Avian Influenza

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Avian Influenza H5-Containing Virus-Like Particles (VLPs): Host-Cell Receptor Specificity by STD NMR Spectroscopy**

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H5N1 influenza virus is a highly contagious pathogen infecting poultry and other birds. The emergence of a human pandemic influenza virus from an avian progenitor appears to involve a switch in preferential binding of the influenza virus hemagglutinin (HA) from α(2,3)-linked *N*-acetylneuraminic acid containing glycans (the major form in the avian enteric tract) to α(2,6)-linked *N*-acetylneuraminic acid containing glycans (the major form in the human upper respiratory tract). Avian influenza viruses such as the H3 virus HA receptor typically contain the amino acids Gln226 and Gly228, which create a narrow binding pocket favoring α(2,3)-linked *N*-acetylneuraminic acid containing glycans.[1] A number of other studies have also investigated HA–glycan recognition.[2,3] Interestingly, the X-ray crystal structure of the reassembled HA of the 1918 Spanish influenza virus shows that its avianlike narrow binding pocket still allows high-affinity binding of α(2,6)-linked *N*-acetylneuraminic acid containing glycans.[4] The mutation of only one amino acid (Asp190) within the HA binding site appears to determine the preference of the avianlike 1918 virus for human α(2,6)-linked *N*-acetylneuraminic acid containing glycans.[1,4,5] A mutation of Asp190 to Glu190 in the HA of the H5N1 strain could potentially switch its binding preference to α(2,6)-linked glycans and consequently result in the emergence of a human pandemic virus.

This fact emphasizes an urgent need to investigate the *N*-acetylneuraminic acid containing glycan recognition requirements by influenza virus HA that may lead to the development of novel antiinfluenza drugs that bind to the viral HA protein and consequently prevent the entry of the virus into human host cells. Rapid access to structural information would provide a detailed understanding of how virus particles interact with host cells on a molecular level and the determinants that prevent interspecies transmission of influenza viruses. Herein we present the first saturation transfer difference (STD) NMR spectroscopic[6–9] study analyzing H5-containing virus-like particles (VLPs) derived from the highly pathogenic avian H5N1 influenza strain in a complex with α(2,6)- and α(2,3)-linked *N*-acetylneuraminides to mimic an in vitro or in vivo virus–host-cell interaction. We recently reported the production of H5 pseudotyped virus particles.[10] To provide larger quantities of suitable VLPs for NMR studies, we have now successfully engineered heterologous H5 influenza VLPs by coexpression of pCDNA–synH5 coding for the hemagglutinin (H5) of H5N1 influenza virus and pCMV-dR8.91-expressing HIV-Gag-pol protein. Coexpression leads to spontaneous assembly of chimeric H5-VLPs, which contain the HA protein. The hemagglutinin, detected as uncleaved HA-0 precursor and HA-1/HA-2 cleaved mature forms, was incorporated on the surface of the viral particles at high levels. Expression of the viral HA protein was characterized by Western blot using specific C-terminal flag-tag M2 antibodies (Figure 1) and detection of VLPs by electron microscopy (Figure 2).

In this study we investigated the capacity of H5-VLPs for competitive selection from a mixture of α(2,6)- and α(2,3)-sialyllactose (6′-SL and 3′-SL, respectively) of a preferred ligand (and therefore linkage) by means of STD NMR spectroscopy. It has been previously demonstrated that STD NMR spectroscopy can be utilized to investigate ligand interactions with whole virus particles,[11] platelets,[12] and intact cells.[13] The large size of viruses and cells makes them attractive for studies with STD NMR spectroscopy because the inherently large line width enables saturation of the particle without affecting ligand signals. Additionally, the larger correlation time of bulky virus particles results in efficient spin diffusion and consequently stronger saturation transfer. To ensure the stability of the influenza H5-VLPs, NMR experiments were performed without prior purification, and 10% D_2O was added for locking purposes.

The ^{1}H NMR spectrum of the influenza H5-VLPs (Figure 3a) shows the signals of the 20% sucrose cushion

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Supporting information (detailed experimental procedures) for this article is available on the WWW under http://www.angewandte.org or from the author.

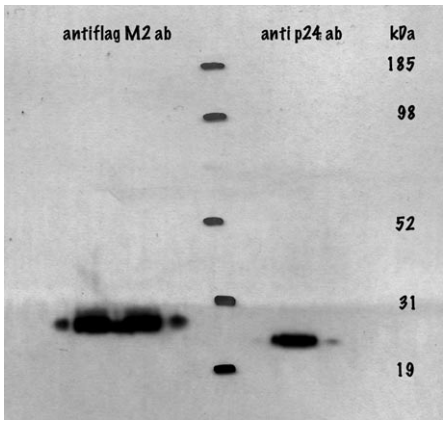

Figure 1. Western blot of the H5-VLP. Under reducing conditions HA-0 is cleaved to provide HA-1 and HA-2. The C-terminal flag tag of HA-2 can then be detected with the antiflag M2–peroxidase conjugate antibody (ab; left). The p24 (HIV-capsid) protein can be detected with a mouse anti-HIV antibody followed by goat antimouse immunoglobulin horseradish peroxidase conjugate (right).

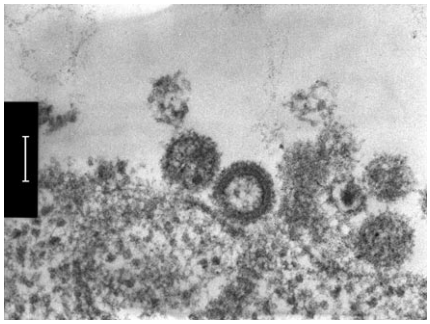

Figure 2. Transmission electron micrograph of an ultrathin section of glutaraldehyde-fixed, epon-embedded transfected HEK293T cells showing the production of H5-VLP on the surface of the cell. The scale bar represents 100 nm. Magnification ×105 000.

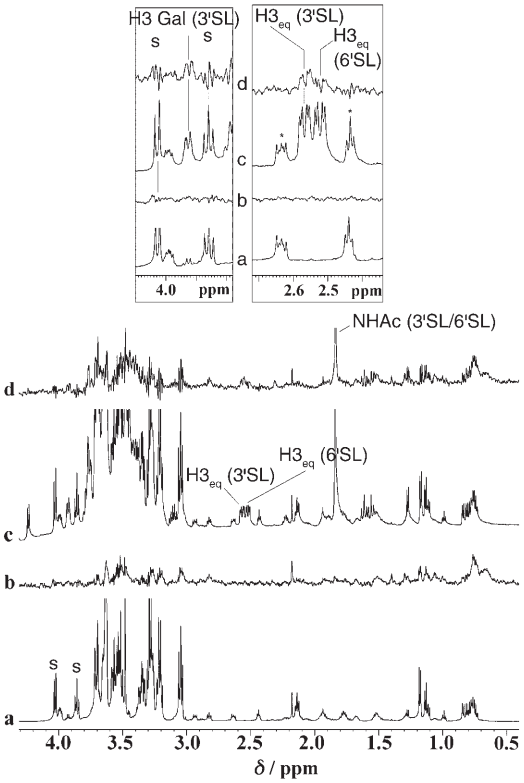

Figure 3. a) ^{1}H NMR spectrum of H5-containing VLPs. b) Control STD NMR spectrum of (a). c) ^{1}H NMR spectrum of H5-containing VLPs in the presence of an equimolar mixture (150 μg each) of α(2,3)-sialyllactose (3′-SL) and α(2,6)-sialyllactose (6′-SL). d) STD NMR spectrum of (c) measured by using the same experimental setup as in (b). The spectral regions $\delta = 3.80$–4.10 and 2.40–2.70 ppm are expanded in the left and right top panels, respectively.

(marked with "s" in Figure 3a) and Dulbecco's modified eagle's medium (DMEM/HIGH). Figure 3b shows the control STD NMR spectrum of the VLPs containing around 6.5 nM expressed HA protein. Some residual effects from the subtraction of strong signals of nonbinding ligands are visible. An equimolar mixture of 3′-SL and 6′-SL was added (Figure 3c), and the corresponding STD NMR spectrum is shown in Figure 3d. A strong STD NMR signal of the methyl protons of the *N*-acetamido group (1.95 ppm) is detectable, indicating that sialyllactose clearly binds to the H5-VLPs. Overlap of the *N*-acetamido signals prevents the distinction between α(2,3)- and α(2,6)-sialyllactose. However, the $H3_{eq}$ protons of 3′-SL and 6′-SL show diagnostic chemical shifts ($\delta = 2.58$ and 2.52 ppm, respectively) and provide an excellent means of differentiating between α(2,3)- and α(2,6)-linkages. This important spectral region is expanded at the top of Figure 3 and unequivocally shows a STD NMR signal for the $H3_{eq}$ proton of 3′-SL, whereas the $H3_{eq}$ proton of 6′-SL show little, if any, authentic STD NMR signal under these experimental conditions. Moreover, further support for this observed preferential selection was provided by extended analysis of the STD NMR spectra (Figure 3), and partial binding-epitope characterization of 3′-SL in a complex with the H5-VLP was possible. This analysis indicates that the H3 proton of the aglycon galactose moiety ($\delta = 3.92$ ppm) also interacts with the influenza H5-VLPs and is consistent with other HA protein structural studies.[1] The signals at $\delta = 4.06$ and 3.86 ppm (marked with "s") attributed to the sucrose present in the VLP buffer (Figure 3a) are not detected in the STD NMR spectrum (Figure 3d). This result confirms that sucrose is a nonbinding ligand and thus has no affinity for the H5 influenza VLPs.

We have shown for the first time that noninfectious H5 influenza VLPs can be subjected, directly in growth media, to STD NMR experiments at low concentrations. Our competition study unequivocally demonstrates that avian influenza H5-VLPs encoding the hemagglutinin HA protein clearly discriminate between the α(2,3)- and α(2,6)-linked *N*-acetylneuraminides (3′- and 6′-SL) and that preferential binding could be seen for α(2,3)-linked compound within the limits of detection. This result is in excellent agreement with the fact that nonhumanized avian influenza strains preferentially recognize α(2,3)-linked *N*-acetylneuraminic acid containing glycans.[1] These data taken together provide strong evidence that the HA lectin function associated with these virus-like

particles behaves in an identical fashion to the HA on the surface of native H5N1 influenza virus.

HA-containing VLPs derived from highly pathogenic H5N1 avian influenza virus are an ideal tool to study cell tropism and the role of sialic acids in H5N1 influenza virus entry. The use of noninfectious H5-VLPs has the advantage that experiments can be performed without high containment laboratory procedures mandatory for the handling of live viruses. The use of VLPs, without the need for additional purification, in STD NMR experiments provides an excellent and rapid model to interrogate the interactions of the virion with host-cell glycans. This experimental development is particularly important in light of our recent report on the possibility of alternative HA-associated carbohydrate binding sites that may facilitate H5N1 infection in the human upper respiratory tract.[14] We are presently extending this approach to investigate in a more quantitative manner, by saturation transfer double difference (STDD) NMR spectroscopic methods,[8,12] sialo- and asialo glycan recognition by wild-type and mutated forms of H5 from the avian influenza virus H5N1. This information is urgently needed for a better understanding of the biological restrictions to interspecies transmission of avian influenza viruses and for the design of antiinfluenza drugs that block the entry of the virus to host-cell receptors.[15]

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