===================== REVIEW =====================

# Mass Spectrometric Approaches to Study Enveloped Viruses: New Possibilities for Structural Biology and Prophylactic Medicine

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**Abstract**—This review considers principles of the use of mass spectrometry for the study of biological macromolecules. Some examples of protein identification, virion proteomics, testing vaccine preparations, and strain surveillance are represented. Possibilities of structural characterization of viral proteins and their posttranslational modifications are shown. The authors' studies by MALDI-MS on S-acylation of glycoproteins from various families of enveloped viruses and on oligomerization of the influenza virus hemagglutinin transmembrane domains are summarized.

Mass spectrometry (MS) is a rapid, sensitive, selective, and universal approach that due to its advantages is widely used for analysis of various chemical compounds. The effectiveness of MS is generally recognized, and many fundamental works in this field have been awarded with Nobel Prizes. The development of soft ionization and desorption of biological macromolecules, electrospray, and MALDI was the most important step for using MS in biology and medicine. Authors of these approaches, John Fenn and Koichi Tanaka, were awarded the Nobel Prize in Chemistry in 2002. In the present review we consider principles of MS and show its possibilities for studies on enveloped viruses.

Enveloped viruses are characterized by the presence of a lipoprotein envelope around the nucleocapsid that the virus acquires on its "budding" from the cell membrane [1]. The group of enveloped viruses includes many dangerous pathogens: viruses of influenza and respiratory infections, hepatites, herpes, hemorrhagic fevers, human immunodeficiency, etc. Many of the data presented here refer to influenza virus, but this does not diminish the generality of using the approaches under consideration.

The influenza A virion, which is a typical representative of the family Orthomixoviridae, includes eight segments of single-stranded negative sense RNA in a complex with nucleoprotein (NP) and polymerase complex proteins (PB1, PB2, PA) [2]. The virion envelope includes two glycoproteins: the major hemagglutinin (HA) responsible for the virus entry into the cell and the enzyme neuraminidase (NA), as well as the minor protein M2, which is an ion channel. Under the lipid membrane there is a layer of molecules of the main structural protein, matrix protein M1. Some copies of protein NS2 (NEP) are also found in virions.

We will present examples of identification and structural characterization of proteins and their posttranslational modifications, consider data on virion proteomics, and show advantages of using MS for strain surveillance and testing vaccines. We will summarize our experience of the last eight years using MALDI-MS for studies on S-acylation, i.e. the modification by fatty acid residues of anchoring segments of viral glycoproteins, and also describe an original approach for assessment of oligomerization of HA transmembrane domains.

**Principles and types of MS.** The main parameter measured by MS is the ratio of the mass of a studied mol-

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*Abbreviations*: HA, hemagglutinin; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; NA, neuraminidase; TM, transmembrane.
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ecule to its charge, *m/z* (Fig. 1a) [3]. The essential scheme of a mass spectrometer includes a source of ions, a system of their separation, and a detector (Fig. 1b). Because the majority of biological molecules (including proteins and peptides) cannot be independently converted into the gas phase, special methods are required to prepare the substance and inject it into the measuring system. At present, proteins and peptides are subjected to soft ionization using two techniques that are more or less associated with co-evaporation of the analyzed biopolymers with an evaporated carrier. These techniques are electrospray and ionization under atmospheric pressure (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Fig. 1, c and d). In both cases, ions of the protein or peptide molecules are produced as a result of detachment of one or a few protons.

For electrospray, the substance in solution in a polar solvent is introduced under voltage and atmospheric pressure through a thin capillary. The excess electrostatic potential produced on the surface of the drop results in its explosion. Charged microdrops lose the solvent molecules through collisions with molecules of air or an inert gas, and they explode repeatedly when the surface charge again overpowers the surface tension. On the entrance into the vacuum system of ion separation, a continuous flow of ions of the analyzed substance is produced. The ions have different charges (up to a maximal one) (Fig. 1c). This ionization technique can be easily combined with a preliminary chromatographic system of separation, e.g. in a configuration with liquid chromatography (LC)-MS/MS, and it is widely used to analyze hydrophilic
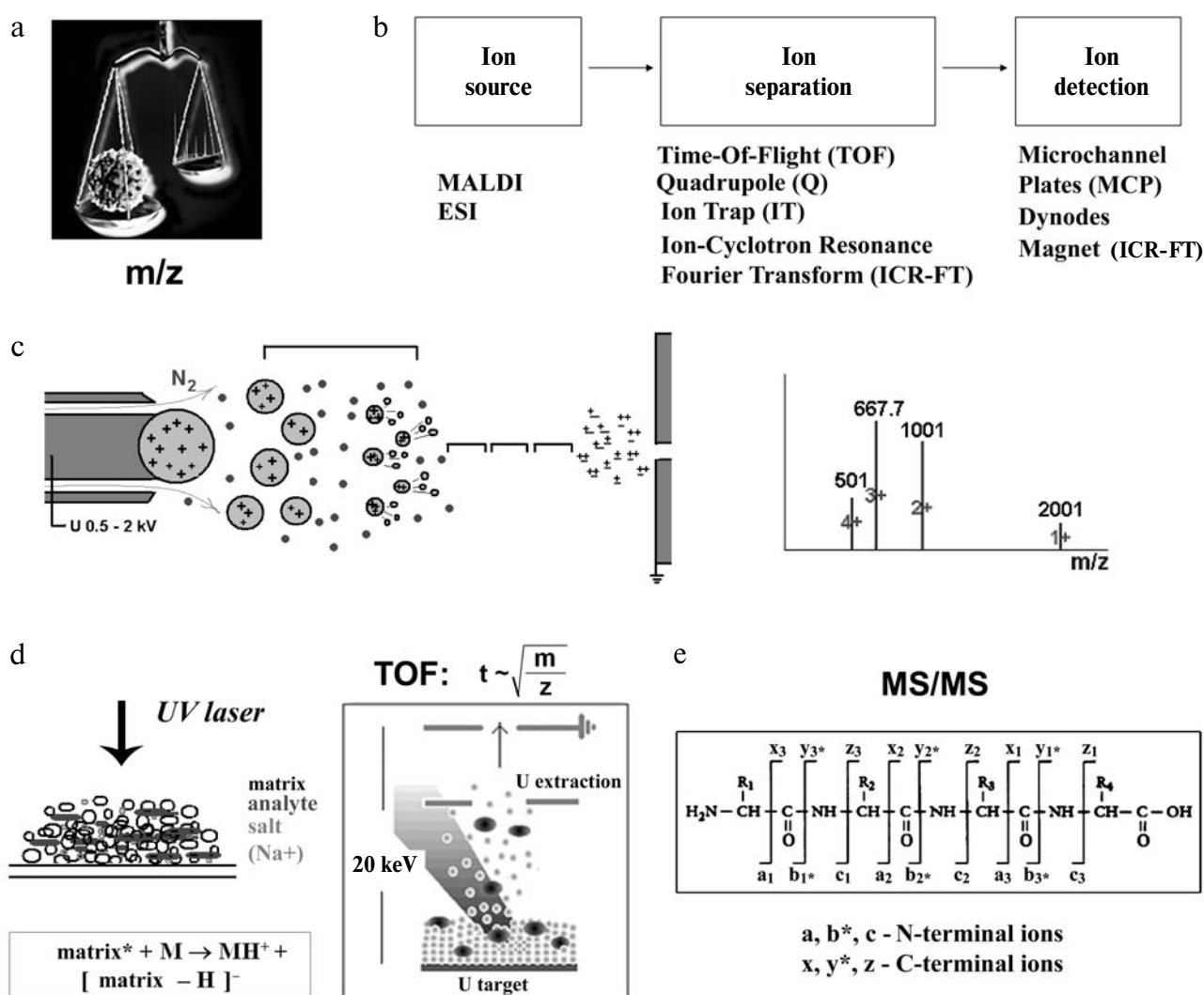


**Fig. 1.** Principles of mass spectrometry. a) *m/z* is a parameter determined by MS, where *m* is the mass of a molecule and *z* is its charge; b) basic scheme of a mass spectrometer. Varieties of ionization systems, ion separation, and detection are presented; c) principle of producing positively charged ions by electrospray and a mass spectrum example (see text for description); d) principle of mass measurement in MALDI-MS. M is a substance analyzed (analyte) that accepts a proton from the matrix; e) principle of tandem MS: masses of *N*- and *C*-terminal fragments of the parent ion are measured. The sign "*" indicates *b*- and *y*-ions resulting on splitting off of a molecule at a peptide bond.

compounds, especially of multicomponent mixtures. On the other hand, on analyzing hydrophobic compounds (especially if they are mixed, e.g. with lipids and detergents) electrospray cannot be used because of strong surface tension and use of nonpolar solvents. For such compounds MALDI can be used for ionization.

In MALDI (Fig. 1d), the studied substance is mixed on a steel target plate with an accessory matrix substance consisting of organic acids, most often cinnamic acid derivatives or 2,5-dihydroxybenzoic acid. Upon desiccation, the matrix co-crystallizes with the studied substance (and also with buffer solution salts), and then the plate with the applied sample is placed into the vacuum chamber of the mass spectrometer. Under the influence of a short pulse of a laser with appropriate wavelength, the matrix is evaporated into the gas phase together with molecules of the studied substance. Moreover, the matrix is also a donor of protons and is responsible for charge transfer onto molecules of the analyte. For unknown reasons, biopolymers (including proteins and peptides) produce mainly singly charged ions. MALDI produces a short pulse ion packet and is well combined with the time-of-flight (TOF) system of ion separation in which the time of ion arrival onto the detector is measured, and this time is proportional to the mass of the ion.

Although both ionization techniques are believed to be soft and nondestructive, some of the initial (parent) ions can be partially fragmented. Peptide bonds are often broken in proteins and peptides and N-terminal b-ions and C-terminal y-ions are produced, but other bonds in the molecule can also be broken (Fig. 1e). The fragmentation can be strengthened by additional influences on the parent ion packet chosen with an ion selector. The additional fragmentation can be induced by collisions with molecules of a specially introduced inert gas (Collision Induced Dissociation (CID)), by photon absorption (Laser Induced Dissociation (LID)), electron transfer (Electron Transfer Dissociation (ETD)), or by electron capture (Electron Capture Dissociation (ECD)) [3]. This determines the possibilities of using the tandem mass spectrometry (MS/MS, and also $MS^3$, $MS^4$, etc.) for more intensive analysis of individual peptides, more accurate description of the primary structure (up to *de novo* sequencing), and determination of posttranslational modifications.

**Identification of proteins.** MS is important for identification of proteins, and this can be done for a complex mixture after preliminary proteolysis of proteins with highly specific protease and for preliminarily separated proteins. One such strategy uses one-dimensional gel electrophoresis plus LC-MS/MS: before being analyzed by MS, a mixture of peptides from different proteins is separated by chromatography, and a database search is performed based on the fragmentation spectra of individual peptides (tandem mass spectrometry, MS/MS). However, handling of individual proteins significantly
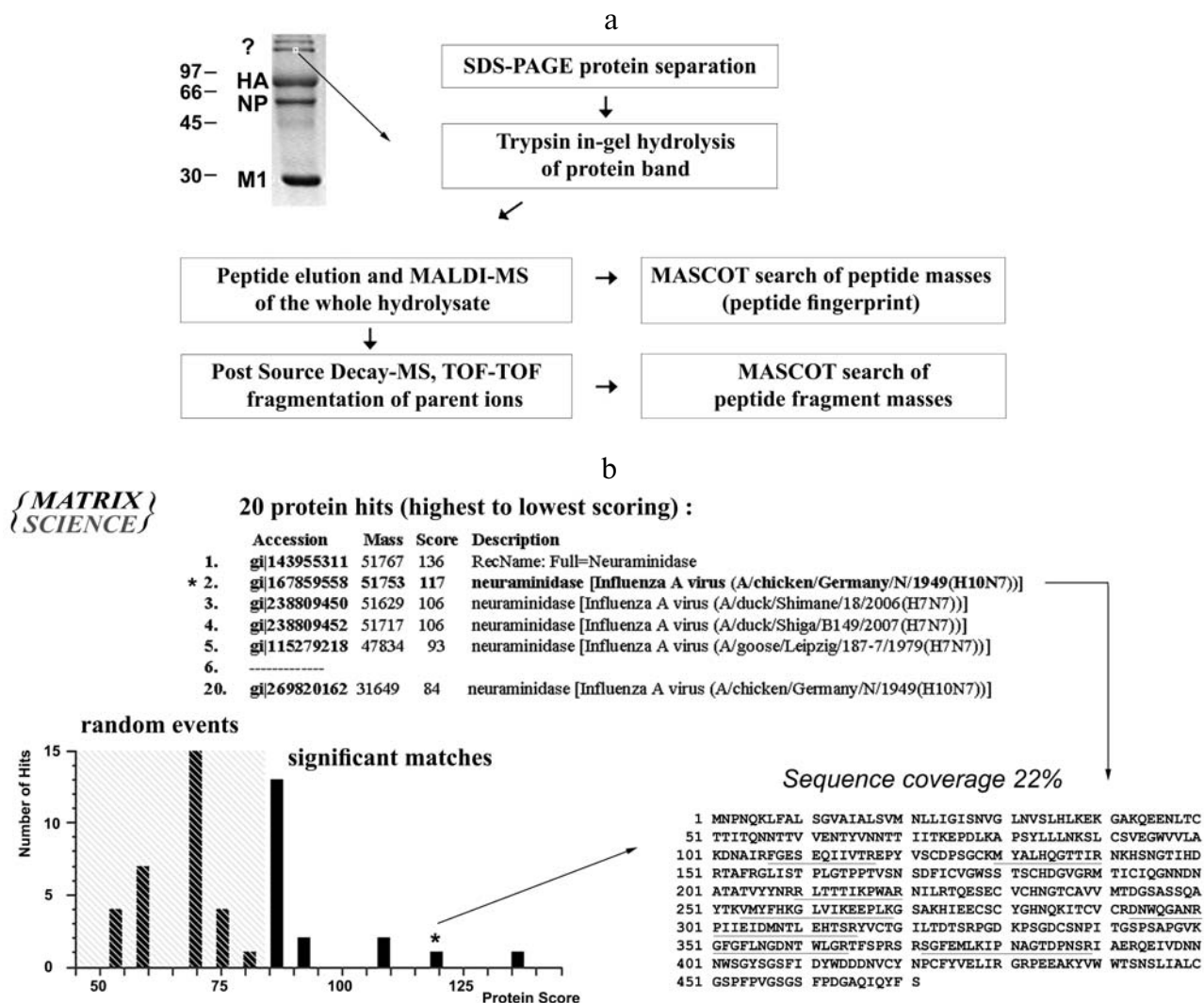
simplifies the subsequent analysis, allowing them to be identified by the peptide fingerprint to reveal in the spectra signals different from the calculated ones, and, thus, to detect *a priori* unknown protein modifications. In this case the studied protein has to be isolated very carefully, e.g. using one-dimensional or two-dimensional gel electrophoresis.

Figure 2a presents the strategy for identification of a dimer $(NA)_2$ in a preparation of virions of influenza virus strain A/Chicken/Germany/49 (H10N7) upon separation of the proteins using one-dimensional gel electrophoresis according to Laemmli and staining with Coomassie. A fragment containing the studied protein is cut from the gel and hydrolyzed *in situ*, usually with highly purified trypsin, and the peptides are eluted from the gel and analyzed by MALDI-TOF MS, or MS/MS in some cases.

The final stage is a search for the correspondence of the resulting set of masses to a homologous protein from the database using the algorithms Peptide/ProteinProphet (Institute for Systems Biology; http://proteinprophet. sourceforge.net/), Mascot (www.matrixscience.com), ProFound [4], and some others. The main pinpoints the Mascot algorithm relies on are the number of "matched" peptides and the protein sequence coverage by the matched peptides (Fig. 2b). In the given case five of 20 candidates found in the NCBI database are in the field of the reliable search, and one exactly corresponds to the NA of the studied strain. In contrast to Mascot, in the search with the ProteinProphet and ProFound algorithms the number of coincident "unique" peptides is a priority criterion. The appropriateness of using one of the various algorithms is also associated with the experiment specificity (preparation of the specimen, MS analysis technique). Because different systems for evaluation of the reliability are used in the search programs, it is reasonable to use two alternative approaches for reliable identification of a new (minor) protein.

**Proteomics of virions.** Proteomics is set of highly technological approaches for studies of proteins and their complexes and interactions, and it is strongly based on the possibility to identify proteins by MS. Although protein identification in a gel slice by MS is considerably less sensitive than Western-blotting, it has major advantages in universality because it does not require preliminary hypotheses regarding the sample protein composition, the time required, and the cost of the experiment.

Recently virions of influenza virus strain A/WSN/33 (H1N1) were analyzed by proteomics approaches using two complementary schemes of protein identification. The first scheme corresponded to the above-described identification of influenza virus NA (Fig. 2). Proteins of the purified virions were fractionated by Laemmli electrophoresis, and then the entire gel lane was cut into thin strips, which were then subjected to tryptic hydrolysis. In the alternative scheme of analysis unseparated purified

a



SDS-PAGE protein separation

↓

Trypsin in-gel hydrolysis
of protein band

↙

Peptide elution and MALDI-MS
of the whole hydrolysate   →   MASCOT search of peptide masses
                                (peptide fingerprint)

↓

Post Source Decay-MS, TOF-TOF   →        MASCOT search of
fragmentation of parent ions              peptide fragment masses

b

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**20 protein hits (highest to lowest scoring):**

|   | Accession | Mass | Score | Description |
|---|-----------|------|-------|-------------|
| 1. | gi|143955311 | 51767 | 136 | RecName: Full=Neuraminidase |
| *2. | gi|167859558 | 51753 | 117 | neuraminidase [Influenza A virus (A/chicken/Germany/N/1949(H10N7))] |
| 3. | gi|238809450 | 51629 | 106 | neuraminidase [Influenza A virus (A/duck/Shimane/18/2006(H7N7))] |
| 4. | gi|238809452 | 51717 | 106 | neuraminidase [Influenza A virus (A/duck/Shiga/B149/2007(H7N7))] |
| 5. | gi|115279218 | 47834 | 93 | neuraminidase [Influenza A virus (A/goose/Leipzig/187-7/1979(H7N7))] |
| 6. | ------------- | | | |
| 20. | gi|269820162 | 31649 | 84 | neuraminidase [Influenza A virus (A/chicken/Germany/N/1949(H10N7))] |

random events     significant matches



*Sequence coverage 22%*

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  1 MNPNQKLFAL SGVAIALSVM NLLIGISNVG LNVSLHLKEK GAKQEENLTC
 51 TTITQNNTTV VENTYVNNTT IITKEPDLKA PSYLLLNKSL CSVEGWVVLA
101 KDNAIRFGES EQIIVTREPY VSCDPSGCKM YALHQGTTIR NKHSNGTIHD
151 RTAFRGLIST PLGTPPTVSN SDFICVGWSS TSCHDGVGRM TICIQGNNDN
201 ATATVYYNRR LTTTIKPWAR NILRTQESEC VCHNGTCAVV MTDGSASSQA
251 YTKVMYFHKG LVIKEEPLKG SAKHIEECSC YGHNQKITCV CRDNWQGANR
301 PIIEIDMNTL EHTSRYVCTG ILTDTSRPGD KPSGDCSNPI TGSPSAPGVK
351 GFGFLNGDNT WLGRTFSPRS RSGFEMLKIP NAGTDPNSRI AERQEIVDNN
401 NWSGYSGSFI DYWDDDNVCY NPCFYVELIR GRPEEAKYVW WTSNSLIALC
451 GSPFPVGSGS FPDGAQIQYF S
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**Fig. 2.** Strategy of protein identification by MS within a band of a polyacrylamide gel. a) Scheme of the experiment; b) search for homolog based on MS data with the Mascot algorithm.

preparations of the virions were fully hydrolyzed with trypsin, and then the peptides were separated by ion-exchange chromatography. In both schemes, the final stage was analysis of the peptides by LC-MS/MS. The search for correspondence of the found masses to homologs from the database revealed that the virions, in addition to nine proteins encoded by the virus genome (HA, NA, NP, PB1, PB2, PA, M1, M2, and NS2), contain 36 proteins of the host cell [5]. Among them are several groups of cytoplasmic and membrane-bound proteins: cytoskeletal proteins, annexins, glycolytic enzymes, and tetraspanins. Many proteins found in the influenza virus virion were identified by MS in virions of other families of enveloped viruses, in particular in retroviruses [6], herpes viruses [7, 8], vesicular stomatitis virus [9], and corona viruses [10, 11].

It seems that some proteins are recruited and "packed" into a virion through interactions with viral proteins or even with the viral genome. Thus, using a proteomics approach combined with biochemical methods, cellular protein partners were identified that interact with the polymerase and ribonucleoprotein complexes of the influenza virus [12, 13]. It seems that some cytosolic proteins can be casually captured during the budding from the virion. Note that influenza virus virions contain a set of proteins detected by the proteomic analysis of rafts (cell membrane domains enriched with sphingolipids and cholesterol) isolated from some types of cells [14, 15]. These proteins include tubulin, actin, annexins, enolase, glypican 4, glyceraldehyde-3-phosphate dehydrogenase, γ-glutamyl transferase, transgenin, and some heat shock proteins. Lipid rafts are structures from which many enveloped viruses are budded, as recently confirmed by MS analysis of the lipid composition of the influenza virus virions [16] and of HIV-1 [17]. On the other hand, such known raft markers as caveolin and flotillin were not

detected either in the influenza virus virions [5] or in the HIV-1 particles [6, 17]. Further investigations are expected to distinguish "accidental co-travelers" from cellular protein partners that are required during stages of virus entrance into the cell, assembly of daughter virions, or are involved in the replication/transcription of the genome. Some of these proteins might be promising targets for antiviral therapy.

The present review was not designed to consider in detail data of comparative quantitative proteomics obtained for healthy and virus-infected cells. Approaches and techniques for such analysis have been considered in review [18], whereas for the system "influenza virus−cell/model animal" some works appeared only recently [19-22]. An interesting result of the proteomics approach for analysis of the mammalian cell line MDCK infected with an avian virus of subtype H9N2 should be mentioned. A shortened form of NP protein that had not been previously described was detected in a spot after two-dimensional gel electrophoresis. This shortened NP protein contained a modification that was identified by the authors as O-sulfonation [23]. Such a modification had not been described earlier for any other influenza virus protein, and its function is unknown.

**Vaccine testing.** Vaccination is currently the most effective preventive measure for limiting the expansion of influenza epidemics. MS allows researchers to qualitatively characterize a vaccine preparation: to determine possible mutations in the antigens and to reveal admixtures of proteins of chick embryo or cell cultures used for growing the virus [24].

During recent years, recombinant virus-like particles (VLPs) are actively used. By proteomics approaches, the virus-like particles produced by insect cells using a baculovirus vector were found to contain, in addition to the expressed viral proteins HA and M1, 37 proteins from the insect cell line SF9 and also 20 vector proteins [25]. Similar proteins (actin, tubulin, heat shock proteins, and glycolytic enzymes) were found in virus-like particles produced by mammalian Vero cells [26] and also in virions of the influenza virus and other enveloped viruses, as already mentioned [5]. This seems to indicate similar pathways for budding of virions and virus-like particles from the cell membrane. Thus, proteomics approaches give useful information for elucidation of mechanisms of production of virus-like particles and also for understanding the principle of inducing protective immunity by these particles.

Introduction into a testing vaccine specimen of specially chosen (evolutionarily restricted) target peptides labeled with stable isotopes (C13- and N15-) in accurately measured quantities allows the determination by MS of the amount of every antigen in the preparation [27]. The isotope dilution approach combined with LC-MS/MS (IDMS) is already shown to be the much more sensitive and less laborious than the SRID (single radial immuno-diffusion assay) routinely used for this purpose [28]. Trivalent seasonal vaccines include strains of two subtypes of influenza A virus, H1N1 and H3N2, and a strain of the type B influenza virus. Target peptides specific for viruses (A/H1, A/H3, and B) are labeled differently to provide for the mass shift in the spectrum relative to the peptide measured to be 10, 8, 7, or 6 units depending on the antigen type/subtype. Every antigen concentration in the vaccine preparation is calculated by the ratio of heights of each pair of peaks. Another variety of the method can be used for determination not only of HA, but also of different NAs in the seasonal vaccine [29], and in the newest modification of the method all antigens are analyzed by MS upon preliminary immune affinity selection with a polyclonal antiserum immunocapture IDMS assay (IC-IDMS) [30].

An accurate quantitative analysis not only of viral but also of cellular components of vaccines seems to be promising for development of proteomics of vaccine preparations, and this can be useful for evaluation of their probable allergenicity.

**Strain surveillance.** Influenza virus has immense potential for variation, and therefore the composition of recommended vaccines is changed every year or every two years based on data of strain surveillance. The World Health Organization (WHO) recommends identifying the circulating strains using the Reverse Transcription Polymerase Chain Reaction (RT-PCR), enzyme immunoassay, and various serological approaches including the hemagglutination inhibition (HI) and microneutralization reactions. In some modern laboratories different MS approaches are now being tested for typing and subtyping of strains and also for mapping antigenic sites of HA.

A method combining RT-PCR with mass spectrometry (RT-PCR/ESI-MS) was introduced in 2005 for identification and subtyping of microbial pathogens [31]. Based on an accurate measurement of molecular weight of PCR products, this combined method allows the number of bases (A, G, C, T) in the amplicon to be calculated. Later the method was adapted for analysis of influenza virus, and combinations of marker clusters of nucleotides in genes of the inner proteins (PB1, PB2, PA, M, NS, and NP) were found to form a "genomic print" of the influenza virus A strains, which allowed the subtyping of the surface antigens of HA and NA [32]. This method is highly specific, rapid (300 specimens can be processed for 24 h), and not expensive because it can be used for analysis of various clinical specimens without preliminary virus replication in a cell culture [31-33]. In addition to systematic analysis of a great flow of specimens during epidemics or pandemics, RT-PCR combined with MS, due to its high sensitivity, can be used for special studies to reveal the transmission pathways of the virus [34].

In addition to gene typing, proteotyping approaches also seem to be promising, and these approaches have

been successfully developed for more than 10 years by a group of Australian researchers [35-39]. The detection by MS of signature peptides in various proteins of influenza virus (HA, NA, M1, and NP) makes possible the typing and subtyping of virus strains [36-43]. The authors have also developed an original combination of the classic immunoassay used in the hemagglutination inhibition reaction with MALDI-MS to locate epitopes of the antigen interaction with monoclonal antibodies [44-47]. Considering antigen–antibody complexes not destroyed during co-crystallization with the matrix and also under the laser bombardment [44, 48, 49], the authors have compared the mass spectra of HA before and after incubation with antibodies. Peptides represented in the spectra as peaks with relative intensity decreased by 15% and more in the experimental specimen relatively to the control seem to be very suitable candidates for interaction with the antibodies [44-47].

**Structural studies on proteins.** Limited proteolysis or chemical modification of certain amino acid residues at the protein globule surface combined with subsequent MS of peptides are helpful to elucidate the spatial structure of proteins. This is especially important if it is difficult to get information about the spatial structure using traditional approaches such as X-ray crystallography or NMR. Thus, notwithstanding many attempts, the 3-D structure was determined only for the NM domain of matrix protein M1 of influenza virus, but not the full-size molecule [50-52]. We found that during the removal with bromelain of HA from the surface of virions in the absence of a thiol reagent (subviral particles were then used for isolation of anchoring segments of HA that we will consider later) the inner matrix protein M1 was fragmented [53]. We supposed that on limited proteolysis the protein M1 regions oriented to the membrane should be hydrolyzed first. In fact, electron microscopic analysis of thin sections of preparations of subviral particles revealed local disruptions of the lipid bilayer [53]. On manually analyzing some minor peaks in the MALDI mass spectra, additionally to peaks of tryptic peptides we localized several possible sites of M1 hydrolysis by bromelain. The main site was localized within the loop connecting the NM- and C-domains of the M1 molecule, whereas the other major sites were localized near one of the poles of the 3-D-model of the protein globule, and we supposed that this pole should be turned to the virion membrane [54].

Organization of glycoprotein E2 of the Sindbis virus (*Alphavirus*) was considered upon surface biotinylation of virions [55]. The modified virions remained infective, and this suggested that the topology of both glycoproteins (E1 and E2) did not change on the virion surface. The viral particles were labeled, denatured, and the proteins were hydrolyzed enzymatically. Biotin-labeled peptides were identified using affinity chromatography on avidin columns or by reversed-phase HPLC-MS/MS. Seven

lysine residues bound with biotin were found in protein E2, whereas protein E1 had only one such site. The authors concluded that protein E1 was virtually completely hidden under protein E2. The most recent data on the reconstruction of Sindbis virus virions based on data of high resolution cryoelectron microscopy [56] do not contradict the results obtained by the MS study.

The capabilities of MS and MS/MS are invaluable for locating mutations in proteins. For example, nanoelectrospray MS of the conservative protein M1 of a highly pathogenic strain of subtype H5N1 revealed six substitutions of amino acid residues in four tryptic peptides of the protein [57]. A similar analysis of nucleoprotein revealed three substitutions in three peptides [58]. It seems that these mutations can affect the protein structure. In another investigation of avian influenza virus subtype H1N1, amino acid structure of HA protein obtained by gene sequencing was compared with results of direct analysis by nanoelectrospray MS [59]. The authors suggested that the detected seven variations could be produced during replication of the viral genome. These approaches can also be used for investigations of the structure of other viral proteins.

**N-Glycosylation.** N-Glycosylation, i.e. the posttranslational addition of carbohydrate chains via the amidic bond to the asparagine residue in the consensus sequence NX(S/T) (X is any residue except proline) is an important feature of viral glycoproteins that determines their antigenic properties and interactions with cell receptors. However, not all potential sites can bind mono- or oligosaccharide chains. The chain length can also vary.

The strategy of MS analysis of glycoproteins usually includes a complex preparation of samples: enzymatic hydrolysis, separation of glycopeptides by affinity chromatography and reversed-phase HPLC, and also using exoglycosidases [60, 61]. The addition of sugars can be revealed by a shift of the glycopeptide mass relatively to the calculated one and on comparing the mass before and after treatment with glycosidase. Objects of MS analysis are either glycopeptides or detached oligosaccharides. Complex mass spectra cannot be interpreted without knowledge of the principles of glycoprotein processing in the cell. Difficulties associated with MS analysis of glycosylation include low tendency of carbohydrates to be ionized, production of ions complexed with sodium or potassium, and also relative instability of the glycoside bond during MS/MS.

To date, N-glycosylation in enveloped viruses has been analyzed by MS in only a few works. In work [62] a strict site-specificity of the addition of oligosaccharide chains to HA in different mutants of strain A/WSN/33 (H1N1) has been shown. Note that the nearer the site is located to the membrane, the higher is the weight of the oligosaccharide chain. In work [63] investigation of three strains of subtype H5N1 showed that all potential sites of N-glycosylation are occupied [63]. MS analysis of N-gly-

cosylation in glycoprotein gp120 of the HIV-1 virus revealed an extremely high content of oligomannose, which is a target for designing microbicides and vaccines [64, 65]. The oligomannose content was significantly decreased in the recombinant glycoprotein as compared to one isolated from the virions [65]. Glycosylation of the matrix protein in a virus from the Bornaviridae family was found using MALDI- and ESI-MS [66].

It should be noted that in addition to hydrophilic modifications, which can be studied by different methods, MS allows hydrophobic and structurally unstable structures to be characterized in detail. While ectodomains of many enveloped viruses have been crystallized and their 3-D structure is known [67-70], there are no data on organization of transmembrane (TM) domains and on S-acylation. We shall consider these two aspects in the last two sections of this review.

**S-Acylation.** Palmitoylation or S-acylation is the posttranslational binding of fatty acid residues to cysteine residues via thioester bonds. This modification occurs in both integral and peripheral membrane proteins [71].

Protein palmitoylation was first found in viruses [72], and the main features of this hydrophobic modification have been studied on viral proteins used as a model [73].

Thus, protein HA of the influenza A virus is S-acylated at three extremely conservative cysteine residues, one of which is located on the C-terminus of the TM domain (at the border with the cytoplasmic or the intraviral domain) and two others are located in the cytoplasmic domain. The modification of HA is important for replication of the virus because (depending on the strain) either virus mutants with more than one acylation site deleted are very poorly reproduced, or they cannot be reproduced by a reverse genetics approach [74, 75].

Initially, S-acylation was demonstrated using metabolic labeling of viruses with [3H]palmitate and subsequent chromatographic analysis of the total pool of bound fatty acids [76]. However, this analysis failed to give accurate information about either the stoichiometry of acylation or the types of attached residues, as well as about their distribution among the potential sites. The type of fatty acid residue is especially interesting because carbo-
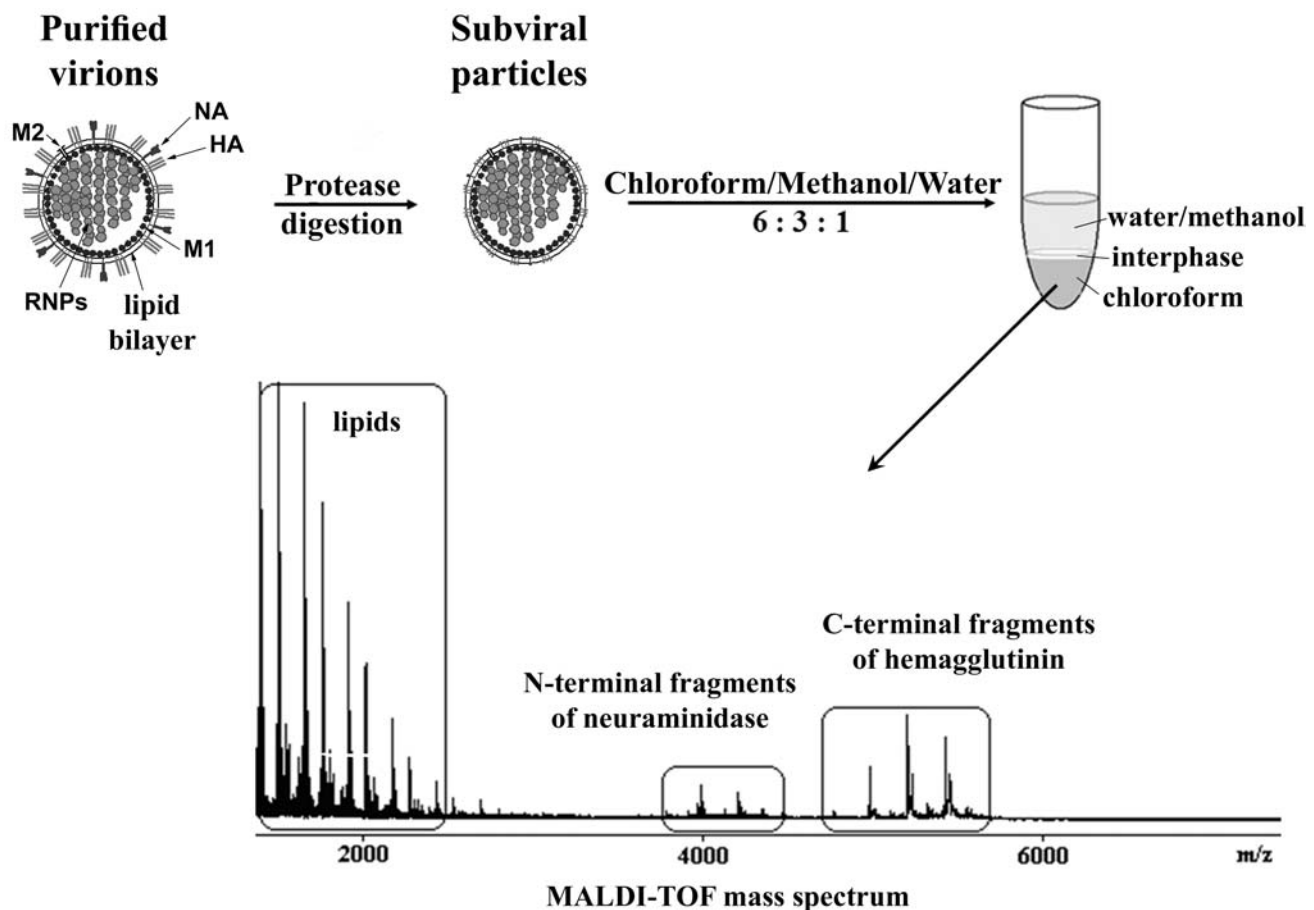


**Fig. 3.** Scheme of isolation and MS analysis of the C-terminal anchoring segment of influenza virus HA. The influenza virus virion components are indicated: lipid bilayer surrounding the viral particle, "spikes" of the envelope glycoproteins − HA homotrimers and NA homotetramers, the ionic channel formed by protein M2, layer of protein M1 molecules under the virion membrane and eight fragments of nucleoprotein particles (RNPs).

hydrate chains different in length by only two carbon atoms are significantly different in their hydrophobicity. This parameter can influence the strength of protein interaction with the membrane and of protein–protein interactions.

We have developed an approach that allows us to analyze the S-acylation of viral proteins on the molecular level (Fig. 3). In the first stage, ectodomains of glycoproteins are removed from the surface of virions with a proteolytic enzyme. Then the subviral particles are extracted with chloroform–methanol mixture according to Folch.

The three resulting phases (aqueous-methanolic, organic, and interphase) were analyzed by MS, and the anchoring segments of glycoproteins (HA and NA) left in the virion envelope were shown to be extracted together with lipid molecules into the organic (chloroform) phase.

We have revealed that in the presence of a reducing agent a partial deacylation of peptides occurs due to destruction of the thioester bond between cysteine and a fatty acid residue [77, 78]. Figure 4a presents a mass spectrum of a preparation of the *C*-terminal segments of HA extracted from subviral particles upon the treatment of
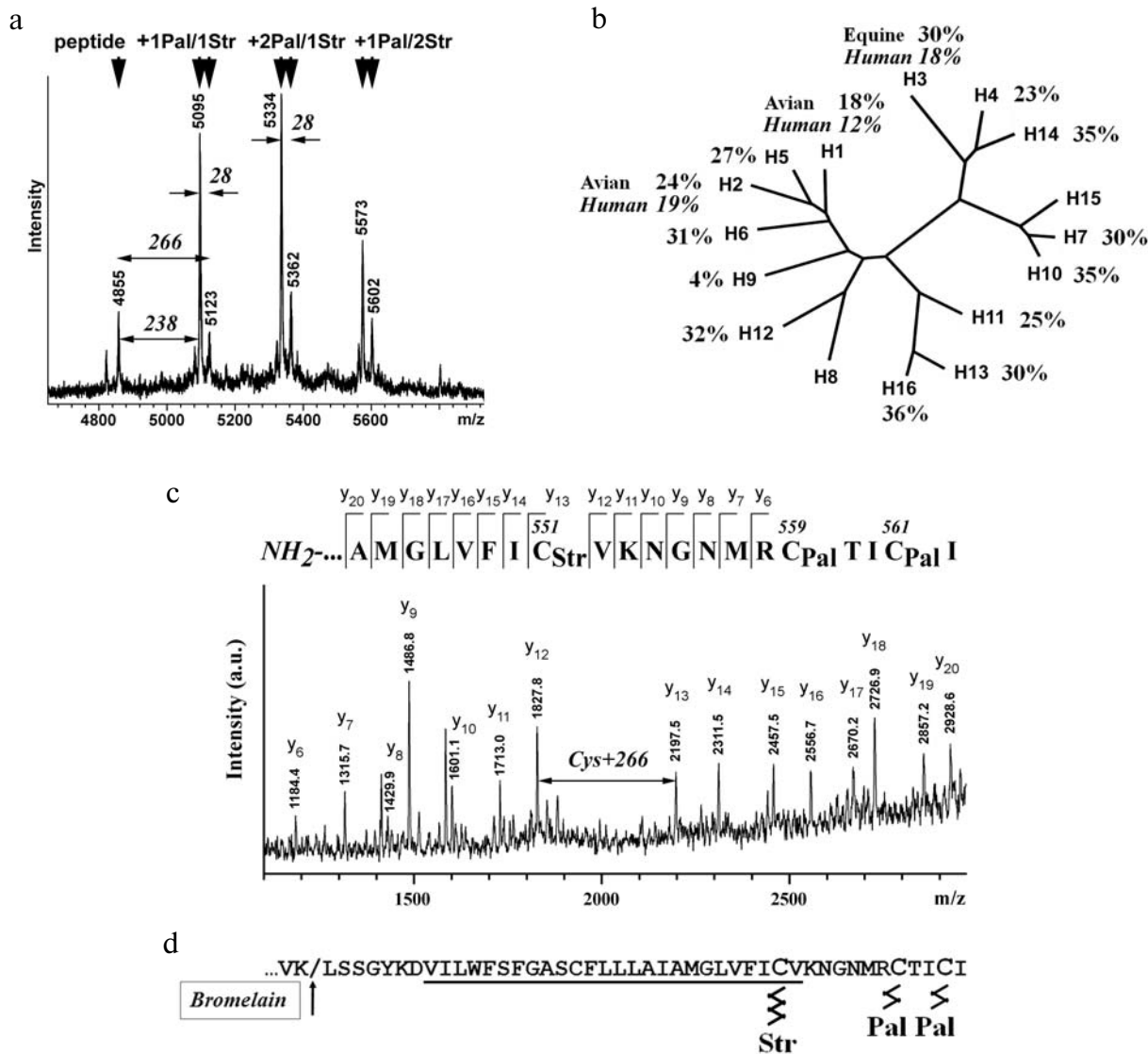


**Fig. 4.** MS analysis of a posttranslational modification of influenza virus hemagglutinin with fatty acid residues. a) Example of MALDI mass spectrum of a preparation of *C*-terminal segments of HA isolated from subviral particles prepared in the presence of 2-mercaptoethanol (strain A/Puerto Rico/8/34(H1N1)). Shifts in the peptide mass by 238 or 266 due to attachment of palmitate (Pal) or stearate (Str), respectively (266 − 238 = 28 *m/z*); b) phylogenetic tree designed for *C*-terminal sequences of HA with the ClustalW algorithm. The relative amount of stearate (C 18:0) bound with HA of different subtypes of the influenza A virus is indicated (the remaining fraction carries palmitate, C 16:0). Differences are shown between human, avian, and horse strains; c) tandem mass spectrum showing identification of the stearate residue covalently bound with the cysteine residue in the transmembrane domain of HA (strain /FPV/Rostock/34(H7N1)). d) Scheme of S-acylation of three conservative cysteine residues with residues of palmitate (Pal) and stearate (Str) (strain A/FPV/Rostock/34(H7N1)). TM domain is underlined. Adapted from [79-81].

virions with bromelain in the presence of 2-mercaptoethanol. Shifts in the peptide mass by 238 or 266 units correspond to addition of saturated fatty acids: palmitic (hexadecanoic, C 16:0) or stearic (octadecanoic, C 18:0), respectively. MS of preparations obtained in the absence of a reducing agent revealed the prevalence (95%) of triply acylated peptides, which suggests stoichiometric occupation of potential sites by fatty acids within the virion [79, 80].

Our approach allowed us to quantitatively evaluate relative fractions of palmitate and stearate based on calculations using a series of mass spectra. The fraction of stearate in different antigenic HA subtypes of influenza A virus varied from 4 to 36% of the total pool of bound fatty acids (Fig. 4b). The relative contents of stearate were different in the strains that were initially isolated from different hosts: HA of human strains of different subtypes (H1, H2, and H3) with identical or almost identical amino acid sequence near acylated cysteines contained significantly less stearate than HA of avian/mammalian strains [81]. The cause of this trend is still unclear.

We also found that HA of influenza B virus that as a result of evolution lacks the conservative cysteine residue in the TM domain but has two sites in the cytoplasmic domain binds only palmitate, whereas the only cysteine of hemagglutinin-esterase-fusion (HEF) glycoprotein of influenza C virus located on the boundary between the TM and cytoplasmic domain is mainly acylated with stearate [80]. The tandem MS analysis and also a series of experiments with recombinant influenza A viruses that include HA with individual substitutions of conservative cysteine residues by serine residues [75] showed that stearate can be attached only to the first of the three potential sites, namely, to the cysteine located on the boundary between TM and the cytoplasmic domain [80] (Fig. 4c). Thus, for HA/HEF of all types of influenza virus (A, B, and C) the stearate residue was found to be located only on the cysteine residue in the TM domain, whereas the cytoplasmic domain cysteines are acylated with palmitate. The number and type of bound fatty acid residues are different in the influenza A, B, and C viruses; therefore, the belonging of an unknown virus strain to one of these three types can be predicted by the acylation character determined by MS.

It was suggested earlier that a short cytoplasmic domain similar to that of HEF of influenza C virus (three amino acid residues, whereas influenza A and B viruses have 10-11 such residues) should act as a molecular signal for attaching stearate (instead of palmitate) [82]. Data of MS indicated that the lipid bilayer boundary could be a signal for stearate attachment. To confirm our hypothesis, we chose several glycoproteins from different groups of enveloped viruses that have different location of cysteine (the acylation site) relatively to the lipid bilayer boundary and that differ also in cytoplasmic domain length. We revealed by MS that vesicular stomatitis virus (VSV) gly-

coprotein G contains palmitate covalently bound to the cysteine residue located in the long (29 residues) cytoplasmic domain. In contrast, glycoprotein F of Newcastle disease virus (NDV) and glycoprotein E1 of Semliki Forest virus (SFV) were stoichiometrically acylated with stearate residues [83]. In both stearoylated glycoproteins the conservative cysteine is located in the TM domain. However, SFV glycoprotein E1 contains a very short cytoplasmic domain consisting of two residues, and the NDV glycoprotein F contains a long cytoplasmic domain consisting of 26 residues. Thus, not the length of the cytoplasmic domain but location of the acylation site relative to the lipid bilayer boundary seems to determine the choice of the modifying residue in different envelop glycoproteins. If viruses are acylated enzymatically similarly to cellular proteins [84, 85], then different enzymes should exist that are responsible for attaching either palmitate or stearate.

**Oligomerization of transmembrane domains.** According to data of 3-D analysis, the HA ectodomain is a trimer [67, 69]. Each ectodomain monomer includes chain HA1 and (partially) chain HA2 containing a fusion peptide on the *N*-terminus, whereas the *C*-terminal region of chain HA2 is anchored to the viral membrane via the TM domain. At acidic pH values, the ectodomain of HA is subjected to pronounced rearrangements resulting in membrane fusion [86, 87]: the HA1 chains get separated as petals of a flower; the fusion peptide is incorporated into the target membrane; the trimer of central helices of the HA2 chain forms a hairpin tightening the target membrane to the virus membrane. This is not associated with a rearrangement of the complex of TM domains; on the contrary, this complex supports the trimer integrity and creates tension required for production of the fusion pore. But the structure of this complex is absolutely unknown. We proposed an alternative approach of extracting anchoring segments of HA for analysis by MS, and this has allowed us to obtain unique information about oligomerization of TM domains of HA.

Our approach includes soft extraction of HA homotrimers from virions with a nonionic detergent and their limited proteolysis in micelles using different enzymes. Subsequent extraction into the organic phase and MS allowed us to determine the *N*-terminus of the anchoring peptide, which is the P1′-site of hydrolysis in the nomenclature adopted for enzymes [88]. Different strains belonging to 14 of 16 known antigenic HA subtypes of influenza A virus and also strains of influenza B virus have been analyzed [81] (Fig. 5a). All varieties of HA of influenza A virus were hydrolyzed with bromelain in the linker region "above" the transmembrane domain, and both the TM domain and cytoplasmic domains remained intact, which suggests the preservation of the homotrimeric structure of HA. Hydrolysis of HA with the serine proteinase subtilisin Carlsberg in the case of sub-

types from phylogenetic Group-2 (H3, H4, H7, H10, H14) also affected only the linker region. In contrast, all HA representatives from Group-1 (H1, H2, H5, H6, H9, H11, H12, H13, and H16) were cleaved with subtilisin in the TM domain. Because the differences we found in hydrolysis character could not be explained by specificity of enzymes, we proposed that the TM domains of HA from Group-2 should have tighter packing of homotrimers than Group-1 (Fig. 5b).

Results of computer-aided modeling performed by original methods based on mapping molecular hydrophobic potential (MHP) on the surface of alpha-helices and calculations of molecular dynamics in membrane-like media are in good agreement with our hypothesis [89]. The homotrimeric model of TM domains of HA of the H14 subtype (Group-2) occurs to be more compact than HA of the H6 subtype (Group-1). We think that this can be due to stacking interactions between aromatic amino acid residues (phenylalanines) located on the inner interface of homotrimer H14 [81].

The type B viruses were cleaved in the TM domain not only with subtilisin, but also with bromelain [81] (Fig. 5a), the surface of which seems to be more hydrophilic [90]. The TM domains of HA of influenza B virus seem to be organized in the homotrimer more "loosely" than in the two groups of type A viruses (Fig. 5b). It could be that
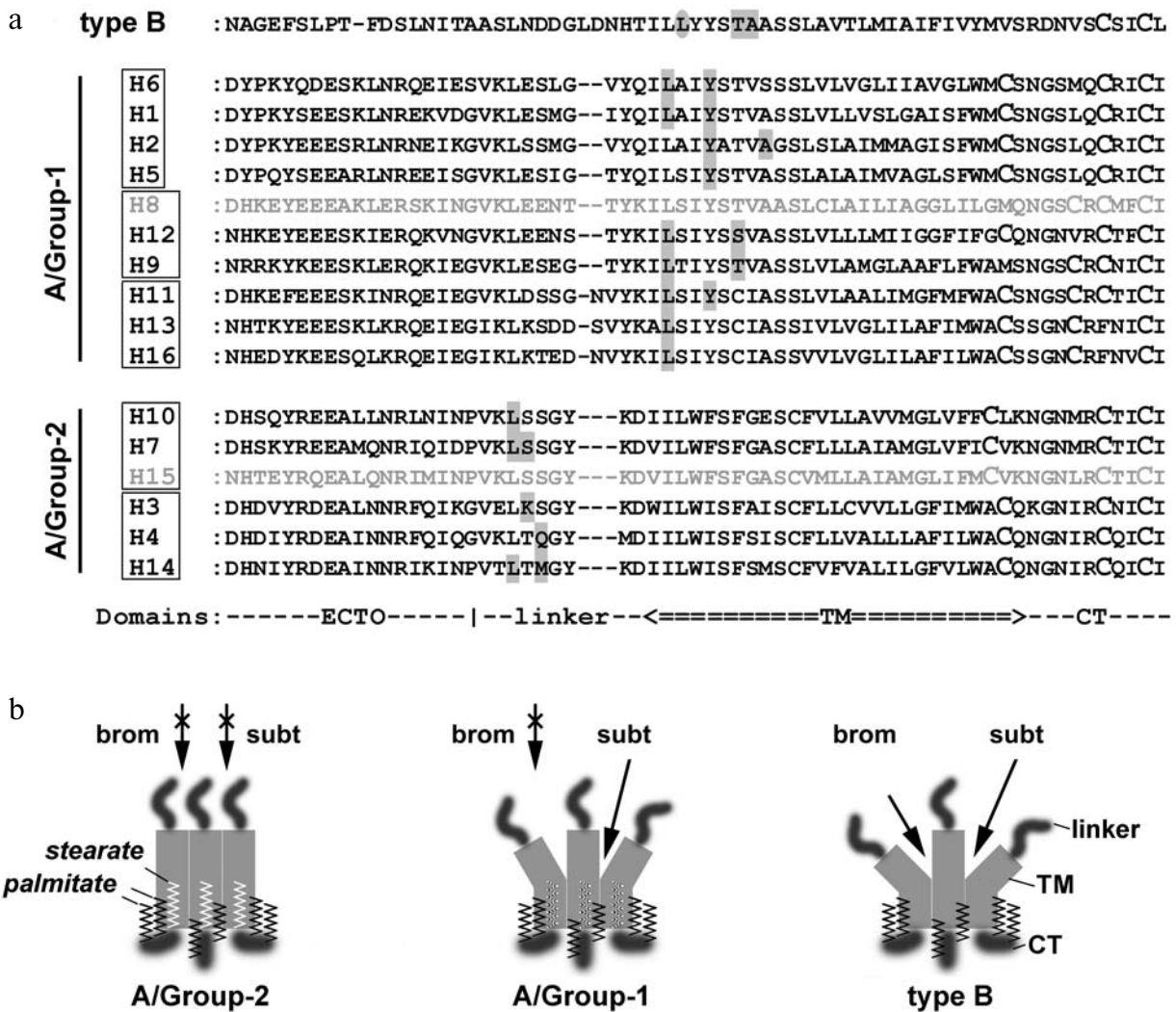


**Fig. 5.** Analysis of oligomerization of transmembrane domains of influenza virus hemagglutinin in micelles. a) Alignment of *C*-terminal region sequences of HA from strains of 16 antigenic subtypes of influenza A virus and from influenza B virus strain. The clusterization of the virus A sequences into Groups 1 and 2 is obvious. The ectodomain (ECTO), linker region (linker), transmembrane domain (TM), and cytoplasmic domain (CT) are indicated. The MS-revealed sites of hydrolysis with subtilisin Carlsberg (P1) are shown in influenza viruses A and B (gray rectangles) and with bromelain in influenza B virus (gray oval); b) hypothetical scheme of packaging of influenza A virus HA homotrimers from Group-1 and Group-2 and of type B virus. Fatty acid residues are shown by zigzags: white (stearate), black (palmitate), or black-white hatched (palmitate/stearate) depending on the virus strain. Brom, bromelain; Subt, subtilisin. Other indications are the same as above. Adapted from [81].

such less tight packaging of the TM domains of HA of influenza B virus compared to the type A virus is associated with the evolutionary absence of the TM-domain-attached stearate in the case of type B virus HA. Our studies on the functional and structural significance of S-acylation in the lifecycle of viruses are now in progress.

Finally, MS analysis of anchoring segments of HA of a series of different subtype strains revealed regularities of the structural organization of linker sequences [90]. Hydrolysis sites were shown to be strictly subtype-specific. Note that in some subtypes hydrolysis occurred at residues that seemed to be not very "suitable" for the specificity of the enzyme used, whereas more "appropriate" residues were located nearby. Thus, the hydrolysis site was determined not by the enzyme specificity, but mainly by the architecture of the "spike neck" to be hydrolyzed and/or adjusting regions of the ectodomain.

Thus, we have shown that MS approaches are often indispensable for fine structural studies of biological macromolecules, system characterization of protein composition of virions, and are useful for solution of some problems of modern preventive medicine, such as strain surveillance and testing of antiviral vaccines. We have considered only some possibilities of using modern MS in virology. In particular, we have not considered in detail data of comparative quantitative proteomics of virus-infected cells and data of lipidomics of virions. No doubt, the prospects for MS in biochemistry are great, but at present MS approaches have only started to be used in various fields of biology and medicine, and this allows us to expect new breaking achievements in the future.

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