

pH-dependent aggregation and secretion of soluble monomeric influenza hemagglutinin

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Summary. We previously reported the expression of soluble A/Victoria/3/75 (H3N2) hemagglutinin in insect cells and the molecular and immunological structure of an aggregated fraction, only observed in cell supernatant when expression was performed at low pH [23]. Here we report that besides this aggregated a monomeric and possibly a trimeric structure is detected in cell supernatant, irrespective of the pH of the medium. Evidence is presented that the aggregated fraction is generated out of monomeric HA0s molecules due to a low intracellular pH encountered during secretion.

Introduction

Hemagglutinin (HA) is the major constituent of the envelope of influenza virus. The functions, structure and structural changes of this protein have been analysed in great detail. HA initiates infection of the cell by binding to sialic acid containing glycoproteins or glycolipids of host cells and by promoting penetration of the viral genome into the cytoplasm through fusion of the viral and endosomal membranes [26]. Native HA is a ~220 kDa trimer of identical subunits, each of which consists of two disulphide-linked glycopeptides, HA1 and HA2. The structure of the soluble ectodomain, obtained by bromelain digestion (BHA), has been determined [27]. HA is built up of two structurally distinct domains, a globular head composed entirely of HA1 and a elongated stem structure consisting of HA2 and part of HA1. The β -structure rich globular domain contains the receptor binding sites. The major component of the stem is a hairpin-like structure consisting of two anti-parallel α -helices of unequal length linked by an extended polypeptide chain, and a membrane proximal, 5-stranded β -sheet. At a lower pH

(5.0–5.5), optimal for membrane fusion, HA undergoes an irreversible conformational change [15, 19]. The distal globular domain loses its trimeric structure and X-ray analysis of TBHA2, a proteolytic fragment of HA in the low pH induced conformation, demonstrates extensive refolding or reorganization, with preservation of the trimeric structure [2, 15]. Based on the irreversibility of this low pH-induced conformational change, increased thermostability of BHA2, and spontaneous folding of a HA2 domain, lacking the membrane spanning domain and fusion peptide, into the low pH-induced conformation in *E. coli* at neutral pH, it was postulated that the HA1 polypeptide kinetically traps the metastable HA2 fold through extensive contacts with parts of HA2 that must refold to achieve the stable state [2, 3, 16, 19]. Therefore, one might expect that expression of anchor-less HA (HA0s) would result in secretion of trimeric molecules. Indeed, Gething and Sambrook [8], initially reported expression and efficient secretion of soluble trimeric A/Japan/305/57 (H2N2) HA in CV-1 cells. However, further research demonstrated that A/Japan HA0s was recovered as a mixture of stable trimers, aggregates and monomers, while A/Aichi/2/68 (H3N2) HA0s was mainly secreted as monomers [18]. Anchor-free H7 HA, when expressed in insect cells or in vertebrate cells remained monomeric and was almost completely retained in the cells, possibly due to a fusion peptide interaction with the membrane [11]. We previously showed that expression of truncated A/Victoria/3/75 (H3N2) HA in Sf9 cells resulted in the secretion of HA0s molecules with molecular masses ranging from almost 1,500 to 100 kDa [23]. More importantly, we demonstrated that this high M_r molecules, called aHA0s, were no longer found in cell supernatant when expression was performed in medium of pH 8.5 instead of pH 6. We now have extended our structural analysis of A/Victoria HA0s present in insect cell supernatant prepared at different extracellular pH-values and investigated the influence of the pH on the folding and oligomerization of anchor-free HA.

Materials and methods

Viruses and cells

Sf9 cells were grown in Tc100 medium (Gibco Bio-Cult, Paisley, UK) supplemented with 10% fetal calf serum, penicillin, streptomycin and glutamine. Tc100 medium (pH 6) was brought to pH 7.5 or 8.5 by adding NaOH. This induced high amounts of precipitates which were removed by filtration. 1 M Tris.HCl pH 7.5 or 8.5 was added to a final concentration of 10 mM. The construction of a recombinant baculo virus expressing anchor-free A/Victoria/3/75 (H3N2) HA has been described elsewhere [23].

Monoclonal antibodies

mAb IIF4, a gift of Dr. F. Kostolansky, was raised against A/Dunedin/4/73 (H3N2) and is a HA2-specific mAb, reacting with the trimeric form of HA in pH 5 conformation [10, 17, 24, 25]. mAb LC89, a low pH-induced HA2-specific mAb, was a gift of Dr. S. Wharton and was raised against detergent-extracted X31 HA in the fusion pH conformation [25]. mAb HC59, kindly provided by Dr. J. Skehel was prepared against A/Port Chalmers/1/73 but reacts also with A/Victoria/3/75 and binds to the native and low-pH induced HA conformation. mAb LMBH5 recognizes a fairly conserved epitope on the H3 hemagglutinin near the receptor-

binding pocket. LMBH5 recognizes the native structure of A/Victoria/3/75 HA, but binding is enhanced after low pH treatment [22]. LMBH6 is a highly cross-reactive mAb binding only trimeric HA. Binding is enhanced after cleavage of the HA. The isolation and characteristics of this mAb will be described elsewhere (Vanlandschoot et al., in prep.).

Preparation of BHA

BHA was obtained by digestion of purified X47 virus with bromelain [1] and purified as described [21].

Expression of HA0s

Sf9 cells were grown in 75 cm² flasks and infected for 5 h at a m.o.i. of 10. Secretion was performed in serum-free Tc100 medium pH 6, 7.5 or 8.5.

Metabolic labeling of HA0s

Sf9 cells were grown in 25 cm² flasks and infected at a m.o.i. of 10. After 5 h the cells were washed with complete Tc100 medium, incubated for 2 days in complete medium, preincubated for 2 h in methionine-free Tc100 medium pH 6 and labeled for 5 h at 28 °C in 2.5 ml methionine-free Tc 100 medium pH 6 or 7.5, containing 100 µCi/ml [³⁵S] methionine.

Gel filtration

Cell supernatant was cleared by centrifugation (10 min, 400 × g), concentrated to ~400 µl with a Centricon 30 (Amicon) and loaded on a Sephacryl S300 HR gel filtration column (30 cm bed height, 0.8 cm diameter). Gel filtrations were performed in PBS at room temperature; fractions of 600 µl were collected. The gel filtration column was calibrated using Blue dextran (2000 kDa), BHA (220 kDa), alcohol dehydrogenase (150 kDa) and BSA (67 kDa).

Trypsin digestion and cross-linking of labeled HA0s

For trypsin treatment 65 µl 100 µg/ml TPCK-treated trypsin (Sigma Chemical Co.) was added to 200 µl of each gel filtration sample. After 1 h at room temperature, the digestion was stopped by adding 32.5 µl 14 mg/ml Pefabloc protease inhibitor. Samples were immunoprecipitated, using polyclonal antiserum. Cross-linking was performed with 5 mM Sulfo-EGS (Pierce, Rockford, USA) in 50 mM Hepes pH 8, 0.5% TX-100 for 1 h at 4 °C. The reaction was stopped by adding 1 M glycine to a concentration of 50 mM. Cross-linked samples were immunoprecipitated, using polyclonal antiserum.

Immunoprecipitation of labeled HA0s

An equal volume of MNT (20 mM Mes, 100 mM NaCl, 30 mM Tris), 1% Triton-X100, 2 × protease inhibitor cocktail (Complete, Boehringer Mannheim) and 2 µl rabbit anti-HA IgG (1 mg/ml) was added to a sample. After incubation for 2–6 h at 4 °C, Protein-A-Sepharose (33%, 60 µl) was added and mixtures were incubated 2–6 h at 4 °C. The immunocomplexes were washed twice with MNT, 0.5% TX-100 and separated by SDS-PAGE [12]. Gels were analyzed using a Phosphor Imager workstation.

ELISA

Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated with rabbit anti-HA IgG (1 µg/ml in PBS). After two washings (0.05% TX-100 in PBS) the wells were blocked with 0.1% bovine serum albumin (BSA) in PBS, followed by two washing steps. HA-containing

fractions were added to the wells and the plates were incubated for 1.5 h at room temperature. After two washings, wells were incubated with mAbs as indicated for 1.5 h at room temperature. After two washings the wells were incubated for 1.5 h at room temperature with alkaline phosphatase conjugated goat anti-mouse IgG (Sigma Chemical Co.) Substrate solution, containing 1 mg/ml Na p-nitrophenol in 10% diethanolamine, 5 mM MgCl₂ pH 9.8, was added and the absorbancy (*A*) was measured at 405 nm at appropriate times. Cleavage of HA was obtained by adding 10 µl TPCCK-treated trypsin (10 µg/ml) to 20 µl of each gel filtration fraction. The mixtures were incubated for 10 min at RT, after which 2 µl of Pefabloc protease inhibitor (7 mg/ml) was added. To induce a low pH conformational change, trypsin treatment was followed by addition of 64 µl 50 mM citrate, 100 mM NaCl pH 5. After 10 min, 10 µl 1 M Tris HCl pH 7.4 was added. All volumes were adjusted to 250 µl and 50 µl was used in ELISA.

Results

Production and analysis of HA0s

48 h after infection with the recombinant HA0s expressing baculo virus, Sf9 cells were incubated for 5 h in serum-free Tc100 pH 6, 7.5 or 8.5. Cell supernatant was concentrated and fractionated on a calibrated Sephacryl S300 HR gel filtration column (see upper left panel of Fig. 1). The presence of HA0s in the different gel filtration fractions was investigated by ELISA using mAb HC59. As reported previously, expression at pH 6 resulted in secretion of immunoreactive HA0s molecules with *M_r* ranging from almost 1,500 to 100 kDa (peak fractions 17,18 and peak fraction 24 respectively), while at pH 8.5 only a fraction between 66–100 kDa (peak fraction 24) was observed. However, Sf9 cells incubated at this high pH rapidly died, therefore the oligomeric structure of HA0s expressed at pH 7.5 was investigated. At this pH, almost only a HA0s fraction between 66–100 kDa (peak fraction 24) was present, while cells survived for a longer time. The oligomeric state of the different HA0s fractions obtained at pH 6 and 7.5 was determined by interaction with conformation-specific antibodies, trypsin digestion and cross-linking.

Characterization with conformation-specific mAbs

Binding of 5 conformation-specific mAbs to native, trypsin treated and trypsin/low pH treated HA0s was determined in ELISA. Using BHA, the specificity and sensitivity of these mAbs in this test was demonstrated (Fig. 1). HC59 recognized native and acid treated BHA equally well. Although we previously demonstrated binding of LMBH5 to native viral HA [22], only binding to low pH-treated BHA, was detected. IIF4 and LC89 only recognized acid treated BHA, while binding of LMBH6 occurred only to trimeric BHA. Highest sensitivity was obtained with mAbs HC59 and LMBH6, while binding of LC89 was fairly weak.

HA0s expressed at pH 6 (Fig. 2a)

Native high *M_r* HA0s, previously called aHA0s [23], was recognized by mAbs HC59, LMBH5, IIF4 and LC89. The binding profile of these latter two mAbs strongly suggested the presence of aHA0s of smaller sizes. After trypsin and

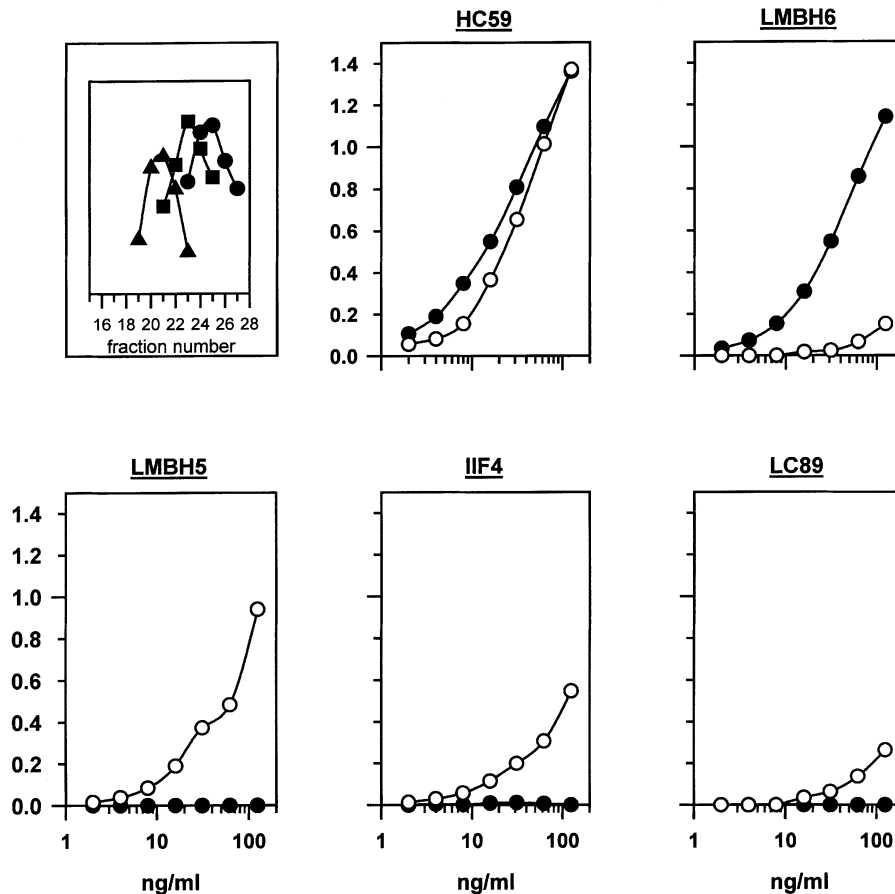


Fig. 1. Hemagglutinin conformation specificity of mAbs HC59, LMBH6, LMBH5, IIF4 and LC89. Binding to (●) BHA and (○) low pH treated BHA was performed in an ELISA assay as described. Upper left panel: calibration of the Sephacryl S300 HR gel filtration column. The column was calibrated with trimeric BHA (▲, 220 kDa, peak fraction 21), alcohol dehydrogenase (■, 150 kDa, peak fraction 23) and BSA (●, 67 kDa, peak fractions 24–25)

trypsin/low pH treatment, binding of HC59, LMBH5, IIF4 and LC89 decreased. The untreated 66–100 kDa form was recognized by HC59 and LMBH5, but not by mAbs IIF4 and LC89. Trypsin and trypsin/low pH treatment did not alter the binding of HC59, but again binding of LMBH5 decreased. Surprisingly, binding of IIF4 already occurred after trypsin treatment only, while an additional low pH treatment did not result in enhanced binding. Weak binding of LC89 after trypsin/low pH treatment to this fraction could sometimes be demonstrated. Binding of LMBH6 to untreated and treated aHA0s and the 66–100 kDa fraction was never observed. Surprisingly, after trypsin treatment a very small third fraction of about 200 kDa (peak fraction 22) was observed using LMBH6, which was no longer seen when trypsin treatment was followed by low pH treatment.

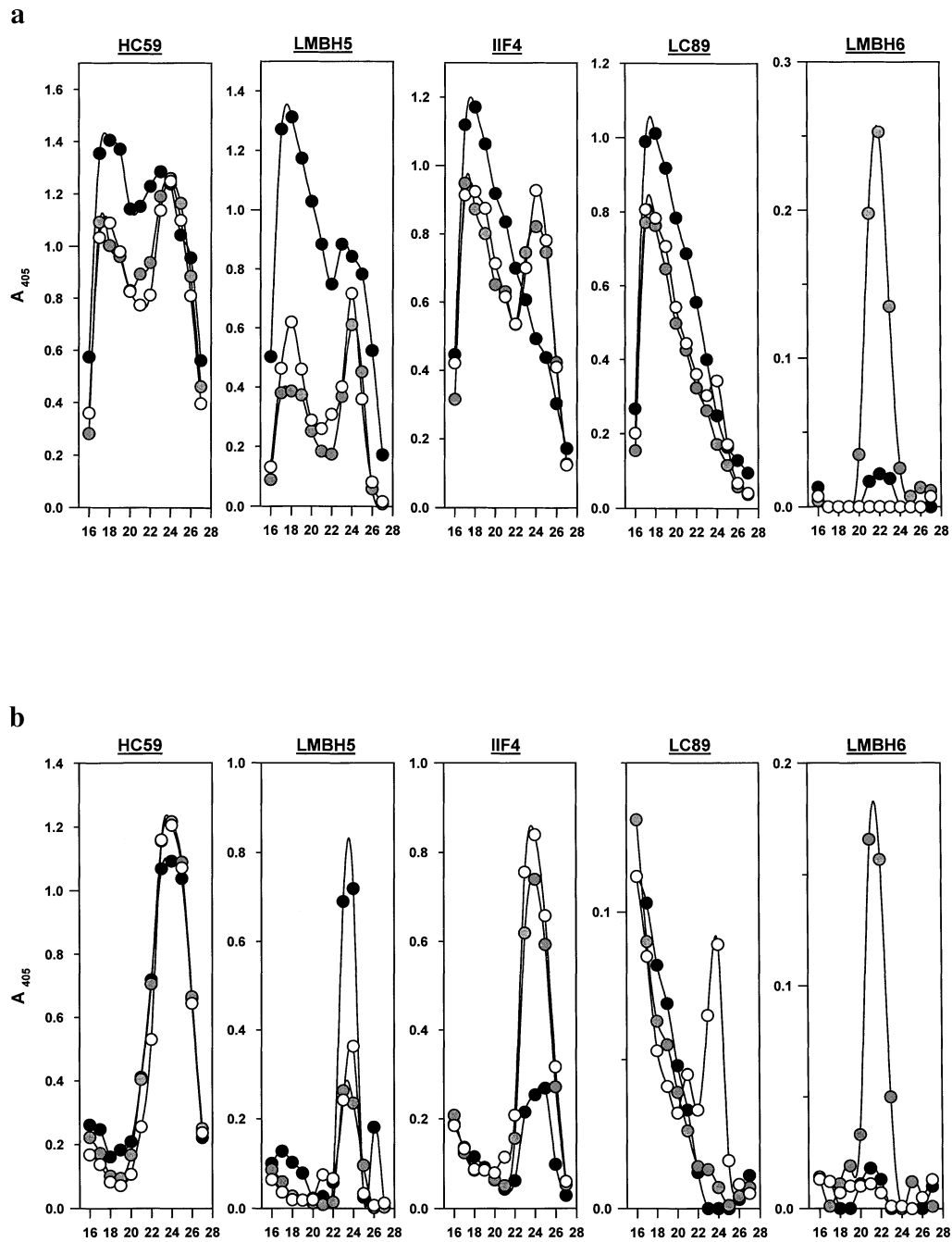


Fig. 2. Binding of mAbs HC59, LMBH5, IIF4, LC89 and LMBH6 to untreated (●), trypsin treated (◐) and trypsin/low pH treated (○) HA0s, expressed at pH 6 **a** and pH 7.5 **b**. Sf9 cell supernatant was concentrated and fractionated on the standardized Sephacryl S300 HR gel filtration column. Trypsin and low-pH treatment and ELISA were performed as described

HA0s expressed at pH 7.5 (Fig. 2b)

The same results were obtained with the 5 mAbs when tested on the 66–100 kDa fractions obtained at pH 7.5, except that no aHA0s was detected. However, compared to the pH 6 expressed 66–100 kDa fraction, binding of LC89 after trypsin/low pH treatment was now detected, although weakly.

Characterization by trypsin digestion and cross-linking

For these experiments metabolically labeled HA0s was used. 48 h after infection, cells were incubated for 2 h in methionine-free medium pH 6 and labeled for 5 h in methionine-free Tc100 medium pH 6 or 7.5, to which 100 $\mu\text{Ci/ml}$ ^{35}S methionine was added. Gel filtration, trypsin digestion, cross-linking and immunoprecipitations were performed as described. Trypsin digestion for 1 h at room temperature never resulted in the generation of HA1 and HA2 fragments as would be expected for native trimeric HA, nor in generation of the four fragments that would be obtained for the low pH induced trimeric HA structure [19]. Instead, rather complex but reproducible patterns of fragments were obtained. It is clear however that at least three different patterns could be distinguished. The pattern of aHA0s differed from that of the 66–100 kDa fraction, while a third pattern was observed for the 200 kDa HA0s fraction (Fig. 3a). Analogous patterns were observed for pH 7.5 expressed HA0s (Fig. 3b). PAGE analysis of cross-linked aHA0s revealed three bands with apparent M_r s consistent with monomer, dimer and trimer, while this was not seen for the 66–100 kDa fractions (Fig. 4a and b), an indication that this 66–100 kDa fraction consisted of monomeric HA0s (mHA0s) molecules. Extensive cross-linking of aHA0s had occurred, as demonstrated by the large amounts still present in the stacking gel (data not shown) supporting our previous results indicating that aHA0s consists of aggregated, monomeric HA0s molecules. Based on the results obtained with the conformation-specific mAbs and the different fragments obtained after trypsin treatment, these monomeric aHA0s molecules are however structurally different from the mHA0s molecules.

Kinetics of aHA0s secretion

The polyhedrin promoter is switched on approximately 12 h after infection and reaches full strength between 24 and 30 h. All experiments and results described until now were performed and obtained with HA0s secreted between 48 and 53 h after infection, when the polyhedrin promoter was still at full strength. To investigate the time dependent secretion of aHA0s, cells were infected as described. Cell supernatant was replaced 15, 24 and 48 h after removal of the inoculum, concentrated and fractionated by gel filtration. The presence of HA0s in the different fractions was determined by ELISA, using mAb HC59. In cell supernatant taken 15 h after infection almost exclusively mHA0s was detected (Fig. 5, left panel). Between 15 and 24 h the production of aHA0s equalled that of mHA0s (Fig. 5, middle panel). A considerable production of HA0s was observed between 24 and 48 h after infection, while the amount of aHA0s was slightly higher than the amount of mHA0s (Fig. 5, right panel).

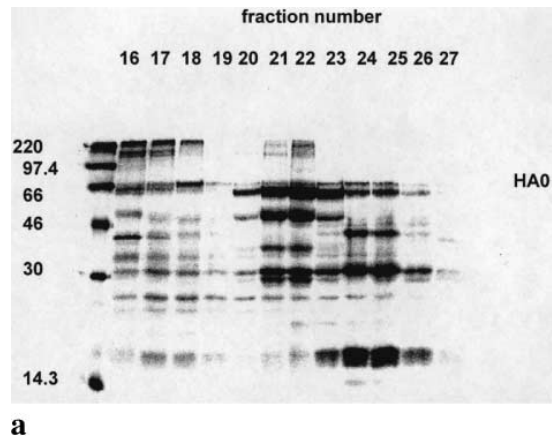
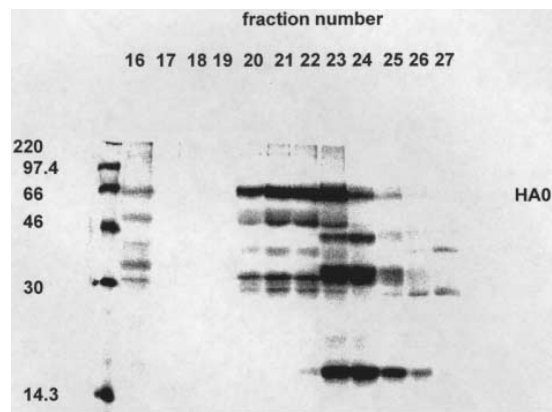
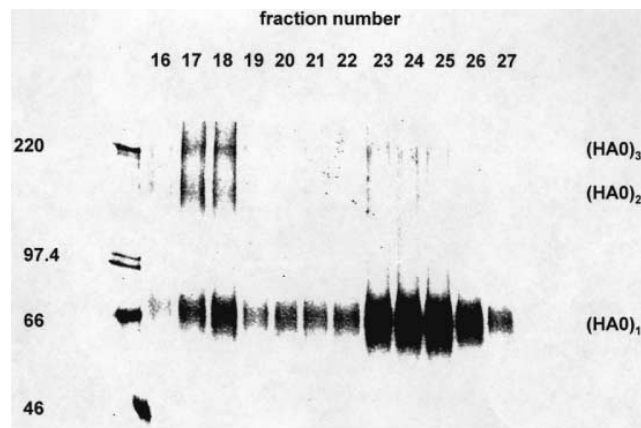
**a****b**

Fig. 3. Trypsin susceptibility of HA0s expressed at pH 6 (**a**) and pH 7.5 (**b**). Supernatant of ^{35}S labeled infected Sf9 cells, was concentrated and fractionated by gel filtration. Prior to immunoprecipitation, trypsin treatment was performed for 1 h at room temperature as described. Proteins were separated on a 12.5% SDS-PAGE and visualized on a Phosphor Imager

**a****Fig. 4** (continued)

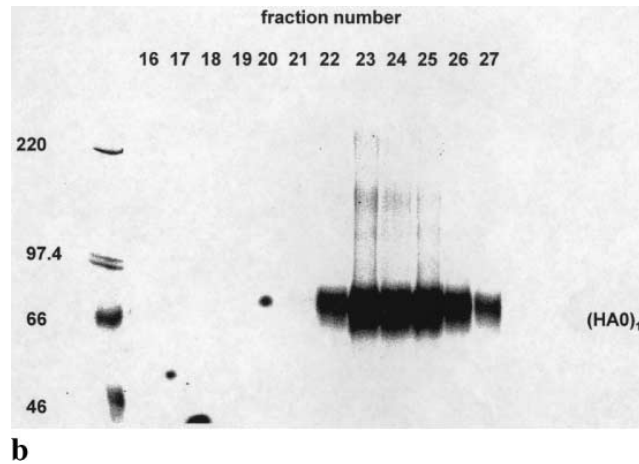


Fig. 4. Cross-linking of ^{35}S labeled HA0s expressed at pH 6 (a) or pH 7.5 (b). Labeling, concentration, gel filtration, cross-linking using Sulfo-EGS and immunoprecipitation were performed as described. Proteins were separated on a reducing 6% SDS-PAGE and visualized on a Phosphor Imager

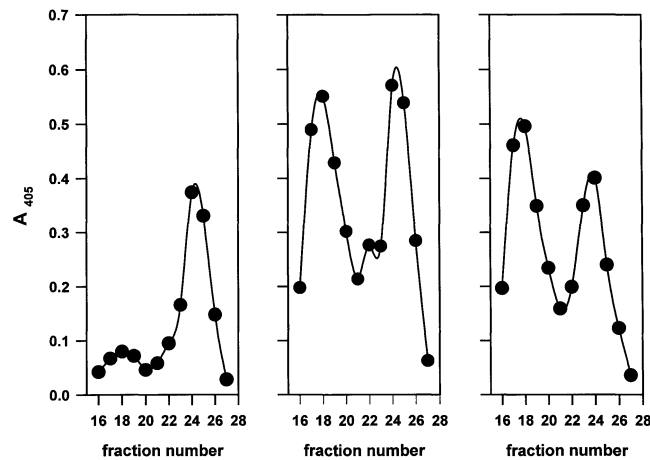


Fig. 5. Time dependent secretion of aHA0s. Cell supernatant was replaced 15 (left), 24 (middle) and 48 h (right) after removal of the virus inoculum, concentrated and fractionated by gel filtration. The presence of HA0s in the different fractions was determined by ELISA, using mAb HC59

Low pH treatment of mHA0s

The results described above strongly suggested the generation of aHA0s out of mHA0s due to a low intracellular pH encountered during secretion. As IIF4 recognizes aHA0s but not mHA0s, the low pH must have resulted in the formation of the corresponding IIF4 epitope. To verify this hypothesis, mHA0s was treated for 15 min at 28 °C with 100 mM NaCl, 50 mM citrate buffer, pH 4, 5 or 6 and neutralized with Tris.HCl pH 8. Binding of IIF4 was tested in ELISA. pH 6 treatment did not result in stronger binding of IIF4, while slightly enhanced

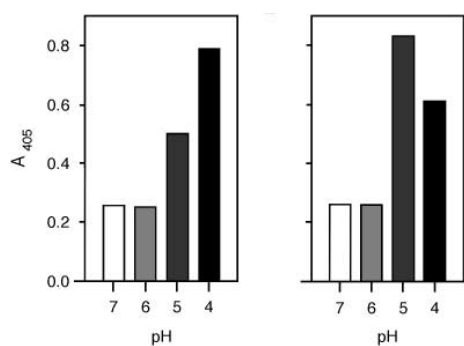


Fig. 6. Binding in ELISA of mAb IIF4 after low pH treatment of mHA0s. mHA0s was treated for 15 min (left) or 60 min (right) at 28 °C with 100 mM NaCl, 50 mM citrate buffer pH 4, 5 or 6 and neutralized with Tris.HCl pH 8

binding was observed after pH 5 treatment. Strong binding was observed after pH 4 treatment (Fig. 6, left panel). A secretion half time of 2 h has been reported for an HA2 lacking HA1 in Sf9 cells [11], suggesting that mHA0s molecules could encounter a low pH for more than 15 min. Incubation for 60 min at pH 6 had again no effect, ruling out that the conformational change occurred in cell supernatant. Strong binding of IIF4 was now observed after pH 5 treatment. Binding of IIF4 to pH 4 treated mHA0s was sometimes slightly reduced compared to the 15 min pH 4 treated mHA0s (Fig. 6, right panel).

Discussion

Expression of anchorless HA, whatever the HA subtype or the cells used, hardly resulted in the secretion of trimeric HA0s. Instead, mainly large aggregates and monomeric molecules were found in cell supernatant. Also inside the cells, trimeric HA0s was never detected, except for the A/Japan/305/57 of H2 HA. Formation of this small secreted trimeric fraction, as well as an aggregated fraction, took place in the medial Golgi or just before arrival at the cell surface and not in ER or the intermediate compartment as is the case for membrane bound HA [6, 11, 18, 20]. The A/Victoria/3/75 H3 hemagglutinin used here resembles the A/Japan/305/57 H2 HA quite well: expression of a truncated form resulted in the secretion of a large amount of aggregated HA0s (aHA0s), a very small amount of most probably trimeric HA0s (HA0s) and again a very large amount of monomeric HA0s (mHA0s). We came to this conclusion based on the results obtained using conformation-specific monoclonal antibodies, cross-linking and trypsin digestion to characterize the HA0s found in Sf9 cell supernatant. The secretion of mHA0s irrespective of the pH, the low extracellular pH-dependent secretion of aHA0s and binding of mAb IIF4 to mHA0s only after low pH incubation, strongly suggest that a low intracellular pH, most probably encountered in *trans* Golgi, induces a conformational change in part of the mHA0s molecules. Due to an enhanced hydrophobicity, these molecules aggregate to form aHA0s. Viral infections can lead to an additional acidification of the secretion pathway and the cytoplasm, coinciding with the synthesis of virus proteins [4, 5, 13]. This could explain why early during infection hardly any aHA0s was secreted, as a low pH, needed to induce the change, had not yet been established. However, it is also possible that expression levels were simply too low for aggregation to occur.

Based on the binding of IIF4 and LC89 and the high susceptibility to trypsin treatment, we previously reported that aHA0s consisted of aggregated monomeric subunits [23]. But mHA0s was not recognized by mAb IIF4, indicating that the monomeric aHA0s molecules were structurally different from the non-aggregated mHA0s molecules. Binding of IIF4 to mHA0s could be induced by trypsin or low pH treatment, indicating that a conformational change had occurred. It has been suggested that alterations in epitope accessibility are responsible for the increased binding of some HA2-specific mAbs to low pH treated HA [10]. However, binding of IIF4 after trypsin or low pH treatment to mHA0s suggests that the exact conformation of the corresponding epitope would not yet be present in native trimeric HA0 but would be generated by either treatment. Alternatively, the binding of IIF4 after trypsin treatment could be a mHA0s-specific property. Because one face of the long alpha-helix is highly hydrophobic it has been proposed that a single folded subunit would not project from the membrane as an erect spike [27]. Structural studies have shown that some very flexible regions must be present in the stem [2, 25], allowing the mHA0s molecules to fold into a stable conformation. As a result of this altered conformation the epitope is not present or accessible. This proposed conformation of mHA0s could perhaps also explain the lack of trimerization observed for soluble HA. Helenius and coworkers demonstrated that extensively folded monomeric HA0 molecules are transported from the ER to the intermediate compartments [20]. During this transport trimerization takes place. Based on the stability of BHA they suggested that the ectodomains alone may contain enough structural information to allow trimerization. They further suggested that the membrane anchor would not be very important in this process but would orient the subunits in the membrane thereby facilitating assembly and contribute to the stability of the final trimeric complex [6, 7]. For anchor-free HA0 they decided that the structural criteria for transport were less stringent; they must fold correctly but do not need to oligomerize [18]. We propose that the quality control in ER and the structural criteria for transport of mHA0s are not less stringent than for membrane bound HA0. We suggest that a mHA0s molecule adopts a structure which is so stable it does not need to oligomerize to be secreted. In the case of monomeric membrane bound HA0 molecules, the membrane anchor prevents folding into such a structure that would no longer need trimerization. This latter process is needed to obtain stable molecules that are allowed to be transported to the Golgi-network. Expression of truncated forms of other viral low-pH fusion dependent membrane proteins also often resulted in secretion of aggregated and monomeric molecules [9,14], suggesting a similar structural behaviour of such proteins.

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