) are infected with vaccinia virus expressing the RNA-polymerase from bacteriophage T7. Then, these cells are transfected with an expression vector, which includes a T7 promoter. The vaccinia virus inside the cells exploits the cellular protein biosynthesis machinery and the T7 polymerase recognizes the T7 promoter and expresses the gene of interest in high quantities.

HA (with inserted point mutations) was subcloned from pHW2000 constructs into the plasmid pTM1 behind the T7 RNA polymerase promoter (4.1.1). Petri dishes with 2\times10^6 CV1 cells (one subconfluent cell monolayer grown in a plastic-dish with a diameter of 6 cm) were infected with recombinant vaccinia virus expressing T7 polymerase (10 plaque forming units /cell). At two hours post infection (p.i.), the cells were washed with PBS prior to transfection with 4 \mu g pTM1-HA plasmids using turbofect (12 \mu L per dish). Five hours p.i. cells intended for [35S]-methionine/cysteine labeling were starved in DMEM medium lacking methionine and cysteine. Cells were labelled at 5.5 hours p.i. with 0.5 mCi/mL [35S]-methionine/cysteine (1175 Ci/mmol, Easy Tag Express Protein labelling Mix (Perkin Elmer, Massachusetts, USA) or with 0.25 mCi/mL [3H]-palmitic acid (30-60 Ci/mmol, 9,10-3H(N); (Perkin Elmer, Massachusetts, USA) for 3 hours. Then cells were lysed: They were washed
4. Methods

with PBS and incubated for 15 min on ice (0 °C) in 600 µL RIPA-buffer. Cell debris was pelleted at 20800 xg at 4 °C for 20 min and the cleared supernatant was subjected to immunoprecipitation (4.1.5).

4.1.5 Immunoprecipitation

In order to selectively isolate individual proteins from a solution, these were prepared using a specific antibody precipitated from cell lysates. Here one employs monoclonal or polyclonal antibodies, which are directed against epitopes. These antibodies can then be bound to agarose- or sepharose beads to allow the purification of bound antigen from complex mixtures, for instance cell lysates. The thus obtained immune-precipitates can be analyzed on other associated proteins.

Cleared supernatant from (4.1.6) was incubated at 4 °C for 16 h with 1 µL of anti-influenza H1N1 virus polyclonal rabbit antibody R309 (Sugita et al., 2011, kindly provided by Y. Kawaoka) per sample. Following a 2.5 h incubation at 4 °C with protein-A-sepharose (40 µL of a 1:1 slurry in RIPA buffer), each lysate was washed with RIPA buffer: The mixtures were spun down at ~700 ×g, the supernatant was removed, the pellet was resuspended in 1 mL RIPA buffer, vortexed and that procedure was repeated four times. 20 µL 2x non-reducing loading buffer was added to the final pellet, boiled at 95 °C for 5 min and analyzed by SDS-PAGE and fluorography.

4.1.6 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a technique by which proteins are separated according to their size. Sodium dodecyl sulfate (SDS) is an anionic detergent, which binds to polypeptides in constant weight ratio. Polymerization of the acrylamide is catalyzed by the oxidating agent ammonium persulfate (APS) together with tetramethylethylenediamine (TEMED).

In this work, virus preparations were heated in the presence of SDS (loading buffer, final concentration 1×, see 3.4) for 5 min at 95 °C for the proteins to denature (and in reducing
conditions separate into their primary polypeptides) and gain an overall identical negative charge density.

**TABLE 4.3**
Reagents and concentrations for SDS-PAGE.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>15% Separating gel (7.5 mL)</th>
<th>4% Stacking gel (2.5 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamid/Bisacrylamid (37.5:1)</td>
<td>3.75 mL</td>
<td>0.415 mL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>1.875 mL</td>
<td>1.46 mL</td>
</tr>
<tr>
<td>4x Tris-SDS</td>
<td>1.875 mL (pH 8.8)</td>
<td>0.625 mL (pH 6.8)</td>
</tr>
<tr>
<td>(Final 0.1% w/v SDS)</td>
<td>(Final 375 mM Tris HCl)</td>
<td>(Final 125 mM Tris HCl)</td>
</tr>
<tr>
<td>10% APS</td>
<td>80 µL</td>
<td>20 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>15 µL</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

The equipment used for casting the gel was wiped with 70% EtOH to remove grease that would obstruct polymerization. The separating gel solution (15% acrylamide) was applied first, up to 1 cm and sealed from surrounding air with 80% isopropanol until the gel solidified. The isopropanol was removed and the stacking gel (5% acrylamide) was cast on top of the separating gel with combs mounted on top. Thawed virus pellets were resuspended by vortexing with equal volumes of 2x SDS-PAGE loading buffer, heated for 5-6 min at 95 °C. Large insoluble particles in the samples were pelleted by centrifugation at 13,000 ×g for 1 min and the supernatant was loaded on to the stacking gel. The samples were applied to the gel at a constant voltage of 70 V in the stacking gel and then separated electrophoretically at 160 V in the separating gel. The polypeptides then migrated in the electric field towards the positive anode and thus could be separated in a porous gel according to their size with smaller proteins migrating faster than the larger ones (Hames, 1998).

Thereafter, the gels were either further processed in a Western blot (see 4.1.8), or dried and purified by Fluorography (4.1.7). To visualize the separation efficiency the gel was stained for 45 min with Coomassie Brilliant blue. Subsequently, gels were destained overnight and photographed.
4. Methods

4.1.7 Fluorography

The signal of radioactively labeled proteins can be captured using X-ray films. To visualize the metabolic labeling in gels they were treated as follows. After SDS-PAGE, gels were fixed, washed with dH₂O twice for 15 min on a shaker, then treated with 1 M salicylate (a scintillator that increases the signal) for 30 min and dried by a vacuum gel drier on Whatman paper. The dried gels were then transferred to a light-protected X-ray film cassette, a piece of X-ray film was placed over them, and the cassette stored at -80 °C. Wherever a labeled protein is present in the gel the radioactive tag would expose the film due to particle decay (autoradiography). The signals of [³⁵S] labeling typically took a few days to become visible, the [³H] palmitate signals several weeks or months. After the necessary time, the films were developed in a dark room (with developing and fixing solution according to the manufacturer), dried and documented. Densitometric quantification of bands was carried out using Bio-1D software (Vilmer-Lourmat).

4.1.8 Western Blot

Western blot denotes the transmission of proteins from a polyacrylamide gel onto a carrier membrane, such as nitrocellulose, nylon, glass fiber or most commonly polyvinylidene fluoride (PVDF). These proteins can then be detected by different reactions. An electric field is applied (electron transfer) which is directed perpendicular to the polyacrylamide gel so that the proteins migrate in the direction of the anode. During transfer, the proteins migrate out of the gel onto a membrane. Using nylon or PVDF proteins adhere due to hydrophobic and polar interactions to the membrane surface, while the adsorption at nitrocellulose or glass fibers is affected by means of ionic and polar interactions.

After the transfer (blotting) of proteins, non-specific protein binding sites on the membrane must first be blocked, as this would cause the antibodies to attach to these sites and make specific detection of antigens impossible. To block free binding sites skim milk powder or bovine serum albumin (BSA) are used, solutions which are unrecognizable for the later used antibodies. The membrane is then treated with a diluted solution of antibodies, which are specifically directed against a protein or against a number of proteins on the membrane. Some
4. Methods

washing steps with a detergent solution to remove weakly adherent, non-specifically bound antibody from the membrane follow. A second antibody solution (the secondary antibody) is added to the membrane, the antibodies are directed specifically against epitopes of the first antibody and bind (typically the Fc region of the antibody) there. After further washing steps, the visualization follows by adding substrate to initiate a chemiluminescent reaction (catalyzed by an enzyme, commonly peroxidase coupled to the secondary antibody).

In this work, before blotting, the PVDF membrane was incubated in 100% methanol for 10 sec, then for 5 min in dH2O and then in transfer buffer (20% methanol in final 1x transfer buffer, 3.2) for 10 min. The blot was performed laying three sheets of Whatman paper, soaked in transfer buffer, the membrane, the gel and on top another three sheets of soaked Whatman paper onto the blot apparatus. The blot was usually run at 230 mA for 70 min. Blocking was performed for 1 h at room temperature or overnight at 4 °C rocking, using 5% skim milk. Washing was performed with PBST (PBS + 0.1% (v/v) Tween-20). The antibodies (see 3.3) were diluted in skim milk and incubated for 2-12 h at 4 °C, rocking. Detection was performed by using Pierce ECL Plus Western Blotting Substrate according to the manufacturer’s instructions (Thermo Scientific, Bonn, Germany).

4.1.9 Protein concentration determination

In order to determine the protein concentration in pelleted virus preparations (from cell culture 4.2.3 or embryonated chicken eggs 4.2.4), the Bradford protein assay was applied. This is an analytical procedure, which relies on spectroscopy. The dye Coomassie Brilliant Blue-G250 exists in three states, each with different absorption wavelengths. By binding to a protein, the dye’s state is transformed from a cationic to an anionic state, the absorption can be measured at 595 nm. This change in absorbance is proportional to the protein concentrations across wide ranges, and was first used by Bradford to determine protein concentrations (Bradford 1976). Coomassie Brilliant Blue-G250 binds mainly to basic amino acids (Chial und Splittgerber 1993). This explains the different degrees of absorption by different proteins. Therefore the absorption measurements were related to a calibration curve with BSA. The assay was conducted as recommended by the manufacturer of Roti®-Quant (Carl Roth, Karlsruhe, Germany).
4. Methods

4.2 Cell and virus culture

4.2.1 Cell cultivation

African green monkey kidney cells (CV1), human embryonic kidney cells (293T) and Madin-Darby canine kidney cells (MDCK II) were maintained in Dulbecco's modified Eagle medium (DMEM; Pan Biotech) supplemented with 10% fetal calf serum, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. All cells were cultured at 37 °C in 5% CO₂ and passaged twice per week, before they had reached full confluency. Cells were propagated in growth medium in 25, 75 or 175 cm² culture flasks. For every passage, the cells were washed twice with PBS (without calcium and magnesium) and detached using trypsin-EDTA at 37 °C for 5-15 min. Trypsinization was stopped by adding growth medium. Depending on the size of the flask, around 1x10^5 to 1x10^6 cells were seeded into a new culture flask.

In preparation for infection, transfection or other cell assays, cells were seeded one night prior to the experiment into 6-12-well plates or petri dishes. To preserve the different cell line stocks, cells from one 75 cm² culture flask were detached, pelleted for 5 min at 300 ×g, resuspended in freezing medium and distributed into three 1 mL cryo-vials. The cells were cooled down by using the Mr. Frosty freezing container, which achieves a rate of cooling very close to -1 °C/minute, and kept at -80 °C for 4 to 24 h. Subsequently, the vials were transferred into a liquid nitrogen tank.

4.2.2 Generation of recombinant virus

In order to generate recombinant virus, A/WSN/33 influenza mutants with point mutations in HA (see nucleotide sequence of HA in materials, 3.2.8), the reverse genetics eight-plasmid system was employed. The plasmids used for the rescue of virus have been described previously (Neumann et al., 1999).

The following amino acids were replaced:
4. Methods

**TABLE 4.4**
Amino acid substitutions in HA.

<table>
<thead>
<tr>
<th>#</th>
<th>Amino acid and position</th>
<th>Codon</th>
<th>Replaced codon</th>
<th>Replaced amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I563</td>
<td>ATA</td>
<td>TTG</td>
<td>L</td>
</tr>
<tr>
<td>2</td>
<td>I563</td>
<td>ATA</td>
<td>CAG</td>
<td>Q</td>
</tr>
<tr>
<td>3</td>
<td>G557</td>
<td>GGG</td>
<td>TAA</td>
<td>Stop</td>
</tr>
<tr>
<td>4</td>
<td>G557</td>
<td>GGG</td>
<td>GCT</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>G557</td>
<td>GGG</td>
<td>GAA</td>
<td>E</td>
</tr>
<tr>
<td>6</td>
<td>Q560</td>
<td>CAG</td>
<td>GAA</td>
<td>E</td>
</tr>
<tr>
<td>7</td>
<td>G547</td>
<td>GGG</td>
<td>ATT</td>
<td>I</td>
</tr>
<tr>
<td>8</td>
<td>G547</td>
<td>GGG</td>
<td>TGT</td>
<td>C</td>
</tr>
<tr>
<td>9</td>
<td>C554 and L559</td>
<td>TGT and TTG</td>
<td>AGC and TGC</td>
<td>S and C</td>
</tr>
</tbody>
</table>

**4.2.2.1 Site-directed mutagenesis**

Site-directed mutagenesis allows a specific change in the DNA by using recombinant DNA. Individual nucleotides of a gene can efficiently be exchanged or entire genes removed. Point mutations in the vicinity of the acylation sites in the cytoplasmic tail of HA were prepared using QuikChange mutagenesis (Stratagene, California, USA) with the pHW2000 HA plasmid, a thermostable DNA polymerase and a thermostable DNA ligase. Stratagene markets Quik change as a ligation-during-amplification (LDA), a method for linear amplification of circular DNA in a PCR-based method. This method is used for the targeted mutagenesis of plasmids in the course of a desired adaptation of the vector as part of the design of the protein. A strand of the circular plasmid DNA (template) is amplified with a mutagenic primer (Table 3.1) linearly in a thermocycler and ligated in several cycles (Tables 4.5-4.8).
4. Methods

Table 4.5
Reagents and concentrations for the primer extension.

<table>
<thead>
<tr>
<th>Components</th>
<th>50 µL reaction volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x Phusion HF buffer</td>
<td>10 µL</td>
<td>1 x</td>
</tr>
<tr>
<td>100 ng/µL DNA template (pHW2000 HA plasmid)</td>
<td>5 µL</td>
<td>10 ng/µL</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1 µL</td>
<td>200 µM</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>33.5 µL</td>
<td></td>
</tr>
<tr>
<td>Phusion polymerase</td>
<td>0.5 µL</td>
<td>0.02 U/µL</td>
</tr>
<tr>
<td>Primer (Table 2.1) 5 µM</td>
<td>2 µL</td>
<td>0.02 µM</td>
</tr>
</tbody>
</table>

Table 4.6
Cycler conditions for the primer extension.

<table>
<thead>
<tr>
<th></th>
<th>98 °C</th>
<th>10 s</th>
<th>Initial denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x</td>
<td>98 °C</td>
<td>10 s</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3 x</td>
<td>69 °C</td>
<td>30 s</td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>3 min</td>
<td>Extension</td>
</tr>
<tr>
<td>1 x</td>
<td>4 °C</td>
<td></td>
<td>Hold</td>
</tr>
</tbody>
</table>

Then 25 µL of each primer pair is mixed on ice and another 0.5 µL Phusion polymerase was added to each tube.

Table 4.7
Cycler conditions for the PCR.

<table>
<thead>
<tr>
<th></th>
<th>98 °C</th>
<th>10 s</th>
<th>Initial denaturation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x</td>
<td>98 °C</td>
<td>10 s</td>
<td>Denaturation</td>
</tr>
<tr>
<td>30 x</td>
<td>69 °C</td>
<td>30 s</td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>3 min</td>
<td>Extension</td>
</tr>
<tr>
<td>1 x</td>
<td>72 °C</td>
<td>10 min</td>
<td>Final extension</td>
</tr>
<tr>
<td>1 x</td>
<td>4 °C</td>
<td></td>
<td>Hold</td>
</tr>
</tbody>
</table>

Then, the parental, methylated plasmid DNA (template) was degraded with the restriction enzyme Dpn I (added to each PCR tube and incubated for 2 h at 37 °C), which cleaves only methylated DNA. Thereafter, the amplified, mutated, single-stranded, circular DNA was transformed into bacteria.
Recombinant influenza A/WSN/33 (H1N1) virus was generated using the eight-plasmid reverse genetics system (Hoffmann et al., 2000), where each plasmid contains cDNA of one viral RNA segment, flanked by suitable promoters. In the HA-encoding cDNA segment, at least two nucleotides (in order to prevent reversions) of the respective codon were replaced to create the mutants (Table 4.4). They were confirmed by sequencing (GATC Biotech). HEK 293T cells in 60 mm dishes were transfected with eight plasmids encoding WSN cDNA (1 µg each) using TurboFect (Fermentas/Thermo, Bonn, Germany) in OptiMEM medium (Gibco/Invitrogen, Carlsbad, USA). 4–6 h after transfection, medium was replaced by infection medium (see 3.2) and incubation was continued at 37 °C. 72 h after transfection, supernatants were harvested and cleared of debris by low-speed centrifugation (2,000 ×g, 5 min, 4 °C). Recombinant viruses were first plaque purified on MDCKII cells and then amplified to obtain a high titer virus stock and its titer was determined in MDCKII cells.

4.2.3 Virus propagation in cells

To prepare virus from cells the virus stock was used to infect MDCKII cells with a m.o.i. of 0.01. The virus supernatant was diluted in infection medium (see 3.2) and applied onto the cells (for infection well of a 6-well-plate 500 µL of virus dilution was used, for T25 flasks 1 mL, for T75 flasks 5 mL and for large dishes and T175 flasks 15 mL). After 1 h of adsorption at 37 °C and 5% CO₂, cells were washed with phosphate-buffered saline (PBS), infection medium was added and incubation was continued until a cytopathic effect (CPE) became evident; then, the supernatant was harvested. For mass spectrometry preparations, the cleared supernatant (cell debris was centrifuged at 3500 ×g for 20 min) was centrifuged at 100,000 ×g for 2 h and the viral pellet was resuspended in 50 µL TNE (see 3.4).

4.2.4 Virus propagation in eggs

Virus (1000 PFU, diluted in 0.9% NaCl, 100 µL per egg) was inoculated with a 0.45×25 mm needle into the allantoic cavity of 11-day-old embryonated chicken eggs. After 48 h to 72 h (dependent on the motility of chicken embryos when candled) at 37 °C, 5% CO₂, the embryos were euthanized by incubating the eggs at 4 °C for 16 h. The allantoic fluid was collected and
4. Methods

centrifuged at 2000 \times g for 20 min at 4 °C to clarify from debris and erythrocytes. The cleared allantoic fluid was centrifuged at 100,000 \times g for 2 h and the viral pellet was resuspended in 50 \mu L TNE (3.4). In some cases, the obtained pellet was centrifuged through 2 mL of a sucrose cushion (20% in TNE) solution and centrifuged at 100,000 \times g for 2 h. The resulting virus pellet was resolved in TNE.

4.2.5 Plaque assay

Plaque assay was performed on MDCKII cells in 6-well plates. Cells were infected with serial 10-fold dilutions of the virus supernatants in infection medium, incubated for 1 h at 37 °C, washed twice with PBS and overlaid with 1.8% SeaPlaque agarose (Lonza, Basel, Switzerland) in 2× Eagle’s minimum essential medium (EMEM; Lonza, Basel, Switzerland) supplemented with 0.2% bovine serum albumin (BSA), 0.1% fetal bovine serum (FBS), 2 mM glutamine, and 1 \mu g/mL TPCK-treated trypsin (this cleaves HA and thus activates the virus particles). After 2-3 days of incubation, cells were stained using neutral red (Biochrom, Berlin, Germany), and plaques were counted.

The tip of a small pipette is inserted into the agar overlay above the plaque. The plug of agar is removed and placed in buffer. The viruses within the agar plug move into the buffer, which can then be used to infect cultured cells.

For plaque purification, the tip of a pipette was inserted into the agar overlaying a single plaque. This plug of agar was then dissolved in 500 \mu L PBS at 4 °C for 16 h and thus the viruses within the plug could move into the buffer. It was then directly used to infect T25 cell culture flasks with MDCKII cells in infection medium. After incubation for 1 h at 37 °C, the cells were washed twice with PBS and infection medium was added. At 1-3 days p.i., when a CPE was visible, supernatants were harvested and cleared of debris by low-speed centrifugation (2,000 \times g, 5 min, 4 °C). Every sample was sequenced to confirm the presence of inserted mutations, before titrating in plaque assay and amplification in large scale in MDCKII cells.
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4.2.6 Growth curve

This test was used to assess growth kinetics of the virus mutants. Therefore, cells were infected with recombinant WSN at an m.o.i. of 0.01. After 1 h of adsorption and subsequent washing with PBS, infection medium (DMEM containing 0.2% bovine serum albumin, 0.1% FBS, 2 mM glutamine, 100 U/mL penicillin/streptomycin and 1 µg/mL TPCK-treated trypsin, Sigma-Aldrich) was added. The supernatant was harvested after a defined incubation time, cleared of debris (5 min at 3000 ×g) and stored at -80 °C before titer determination by plaque assay.

4.2.7 HA assay

Influenza virus possesses the capability to interconnect erythrocytes via its surface proteins and thus agglutinates them. This process can be used as a hemagglutination (HA) assay in order to find out at which dilution no agglutination of erythrocytes occurs and can be employed to detect virus.

At 4 °C the aggregates are stabilized, which can dissolve at higher temperatures. The binding is pH dependent and host cell specific. The HA assay is not a measurement for infectivity of a solution, because agglutination of erythrocytes occurs also without infectious RNA.

A blood sample of a hen was taken from the vena ulnaris and sodium citrate was added to a final concentration of 25%. Before using, the blood was centrifuged for 5 min at 2000 ×g/min, the supernatant was removed and the remaining erythrocyte pellet was dissolved in 0.9% sodium chloride. This washing process was repeated 2-3 times. The blood was diluted 1:100 in PBS and used for the HA assay, adjusted to a working solution of 0.5% erythrocytes. For this purpose, 50 µL of a 0.9% NaCl solution were given in each well of a 96-well plate with conical bottom. 50 µL of the virus was given into the wells of the first row and diluted by twofold serial dilutions up to row twelve. Two each well 50 µL of the blood dilution were given, mixed and incubated at 4 °C for 20 min. The analysis was performed by tilting the plate and comparing the agglutinated complexes to the non-agglutinated samples, which run downwards.
4. Methods

4.2.8 Reverse transcription (RT)-PCR and sequencing

To check for correctness of HA sequences in the recombinant viruses, RNA was isolated from 200 µL cell culture supernatants or virus preparations with Invisorb Spin Virus RNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) followed by reverse-transcription and polymerase chain reaction (RT-PCR) using HA-specific primers and OneStep RT-PCR kit (Qiagen, Hilden, Germany). Either PCR products were purified from agarose gels using the Fragment CleanUp Kit (STRATEC Molecular GmbH, Berlin, Germany) or directly treated with the ExoSAP-IT Cleanup Kit (Affymetrix, California, USA) to remove excess of primers and dNTPs, which might hinder the sequencing reaction. Here, one assay contained 5 µL PCR product plus 2 µL ExoSAP-IT reagent and was incubated at 37 °C for 15 min, then 15 min at 80 °C to deactivate the enzyme. ExoSAP-IT is based on Exonuclease I and Shrimp Alkaline Phosphatase and degrades primers and dephosphorylates dNTPs that were not consumed in the PCR reaction. Sequencing was performed by GATC Biotech. For the vRNA segment 4 (HA) specific primers for RT-PCR of viral RNA were used.

Sense:   CGGGATTACAAACAAGG
and Antisense:   TTATATTTCTGAAATCC
and for sequencing:   TGGGACTTATGATTATCC.
They bind to the 5’ end of the HA gene and produce approx. 300 nucleotides.

4.2.9 Metabolic labeling of viruses with $^{35}$S methionine/cysteine

In order to analyze the protein composition of the different virus mutants, radioactively labeled viruses were generated as follows: MDCKII cells in a 15 cm dish were infected at an m.o.i. of >2 with the wild-type virus and the mutants, respectively. 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 L-glutamine, 1 mg/mL TPCK-treated trypsin and 0.3 mCi (11.1 kBq) $^{35}$S methionine/cysteine (EasyTag™ EXPRE35S35S Protein Labelling Mix; PerkinElmer, Massachusetts, USA) ml-1. At 24 h after infection, the supernatants were harvested, cell debris was removed (2000 ×g, 10 min, 4 °C), and the virus was pelleted from
4. Methods

the supernatant by ultracentrifugation (Beckman SW-28 rotor, 28 000 r.p.m., 2 h, 4 °C), resuspended in 100 mL PBS and analyzed by SDS-PAGE under non-reducing conditions and fluorography or by SDS-PAGE followed by Western blotting as described previously (see 4.1.6-4.1.8). For Western blotting a polyclonal antiserum against fowl plague virus, which cross-reacts with WSN M1 was used. To detect chemiluminescence HRP-coupled secondary antibodies were used. The reaction was visualized with ECL Plus substrate (GE Healthcare, Uppsala, Sweden) and a Fusion SL camera system (Peqlab, Erlangen, Germany), which detects photons over a wide linear signal-response range. Densitometric quantifications were carried out using Bio-1D software (Vilmer-Lourmat).

4.2.10 Quantitative real-time RT-PCR

First, an HA assay was performed with the virus-containing supernatant obtained from infected cells in order to analyze an equivalent number of virions. The supernatants from infected MDCK cells were adjusted to an HA titer of 2^6. Then, RNA was extracted utilizing the Invisorb Spin Virus RNA Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) and cDNA synthesized applying the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA). The following components were added per tube:

**TABLE 4.8 Reverse transcription.**

<table>
<thead>
<tr>
<th>Total reaction volume</th>
<th>20 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT buffer</td>
<td>4 µL</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Oligo(dT) (100 µM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Maxima H Minus enzyme mix</td>
<td>1 µL</td>
</tr>
<tr>
<td>RNA</td>
<td>13 µL</td>
</tr>
</tbody>
</table>

The reaction was incubated at 50 °C for 30 minutes for reverse transcription and terminated by incubating for 5 min at 85 °C. The reaction product was used directly in a quantitative RT-PCR with primers specific for the influenza A virus M segment:
4. Methods

**TABLE 4.9**
Quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total reaction volume</td>
<td>25 µL</td>
</tr>
<tr>
<td>5x OneTaq buffer</td>
<td>5 µL</td>
</tr>
<tr>
<td>RNase free water</td>
<td>16 µL</td>
</tr>
<tr>
<td>dNTPs (100 µM)</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>100 µL Forward primer (AGATGAGTCTTTAACCGAGGTCG)</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>100 µL Reverse primer (TGCAAAACATCTTCAAGTCTCTG)</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>Probe (6FAM-TCAggCCCCTCAGAgCCa-TMR, 10 µM)</td>
<td>0.75 µL</td>
</tr>
<tr>
<td>OneTaq DNA polymerase (0.2 U/µL)</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>cDNA template (concentration not measured, adjusted to HA titer in the beginning)</td>
<td>2 µL</td>
</tr>
</tbody>
</table>

Genome copy numbers were quantified using a TaqMan fluorescent quantitative (q) PCR assay. The real-time RT-PCR assay was performed making use of a TaqMan Probe, OneTaq DNA polymerase (NEB, Massachusetts, USA) and the iCycler-iQ5® (Multicolor Real Time PCR) detection system (BIO-RAD, California, USA).

**TABLE 4.10**
Cycler conditions for qPCR.

<table>
<thead>
<tr>
<th>x</th>
<th>Initial temperature</th>
<th>Initial time</th>
<th>Final step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x</td>
<td>95 °C</td>
<td>3 min</td>
<td>Denaturation</td>
</tr>
<tr>
<td>40 x</td>
<td>95 °C</td>
<td>10 s</td>
<td>Annealing and elongation</td>
</tr>
<tr>
<td></td>
<td>55 °C</td>
<td>30 s</td>
<td>Final step</td>
</tr>
<tr>
<td>1 x</td>
<td>4 °C</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

Fluorescence values (FAM) were collected during the annealing step. A standard curve was generated by using serial dilutions of an *in vitro* transcribed M segment derived RNA transcript. Expression levels were calculated using the Delta-Delta-Ct-Method calculation (Livak *et al.*, 2001) Method.
4. Methods

**Figure 4.1**
Quantitative PCR output of M-gene in wild-type and mutants. Here, as an example are depicted the curves and cycle thresholds (Ct) of the different samples of the qPCR. The positive control (pink) samples (20 ng M plasmid) reached the threshold after 7-8 cycles. The virus samples (various colors) reached the threshold after 25-29 cycles. And the negative control (orange), water reached the threshold after 31-33 cycles.

Figure 4.1 depicts the output of real-time qPCR experiment. As positive control, 20 ng of the plasmid of the M gene in the pHW2000 vector was used. The cycle threshold is set to the change of steepness in the curve, when the amplification in the PCR has reached a predefined number. PCR of virus samples was applied in triplicate. As negative control, Millipore water was used to define the cycle number that is needed to initiate PCR without the template. Primer dimers appear very late in the amplification cycles, and mark unspecific amplification.

### 4.2.10.1 Statistical analysis

Viral protein compositions were statistically evaluated by an analysis of variance (ANOVA). The results of quantitative real-time PCR were analyzed by Student’s t-test. Statistically significant differences of p <0.05 are indicated, and all analyses were performed with Prism 6.0 (GraphPad Software Inc.).
4. Methods

4.2.10.2 Mass spectrometry

All protocols used to perform mass spectrometry are described in the article, where these results were published (Brett et al., 2014) and were developed and performed by Dr. Larisa Kordyukova and Marina Serebryakova.
5.  RESULTS

I first explain how I came to choosing the mutation sites for the rescue of recombinant influenza virus and describe these sites. Then I depict the characteristics measured of viable virus mutants. I will then describe the analysis of lethal mutants. Finally, I will focus on experiments made by means of mass spectrometry to examine the acylation pattern of the mutants. Chapters 5.1, 5.3 and 5.5 are composed of results shown in my previously published study (Brett et al., 2014) and descriptions are therefore very similar.

5. 1  Mutagenesis

5.1.1  Conserved amino acids in the cytoplasmic tail of Hemagglutinin

As previously mentioned, hemagglutinin (HA) is differentially S-acylated: with palmitate attached to the two cysteines in the cytoplasmic tail (CT) and (mostly) stearate to the cysteine located membrane-proximal. In principle, the differential acylation pattern for HA might be influenced by the location of the acylated cysteine relative to the transmembrane span or (but mutually not exclusive) by the presence of specific amino acids near the acylation site. Before thinking of adequate mutations, a bioinformatics comparison was performed by Ramil R. Mintaev (I. I. Mechnikov Research Institute of Vaccines and Sera, Russian Academy of Medical Sciences, Moscow) and Andrei V. Alexeevski (Department of Bioengineering and Bioinformatics, Lomonosov Moscow State University, Moscow) using all HA-sequences of influenza A virus present in the NCBI-database. This comparison revealed conserved residues in the transmembrane region (TMR) and CT that might be part of a complex acylation signal (Figure 5.1).
5. Results

Briefly, there is a hydrophobic patch with the sequence ICI at the very end, a position with either glutamine or arginine in the middle and a completely conserved glycine at the beginning of the CT (Figure 5.1). Located in the TMR there is a conserved glycine, which is shifted 2-3 amino acids in some subtypes (Pica et al., 2013).

5.1.2 Mutations introduced into HA’s membrane-anchoring domain

I aimed to assess the interaction of an acylation enzyme (i.e. the active center of a DHHC-protein) at the three acylation-sites of HA. For this purpose, several conserved residues in the HA from the WSN-strain (H1-subtype) were mutated. The mutations were inserted in the vicinity of the cysteines, i.e. located in the inner part of the TMR or in the CT (Table 5.1).
One of the latter is the hydrophobic sequence ICI at the C-terminus where the terminal isoleucine 565 and the palmitoylated cysteine 564 are present in each HA-variant. The adjacent isoleucine 563 is almost completely conserved through all HA subtypes, in some subtypes it is replaced by other large hydrophobic amino acids. To investigate whether the acylation pattern of HA is determined by the C-terminal hydrophobic patch, I563 was exchanged by the polar residue glutamine (I563Q) or the hydrophobic residue leucine (I563L).

Glycine at position 557 in the cytoplasmic tail is also completely conserved through all HA-sequences. I exchanged it by the small residue alanine (G557A) or the negatively charged glutamic acid (G557E). In another mutant, the whole cytoplasmic tail (including the two palmitoylated cysteines) was deleted by replacing the codon for glycine 557 with a Stop-codon (G557Stop). Position 560 located adjacent to a palmitoylated cysteine is not conserved through all HA sequences, but accommodates only two consensus amino acids, either a glutamine or an arginine, depending on the subtype. The glutamine present in HA of the WSN strain was replaced by glutamic acid (Q560E).

Each HA subtype (except H4) contains in the middle of its TMR a glycine that has a very low propensity to be present in a α-helical structure. It thus might induce a kink that presents the
cysteine at the end of the TMR to an acyl-transferase. I exchanged glycine 547 by serine (G547S), which is better compatible with a α-helical structure.

To determine whether the location of a cysteine relative to the transmembrane span is the main determinant for attachment of stearate, I shifted the cysteine at the end of the TMR to a cytoplasmic location. In order to do so cysteine 554 was replaced by a serine and leucine 559 was exchanged by cysteine (C554S+L559C). The resulting mutant thus contains an identical location of cysteines as in H8 and H9 subtype HAs from naturally occurring avian, equine, swine and human virus strains that have very low stearate content (Kordyukova et al., 2008).

5. Results

5.2 Properties of viable HA virus mutants

5.2.1 Rescue of HA virus mutants and their plaque sizes

To investigate what influence the mutations in HA have on the virus rescue, I utilized reverse genetics. Having verified the mutation by sequencing, the mutant HA-plasmid together with seven plasmids encoding the other viral proteins were transfected into HEK 293T cells. The supernatant harvested at 72 h post-transfection was then inoculated into MDCKII cells to amplify recombinant virus particles. After three days of incubation, an extensive cytopathic effect (CPE) was observed in infected cells. The supernatant was tested for virus particle release by a plaque assay.

However, for two mutants, G557Stop and I563Q, infectious virus particles were never rescued, although control experiments using wild type (WT) HA done in parallel showed that transfection was successful. Thus, the mutation might have destroyed one of the essential activities of HA, i.e. virus assembly and budding and/or membrane fusion during virus entry. All the other mutations in HA were not lethal for virus replication.

Viral plaque sizes, a measure how fast a virus replicates, were compared between WT virus and mutants (Figure 5.2). MDCKII cells were infected with virus supernatant and incubated at 37 °C. At 72 h post infection (p.i.), an unambiguous CPE became visible; i.e. clear zones
formed in a lawn of cells due to lysis. They were visually detected by staining with neutral red and photographed.

Two parallel infection experiments were performed with the WT and the mutants (Figure 5.2). In the upper row, all plaque sizes were comparable; the mutants G557A, Q560E and C554S+L559C never showed significantly bigger or smaller sizes than WT, whereas the plaques shown here are large, in general. Likewise, in the lower row, no difference in size or shape between the mutants G547S, G557E, I563L and WT was detected, but the plaques were overall much smaller than in the upper row. This demonstrated that the plaque size in general varied. Different cell densities due to cell passaging might have been responsible for these differences. Nevertheless, an effect of a mutant to produce permanently smaller or larger plaques compared to WT was never observed.

5.2.2 Serial passage of mutant virus resulted in reversions

In some cases, the rescue of virus mutants with the mutation of interest in HA was not or not always possible. Routinely, after every passage of virus propagation, the HA sequence was checked to confirm the presence of the inserted mutation. In order to do so, supernatants were collected, the viral RNA was isolated and subjected to RT-PCR and sequencing. Interestingly,
5. Results

I observed in three viable virus mutants nucleotide reversions at the mutated codons: in the mutants G547S, C554I+L559C and G557G.

As depicted in Figure 5.3, the nucleotide changes in these revertants happen gradually. For mutant G547I, virus particles with an exchange in the TMR (G547I) could never be rescued and propagated. I observed a single nucleotide exchange from ATT (isoleucine) to AGT (serine). A partial exchange of T to G, reflected by superimposed peaks for the respective bases, was already visible in the viral genomes prepared from the transfection supernatant and the resulting virus G547S completely outgrew G547I after two amplifications. The reversion from the chosen mutation G547I to the unanticipated mutation G547S always happened in five transfections.

In the second case, for the mutant with the shifted acylation site (C554S+L559C), I observed that the codon AGC (serine 554) changed to ATC (isoleucine). The C554S+L559C to C554I+L559C reversion also takes place in two steps, while superimposed peaks of the nucleotide exchange at the position of the G to a T appear already in the transfectant supernatant. C554S+L559C to C554I+L559C reversion occurred three times out of five. Yet, the inserted C554S+L559C mutation was dominant and resulted in large-scale propagation.

Finally, I observed the reversion from G557E (codon GAA) to the WT G557G (codon GGA) only once in five experiments. This led to a mutation to glycine, the amino acid present in WT HA where it is specified by a different codon.
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The occurrence of these reversions suggests that the sequence context in this region has an impact on the fitness of the virus. Since the G547I designed mutant reverted to G547S in all cases, this strongly indicates that it has a competitive fitness advantage. Similarly, both other resulting revertants rapidly outgrew the mutant one, but for these I obtained stable virus with the desired mutation that could be characterized. The remaining three recombinant virus mutants (G557A, Q560E, I563L) were stable for at least three passages, reversions at the mutated codon or other sites in the C-terminal ~100 amino acids of HA were never observed.

**FIGURE 5.3**

Reversions of the mutants G547I, C554S+L559C and G557E. Sequencing chromatograms of cDNA of reverted mutants after several propagation steps. (A) The designed point mutations of all mutants were inserted into the plasmid by site-directed mutagenesis, the obtained sequence is shown. (B) First DNA sequence obtained after virus was rescued from 293T supernatant (after 72 h). (C) DNA sequence obtained after the first amplification of the virus in MDCKII cells. (D) DNA sequence after a second propagation of the virus in MDCKII cells. MDCKII cells were infected at a multiplicity of infection of 0.1. After 24 h the supernatants were collected, the viral RNA was isolated and subjected to RT-PCR and sequencing. Nucleotide changes are highlighted by colored boxes.
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5.2.3 Growth kinetics of the viable HA mutants

Multiple-step growth cycles were performed to assess the virus production kinetics of all viable mutants. MDCKII cells were infected with WT WSN or one of the mutants at a low multiplicity of infection (m.o.i.), the supernatants were collected at various times and virus titers were measured by using plaque assay on MDCKII cells.

![Graph A](image1.png)  
**A** Titre/(pfu ml⁻¹)  
WT - Black  
G557A - Blue  
Q560E - Red  

![Graph B](image2.png)  
**B** Titre/(pfu ml⁻¹)  
WT - Black  
G547S - Green  
G557E - Pink  
I563L - Dark Blue  
C554S+L559C - Orange  

**FIGURE 5.4**  
Growth of recombinant influenza virus (WSN strain) with mutations in the cytoplasmic tail of hemagglutinin and wild type (WT). MDCKII cells were inoculated with recombinant virus mutants at an m.o.i. of 0.01 and were incubated at 37°C. Supernatants of infected cells were harvested at the indicated times (8 h, 16 h, 24 h, 48 h, 72 h). Virus titers in the supernatant were determined by plaque assay on MDCKII cells. The data points represent the means +/- standard error of the mean.  
(A) Recombinant WSN WT virus and mutants G557A and Q560E in parallel. (B) Recombinant WSN WT virus and mutants G547S, G557E, I563L and C554S+L559C in parallel. pfu= plaque-forming units.

Overall, all virus mutants tested in a growth cycle revealed only subtle growth differences compared to WT virus (Figure 5.4). Mutants G557A and G557E showed a slight growth retardation compared to WT virus, but at 72 h p.i. the titer difference to WT did not exceed ~1.5 logs. Mutant Q560E on the other hand, demonstrated a rapid growth in the first 8 hours p.i., even faster than the WT reference (three log differences). Eventually, after 72 hours p.i. it
settled at almost the same final titer as the WT. Likewise, mutant C554S+L559C overtook the WT in the first 16 h, but yielded comparable titers at later time points. The mutants G547S and I563L displayed slightly increased titers of 10E3 at 8 hours p.i., and at later times titers were reduced by one log.

These results suggest that the amino acids at the cytoplasmic tail in the vicinity of the acylation sites have some effect, but do not play any critical role in infectious virus production.

5.2.4 Morphology of viral particles characterized by electron microscopy

One of the properties of the rescued virus mutants can be a failure in the budding and scission of the particles from the cell membrane. Aberrant morphology of virions was found as a result of such budding defects (Nayak et al., 2009). This was already observed when the cytoplasmic tails of both HA and NA were deleted from the Udorn strain (Jin et al., 1997).

**FIGURE 5.5**

Virus morphology characterization by transmission electron microscopy. Virions were grown in MDCKII cells, harvested from the supernatant and purified by centrifugation through 30% sucrose. The virions were negatively stained with 1% uranyl acetate and observed under a transmission electron microscope. Bars indicate 100 nm. WT= wild type
Thus, to examine whether the introduced point mutations in the cytoplasmic tail of HA in WSN have an influence on the virion shape, particles were harvested from the supernatant of infected MDCKII cells purified. Virus particles were visualized by means of negative staining transmission electron microscopy by Lars Möller (Group of Advanced Light and Electron Microscopy at the Robert Koch Institute).

Figure 5.5 shows that the virus particles were spherical and mostly round shaped in all cases, yielding no evidence for any perturbation of virus morphology by mutations in HA. In comparison with the WT, no increased numbers of damaged or broken virus particles could be spotted in the mutants. Furthermore, one can see each virus particle clearly and the surface covered densely with distinct spikes, in all mutants and WT. No reduction in the amount of protein spikes on the virions was observed, but a more precise counting of spikes is only possible with cyro-EM. To conclude, mutations in HA inserted at this location yielded no evidence for any aberration of virus morphology.

### 5.2.5 Protein composition of recombinant virus particles

To check whether there is an influence of the mutations in the CT of HA (WSN strain) on the expression and/or incorporation of viral proteins, I analyzed the protein composition of the virions. The content of the individual virus components in each mutant virus particle was assessed. So, if there was an influence of the individual amino acids in the CT of HA on the recruitment of M1 (and as a consequence incorporation of RNPs), there should be a difference in their relative abundance. To determine the protein composition, I propagated wild-type and mutant virus particles by infecting MDCKII cells with an m.o.i. of five and labeled the cells three hours p.i. with $[^{35}\text{S}]$-methionine/cysteine. The cell culture supernatant was removed 16 hours p.i., cleared from cell debris, and virus particles were pelleted and subjected to non-reducing SDS-PAGE, and fluorography.
5. Results

The resulting X-ray film in Figure 5.6 A illustrates three bands, which refer to the three most abundant structure proteins of influenza particles: HA, NP and M1 with approximate molecular weights of 77, 50 and 25 kDa. This experiment was repeated three times; Figure 5.6 A demonstrates a band pattern representation of each mutant. To quantify the protein composition of virus particles from this the corresponding band intensities were quantified by densitometry on scanned films.

The most obvious difference is the higher variability in the protein composition of most mutants compared to wild-type virus, especially evident in the mutants I563L and G557A, where the proportion of NP and M1 varied between ~15% and ~60%. However, the
proportion of HA in the mutants G557E and Q560E was rather consistent. In general, the relative abundance of M1 seems to be decreased and of NP increased in all mutants, whereas the proportion of HA was mostly not changed compared to wild-type virus.

The results revealed considerable variability in the relative protein proportions between virus preparations. Because of this high variation, an additional experiment was performed with seven different WT preparations in parallel (Figure 5.7). The proportion of NP varied between 28% and 45%, of HA between 18% and 35% and of M1 between 25% and 42%. Apart from that, I also divided band intensities by the number of methionine and cysteine residues present in the respective protein and normalized the results relative to M1.

**FIGURE 5.7**

Deviation of the protein composition between different wild-type (WT) preparations. MDCKII cells were infected with WSN recombinant WT virus at an m.o.i. of 4; virus particles were radioactively labeled with $^{[35]S}$ methionine/cysteine. After 16 hours, the cell-culture supernatant was harvested and the virus particles were pelleted by ultra centrifugation. The samples were subjected to SDS-PAGE and a fluorography was produced. (A) Fluorograms from seven different virus preparations are shown. (B) The band intensities were calculated by densitometry on scanned films. All three proteins (HA, NP, M1) of each preparation make up 100%. The bars display arithmetic means ±SD. (C) The band intensities calculated as in B were divided by the number of methionine and cysteine residues present in the respective protein (HA, NP and M1). The results were normalized relative to M1 and are shown in a bar diagram.
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The resulting graph (Figure 5.7) shows that M1 is the most abundant protein in virus particles, in accordance with previous estimates (Schulze, 1970). The relative abundance of NP and HA is calculated as 0.7 and 0.65, respectively. It is thus in the same range as estimated by SDS-PAGE and Coomassie-staining for the same virus strain, but higher as recently determined by mass spectrometry (NP: 0.31, HA: 0.17) (Hutchinson et al., 2014). The relatively large difference, especially for HA, might be due to less efficient detection of glycoproteins by mass spectrometry and/or differences in host cells and sample preparation.

Noticeably, the values did not fluctuate as much between the different WT preparations as between the mutants. The experiment in Figure 5.7 underlines that the protein composition of these virus preparations varied a lot, in general. Therefore, slight differences should not be mistaken to be due to significant effects of the mutations. These mutations surrounding the acylation sites have only a negligible effect on the incorporation of viral structural proteins into the virions. The marginal effect of the mutations on the growth goes along with this observation (Figure 5.4, growth curves), since a reduction of structural proteins would eventually lead to a growth defect.

However, (probably due to the high variability) no statistically significant difference (Anova-test) in the protein content between wild type and mutant virus particles was estimated.

5.2.6 Packaging of M1 and RNPs into virions

The protein composition of assembled virus particles may vary in mutants due to the introduced mutations. These mutations might have an impact on the expression, the packaging of vRNA or the interaction of the viral proteins with each other and subsequent assembly of the virions.

To determine the ratio between HA and M1 I adjusted culture supernatants from virus infected MDCKII cells to a HA titer of 2^6. Then I subjected aliquots to SDS-PAGE and western blotting with polyclonal antiserum against fowl plague virus, which cross-reacts with WSN M1. Quantification of M1 bands by chemiluminescence imaging from three different experiments revealed that the amount of M1 is increased relative to HA in the mutant G557E and decreased in I563L and Q560E, but the calculated means are not statistically significantly (according to t-test) different from WT. The high error bars of the standard deviation again suggest that there is a high variance between virus preparations (Figure 5.8 B).
Another aspect regarding the budding process of influenza virus, is the association of M2 to HA and the edge of rafts, where the virus contents are assembled (see Introduction 1.2.3.2 Assembly and release of viral particles) M2 serves to cut off the newly formed virion. When 20fold higher concentrated virus samples, (compared to the loaded samples in the experiment shown in Figure 5.8), were subjected to western blotting, the M2 protein was detected in wild type virus and in each mutant (Figure 5.9). M2 was detected as a double band on the western blot, which has been previously already been observed is supposed to be due to cleavage by cell proteases (Thaa et al., 2012; Grantham et al., 2009; Zhirnov et al., 1999). Thus, the mutations in the cytoplasmic tail of HA did not per se abolish recruitment of M2 to the budding site. The large difference in the band intensities between some virus mutants and WT could not be reproduced and does therefore not direct to a changed expression of M2. However, the general low abundance of M2 in virus particles precludes a precise quantification.
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The M1 gene segment was here used as a reporter marker that indicates whether the viral genome is packaged fully into the virions. Thus, measuring the M1 gene was employed as a means to quantify the amount of vRNPs into newly produced virus particles.

To analyze this question, quantitative PCR with M1 specific primers on virus samples adjusted to a HA-titer of $2^6$ was performed (Figure 5.8 C). In Figure 5.8 C, the cycle thresholds (Ct) are demonstrated. The higher the Ct was compared to the WT, the less M-genome segment was originally present. The results from three different experiments revealed that two mutants have the same (G547S, Q560E) or a slightly higher cycle threshold value (28.2-28.6) compared to WT WSN (27.5). However, a statistically significant difference was obtained only for the mutant I563L that has a Ct value of 29.1 (according to t-test). One cycle difference in the Ct value indicates a twofold difference in the amount of DNA suggesting that the content of RNPs in mutant I563L is reduced to ~30% compared to WT.

In summary, whereas deletion of the two cytoplasmic palmitoylation sites and exchange of isoleucine by glutamine prevented rescue of infectious virus, the other mutants showed only subtle defects in growth kinetics, higher variability of the protein and RNP content of virus particles.

5.3 Analyzing S-acylation of HA with lethal mutations

The lethal replication defect of virus mutants containing the HA mutants I563Q and G557Stop might be due to a complete defect in HA’s acylation, being an essential modification for virus replication. To address whether the cytoplasmic tail is an essential part
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of an acylation signal, I expressed mutant HA with a vaccinia virus T7 system in CV1-cells and analyzed acylation by metabolic labeling with \([^{3}H]\)-palmitate.

Labeling with \([^{35}S]\)-methionine/cysteine done in parallel, immunoprecipitation, SDS-PAGE under non-reducing conditions and fluorography showed two HA bands. The lower HA-band is likely to represent the mannose-rich precursor with higher SDS-PAGE mobility and the slower migrating product with complex glycans that has been described for HA for WSN (Rodriguez-Boulan et al., 1984). The upper band is the mature product of HA. The intensity of the upper and lower bands differed between WT HA and the mutants, especially HA with a deleted tail exhibits more of the precursor relative to the mature form indicating that its intracellular transport is retarded. The fluorogram of the \([^{3}H]\)-palmitate labeled samples revealed that each HA mutant is labeled, but with varying intensity. Densitometric quantification of \([^{35}S]\)-methionine/cysteine and \([^{3}H]\)-palmitate labeled bands revealed no meaningful reduction in palmitoylation (Figure 5.10). Both \([^{35}S]\)-methionine/cysteine labeled bands and the \([^{3}H]\)-palmitate band revealed a \([^{3}H]\) to \([^{35}S]\) ratio of 90% for Q560E and 86% for I563Q relative to WT HA (100%). The G577Stop mutant has a \([^{3}H]\) to \([^{35}S]\) ratio of 62%, but contains only one acylated cysteine. Meaning that the (non-lethal) HA-mutant Q560E and the mutant I563Q are acylated with only slightly reduced efficiency (~90%) relative to WT HA (100%). Acylation of G577Stop, where two acylated cysteines are deleted, is reduced to ~60%, suggesting that the one remaining acylation site at the end of the TMR is efficiently used. Thus, the mutations introduced into HA did not prevent acylation, but subtle differences in the occupancy of individual acylation sites cannot be excluded.
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5.4 Mass spectrometry

The main focus of the project was to analyze the acylation pattern of the created mutants. In order to do this, rescued virus mutants and WT virus were propagated in cell culture and in embryonated chicken eggs and then prepared by ultracentrifugation (see methods for details). The samples were prepared for mass spectrometry analysis using different methods, as described in the following.

5.4.1 Establishment of a new sample preparation procedure for mass spectrometry

In previous sample preparation protocols for MS analysis the HA-ectodomain was removed by digestion of virus particles with bromelain, shaved particles were pelleted, the membrane-anchoring fragments of HA were extracted to chloroform phase and analyzed by MALDI-TOF MS. In order to remove impurities that might affect proteolytic cleavage of HA, virus particles were purified from the allantois fluid of embryonated chicken eggs by sucrose-gradient purification. Since the viral mutants were compromised in growth, Dr. Larisa Kordyukova (A. N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University) developed an alternative procedure. This procedure does not require the material demanding step of gradient purification and can thus be performed with virus pelleted from the supernatant of MDCK-cells. In the new approach virus particles are separated by non-reducing SDS-PAGE, proteins are blotted onto PVDF-membranes and are visualized by Ponceau S staining. The HA-band is cut from the membrane, digested with trypsin and the resulting peptides are eluted and analyzed by MALDI-TOF MS (Figure 5.11, A).
To test whether the new approach yields reliable results both procedures were compared with the same virus preparation, WSN WT virus grown in MDCKII cells. Each procedure utilizes a different protease and therefore different peptides were observed in the MS-spectra (Figure 5.11 B). According to its specificity trypsin can cleave the polypeptide chain after arginine or lysine. It cuts HA mainly after arginine 513 located 15 amino acid residues above the TMR.
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resulting in a peptide that matches to the complete membrane anchoring plus linker region spanning amino acids 514 to 565. Two other cleavage sites, after lysines 515 and 520 are also present in lower proportion (Figure 5.11 C).

Bromelain possesses a wider substrate specificity compared to trypsin, but only glycine 518 and lysine 520 located in the linker region connecting the TMR with the well-structured HA ectodomain are spatially accessible for the enzyme. Thus, the resulting peptides are shorter, spanning amino acids 519 to 565 and 521 to 565, respectively. Each of the mentioned peptides is present as two main peaks representing triple palmitoylated peptide and a peptide containing two palmitates and one stearate. A very small third peak representing a peptide containing one palmitate and two stearates also appears sometimes. Calculation of the amount of stearate revealed very similar results, 20.1% for the new blotting procedure and 21.1% for the old extraction method. Hence, the new method is reliable and able to detect acylated peptides using ~5-10 µg of protein, i.e. a Ponceau S stained band.

Surprisingly, however, the stearate content from HA of the WSN virus was much higher as the amount previously determined for the closely related PR8 virus (12.6%, Kordyukova et al., 2008) that contains a very similar C-terminal amino acid sequence. Since the virus was grown in MDCKII cells and embryonated eggs, respectively, the host cells supposedly affect the acylation pattern of HA.

5.4.2 Comparison of acylation patterns of HA synthesized in avian and mammalian cells

All previous MS-analysis (by Larisa Kordyukova and Marina V. Serebryakova, A. N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University) was performed with virus strains purified from an avian host, embryonated chicken eggs. In this system, HAs from human virus strains have less stearate (12-18%) compared to HAs from avian strains (18%-35%, Kordyukova et al., 2008; Kordyukova et al., 2011). In order to analyze whether HA from human virus strains contains more stearate if grown in mammalian cells MS-analysis was performed with WSN using a variety of samples. Recombinant WSN (generated from transfected MDCKII cells and then further amplified either in eggs or in MDCKII cells) was compared to a natural WSN virus strain amplified in eggs or in MDCKII cells using both the old extraction method and the new blotting procedure. In each case WSN virus grown in eggs had less HA-bound stearate (8% to 12%) compared to WSN grown in
5. Results

MDCKII cells (20.1% to 22.9%, results are summarized in Table 5.2). Thus, neither the sample preparation for MALDI-TOF MS nor the origin of the virus (from plasmid or from virus strain) significantly affected the result indicating that mammalian cells attach more stearate to human virus strains compared to cells of embryonated chicken eggs.

**TABLE 5.2**

HA-bound stearate in virus grown in avian and mammalian cells. Determined by MALDI-TOF MS. Results are represented, as mean ± SD. Remainder is palmitate.

<table>
<thead>
<tr>
<th></th>
<th>WSN</th>
<th>PR8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant virus</td>
<td>Natural virus strain</td>
<td>Natural virus strain</td>
</tr>
<tr>
<td>Eggs$^2$</td>
<td>MDCK$^2$</td>
<td>Eggs$^2$</td>
</tr>
<tr>
<td>7.9±4.7</td>
<td>22.9±1.2</td>
<td>7.6±1.5</td>
</tr>
</tbody>
</table>

1: New blotting protocol. 2: Old extraction protocol. A: Published in (Kordyukova et al., 2008).

Additionally, the analysis of the HA from the PR8 strain was of interest. This HA is used as a backbone to make seasonal Flu-vaccines, i.e. the glycoproteins of circulating human strains are reassorted into PR8 and the resulting virus strain is amplified in embryonated eggs for vaccine production. Identical to HA of WSN a tryptic peptide that matches to the complete membrane anchoring region spanning amino acids 514 to 565 was observed (Figure 5.12).

**FIGURE 5.12**

MS result from PR8 virus grown in avian and mammalian cells. (A) Non-reducing SDS-PAGE and Coomassie staining of A/Puerto Rico/34 (H1N1) viirons grown in MDCKII cells or in embryonated eggs. The position of viral proteins (HA, NP, M1) and molecular weight markers in kDa is indicated. The strong band below NP is probably albumin from the serum used to grow virus in cell culture. HA was analyzed with the new blotting-MS protocol. (B) MALDI-TOF MS analysis. Mass spectra obtained in reflector mode are depicted. Indicated are the m/z values of the peaks, the first and last amino acid residue of the corresponding peptides and the number and type of HA-bound fatty acid. For simplicity, additional minor peaks producing the peptides HA [516-565] and HA [521-565] with lower m/z values are neither shown nor used for stearate calculation. The calculated stearate content found for the HA peptides [514-565] from several spectra is listed in Table 5.2. (C) Amino acid
5. Results

sequence of the HA C-terminal region indicating the prevailing site of cleavage by trypsin. Cysteines as potential acylation sites are enlarged and the TMR is underlined.

Calculation of the stearate amount in the two peaks revealed low stearate content in HA if the virus was grown in eggs (8.2%) compared to virus obtained from MDCKII cells (21.2%). A low stearate amount was also previously determined with egg-grown PR8 virus using the old extraction method (Kordyukova et al., 2008). Thus, it appears that HA from human strains is incompletely stearoylated if the virus is amplified in avian cells. However, no under-acylated peptides were observed indicating that the stearoylation site at the end of the TMR is occupied by palmitate. This was earlier confirmed by MALDI-TOF-TOF fragmentation analysis (Serebryakova et al., 2006).

5.4.3 Effects of point mutations on the acylation pattern

Next the fatty acid pattern of mutant HAs from recombinant WSN virus grown in MDCKII cells was determined using the blotting procedure. Since the error rate of the viral polymerase is high and virus with nucleotide reversions to the WT sequence rapidly outgrows the mutant virus, I sequenced the genomic RNA encoding the C-terminus of HA and only virus preparations with the desired sequence were analyzed by MALDI-TOF MS. Similar to the WT HA, trypsin cleaves the polypeptide chain after arginine 514, but also after arginine 516 such that two peptides appear in the spectra that encompass from the cleavage site until HA’s C-terminus (Figure 5.13).
The observed peptide masses are also consistent with the expected sequences shown in Table 5.1 indicating that no reversion to WT (or any other amino acid) occurred.

Each of the mentioned peptides is present as two main peaks representing triple palmitoylated peptide and a peptide containing two palmitates and one stearate. However, the intensities of the peaks clearly differed between the HA mutants indicating that the introduced amino acid
replacements changed the acylation pattern. The stearate content for this and usually one other virus preparation was calculated; the results are graphically shown in Figure 5.14.

A meaningful reduction in the stearate content was observed only for G557E, which contains 15% of the longer acyl chain in comparison to 23% stearate calculated for WT HA. Since the conservative replacement of the completely conserved glycine 557 by an alanine has no significant effect (24% stearate) it is rather the insertion of the acidic glutamic acid that is responsible for the observed stearate reduction in G557E. Exchange of glutamine by glutamic acid in position 560 caused a very minor increase in the stearate proportion (26%) indicating that the insertion of a negatively charged residue into the cytoplasmic tail does not reduce stearoylation per se. However, the conservative replacement I563L at the hydrophobic C-terminus of HA significantly raised the stearate proportion to 31%. The largest increase in the stearate content to 33% was observed with the HA-mutant that contains a serine instead of the glycine located in the middle of the TMR.

One of the HA-mutants (G557A) with egg-grown virus was also analyzed. Here, the old, better-established procedure for sample preparation was used (Figure 5.15).
5. Results

Around 12\% of stearate was determined, which is identical to the WT HA analyzed with the same procedure, but half as much as was calculated for the same mutant virus grown in MDCKII cells. This result reinforces our notion, that avian cells attach less stearate to the HA of human virus compared to mammalian cells. In addition, non- or under-acylated peptides were not detected suggesting that the mutation did not alter the stoichiometry of S-acylation.

**FIGURE 5.15**

MS results from WSN wild type (WT) and G557A mutant grown in avian cells. (A) Non-reducing SDS-PAGE and Coomassie staining of A/WSN/33(H1N1) virions (control) and WSN G557A recombinant viral particles grown in eggs before (-) and after (+) digestion with bromelain and pelleting (100 000×g, 1.5 h, 4°C). The bromelain digested viral particles were further analyzed with the old extraction-MS protocol. The position of viral proteins (HA, NP, M1) and molecular weight markers in kDa is indicated. (B) MALDI-TOF MS analysis. Mass spectra obtained in reflector mode are depicted. Indicated are the m/z values of the peaks, the first and last amino acid residue of the corresponding peptides and the number and type of HA-bound fatty acid. Indicated is the percentage of HA-bound stearate (mean ± S.D.) calculated from 4-6 spectra obtained for each of 1-3 digestions performed for three (control) or one (G557A) virus preparations. Note that non- or mono-acylated HA peptides (peaks with m/z values around 4845 and 5083, respectively) were not detected in the spectra, and only very tiny peaks of doubly acylated peptides (m/z around 5321) were observed. (C) Amino acid sequence of the HA C-terminal region indicating the amino acid exchange and the site of cleavage by bromelain. The arrow lengths approximately correspond to the respective peak heights in the spectra. Cysteines as potential acylation sites are enlarged and the TMR is underlined.
5. Results

In summary, point mutations introduced into the TMR or cytoplasmic tail of HA modestly affected the fatty acid pattern of HA, but did neither abolish stearoylation nor S-acylation per se.

5.4.4 Shifting of a cysteine from the TMR to the cytoplasmic tail

Finally, the fatty acid pattern of the C554S+L559C mutant grown in MDCKII cells where the usually stearoylated transmembrane cysteine was shifted to a cytoplasmic location was determined. Only very small peaks representing peptides with two palmitates and one stearate were detectable, the by far predominant peptide contains three palmitates (Figure 5.16).

**FIGURE 5.16**
MS results from a WSN mutant and a natural H9 subtype HA with shifted cysteine. The A/WSN/33 (H1N1) recombinant virus mutant [C554S + L559C] was grown in MDCKII cells and analyzed using the blotting approach while the A/Teal/Primorie3631/02 (H9N2) virus was grown in embryonated chicken eggs and analyzed using the old protocol based on bromelain digestion of virions. Mass spectra obtained in reflector mode are depicted. Indicated are the m/z values of the peaks, the first and last amino acid residue of the corresponding peptides and the number and type of HA-bound fatty acid. The stearate content was calculated as 3.3% for the H1 subtype mutant and 4.5% for H9 subtype HA. The peaks of both H1 subtype HA [516-565], HA [514-565] and H9 subtype HA [518-560], HA [516-560] were used for calculations. Depicted below are the spectra are the C-terminal amino acid sequences of H1 and H9 subtype HA indicating the cleavage sites by trypsin and bromelain, respectively. Cysteines as potential acylation sites are enlarged and the TMR is underlined.
Calculation of the stearate content revealed that it was reduced to 3.3% in the HA of the C554S+L559C mutant compared to 23% stearate calculated for WT HA. Thus, the location of an acylated cysteine relative to the end of the transmembrane span is the decisive factor for the attachment of palmitate versus stearate as demonstrated both for an egg-grown avian virus as well as for a human virus from MDCKII cells.
6. DISCUSSION

In this study I used reverse genetics and mass spectrometry to define the molecular signals that determine site-specific attachment of palmitate and stearate to the transmembrane region (TMR) and the cytoplasmic tail (CT) of hemagglutinin (HA) of Influenza virus and analyzed their influence on the properties of the virus. I generated seven mutants with amino acid exchanges in the vicinity of the three acylation sites and one mutant with a shifted acylation site. Two of the mutants with drastic amino acid exchanges did not result in viable virus. The viable mutants were examined for their growth properties, viral protein composition, packaging of RNPs and fatty acid attachment to HA by mass spectrometry. The HA of the two non-viable mutants was studied on the protein expression level for palmitoylation.

**Are only the acylation sites or also other amino acids in the CT essential for virus growth?**

I observed that without a functional CT, no viable virus could be generated. The mutation G557Stop, which inserts a stop codon directly downstream of the end of the TMR, never resulted in rescue of infectious virus. This finding is in accordance with a previous study, where the deletion of the CT in HA of influenza B resulted in 5-log titer reduced virus replication compared to wild-type virus (Imai *et al.*, 2012). In another study with Influenza A virus however (Jin *et al.*, 1994), where the CT was deleted, rescued virus assembled and replicated almost as efficiently as virions with wild-type HA. Here, it was concluded that the CT is not essential for the replication. Yet, since at the time no efficient plasmid-based reverse genetics system was available, they utilized HA of the A/Udorn/72 (Ud) strain, a H3N2 virus, in a helper virus (WSN) background and the result was that revertant virus was isolated, proposing that the CT did present an advantage. My finding on the other hand is the consequence of modern reverse genetics using the eight plasmid-based expression system for WSN (Hoffmann *et al.*, 2000). The combination of genes used from different viral strains in the mentioned study (Jin *et al.*, 1994) might be the reason for this rather mild phenotype. In addition, it was later reported
that when M1 derived from WSN expressed in the Ud background, recombinant virus could be generated, even without HA acylation (Chen et al., 2005). Evidently, the CT is essential for the virus to amplify. Regarding the CT’s functions, it might influence the interaction with other proteins e.g. M1 and RNPs or direct the protein into rafts at the plasma membrane for correct assembly and budding of newly formed virions.

From a study where the acylation sites (Ac) were deleted in the Ud strain (Chen et al., 2005) it is known that no viable virus could be rescued when all three (Ac1, Ac2 and Ac3) or both of the cysteines in the CT (Ac2 and Ac3) were exchanged by a serine. In Figure 5.1 one can see the position of the three acylation sites (the three cysteines, here in the WSN strain background). Other combinations of the substitution of two acylation sites (Ac1 and Ac2 or Ac1 and Ac3) and the substitution of one cysteine led to viruses with smaller plaques, whereas the substitution of only Ac1 (situated in the TMR) had no influence whatsoever and led to plaque sizes comparable to wild-type. In the same study, deletions in the WSN strain at Ac2 led to highly attenuated viruses and in Ac3 mutants failed to yield infectious particles. This undermines the assumption that the region of Ac2 and Ac3 (the CT) as well as these cysteines are essential for virus recovery. But the report also mentions that an altered M1 protein could counteract a deficiency in virus assembly caused by deleted acylation sites.

In another study shown on the WSN background (Zurcher et al., 1994) deletion of the cysteine at Ac2 or double or triple mutations on the acylation sites never resulted in any infectious-particle formation. From newer results with plasmid-based reverse genetics conducted in WSN by my colleague Stefanie Siche, this could be confirmed: Only the Ac1 mutant yielded viable virus. All this considered, the CT and the here-situated acylation sites seem to be the determining factor for virus growth. However, it remains to be elucidated whether other amino acids in the CT apart from the cysteines disturb virus assembly and replication.

My truncated tail mutant is de facto an Ac2 and Ac3 double mutant that, as mentioned before does not yield viable virus. The palmitoylation of this mutant was analyzed on protein expression level (Figure 5.10) and the results revealed that it is regularly acylated at its one remaining acylation site situated in the TMR (Ac1).
Furthermore, the mutation I563Q never yielded any viable virus. This mutant is interesting, because its failure to produce viable virus particles might be due to two different reasons: The inhabited acylation site and/or the hydrophobic region. The bulky and hydrophilic amino acid glutamine, which was inserted in replacement of the hydrophobic amino acid isoleucine, might have made it impossible for an acyl-transferase to acylate the adjacent cysteine. The mutant I563L on the other hand was rescuable and produced viruses that grew similar to wild-type. The inserted amino acid leucine is a conservative exchange of isoleucine and did not provoke any substantial biophysical changes that would lead to sterical problems at that position nor did it change chemical properties being also hydrophobic. The palmitoylation of this mutant was also assessed on protein expression level and showed that the acylation was only merely reduced, compared to wild-type, as could be demonstrated by metabolic labeling (Figure 5.10). So the assumption that a prevented acylation leads to a failure in virus particle formation is rebutted. This indicates that the hydrophobicity of the region weighs more than the acylation itself. In order to emphasize this, one could measure the palmitoylation of remaining acylation sites in Ac2, Ac3 and double mutants on the protein expression level.

Another process, which might be disturbed in the virus life cycle, is membrane fusion. In one study the TMR and CT were replaced by a glycolipid anchor (Kemble et al., 1994), in another the acylation sites of HA of the Fowl Plaque Virus (H7N1) were deleted (Wagner et al., 2005). In both cases with a non-functional CT, after reaching the state of hemifusion, the full fusion and the formation of a fusion pore were not achieved. A similar observation could apply to my tailless mutant. However, the interpretation of the G557Stop mutant does not confer what exactly in the deleted CT sequence is necessary for the virus to amplify. Either the acylation sites situated in the CT or the chemical properties of the entire sequence surrounding (and including) the cysteines could be the determining factor for the virus growth defect, in both processes, budding and fusion.

**Amino acids in the TMR of HA affect virus fitness**

While rescuing virus mutants, I observed three kinds of reversions: One was in the CT and occurred only once, resulting in a back mutation to the wild-type sequence (G557G) and the other two happened in mutations inserted into the TMR (C554I+L559C and G547S) (Figure 5.3). Table 6.1 displays the other possible amino acid exchanges for the three observed reversions, which would result from a single nucleotide substitution in the
6. Discussion

respective codon.
In the mutant C554S+L559C a substitution of C554 would have been possible with alternative amino acids such as arginine, asparagine, threonine, glycine and cysteine. A substitution that would lead to arginine is unlikely to happen, since it would insert a long residue into the thus already packed region. Additionally, this would introduce a positive charge, while the TMR generally does not tolerate any charges. The nucleotide exchange that would lead to the amino acids of asparagine, threonine or glycine, would insert an uncharged, but polar amino acid. Isoleucine is the only hydrophobic amino acid that may arise from a single nucleotide exchange at that position. The C554I+L559C revertant arose three out of five times. This suggests that this mutant tolerates a hydrophobic amino acid (isoleucine) at position 554 rather than a hydrophilic one. In the functional virus, this position is acylated and together with the attached fatty acid, the cysteine residue is enlarged and becomes a hydrophobic character. Thus, the introduced isoleucine confers the same characteristics, as an otherwise acylated cysteine residue at this position would have. At the second point mutation in this double mutant a hydrophobic amino acid (leucine) is exchanged against a hydrophilic amino acid (cysteine). The first point mutation in this double mutant (which evokes no biophysical change, cysteine to serine) is reverted into a hydrophobic amino acid (isoleucine). Interestingly, the reversion from serine back to cysteine does not occur.

Perhaps the exchange to isoleucine is preferred in combination with the exchange from a hydrophobic (leucine) to a hydrophilic (cysteine) amino acid at position 559. This reversion at position 554 might act as compensation for the exchange at 559.

The revertant G547S completely outgrew G547I after two amplifications (Table 6.1). Besides this serine, only two hydrophilic amino acids could arise (asparagine, threonine) by a single nucleotide exchange. Other possible single nucleotide exchanges in that codon would specify a large hydrophobic amino acid (isoleucine, leucine, phenylalanine, valine, methionine). Since the latter are typical constituents of transmembrane regions, the repeated reversion to serine strongly suggests that a small amino acid is preferred at that position. Additionally, at this position the protein needs a neutral or hydrophilic amino acid, possibly due to chemical interactions. Of the three possible ones, serine has the smallest residue and might possibly be favored sterically.
6. Discussion

**TABLE 6.1**
Possible single nucleotide changes in each codon of the three mutants in which reversions were observed. Apart from the reversions that actually happened, each of three nucleotides of the mutated codons could have theoretically reverted to a different one. These different nucleotide exchanges would lead to altered amino acids at that position, which again have various properties, e.g. hydrophobicity. “+” stands for a hydrophobic, “-” for a hydrophilic and “n” for a neutral amino acid.

<table>
<thead>
<tr>
<th>1st Nucleotide</th>
<th>Amino acid</th>
<th>2nd Nucleotide</th>
<th>Amino acid</th>
<th>3rd Nucleotide</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTT</td>
<td>Leucine</td>
<td>+</td>
<td>AAT</td>
<td>Asparagine</td>
<td>-</td>
</tr>
<tr>
<td>GTT</td>
<td>Valine</td>
<td>+</td>
<td>ACT</td>
<td>Threonine</td>
<td>-</td>
</tr>
<tr>
<td>TTT</td>
<td>Phenylyalanine</td>
<td>+</td>
<td>AGT</td>
<td>Serine</td>
<td>-</td>
</tr>
</tbody>
</table>

**1. G547I / G547S**

Wild-type GGG (glycine) → **Mutagenesis** ATT (isoleucine) → **Reversion** AGT (serine) →

**2. C554S+L559C / C554I+L559C**

Wild-type TGT (cysteine) → **Mutagenesis** AGC (serine) → **Reversion** ATC (isoleucine) →

<table>
<thead>
<tr>
<th>Wild-type</th>
<th>TGT (cysteine)</th>
<th>Mutagenesis</th>
<th>AGC (serine)</th>
<th>Reversion</th>
<th>ATC (isoleucine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGC</td>
<td>Arginine</td>
<td>-</td>
<td>AAC</td>
<td>Asparagine</td>
<td>-</td>
</tr>
<tr>
<td>GCC</td>
<td>Glycine</td>
<td>n</td>
<td>ACC</td>
<td>Threonine</td>
<td>-</td>
</tr>
<tr>
<td>TGC</td>
<td>Cysteine</td>
<td>-</td>
<td>ATC</td>
<td>Isoleucine</td>
<td>+</td>
</tr>
</tbody>
</table>

**3. G557E / G557G**

Wild-type GGG (glycine) → **Mutagenesis** GAA (glutamic acid) → **Reversion** GGA (glycine) →

<table>
<thead>
<tr>
<th>Wild-type</th>
<th>GGG (glycine)</th>
<th>Mutagenesis</th>
<th>GAA (glutamic acid)</th>
<th>Reversion</th>
<th>GGA (glycine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAA</td>
<td>Lysine</td>
<td>-</td>
<td>GCA</td>
<td>Alanine</td>
<td>+</td>
</tr>
<tr>
<td>CAA</td>
<td>Glutamine</td>
<td>-</td>
<td>GGA</td>
<td>Glycine</td>
<td>n</td>
</tr>
<tr>
<td>TAA</td>
<td>Stop</td>
<td>n</td>
<td>GTA</td>
<td>Valine</td>
<td>+</td>
</tr>
</tbody>
</table>

The third observed reversion occurred only once out of five attempts and resulted in a back mutation to wild-type. Exchanges to other nucleotides in that codon could specify charged, hydrophobic or hydrophilic amino acid residues (Table 6.1). However, at this position the pressure to revert obviously is not as great as in the other two cases.

Altogether, the reoccurring amino acid reversions propose that the conserved residues in TMR and CT confer a growth advantage. Since I have only created two viruses with mutations in the transmembrane span, I conjecture very tentatively from the resulting reversions. I have not detected (with a one-time exception of the silent mutation that lead to the wild-type reversion G557G) any such reversions in viruses with mutations in the CT. Consequently, I assume that alterations in the amino acid context within the region of the transmembrane span of HA might be less tolerated than in the CT. The TMR is inserted into the membrane and hence might likely be more compact and less flexible in its chemical and sterical composition. Furthermore, the essential amino acids in the CT are the cysteines at Ac2 and Ac3, which are palmitoylated.
6. Discussion

**Point mutations in the CT hardly have an impact on replication and viral protein composition**

I could not observe any substantial impairment in the growth kinetics of any of the virus mutants generated, including the one with the shifted cysteine (Figure 5.3), indicating that mutations in the CT do not have any drastic impact on the replication. Neither could any smaller plaques compared to wild-type be identified (Figure 5.2), nor any aberrant virions be detected by electron microscopy (Figure 5.5).

Additionally, the viral protein composition, as presented in Figures 5.6 and 5.7, varies somewhat between mutants and wild-type virus particles, but also among different preparations of the same mutant. Therefore, a statistically significant difference of the three proteins (M1, NP or HA) is significantly elevated or reduced proportionally compared to wild-type virus. Consequently, I conjecture that these mutations do not impact the protein composition of virus particles.

In all mutated virus particles a higher variability of the protein and RNP content was observed compared to wild-type virus (Figure 5.8 and 5.9). The quantification of the amount of RNPs present in the mutant virions demonstrated that two mutants (G547S, Q560E) have approximately the same or comparable amounts to wild-type. Nonetheless, a statistically significant difference was obtained only for the mutant I563L with a RNP content reduced to app. 30% compared to wild-type.

It is supposed that recruitment of M1 to the viral assembly site occurs by hydrophilic binding forces, such as salt bridges or hydrogen bonds. Together with the second acylation site (Ac2) the hydrophobic patch ICI at the C-terminal end possibly anchors the CT to the inner leaflet of the lipid bilayer (Figure 6.1). As mentioned above, virus rescue is not possible without Ac2 or/and Ac3 or a sufficiently hydrophobic C-terminal end of the CT: The insertion of the hydrophilic residue glutamine in the I563Q mutant counteracts virus rescue, while its exchange by a long and hydrophobic leucine chain affects it only marginally.

In close proximity to this area is the conserved basic amino acid arginine (Figure 6.1), which is able to cooperate with the head groups of negatively, charged lipids at the inner side of the membrane (van Meer et al., 2008). Consequently, if many amino acid residues (or attached fatty acids) of the CT of HA interact with the plasma membrane, only four of them (N, G, S and Q) remain to specifically bind M1 (Figure 6.1). Still, substituting two
of them (G557 and Q560) did not reduce virus growth. This suggests that they do not bind to M1, at least not with high-affinity since one would assume that replacing an amino acid making an essential contact with M1 would more drastically impair virus growth.

![Diagram of HA and M1 interaction](image)

**FIGURE 6.1**
**Model of the cytoplasmic tail of HA.** Amino acids, indicated in single letter code, which were exchanged in this study, are underlined. Essential amino acids, i.e. the non-conservative exchange of which prevented rescue of infectious virus particles, are in red. Hydrophobic or positively charged amino acids that probably interact with the acyl chains or head groups of (negatively charged) lipids are in purple. The transmembrane proximal acylation site (Ac1) is in black, whereas the cytoplasmic acylation sites (Ac2 and Ac3) are in red.

However, it was explicitly proven that HA functionally cooperates with M1 during budding (Chen et al., 2005). Supposedly, M1 reacts with amino acid side chains situated in the rather variable interior of the TMR of HA. This notion might help to understand why, as mentioned previously, lethal HA acylation mutants in Ud yield virus when expressed with M1 from WSN (Chen et al., 2005). Otherwise, the CT of HA and M1 might not physically interact, but promote virus budding in an indirect manner. They might attract other lipid components to the plasma membrane and thus alter its fluidity to establish a firm and stable assembly site.

**Virus growth in avian cells results in a different acylation pattern**

Employing both the new and old procedure for sample preparation revealed that HA from two human H1N1 strains (WSN and PR8) contains more stearate (20-23%) if grown in mammalian MDCK cells compared to the same virus amplified in avian cells or embryonated chicken eggs (8-12%, Figure 5.12, Table 5.1). Moreover, an earlier study
presented that HA from avian viruses already contains a high proportion of stearate (22-35%) when the virus was grown in eggs (Kordyukova et al., 2008; Kordyukova et al., 2001). From these two observations, I speculate that viruses have adapted to their natural host cells such that they receive a high stearate proportion. However, before any firm conclusions can be drawn more experiments with avian, porcine, equine and human Influenza viruses grown in natural and non-natural host cells ought to be performed. Nevertheless, these observations already signify that there are cell type specific differences in the acylation pattern of HA. The same observation might also be valid for cellular proteins, since viruses rely on the cellular acylation machinery. On top of that, the results emphasize the very recent discovery that influenza virions produced by mammalian and avian cells have distinct protein compositions (Hutchinson et al., 2014). These findings imply that the host cell influences the composition of virus particles.

The introduced mutations never prevented S-acylation

I have also showed that none of the introduced mutations prevented S-acylation of HA. The deleterious mutations in HA that did not allow producing infectious virus particles, i.e. I563Q and G557Stop, are acylated as efficiently as wild-type HA that can be deduced from metabolic labeling with $[^3]$H-palmitate (Figure 5.10). Thus, the functional defect in HA introduced by the mutation is not due to a significant blockade of acylation. In the MS-spectra from HA-mutants for which I could generate infectious virions peaks that correspond to non-acylated or under-acylated peptides were never detected (Figs. 5.12-16). It is even possible to shift a cysteine from the TMR to a cytosolic location without compromising attachment of fatty acids at any of the three acylation sites.

However, a word of caution regarding the quantitative nature of MS-data is necessary. Although in principle non- and under-acylated peptides with this MALDI-TOF setting could be detected (Kordyukova et al., 2004), presently I cannot exclude that non- or under-acylated peptides containing exchange of crucial amino acids “do not fly” or are lost during sample preparation. Thus, I cannot rule out that some peptides are under-acylated, i.e. that the mutations reduced the efficiency of acylation at certain sites. Nevertheless, it is fair to conclude that a putative acyl-transferase does not need to recognize and interact with amino acids in the vicinity of acylation sites as an essential step of its catalytic cycle. Most likely, a reactive cysteine, i.e. reduced and deprotonated,
which is accessible to the active site of an enzyme, i.e. exposed near the cytoplasmic face of the membrane, is sufficient for acylation to occur (Rodriguez-Boulan et al., 1984).

The location of the cysteine determines the type of S-acylation

Among all the generated point mutations, only one resulted in a significant change of fatty acid pattern: the one with the TMR proximal acylation site shifted to the CT (C554S+L559C). This shifting of the cysteine to a cytoplasmic location drastically reduced the attachment of the longer chain fatty acid to 3%. The same proportion of stearate was found in the HA of a natural H9 subtype virus strain (Table 1.1, Figure 5.16), in the HA of H7 subtype if the TMR cysteine was exchanged for a serine and in the HA of Influenza B virus that evolutionary lacks a cysteine residue at the end of the TMR (Kordyukova et al., 2008). Consequently, I conclude that the location of a cysteine relative to the end of the TMR is the decisive factor for the attachment of stearate.

It is now obvious that the length of the CT originally proposed to be important for stearoylation (Veit and Reverey, 1996) plays no role at all. The fusion protein of Newcastle Disease virus for instance is stearoylated at a TMR cysteine despite containing a long (27 aa) CT (Kordyukova et al., 2010).

The amino acid context has a marginal effect on differential S-acylation

An exchange of amino acids only subtly influences the proportion of stearate attached to HA (Figs. 5.13-5.15). The most prominent effect was calculated for the mutant G547S showing an increase in the stearate proportion from 23% (wild-type HA) to 33%. A serine is more compatible with a α-helical conformation the TMR of HA likely adopts (Mineev et al., 2013). Thus, I hypothesize that the exchange of the conserved glycine in the TMR by a serine straightens the transmembrane span and places the TMR cysteine in a better position for stearoylation (Figure 6.2). Alternatively, it was proposed that glycine residues in the transmembrane region are oriented toward the helix-helix interface to mediate interactions (Javadpour et al., 1999). Indeed, molecular modeling of the closely related H6 subtype HA implies that this is the case for the HA TMR (Kordyukova et al., 2011). Substitution of glycine by serine might alter these interactions and/or rotate one
TMR-helix against the other. In all these cases the cysteine at the end of the TMR might become better accessible by a putative stearate-specific acyl-transferase.

The conservative substitution of isoleucine with leucine at the hydrophobic patch at the C-terminus of HA (and thus in a distance of nine amino acids from the TMR cysteine) increased the stearate proportion to 31%. Due to limited sample material the fatty acid pattern for each acylation site could not be identified with MS/MS sequencing as in previous analyses (Kordyukova et al., 2008). However, in previous analyses attachment of stearate at sites other than the TMR cysteine was never observed.

**FIGURE 6.2**
Model of what effect the point mutations in the transmembrane region (TMR) and cytoplasmic tail (CT) of HA might have on the S-acylation. Depicted is the membrane (yellow and blue), the TMR (blue bar spanning the lipid bilayer) and the CT of HA (blue stripe). The Cs represent cysteines, which are S-acylated by palmitate (black crinkled line) or stearate (red crinkled line). Shown are wild-type (WT) and the four point mutations analyzed by MS.
Thus, it is likely that an additional stearate (relative to the wild-type HA) is also attached here and not at the C-terminal cysteine 564. One might speculate that the hydrophobic patch at the C-terminus of the CT interacts with the TMR thereby contributing to a subtle increase of the stearate content at cysteine 554.

A reduction in the stearate content to 15% was observed in the mutant G557E. This is not due to replacement of the completely conserved, cytosolic glycine residue since its exchange by an alanine had no effect on the stearate content.

It is also not caused by the presence of glutamic acid per se since in the mutant Q560E a slight increase in the stearate content was noticed. I suppose that insertion of a hydrophilic residue at the membrane-cytosol interface pulls the cysteine out of the membrane and exposes it to the cytosol such that it is in a less favorable location for stearate attachment (Figure 6.2). Since the TMR of HA is very long (27 aa), it has a wide range to move perpendicular to the plane of the membrane, especially at the intracellular site of acylation, the late ER or early-Golgi region, where the membrane bilayer is supposed to be thinner. In summary, conservative as well as drastic exchange of amino acids in various regions at HA’s C-terminus modestly alter the stearate proportion in a rather unpredictable manner. In summary, I assume that the mutations alter the location of the cysteine within the transition region between the membrane bilayer and the cytosol thereby affecting its accessibility for a putative stearate specific acyl-transferase.

Could S-Acylation have a similar effect on the adaptation of influenza viruses to new hosts such as N-linked glycosylation?

In 2012, a study on the potential for respiratory droplet A/H5N1 influenza virus to evolve in a mammalian host and become transmissible among ferrets alerted scientists all over the world (Imai et al., 2012). It was elucidated that the minimal set of amino acid substitutions for the avian virus to adapt to a mammalian host includes two in the receptor binding site of HA, known to convert the virus from a α-2–3 linked sialic acid (found rather in birds) to a α-2–6 linked sialic acid (found rather in humans) specificity (Herfst et al., 2012), two others in HA of which one disrupts a N-linked glycosylation sequon and one in PB2 (Hatta et al., 2007; Imai et al., 2012). Some of the substitutions identified demonstrated an increase of virus fitness within the same host, specifically due to the loss of glycosylation. Apparently, these amino acid substitutions can yield airborne-transmissible A/H5N1 viruses by affecting glycosylation (among other biological traits as
receptor binding and replication). Additionally, it was observed for other influenza strains that in order to escape immune pressures from their hosts, they alter their protein glycosylation by glycosylation sites migration and increase of their numbers as a possible evolutionary factor (Sun et al., 2012). Genome-based glycosylation site prediction and modeling of glycoprotein structures could indicate that influenza H1N1 viruses underwent diverse alterations of protein glycosylation in different hosts (Sun et al., 2011). Furthermore, it could be shown that HA N-glycosylation patterns were dependent on the cell line the virus was propagated in. By adaptation to new cell lines the patterns stabilized fast within one or two following passages (Roedig et al., 2011).

Overall, N-linked glycosylation is a post-translational modification attached to the virus by the cellular machinery, which can be altered by mutations in the amino acid sequence of the viral protein. Hence, the virus may indirectly modify its glycosylation pattern and thus influence its virulence and response to the host. The shift of the S-Acylation pattern of HA 1 observed when having grown viruses in avian cells compared to mammalian cells on the other hand was not due to mutations in the amino acid sequence, which remained the same. So, as mentioned earlier, in this aspect it is rather the cellular availability of specific fatty acids that determines the acylation pattern of the virus in contrast to N-linked glycosylation.

Yet, as previously mentioned an adaptation to the host was observed and one could imagine that it might have an impact on the overall pathogenicity of a viral strain. But has a correlation between pathogenicity of the strain and acylation pattern ever been observed? In a previous study, the fatty acid pattern in 10 different influenza strains from human and avian origin has been measured (Kordyukova et al., 2008). The amount of stearate varied from 12% to 35% in HAs from strains of subtypes H1, H3, H5, H7 and H10 (see Introduction, Table 1.1). Some of these strains were highly pathogenic, such as avian influenza (HPAI) viruses (A/FPV/Rostock/34 and A/Vietnam/1194/04) with stearate amounts of 30% and 23%, respectively. Others were low pathogenic, e.g., avian influenza (LPAI) viruses (Duck/Vietnam/342/2001, Mallard/Penn/1024/84 and A/chicken/Germany/N/49) with stearate amounts of 32%, 29% and 35%, respectively.
Additionally, HA was always completely acylated, i.e. the acylation did not vary between HPAI and LPAI viruses. As a conclusion, this does not speak for a higher or lower stearate amount in HPAI compared to LPAI strains in general.

**Different enzymes might catalyze attachment of palmitate and stearate**

My next conclusion alludes to the mechanism of S-acylation of HA, i.e. whether attachment of palmitate and/or stearate requires an enzyme or only the presence of the lipid donor, i.e. Pal-CoA or Stear-CoA. None of my virus mutants was comprised in the attachment of fatty acids at any of the three acylation sites, just as the wild-type. The site-specific attachment of stearate versus palmitate observed for viral spike proteins argues clearly against a uniquely non-enzymatic reaction (Kordyukova *et al.*, 2008). On the contrary, probably even individual enzymes exist that differ in their acyl-CoA specificities. This could be demonstrated for the candidates DHHHC2 and 3 (Jennings and Linder, 2012). One possibility would be that the active site of such a DHHHC enzyme would penetrate deeper into the membrane to attach stearate to a transmembrane cysteine, whereas an enzyme candidate with specificity for palmitoyl-CoA only acts on the membrane surface. Such enzymes might not recognize amino acids in the vicinity of the acylation site, but rather the position of the cysteine within the lipid bilayer. Until now, there have been no experimental advancements to undermine this idea and this remains as an open question to be studied in future works.

Since acyl-CoAs are sequestered with very high affinity (~0.1 nM) by the acyl-CoA binding protein (Veit and Reverey, 1996), their cytosolic concentration is presumably too low to facilitate spontaneous acylation. However, acyl-CoA partitions into lipid bilayers with a high partition constant (1-5x10^5/M) by insertion of its acyl chain. Using atomic force microscopy and supported lipid bilayers it was found that acyl-CoA forms lateral aggregates in the plane of the bilayer (Javadpour *et al.*, 1999), which might represent the lipid donor for non-enzymatic S-acylation if such clusters exist in living cells. One might imagine that a fatty acid is spontaneously transferred to a protein if acyl-CoA encounters a cysteine at the end of the TMR of a protein in transit along the exocytic pathway.

However, such a non-enzymatic reaction would not show any preference for the attachment of a particular fatty acid to a certain site, but should rather reflect the concentration of individual acyl-CoAs at the intracellular site of acylation, the membranes of the late ER or early Golgi (Veit and Schmidt, 1993). Although very little is
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known about the distribution of acyl-CoAs in cellular membranes, there is no indication that the concentration of Pal-CoA and Stear-CoA grossly differ in the ER/Golgi. Fatty acids biosynthesis takes place in the cytosol and is conducted by fatty acid synthases that repeatedly attaches C2 units to a growing acyl chain (Chirala et al., 2004) until palmitic acid (C 16:0) is synthesized. The elongation to stearate (and longer chains) happens at membrane-bound enzymes of the ER. Basically there is no evidence that either one of the two, palmitate or stearate, are in higher stock than the other, since both are also used for the synthesis of phospholipids, which contain them as major types of fatty acids.

In addition, the cell type specific heterogeneity I found in the acylation pattern of HA is also difficult to reconcile with a non-enzymatic mechanism of S-acylation. Moreover, although insect cells are able to acylate HA when expressed with the baculovirus system (Kuroda et al., 1991; Faergeman and Knudsen, 1997), attachment of stearate was observed neither for HA nor for endogenous insect proteins (Reverey et al., 1996) (Simonsen et al., 2003). Thus, stearoylation is apparently an evolutionary achievement of higher cells and the responsible enzyme(s) might have then acquired slightly different substrate specificities in mammalian and avian cells.

In sum, it is more likely that (at least) two enzymes exist that differ in their acyl-CoA specificities, as recently demonstrated for DHHC 2 and 3 (Jennings and Linder, 2012). One can imagine that the enzyme responsible for palmitoylation recognizes cysteine exposed to the hydrophilic milieu of the cytosol. In contrast, the active site of a DHHC protein with a strong preference for Stear-CoA might have a certain affinity for the unique biophysical environment of the border region between the hydrophobic and hydrophilic part of the lipid bilayer. However, it cannot penetrate deeply into the lipid bilayer since HAs of H7 and H10 subtypes contain one and HAs of H3, H4 and H14 subtypes contain two cysteines in the middle of their TMR (Figure 5.1), which are not acylated (Veit et al., 1991; Kordyukova et al., 2011).

**DHHC inhibitors are potential Influenza drug candidates**

Identification of the DHHC proteins that acylate HA is a prime aim of future investigations. Albeit 23 or 24 different DHHCs with distinct, partly overlapping substrate specificities are encoded by the mammalian genome (Greaves and Chamberlain, 2011; Linder and Deschenes, 2007; Roth et al., 2006), only a few might catalyze
acylation of HA. The first key goal would be to identify the particular enzyme that specifically acylates Influenza proteins. One approach would be to identify DHHC proteins would be to search for transcribed genes by means of PCR that are expressed in the cells of lung tissue, the main target of influenza virus infection. From a pool of these cells identified DHHC proteins one then would have to determine, which ones acylate HA, e.g. by inhibiting these candidates individually with siRNA and then infecting with influenza virus. If an influenza virus infection can be significantly reduced by such a directed inhibition of an identified DHHC candidate, one could design a drug directed explicitly at this enzyme. This medication should inhibit the virus’s acylation and thus prevent further propagation of the virus when given to the patient.

Currently licensed Influenza treatments are directed against the virus’s proteins. Neuraminidase inhibitors (Oseltamivir and Zanamivir) bind the active center in the NA and thus inhibit the cleavage of newly budded virions from the cell (Colman, 1994). M2 inhibitors (Amantadine and Rimantadine) prevent the uncoating of viral genome into the cytoplasm by interfering with the proton channel function of M2 (Jing et al., 2008). M2 inhibitors are not prescribed anymore since they rapidly lead to resistances (Deyde et al., 2007), but resistances are also increasingly reported by medical use of neuraminidase inhibitors (Thorlund et al., 2011). A specific DHHC inhibitor on the other hand would not give rise to resistances that easily, because DHHCs are cellular proteins. The viral RNA polymerase works fast, but its poor proofreading ability (Liu et al., 2009) allows much more errors in the transcript than the cellular RNA polymerase and thus can give rise to mutations much quicker.

Cellular drug resistance can occur in two ways. One speaks of resistance when the cells do not respond to the therapy likely due to an unanticipated immune response or a wrong target pathway of the drug. However, usually resistances occur only in tumor cells and this would require a cancer to be already present. Here, resistance can be a characteristic of a subpopulation of heterogeneous cancer cells or acquired as a cellular reaction to the exposure of the medication. Often several different mechanisms are switched on in the cells, such as the activation of transmembrane proteins effluxing chemical substances out of the cell, activation of the enzymes of the glutathione detoxification system and alteration of genes and proteins involved in the control of apoptosis (Stavrovskaya, 2000; Longley and Johnston, 2005). It is unlikely that such resistances are provoked in non-
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tumor cells upon medication with a hypothetical DHHC inhibitor in such a short treatment period (an Influenza infection is usually treated for about a week). Therefore, DHHC inhibitors are promising drug candidates. Their inhibition might not compromise acylation of most remaining cellular proteins and presumably, the toxicity of a DHHC inhibitor medication should not be an issue.


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Eidesstattliche Erklärung


Ein Teil der beschriebenen Ergebnisse wurde in Zusammenarbeit mit Wissenschaftlern aus Kollaborationen erzielt und ist entsprechend gekennzeichnet.

Ich bin nicht in Besitz eines entsprechenden Doktorgrades und habe mich nicht anderwärts um einen Doktorgrad beworben.

Die dem Promotionsverfahren zugrunde liegende Promotionsordnung ist mir bekannt und ich erkläre hiermit nach der aktuellen Promotionsordnung zu promovieren.

Katharina Brett

Berlin,