Co-translational Processing of Glycoprotein 3 from Equine Arteritis Virus

N-GLYCOSYLATION ADJACENT TO THE SIGNAL PEPTIDE PREVENTS CLEAVAGE*

Anna Karolina Matczuk, Dušan Kunec, and Michael Veit

From the Institut für Virologie, Free University Berlin, 14163 Berlin, Germany

Signal peptide cleavage and N-glycosylation of proteins are co-translational processes, but little is known about their interplay if they compete for adjacent sites. Here we report two unique findings for processing of glycoprotein 3 of equine arteritis virus. Glycoprotein 3 (Gp3) contains an N-terminal signal peptide, which is not removed, although bioinformatics predicts cleavage with high probability. There is an overlapping sequon, NNTT, adjacent to the signal peptide that we show to be glycosylated at both asparagines. Exchanging the overlapping sequon and blocking glycosylation allows signal peptide cleavage, indicating that carbohydrate attachment inhibits processing of a potentially cleavable signal peptide. Bioinformatics analyses suggest that a similar processing scheme may exist for some cellular proteins. Membrane fractionation and secretion experiments revealed that the N-terminal signal peptide of Gp3 does not act as a membrane anchor, indicating that it is completely translocated into the lumen of the endoplasmic reticulum. Membrane attachment is caused by the hydrophobic C terminus of Gp3, which, however, does not span the membrane but rather attaches the protein peripherally to endoplasmic reticulum membranes.

Significance: N-Glycosylation and signal peptide cleavage are co-translational modifications of membrane proteins.

Results: Attachment of carbohydrates adjacent to a potentially cleavable signal peptide inhibits its processing in a glycoprotein from an arterivirus.

Conclusions: Glycosylation occurs prior to signal peptide cleavage, contrary to other proteins analyzed in this regard.

Arteriviruses are a family of enveloped RNA viruses comprising the prototype member equine arteritis virus (EAV)2; porcine reproductive and respiratory syndrome virus (PRRSV), the main pathogen in the pork industry; and lactate dehydrogenase-elevating virus of mice (1, 2). Only very limited information is available on the structure and membrane topology of the glycoprotein complex Gp2/3/4, which is required for cell entry (3, 4) via clathrin-mediated endocytosis (5). Both in transfected and virus-infected cells, the glycoproteins are retained in the ER and/or pre-Golgi region, the viral budding site, by means of unidentified retention signals (6).

Gp2 and Gp4 are typical type I transmembrane proteins (6, 7) that form an intermolecular disulfide linkage in the ER (8). Gp3, the focus of this study, consists of an N-terminal signal peptide, an ectodomain containing six potential N-glycosylation sites, and a C-terminal hydrophobic region. In virus particles, Gp3 is present in a disulfide-linked trimer with Gp2/4. Uniquely, disulfide linkages between Gp3 and Gp2/4 are not formed in the ER but only after release of virus particles from infected cells (8). In vitro translation experiments in the presence of microsomes showed that the N-terminal signal peptide is not cleaved from Gp3, indicating that it might function as a signal anchor domain (6, 9). In addition, the hydrophobic C terminus might also span the membrane, creating a protein with two transmembrane regions. However, neither a membrane anchor function of the signal peptide of Gp3 nor the location of its C-terminal region relative to the membrane, i.e. either luminal or cytoplasmic, has been investigated so far.

Surprisingly, the bioinformatics tool SignalP 4.0 (10) predicts signal peptide cleavage from Gp3 with high probability. However, SignalP does not take into account possible protein modifications that may affect cleavage. Here we analyzed whether attachment of carbohydrates to an overlapping sequon (NNTT) located just two amino acids downstream of the predicted signal peptide cleavage site prevents cleavage.

Overlapping sequons are rare in natural proteins, and if present, only one (or even none) of the asparagine residues is used for attachment of a carbohydrate side chain (11, 12). The smallest separation between neighboring glycosylation sites found so far in a natural protein is three residues, i.e. NHSENAT. Likewise, engineering the distance between potential glycosylation sites in a model protein revealed that separation by at least two residues (e.g. NSTNST) is required to achieve at least partial glycosylation of both sites. In contrast, the sequence NNTT was glycosylated exclusively at only one site per molecule (11, 13).

N-Glycosylation and signal peptide cleavage are performed by multimeric protein complexes termed oligosaccharyltransferase (OST) (13, 14) and signal peptidase complex (15), respectively. Both OST and signal peptidase complex can associate

* This work was supported by the European Union (FP7, Initial Training network “Virus entry”).

1 To whom correspondence should be addressed: Inst. für Virologie, Fachbereich Veterinärmedizin, Zentrum für Infektionsmedizin-Robert-von-Ostertag-Haus, Freie Universität Berlin, Robert-von-Ostertag-Strasse 7–13, 14163 Berlin, Germany. Tel.: 49-30-838-51891; Fax: 49-30-838-451847; E-mail: mveit@zedat.fu-berlin.de.

2 The abbreviations used are: EAV, equine arteritis virus; Gp, glycoprotein; ER, endoplasmic reticulum; OST, oligosaccharyltransferase; PRRSV, porcine reproductive and respiratory syndrome virus; NA, neuraminidase; BHK, baby hamster kidney; PNGase F, peptide-N-glycosidase F; Endo H, endoglycosidase H.
with the translocon such that nascent polypeptide chains emerging on the luminal site of the ER are immediately processed (16, 17). It is largely unexplored how these co-translational processes affect each other and whether they compete for overlapping or neighboring sites in a protein. Glycosylation sites located in the vicinity of the signal peptide generally require signal peptide cleavage for efficient glycosylation, indicating that signal peptidase and OST act in a sequential and dependent manner on their substrate (18).

Here we report that for Gp3 from EAV an overlapping, efficiently double-glycosylated sequon located just downstream of a potentially cleavable signal peptide inhibits its processing. Furthermore, we present experiments that indicate that the signal peptide does not function as a signal anchor. Membrane attachment is caused by the hydrophobic C terminus of Gp3, which, however, does not span the membrane but rather attaches Gp3 peripherally to ER membranes.

**EXPERIMENTAL PROCEDURES**

**Cloning and Transfection**—Full-length Gp3 and Gp4 from the Bucyrus strain of EAV were cloned between the Xhol and BamH1 sites of pEYFP-N1 (Clontech) to produce EAV proteins C-terminally tagged with yellow fluorescent protein (Gp3-YFP and Gp4-YFP).

Cloning introduced the amino acid sequence GMAPGRDP-PVAT between the C terminus of Gp3 and Gp4, respectively, and the start of YFP. Gp3-HA was generated by PCR using a primer that encodes the amino acid sequence YPYDVPDYA and was subcloned between the Xhol and Xba1 sites of pCMV-TNT (Promega). To delete the two first N-glycosylation sequons, the N28Q, N29Q, and N28Q/N29Q mutations as well as the mutations N28H, N29H, and N28H/N29H were introduced by site-directed mutagenesis (19). The ΔC-Gp3-HA constructs lacking the C-terminal hydrophobic domain (Gp3 amino acids 1–136 directly fused to the hemagglutinin (HA) tag) were created by overlap extension PCR from the Gp3 wild type (WT) and Gp3-N28Q/N29Q templates in pCMV-TNT. To delete the two first N-glycosylation sequons, the N28Q, N29Q, and N28Q/N29Q mutations as well as the mutations N28H, N29H, and N28H/N29H were introduced by site-directed mutagenesis (19). The ΔC-Gp3-HA constructs lacking the C-terminal hydrophobic domain (Gp3 amino acids 1–136 directly fused to the hemagglutinin (HA) tag) were created by overlap extension PCR from the Gp3 wild type (WT) and Gp3-N28Q/N29Q templates in pCMV-TNT. To delete the two first N-glycosylation sequons, the N28Q, N29Q, and N28Q/N29Q mutations as well as the mutations N28H, N29H, and N28H/N29H were introduced by site-directed mutagenesis (19).

**Metabolic Labeling, Tunicamycin Treatment, Immunoprecipitation, and Fluorography**—Twenty-four hours after transfection, CHO cells were starved for 2 h in medium lacking methionine and cysteine (Eagle’s minimal essential medium with Earl’s balanced salt solution; PAN-Biotech, Aidenbach, Germany). Cells were labeled in the same medium for 2 h with EasyTag Expre35S35S protein labeling mixture containing [35S]methionine and [35S]cysteine (30 μCi/ml; PerkinElmer Life Sciences). For the pulse-chase experiment, cells were starved for 15–30 min and labeled with 100 μCi/ml 35S protein labeling mixture for 30 s. Cells were then washed once with medium containing 20 mM cysteine and 20 mM methionine (Sigma-Aldrich) and incubated in the same medium for the indicated time at 37 °C. After metabolic labeling, the medium was removed; cells were washed twice with ice-cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4) and lysed in radioimmune precipitation assay buffer (20 mM Tris/HCl (pH 7.4), 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 10 mM EDTA, 10 mM iodoacetamide, 1 mM PMSF). To inhibit N-glycosylation, cells were preincubated with tunicamycin (5 μg/ml; Sigma-Aldrich) for 30 min and labeled in the presence of the same concentration of tunicamycin for 45 min. Labeling was terminated by washing with ice-cold PBS containing 20 mM iodoacetamide followed by cell lysis in radioimmune precipitation assay buffer.

Cleared lysates (5000 × g, 5 min, 4 °C) were incubated overnight with 2 μg (0.8 μl) of rabbit anti-GFP antibody (A11122, Molecular Probes/Invitrogen) under shaking at 4 °C. Next, 40 μl of a 1:1 slurry of protein A-Sepharose (Sigma-Aldrich) in radioimmune precipitation assay buffer was added, and samples were shaken at 4 °C for an additional 2.5 h. The protein A-Sepharose with bound antigen-antibody complexes was pelleted (2000 × g, 3 min), washed four times in 800 μl of radioimmune precipitation assay buffer, and resuspended in reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer.

**Glycosidase Treatment**—For deglycosylation of immunoprecipitates, protein A-Sepharose with bound antigen-antibody complex was resuspended in 20 μl of 1× glycoprotein denaturing buffer (0.5% SDS, 40 mM DTT). For deglycosylation of whole cell lysates, cells were washed with PBS, detached from the dish with trypsin-EDTA (PAN-Biotech), pelleted, resuspended in 80 μl of 1× glycoprotein denaturing buffer, and boiled for 10 min at 100 °C. Typically, 20 μl of this lysate was digested with peptide-N-glycosidase F (PNGase F; 2.5–5 units/μl) or endoglycosidase H (Endo H; 2.5–5 units/μl) according to the manufacturer’s instructions (New England Biolabs, Frankfurt am Main, Germany) for 1 h at 37 °C. For limited Endo H or PNGase F digestion, serial 2-fold dilutions of the enzymes (starting with 1 unit/μl) were incubated with the substrate. After the deglycosylation reaction, samples were supplemented with reducing SDS-PAGE buffer and subjected to SDS-PAGE and fluorography or Western blotting.

**In Vitro Transcription/Translation**—Unprocessed Gp3-HA was generated by in vitro transcription/translation using the TnT Quick Coupled Transcription/Translation System (Promega, Mannheim, Germany). A reaction (25 μl) was composed of 20 μl of rabbit reticulocyte lysate (TnT Master Mix, also including T7 RNA polymerase), 2.2 μl of EasyTag Expre35S35S protein labeling mixture, and 1 μg of pCMV-TNT-GP3-HA plasmid DNA, which contains a T7 promoter. Reactions were incubated for 90 min at 30 °C, supplemented with reducing SDS-PAGE buffer, and subjected to SDS-PAGE and Western blotting.

**Preparation of Membranes and Soluble Fractions from Microsomes**—CHO-K1 cells were treated with digitonin (30 μM) for 10 min on ice, cell debris was removed by low speed centrifugation (1000 × g, 5 min, 4 °C), microsomes were pelleted by centrifugation (100,000 × g, 1 h, 4 °C), and resuspended in PBS. After homogenization of cells using ultrasonication (Misonix XL-2000), membranous and soluble fractions were prepared by centrifugation (100,000 × g, 1 h, 4 °C). The pellet was washed once in PBS and suspended in reducing SDS-PAGE buffer.
Glycosylation and Signal Peptide Cleavage of Gp3

buffer. Proteins present in the supernatant were precipitated with trichloroacetic acid (TCA; 10% final concentration) and pelleted (14,000 × g, 30 min, 4 °C). Pellets were washed three times with 100% ethanol, dried, resuspended in reducing SDS-PAGE buffer, and analyzed by Western blotting.

Experiments to Analyze Secretion of Gp3 from Cells—Twenty-four hours after transfection of BHK-21 cells, the supernatant was removed, and cell debris was pelleted by low speed centrifugation (1000 × g, 5 min, 4 °C). Proteins were concentrated with a 9000 molecular weight-cutoff column (Thermo/Pierce) and resuspended in reducing SDS-PAGE buffer. The cells were washed with PBS, detached from wells, and lysed in reducing SDS-PAGE buffer.

SDS-PAGE, Western Blotting, and Fluorography—Samples in reducing SDS-PAGE buffer were boiled for 5 min at 95 °C. After SDS-PAGE using 12 or 17% polyacrylamide gels, gels were blotted onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare) using standard methodology. After blocking of membranes (blocking solution: 5% skim milk powder in PBS with 0.1% Tween 20 (PBST)) for 1 h at 25 °C, rabbit-anti-HA tag (ab9110, Abcam, Cambridge, UK; 1:4000), rabbit anti-GFP (A11122, Invitrogen; 1:1000), or rabbit anti-calreticulin (Calbiotech, Amsberg, Germany; 1:4000) was applied for 45 min at 25 °C. The bands were visualized with Kodak X-Omat AR films (BioMax, Sigma-Aldrich).

Fluorescence Protease Protection Assay—The fluorescence protease protection assay was performed as described (20). Briefly, CHO-K1 cells seeded on 35-mm dishes with glass coverslips (MatTek Corp., Ashland, MA) were transfected with plasmids Gp3-YFP or Gp4-YFP as described above. Twenty-four hours after transfection, cells were treated with digitonin (20–40 µM) for 60 s, and then proteinase K (50 µg/ml; Sigma-Aldrich) was added. The pictures were taken using an Olympus IX-81 epifluorescence microscope.

Bioinformatics Identification of Proteins That Contain a Glycosylation Site Adjacent to Signal Peptide Cleavage site—The human (Homo sapiens) and mouse (Mus musculus) reference proteome sets were downloaded from the UniProt database (release 2013_09). These sets contained 68,936 and 43,284 protein records of all proteins that are thought to be expressed by humans and mice, respectively. Protein sequences that were extracted, and a 10-amino acid region immediately behind the signal peptide sequence was scanned for the presence of a potential glycosylation site (NX(S/T); X ≠ Pro). The proteins with a signal peptide in UniProt contain one of three qualifiers in the database annotation: “potential,” “probable,” or “by similarity.” If there is no qualifier present, experimental evidence has been published that the signal peptide is cleaved. The by similarity qualifier indicates that experimental evidence is available for a protein from the same family within a certain taxonomic range. For our analyses, we used only sequences with the potential qualifier where cleavage of the signal peptide is based on bioinformatics prediction only. Protein sequences with the probable qualifier have a somewhat higher probability of signal peptide cleavage and were not included in the analyses.

RESULTS

The Overlapping Sequon Near the Signal Peptide Is Glycosylated at Both Asparagines—SignalP 4.0 (10) predicts signal peptide cleavage of Gp3 with a probability of 82% (D score in Table 1), but the signal peptide is retained (6, 9). Two possible cleavage sites, between residues 26 and 27 and between amino acids 27 and 28, are calculated with similar probabilities (Y scores in Table 1). However, SignalP does not take into account protein modifications that may affect cleavage. We hypothesized that attachment of carbohydrates to the overlapping sequon (NNTT) located just two amino acids downstream of the predicted signal peptide cleavage site prevents cleavage (Fig. 1A).

We analyzed first whether asparagines of the overlapping sequon are glycosylated. When Gp3-YFP was expressed in CHO cells, it ran as two bands upon labeling with [35S]methionine/cysteine in SDS-PAGE. Because only one band was observed after complete digestion of samples with Endo H, the two bands are due to heterogeneity in N-linked glycosylation (Fig. 1B). Stepwise reduction of the amount of Endo H yielded a ladder-like band appearance that allows counting of six carbohydrates. Thus, all six potential glycosylation sites in Gp3, including both in the overlapping sequon, are (at least partially) glycosylated. To directly demonstrate that both sites of the overlapping sequon are glycosylated, mutants in which either one or both
Glycosylation and Signal Peptide Cleavage of Gp3

Removal of Two Glycosylation Sites Allows Cleavage of the Signal Peptide—To analyze whether removal of one or both of the glycosylation sites allows signal peptide cleavage, the SDS-PAGE mobility of the Gp3-HA constructs after digestion with glycosidases was compared. As a size marker for unglycosylated Gp3 containing the signal peptide, Gp3-HA-WT was in vitro translated in the absence of microsomes. The resulting product ran to the same position in the gel as Gp3-HA-WT expressed in BHK cells and deglycosylated with Endo H (Fig. 2A). This confirms previous results with native Gp3 demonstrating that the signal peptide is not cleaved from Gp3 (6, 9). However, the deglycosylated double mutants Gp3-HA-N28Q/N29Q and Gp3-HA-N28H/N29H ran significantly faster in the gel. The mutants where only one of the two glycosylation sites in the overlapping sequon was exchanged ran as wild type (Fig. 2A).

Endo H cleaves between the two innermost N-acetylglucosamine (GlcNAc) residues of the carbohydrate side chain, resulting in the retention of one GlcNAc residue per glycosylation site. Thus, one may argue that the number of residual GlcNAc residues, i.e. six or five in Gp3-HA-WT and the single mutants but only four in the double mutant, causes the observed change in SDS-PAGE mobility. Therefore, we digested the samples with PNGase F, which removes the complete carbohydrate from Gp3, but the same result was obtained (Fig. 2B). Likewise, when wild type Gp3-YFP and double and single glycosylation mutants were expressed in CHO cells and digested with Endo H and PNGase F, respectively, an identical result was observed (Fig. 2C). Thus, neither the nature of the antibody tag nor the cell type used for expression of Gp3 affected signal peptide cleavage upon replacement of two, but not one, glycosylation site. The increased SDS-PAGE mobility

asparagine residues were exchanged by glutamine were analyzed next. Metabolic labeling again showed the characteristic double band for wild type Gp3-YFP but only one band for each of the three mutants. The SDS-PAGE mobility of the single mutants (N28Q and N29Q) corresponded to that of the lower band of the wild type protein, whereas the double mutant N28Q/N29Q ran faster (Fig. 1C). An identical result was observed when Gp3 fused at its C terminus to an HA tag was expressed in BHK cells and the resulting lysates were analyzed by Western blotting. In addition, mutants with an exchange of asparagines of the overlapping sequon by histidines exhibited the same band pattern (Fig. 1D). A double band was also observed by others for native Gp3 in vitro translated in the presence of microsomes and for Gp3 in virus particles (6, 9), indicating that the double band is not caused by the tags we attached to detect Gp3.

We next asked whether the signal peptide of Gp3 is required for double glycosylation of the overlapping sequon. The first 41 N-terminal amino acids of the influenza virus NA, a type II membrane protein with an uncleaved signal peptide, allow translocation and intracellular transport of transplanted sequences (21). Therefore, this sequence containing the signal peptide plus the following linker region (14 amino acids) was used to replace the signal peptide of Gp3. After expression of the construct NA-Gp3-HA, only one protein band appeared in the blot, and limited digestion with PNGase F showed that only five glycans were attached to the protein. Although it is formally possible that one of the four C-terminal glycosylation sites in Gp3 is not used in the NA-Gp3-HA construct, the result suggests that the overlapping sequon was glycosylated only once (Fig. 1E).
Glycosylation and Signal Peptide Cleavage of Gp3

is also not due to proteolytic processing of the C terminus of Gp3 because in that case the HA tag would have been removed.

The amino acid substitutions introduced into Gp3 might cause an aberrantly increased SDS-PAGE mobility of the protein that is not due to cleavage of the signal peptide. To exclude that our conclusion is based on such an artifact, we in vitro translated wild type Gp3-HA and the glycosylation mutants to compare their SDS-PAGE mobility. This procedure yields proteins without any modifications. However, in that case, all Gp3-HA constructs ran to the same position in the gel (Fig. 2D). Thus, substitutions of asparagines, either by histidines or by glutamines, did not change the SDS-PAGE mobility of Gp3 progressively; therefore, the mobility increase of cell-expressed Gp3 must be due to cleavage of the signal peptide. In addition, SignalP predicts signal peptide cleavage from all Gp3 mutants with probabilities similar to wild type Gp3, indicating that the amino acid substitutions alone have no significant effect (Table 1).

Glycosylation of the Overlapping Sequon Occurs Co-translationally and Prevents Signal Peptide Cleavage—To prevent access of the signal peptidase to the cleavage site, carbohydrates must be added very early to the nascent polypeptide chain. However, in some proteins, carbohydrates are attached only after completion of protein synthesis (22). Therefore, we asked whether we can discern glycosylation of individual sites of Gp3-YFP using pulse-chase experiments. After short labeling, the typical double band pattern was already visible in the fluorogram (Fig. 3A). Neither the relative abundance nor the SDS-PAGE mobility of both bands changed significantly for up to 10 min of chase. Thus, glycosylation of all sequons of Gp3-YFP occurs co-translationally.

Finally, we used tunicamycin in labeling experiments to analyze whether signal peptide cleavage is achieved upon inhibition of carbohydrate attachment. In the absence of this inhibitor of \( N \)-glycosylation, the characteristic band pattern was observed for wild type Gp3-YFP and Gp3-YFP-N28Q/N29Q (Fig. 3B). After deglycosylation, Gp3-YFP-N28Q/N29Q again ran faster than wild type Gp3-YFP. In the presence of tunicamycin, a non-glycosylated band appeared at the expense of the glycosylated bands, demonstrating that glycosylation was inhibited. These non-glycosylated bands had exactly the same size for wild type Gp3-YFP and the double mutant. The non-glycosylated proteins also had the same SDS-PAGE mobility as deglycosylated Gp3-YFP-N28Q/N29Q but ran faster than deglycosylated wild type Gp3-YFP. Thus, this result is direct proof that inhibition of glycosylation allows cleavage of the signal peptide from Gp3.

The Signal Peptide of Gp3 Does Not Function as a Membrane Anchor—Uncleaved signal peptides usually function as a signal anchor, causing the protein to adopt a type II topology with the N terminus inserted into the membrane and the C terminus exposed to the lumen of the ER. We used a fluorescence prote-

FIGURE 2. The signal peptide is cleaved from Gp3 after removal of two, but not one, glycosylation site of the overlapping sequon. A, Gp3-HA-WT and mutants were expressed in BHK cells, and cell lysates were completely digested with Endo H prior to Western blotting. Left lane, Gp3-HA was translated in rabbit reticulocyte lysate as a size marker for unmodified Gp3. B, Gp3-HA-WT and mutants were expressed in BHK cells, and cell lysates were completely digested with PNGase F prior to Western blotting. C, Gp3-YFP-WT and mutants were labeled for 2 h with \(^{35}S\)methionine/cysteine in transfected CHO cells, immunoprecipitated, and digested with Endo H or PNGase F as indicated prior to SDS-PAGE and fluorography. D, Gp3-HA-WT and mutants were translated in rabbit reticulocyte lysates in the presence of \(^{35}S\)methionine/cysteine, and samples were subjected to SDS-PAGE and fluorography.

FIGURE 3. Glycosylation of the overlapping sequon occurs rapidly, and its inhibition by tunicamycin allows signal peptide cleavage. A, Gp3-YFP-WT was expressed in CHO cells, labeled for 30 s with \(^{35}S\)methionine/cysteine, and chased for the indicated time periods prior to immunoprecipitation. Band intensities of the fluorogram were quantified with Bioprofile software. The upper band, double glycosylated at the overlapping sequon, accounts for 50% (1 min), 60% (2 min), 65% (6 min), and 59% (10 min) of both bands; indicating that very little, if any, post-translational glycosylation occurred. B, Gp3-YFP-WT was expressed in CHO cells and labeled for 45 min with \(^{35}S\)methionine/cysteine in the presence or absence of tunicamycin (5 \( \mu \)g/ml) prior to immunoprecipitation with anti-GFP antibodies, treatment with PNGase F as indicated, SDS-PAGE, and fluorography. Cells labeled in the presence of tunicamycin were also preincubated with this compound for 30 min prior to labeling.
Glycosylation and Signal Peptide Cleavage of Gp3

FIGURE 4. The C terminus of Gp3 is translocated into the lumen of the ER. CHO cells expressing Gp4-YFP or Gp3-YFP were treated with digitonin (30 μM) for 1 min and with proteinase K (50 μg/ml) for 2 and 6 min. After each time point, the same microscopic field was recorded with an epifluorescence microscope.

FIGURE 5. Signal peptide cleavage does not convert Gp3 into a soluble protein. CHO cells were transfected with Gp3-YFP wild type, Gp3-YFP-N28Q/N29Q, or transmembrane protein HA-TMD-YFP (35). Twenty-four hours after transfection, the cells were gently opened by digitonin, and microsomes were prepared by ultracentrifugation, resuspended in buffer, and opened by ultrasonication. Membranous (M) and luminal (L) fractions were separated by ultracentrifugation and subjected to Western blotting with antibodies against GFP or against calreticulin, a luminal protein of the ER. The SDS-PAGE mobility of molecular weight markers is indicated at the left side of the blot. M, mock, untransfected cells.

FIGURE 6. Deletion of the C terminus allows secretion of Gp3 from transfected cells. A, BHK cells were transfected with Gp3-HA wild type, Gp3-HA-N28Q/N29Q, or ΔC-Gp3-HA-N28Q/N29Q, a mutant in which the hydrophobic C terminus of the protein was deleted. Twenty-four hours after transfection, the concentrated cell culture supernatant (sup) and 25% of a cell lysate (cell) were analyzed by Western blotting with anti-HA tag antibodies. Mock, untransfected cells. Both parts of the figure are derived from the same blot, but the left part was exposed for 10 min, and the right part only exposed for 10 s. B, BHK cells were transfected with ΔC-Gp3-HA wild type or ΔC-Gp3-HA-N28Q/N29Q, and the concentrated supernatant and 10% of a cell lysate were analyzed by Western blotting with anti-HA tag antibodies. C, BHK cells were transfected with Gp3-HA wild type, Gp3-HA-N28Q/N29Q, ΔC-Gp3-HA wild type, or ΔC-Gp3-HA-N28Q/N29Q. Twenty-four hours after transfection, cell lysates were completely digested with PNGase F and analyzed by Western blotting with anti-HA tag antibodies.
Glycosylation and Signal Peptide Cleavage of Gp3

deleted from Gp3 with exchanged glycosylation sites to analyze possible secretion from transfected cells. In contrast to the full-length protein, a substantial fraction of ΔC-Gp3-HA-N28Q/N29Q was secreted into the supernatant (Fig. 6A). Similarly to Gp3 present in extracellular virus particles (6), the Gp3 band representing secreted protein had a higher molecular weight than its cellular counterparts. This indicates that Gp3 lacking the C terminus passed through the whole Golgi region where terminal glycosylation occurs.

We next investigated whether Gp3-HA containing the signal peptide is also secreted after deletion of the C terminus. Western blotting from supernatants of transfected cells revealed that this is the case; the fraction of secreted protein was even larger compared with the secreted fraction of ΔC-Gp3-HA-N28Q/N29Q (Fig. 6B). More efficient secretion of ΔC-Gp3-HA might be due to the presence of the signal peptide that might allow more efficient folding, a prerequisite for export of proteins from the ER (23, 24).

Intracellular ΔC-Gp3-HA-WT was present as two bands, but the lower band was more prominent, which is different from the band pattern obtained for Gp3-HA-WT. Furthermore, ΔC-Gp3-HA-N28Q/N29Q also formed two bands, which were never observed for Gp3-HA-N28Q/N29Q. To exclude that removal of the C terminus affected co-translational processing of Gp3, we analyzed N-glycosylation and signal peptide cleavage of these constructs. Limited digestion with PNGase F revealed that ΔC-Gp3-HA-WT contains six and ΔC-Gp3-HA-N28Q/N29Q contains four carbohydrates (data not shown), comparable to the corresponding proteins containing the complete C terminus. Thus, the double bands might be due to inefficient glycosylation of a site other than the overlapping sequon. Because glycosylation at sites close to the C terminus is limited by rapid folding of the protein (22), we speculate that the most C-terminal site at position 118 is incompletely glycosylated when the C terminus is removed from Gp3.

Finally, we analyzed the SDS-PAGE mobility of deglycosylated Gp3 proteins with and without the C-terminal domain to investigate signal peptide cleavage. The results again revealed that Gp3-HA-N28Q/N29Q runs faster than Gp3-HA-WT (Fig. 6C). ΔC-Gp3-HA, both wild type and N28Q/N29Q, was present as one band after deglycosylation, confirming that the variety of bands observed for intracellular and secreted forms of the proteins is due to heterogeneous glycosylation. Importantly, ΔC-Gp3-HA-N28Q/N29Q had a higher SDS-PAGE mobility compared with ΔC-Gp3-HA-WT. Thus, the signal peptide is present in ΔC-Gp3-HA-WT but not in ΔC-Gp3-HA-N28Q/N29Q. This indicates that removal of the C terminus does not affect inhibition of signal peptide cleavage by adjacent carbohydrates.

In summary, the last set of experiments provides evidence that the signal peptide of Gp3 does not function as a membrane anchor. This implies that the signal peptide is completely translocated into the lumen of the ER. Membrane attachment is caused by the hydrophobic C terminus of Gp3, which, however, does not span the membrane but rather attaches the protein peripherally to ER membranes. These results suggest a new model for the membrane topology of Gp3 that is shown in Fig. 7.

Bioinformatics Analyses on the Occurrence of Glycosylation Sites Adjacent to Signal Peptides—Viral proteins completely rely on the cellular processing machinery for their synthesis. It is unlikely that cells possess a molecular mechanism that is beneficial only for a protein of their parasites but would not work on at least some of their own proteins.

Therefore, we were interested to estimate by a bioinformatics approach how many proteins possess a potential N-glycosylation site in the vicinity of the N-terminal signal peptide. Of the 68,936 H. sapiens and 43,284 M. musculus proteins that are listed in the UniProt reference proteome sets (September 2013), 3,453 human and 3,082 mouse proteins are annotated to contain a confirmed/predicted signal peptide at their N terminus. Of these 3,082 mouse proteins, 148 (4.8%) contain a predicted N-glycosylation site (NX(S/T); X ≠ Pro) within 10 amino acids, and 52 (1.7%) contain a predicted N-glycosylation site within five amino acids behind the signal peptide cleavage site. Of the 3,514 human protein sequences that are annotated to contain a signal peptide, 154 (4.5%) and 58 (1.7%) contain an N-glycosylation motif within 10 and five amino acids behind the signal peptide cleavage site, respectively. None of the proteins contain an overlapping sequon (NN(S/T)(S/T)) in the vicinity of the signal peptide. However, at least one overlapping sequon was present in the complete sequence of 1.0% of all human and 1.8% of all mouse proteins containing an N-terminal signal peptide, demonstrating that overlapping sequons are rare (11).

The UniProt database annotates the existence of experimental evidence for signal peptide cleavage for 32 (human) and 12 (mouse) of the signal peptide sequences with nearby glycosylation sites. In addition, four human and 11 mouse signal peptides are likely to be cleaved because experimental evidence has been obtained for a member of the same protein family within the same taxonomic range. However, no processing experiments have been performed for the majority of the proteins containing...
a signal peptide predicted to be cleaved plus a glycosylation sequon nearby. Some of these proteins might behave like Gp3 from EAV; i.e. glycosylation might inhibit signal peptide cleavage. Tables 2–4 list the proteins identified in both the mouse and human proteomes that contain a predicted signal peptide cleavage site and a potential glycosylation site located within five amino acids downstream (human sequences listed).

**DISCUSSION**

Our studies on Gp3 of EAV provide two novel aspects to N-glycosylation of proteins. This is the first demonstration that both asparagines of the overlapping sequon NNTT are efficiently glycosylated (Fig. 1) and that glycosylation near the signal peptide cleavage site inhibits processing of the protein by signal peptidase (Figs. 1 and 2).

So far, overlapping sequons have been shown to be glycosylated only at one asparagine per molecule (11, 12). This observation was attributed to steric hindrance because a bulky carbohydrate is likely to prevent access of OST to a second site in its vicinity. This assumption is supported by the crystal structure of PglB (the homolog of the catalytic subunit STT3 of OST) from Campylobacter. An acceptor peptide bound to one side of PglB forms a loop that almost completes a 180° turn. Asparagine located at the tip of the loop is presented through a small tunnel ("porthole") to the oligosaccharide-binding cavity, which is located on the other side of PglB (25). Assuming that mammalian OST has a similar structure, it is difficult to understand how an already glycosylated peptide could be accommodated in such a narrow acceptor binding pocket to achieve glycosylation of the second asparagine in the (S) overlapping sequon. Because our data unequivocally show double glycosylation of the overlapping sequon for the majority of Gp3 molecules, a mechanism must exist so OST can achieve this. Because Gp3 construct containing the signal anchor (27 amino acids) plus 14 amino acids from the stalk region from NA of influenza virus is glycosylated only once, one may speculate that the signal peptide of Gp3 (26 amino acids) is required for double glycosylation at the overlapping sequon. Alternatively, because the distance between the transmembrane region and the overlapping sequon is longer in NA-Gp3-HA, a location of the overlapping sequon near the translocon might be required to facilitate double glycosylation.

Membrane fractionation and secretion experiments revealed that signal peptide of Gp3 does not act as a membrane anchor, indicating that it is completely translocated into the lumen of the ER (Figs. 5 and 6). Membrane attachment is caused by the hydrophobic C terminus of Gp3, which, however, does not span the membrane but rather attaches the protein peripherally to ER membranes (Fig. 4 and schematic representation in Fig. 7).

It is not obvious from its biophysical properties how the signal peptide of Gp3 does not function as a signal anchor domain. Integration of helices into the bilayer appears to be a physicochemical partitioning process between translocon and bilayer membranes.

### Table 2

| ID     | Protein                  | Amino acid sequence                          |
|--------|--------------------------|----------------------------------------------|
| P28893 | Gp3 of EAV (arterivirus) | MGRAYGVPALLCFLLYFCICGSVG | SNNTTICMHTTSDTVH |
Glycosylation and Signal Peptide Cleavage of Gp3

that can be described quantitatively by apparent free energies (26). Using such a calculation (ΔG Prediction Server v1.0) it was predicted that residues 3–22 of Gp3 (MGRAYSGPVALLCFF-LYFCFICGSVGSNNTT) may form a transmembrane region. Note, however, that the presence of a proline residue (bold) conserved in 95% of all EAV strains may prevent the formation of an α-helix, which is also critical for membrane insertion (26). Furthermore, if the signal peptide were a stable transmembrane region, then the glycosylation sites of the overlapping sequon would be too close to the membrane to allow glycosylation. At least 13 amino acids must be present between the end of a transmembrane span and a sequon to allow glycosylation (27), but in Gp3, there are only six residues between the membrane and the sequons (assuming transmembrane configuration of the signal peptide). Furthermore, Gp3 from PRRSV, which does not contain a hydrophobic C terminus, is (partly) secreted in a soluble form from cells (28), reinforcing the notion that the signal peptide of Gp3 does not act as a signal anchor domain.

The most remarkable finding, probably with significance for glyobiology in general, is that co-translational carbohydrate attachment adjacent to the signal peptide of Gp3 prevents processing. Removal of both (but not one) glycosylation sites of the overlapping sequon increased the SDS-PAGE mobility of deglycosylated Gp3 (Fig. 2). This change was independent of the enzyme used for deglycosylation, the mutation introduced, and the antibody tag attached. In addition, wild type Gp3 and the mutants with one and two deleted glycosylation sites had identical SDS-PAGE mobility when synthesized in vitro, a procedure that yields protein without modifications. This demonstrates that the amino acid exchange per se does not alter the SDS-PAGE mobility of Gp3. Furthermore, glycosylation of Gp3 is very rapid; no difference in carbohydrate attachment to individual sites was observed in pulse-chase experiments (Fig. 3A).

Finally, treatment of Gp3-expressing cells with tunicamycin allowed cleavage, demonstrating that glycosylation inhibits signal peptide cleavage (Fig. 3B).

The glycoproteins of arteriviruses belong to the most variable proteins of all viruses; especially glycosylation sites are acquired or lost during evolution (2). However, comparison of 252 Gp3 sequences from the NCBI database showed that Gp3 contains the overlapping sequon adjacent to the predicted signal peptide cleavage site in two-thirds of all EAV strains. The remaining EAV strains have just one site as do the multitude of Gp3 variants from the other members of the Arteriviridae. We observed that the presence of one glycosylation site is sufficient to prevent signal peptide cleavage from Gp3 of the Bucyrus strain of EAV. Thus, one glycan in this region appears to be sufficient to block access of the signal peptidase despite the fact that the signal peptide from Gp3 of all arteriviruses is predicted to be cleaved with high probability (Table 1). Retention of the signal peptide also has been experimentally proven for Gp3 from lactate dehydrogenase-elevating virus (29). Thus, this unusual mode of co-translational processing of Gp3 is probably important for the biology of all arteriviruses.

Inhibition of signal peptide cleavage by addition of nearby glycans may be a more general principle, possibly occurring in other proteins as well. p62, the precursor for the E2 spike of Semliki Forest virus, is targeted to the ER by means of the N-terminal E3 protein, which functions as an uncleaved signal peptide. However, E3 does not act as a signal anchor but is completely translocated into the lumen of the ER. Cleavage occurs only later by furin in the trans-Golgi network at a polybasic site (30, 31). Interestingly, SignalP predicts that the signal peptide is cleaved from E3 (albeit with a probability just above the threshold level; Table 1), but an efficiently used glycosylation site overlaps the predicted cleavage site. Because cells do not preserve mechanisms that are beneficial only for their parasites, we propose that glycosylation adjacent to the signal peptide will also inhibit processing of some cellular proteins. Tables 2–4 list a multitude of human proteins that contain a glycosylation sequon within five amino acids after the (predicted but experimentally not verified) signal peptide cleavage site. At least some of them may be processed similarly as shown here for Gp3. This remains to be proven experimentally.

However, for proteins previously investigated in this regard, it has been clearly demonstrated that the attachment of carbohydrates in the vicinity of the signal peptide does not prevent cleavage. We have recently shown that the presence of one (and even the insertion of a second) efficiently used glycosylation site located just three amino acids downstream of the signal peptide cleavage site does not inhibit processing of Gp5 from PRRSV (32). In the case of prepro-α-factor and preinvertase, glycosylation at sequons located four amino acids downstream of the signal peptide requires prior cleavage of the signal peptide. This indicates that signal peptidase and OST act in a sequential and dependent manner with their substrate; i.e. signal peptide cleavage occurs first (18, 22).

Therefore, we speculate that the order of events is reversed during translocation of Gp3 such that glycosylation occurs first and inhibits signal peptide cleavage. We further speculate that it must be a subject of regulation whether signal peptidase or OST has privileged access to the growing polypeptide chain if they compete for neighboring sites. This is evident if signal peptide cleavage is a requirement for efficient glycosylation (18, 22) or especially if glycosylation inhibits signal peptide cleavage (this study). In the absence of regulation, i.e. if OST and signal peptidase would have random access to neighboring sites, a mixed protein population would be produced: a fraction of proteins would and the other would not contain the signal peptide.

How the sequence of co-translational, possibly competitive modifications is regulated is unknown, but the signal peptide itself may play a role. It has emerged recently that different signal peptides interact with different binding sites within the translocon and that these differences can substantially affect protein biogenesis (15). Because the translocon may associate with other proteins (16) and multiple OST complexes with different protein composition have been described (17), one can imagine that the signal peptide of Gp3 selects or recruits a specific complex for its biosynthesis that is able to perform rapid double glycosylation of the overlapping sequons to prevent signal peptide cleavage. It was proposed recently that signal anchors insert head first into the translocon and subsequently but still at the translocon rotate 180° to expose the N terminus to the cytoplasm (33). To facilitate this inversion, the N terminus should contain a net charge of +3 to fulfill the “positive inside rule” (34). However, the signal peptide of Gp3 has only
one positively charged amino acid, which is only even present in around one-third of all Gp3 sequences. Thus, one can speculate that the signal peptide of Gp3 may not loop back but could slip out from the transloco into the lumen of the ER such that the overlapping sequon becomes accessible to OST, similar to what was proposed for E3 of Semliki Forest virus (30).

It seems somewhat mysterious why evolution created such a complicated procedure to prevent signal peptide cleavage from Gp3. However, the genomic region encoding the N terminus of Gp3 overlaps with the C terminus of Gp2 (2), and therefore, the resulting amino acid sequence may be a compromise to fulfill the functional requirements of both Gp2 and Gp3. Furthermore, regulation of signal peptide cleavage by glycosylation may allow variability of processing. One may speculate that during virus infection of animals the signal peptide is cleaved in certain cell types only. This possibly creates a protein with different antigenic properties such that existing antibodies no longer bind to Gp3. This hypothesis may partially explain viral persistence, a characteristic hallmark of arterivirus infections.

Acknowledgments—We thank Bastian Thaa for performing the sequence comparison of Gp3 proteins, many helpful suggestions, and correction of the manuscript and Peter Rottier (Virology, Utrecht University) for providing cDNA of EAV genes.

REFERENCES

1. Balasuriya, U. B., Go, Y. Y., and Maclachlan, N. I. (2013) Equine arteritis virus. Vet. Microbiol. 10.1016/j.vetmic.2013.06.015
2. Snijder, E. J., and Meulenberg, J. J. (1998) The molecular biology of arterivirus. J. Gen. Virol. 79, 961–979
3. Tian, D., Wei, Z., Zevenhoven-Dobbe, J. C., Liu, R., Tong, G., Snijder, E. J., and Yuan, S. (2012) Arterivirus minor envelope proteins are a major determinant of viral tropism in cell culture. J. Virol. 86, 3701–3712
4. Wieringa, R., de Vries, A. A., van der Meulen, J., Godeke, G. J., Onderwater, J. I., van Tol, H., Koerten, H. K., Moomaa, A. M., Snijder, E. J., and Rottier, P. J. (2004) Structural protein requirements in equine arteritis virus assembly. J. Virol. 78, 13019–13027
5. Nitschke, M., Korte, T., Tiesles, C., Ter-Avetisyan, G., Tünnemann, G., Cardoso, M. C., Veit, M., and Herrmann, A. (2008) Equine arteritis virus is delivered to an acidic compartment of host cells via clathrin-dependent endocytosis. Virology 377, 248–254
6. Wieringa, R., de Vries, A. A., Raamsman, M. J., and Rottier, P. J. (2002) Characterization of two new structural glycoproteins, GP3 and GP4, of equine arteritis virus. J. Virol. 76, 10829–10840
7. de Vries, A. A., Raamsman, M. J., van Dijk, H. A., Horzinek, M. C., and Rottier, P. J. (1995) The small envelope glycoprotein (GS) of equine arteritis virus folds into three distinct monomers and a disulfide-linked dimer. J. Virol. 69, 3441–3448
8. Wieringa, R., de Vries, A. A., and Rottier, P. J. (2003) Formation of disulfide-linked complexes between the three minor envelope glycoproteins (GP2b, GP3, and GP4) of equine arteritis virus. J. Virol. 77, 6216–6226
9. Heudes, J. F., Balasuriya, U. B., and Maclachlan, N. I. (1999) The open reading frame 3 of equine arteritis virus encodes an immunogenic glycosylated, integral membrane protein. Virology 264, 92–98
10. Petersen, T. N., Brunak, S., von Heijne, G., and Nielsen, H. (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8, 785–786
11. Karamyshev, A. L., Kelleher, D. J., Gilmore, R., Johnson, A. E., von Heijne, G., and Nilsson, I. (2005) Mapping the interaction of the STT3 subunit of the oligosaccharyl transferase complex with nascent polypeptide chains. J. Biol. Chem. 280, 40489–40493
12. Reddy, A., Gibbs, B. S., Liu, Y. L., Coward, J. K., Changchien, L. M., and Maley, F. (1999) Glycosylation of the overlapping sequons in yeast extracellular invertase: effect of amino acid variation on site selectivity in vivo and in vitro. Glycobiology 9, 547–555
13. Kelleher, D. J., and Gilmore, R. (2006) An evolving view of the eukaryotic oligosaccharyltransferase. Glycobiology 16, 47R–62R
14. Yan, A., and Lennarz, W. J. (2005) Unraveling the mechanism of protein N-glycosylation. J. Biol. Chem. 280, 3121–3124
15. Hegde, R. S., and Bernstein, H. D. (2006) The surprising complexity of signal sequences. Trends Biochem. Sci. 31, 563–571
16. Rapoport, T. A. (2007) Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. Nature 450, 663–669
17. Yan, A., and Lennarz, W. J. (2005) Two oligosaccharyltransferase complexes exist in yeast and associate with two different translocons. Glycobiology 15, 1407–1415
18. Chen, X., Van Valkenburgh, C., Liang, H., Fang, H., and Green, N. (2001) Signal peptidase and oligosaccharyltransferase interact in a sequential and dependent manner within the endoplasmic reticulum. J. Biol. Chem. 276, 2411–2416
19. Veit, M., Ponimaskin, E., and Schmidt, M. F. (2008) Analysis of S-acylation of proteins. Methods Mol. Biol. 446, 163–182
20. Lorenz, H., Hailey, D. W., Wunder, C., and Lippincott-Schwartz, J. (2006) The fluorescence protease protection (FPP) assay to determine protein localization and membrane topology. Nat. Protoc. 1, 276–279
21. Ernst, A. M., Zacherl, S., Herrmann, A., Hacke, M., Nickel, W., Wieland, F. T., and Brügger, B. (2013) Differential transport of influenza A neuraminidase signal anchor peptides to the plasma membrane. FEBS Lett. 587, 1411–1417
22. Ruiz-Canada, C., Kelleher, D. J., and Gilmore, R. (2009) Cotranslational and posttranslational N-glycosylation of polypeptides by distinct mammalian OST isoforms. Cell 136, 272–283
23. Braakman, I., and Bulleid, N. J. (2011) Protein folding and modification in the mammalian endoplasmic reticulum. Annu. Rev. Biochem. 80, 71–99
24. Ellgaard, L., and Helenius, A. (2003) Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol. 4, 181–191
25. Lizak, C., Gerber, S., Numao, S., Aebi, M., and Locher, K. P. (2011) X-ray structure of a bacterial oligosaccharyltransferase. Nature 474, 350–355
26. White, S. H., and von Heijne, G. (2008) How translocons select transmembrane helices. Annu. Rev. Biophys. 37, 23–42
27. Nilsson, I. M., and von Heijne, G. (1993) Determination of the distance between the oligosaccharyltransferase active site and the endoplasmic reticulum membrane. J. Biol. Chem. 268, 5798–5801
28. Mardassi, H., Gonin, P., Gagnon, C. A., Massie, B., and Dea, S. (1998) A subset of porcine reproductive and respiratory syndrome virus GP3 glycoprotein is released into the culture medium of cells as a non-virion-associated and membrane-free (soluble) form. J. Virol. 72, 6298–6306
29. Faaberg, K. S., and Plagemann, P. G. (1997) ORF 3 of lactate dehydrogenase-elevating virus encodes a soluble, nonstructural, highly glycosylated, and antigenic protein. Virology 227, 245–251
30. Garoff, H., Huylebroeck, D., Robinson, A., Tillman, U., and Liljestrom, P. (1990) The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. J. Cell Biol. 111, 867–876
31. Jose, J., Snyder, J. E., and Kuhn, R. J. (2009) A structural and functional perspective of alphavirus replication and assembly. Future Microbiol. 4, 837–856
32. Thaa, B., Sinhadri, B. C., Tiesles, C., Krause, E., and Veit, M. (2013) Signal peptide cleavage from GP5 of PRRSV: a minor fraction of molecules retains the decoy epitope, a presumed molecular cause for viral persistence. Future Microbiol. 8, 655–658
33. Devaraneni, P. K., Conti, B., Matsumura, Y., Yang, Z., Johnson, A. E., and Skach, W. R. (2011) Stepwise insertion and inversion of a type II signal anchor sequence in the ribosome-sec61 translocon complex. Cell 146, 134–147
34. von Heijne, G. (2006) Membrane-protein topology. Nat. Rev. Mol. Cell Biol. 7, 909–918
35. Scolari, S., Engel, S., Krebs, N., Plazzo, A. P., De Almeida, R. F., Prieto, M., Veit, M., and Herrmann, A. (2009) Lateral distribution of the transmembrane domain of influenza virus hemagglutinin revealed by time-resolved fluorescence imaging. J. Biol. Chem. 284, 15708–15716