



# Folding of influenza virus hemagglutinin in insect cells is fast and efficient



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## ABSTRACT

Folding of influenza virus hemagglutinin (HA) in the endoplasmic reticulum has been well defined in mammalian cells. In different mammalian cell lines the protein follows the same folding pathway with identical folding intermediates, but folds with very different kinetics. To examine the effect of cellular context on HA folding and to test to which extent insect cells would support the HA folding process, we expressed HA in Sf9 insect cells. Strikingly, in this invertebrate system HA folded faster and more efficiently, still via the same folding intermediates as in vertebrate cells. Our results suggest that insect cells provide a highly efficient and effective folding environment for influenza virus HA and the ideal production platform for HA (emergency) vaccines.

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## 1. Introduction

Insect cells have been used from the early 1980s to produce foreign proteins (Smith et al., 1983; Pennock et al., 1984; Luckow and Summers, 1988). Using baculovirus vectors, lepidopteran insect cells can generate vast amounts of foreign protein, a system that can easily be expanded to large-volume bioreactors (e.g. Bernal et al., 2009; Carinhas et al., 2010). For example Sf9 cells, a clonal isolate of Sf21 cells and derived from *Spodoptera frugiperda* (order Lepidoptera), are commonly used to produce recombinant proteins (Smith et al., 2013; reviewed by van Oers et al., 2014). Quite a few proteins have been expressed in insect cells, for various purposes ranging from fundamental research – such as structure determination – to therapeutic protein development and vaccine production. Many viral antigens, including envelope proteins, complex subunits, and virus-like particles have been produced (reviewed by van Oers, 2006; Metz and Pijlman, 2011). An influenza virus hemagglutinin-subunit vaccine (Flublok®), yielded from Sf9-derived cells infected with a baculovirus vector (Cox and Hashimoto, 2011), was recently approved by the USA Food and Drug Administration. One of the cervical

cancer vaccines (Cervarix®, from GlaxoSmithKline), consisting of human papillomavirus VLPs (Harper, 2008), is also produced in baculovirus-infected insect cells, as well as a number of veterinary vaccines.

To enable use of insect cells for secretory protein production, the produced protein needs to fold into its biologically active conformation. Whereas insect cells are considered ideally suited to produce proteins that require complex folding and post-translational modifications, how and whether proteins are folded properly in insect cells has been a trial and error venture that lacks a fundamental understanding. Secretion alone usually is considered a representative parameter for protein quality, but often most of the produced protein is stuck in intracellular compartments, in particular the endoplasmic reticulum (ER).

Protein folding in mammalian cells has been studied for decades. It is the process in which a linear polypeptide acquires its biologically active three-dimensional conformation. Although all information needed to reach the native conformation is encoded in the amino-acid sequence of the polypeptide (Anfinsen, 1973), protein folding inside the cell requires molecular chaperones and folding enzymes (Gething and Sambrook, 1992; Hartl, 1996; Kleizen and Braakman, 2004; Braakman and Bulleid, 2011). Nascent chains and newly synthesized proteins need molecular chaperones and folding enzymes during their folding process to protect them from misfolding and aggregation. The ER is highly specialized for the folding of membrane and secretory proteins, and the molecular chaperones and folding enzymes retain a secretory protein in the ER until it is properly folded and assembled (Hurtley and Helenius, 1989).

Abbreviations: ER, endoplasmic reticulum; HA, influenza virus hemagglutinin.

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**Fig. 1.** HA folding intermediates are similar in Sf9 and HeLa cells. (A) Diagram of the three-dimensional structure of monomeric HA0 (X31) as it is present in the trimer, at neutral pH (Wilson et al., 1981), consisting of a top globular domain composed mainly of  $\beta$ -sheets and a stem domain composed of  $\alpha$ -helices. Conformational epitopes are indicated as A, B, E, F1, and F2. The left box presents the 12 cysteines (in yellow balls on sticks in the structure) involved in disulfide bonding and the right box presents the position of the 7 N-linked glycosylation sites (red triangles: 5 visible, 2 behind the strands). Cells expressing HA were [ $^{35}$ S]-radiolabeled for 2 min at 37°C (Hela) or 10 min at 28°C (Sf9), followed by a chase for the indicated times (B and C). In (D) Sf9 cells expressing HA were [ $^{35}$ S]-radiolabeled for 10 min at 28°C in the presence of 5 mM DTT, followed by a chase for the indicated times without DTT. Cells were lysed in Triton X-100, and HA was immunoprecipitated with a polyclonal antiserum P that recognizes all forms of HA. Samples were analyzed using nonreducing (NR, top panels) and reducing (R, bottom panels) 7.5% SDS-PAGE. IT1, first HA folding intermediate; IT2, second HA folding intermediate; NT, native, folded HA; G, HA containing Golgi-modified glycans; R, reduced HA. The black dot indicates an additional HA folding intermediate or an HA nascent chain.

cell system is an excellent platform for HA protein and vaccine production.

## 2. Materials and methods

### 2.1. Reagents

HeLa cells were cultured in MEM (Life Technologies, Rockville, MD) supplemented with 10% FBS (Life Technologies), penicillin, and streptomycin (100 U/ml), 2 mM glutamax (Life Technologies) and nonessential amino acids (Life Technologies). Sf9 cells were grown in Sf-900 II SFM (Invitrogen) with 5% FBS (Life Technologies) and Gentamicin (50 µg/ml, Life Technologies).

DMEM (No methionine, no cysteine, Life Technologies) was used for depletion of HeLa cells. Grace's Insect Medium (Unsupplemented, Life Technologies) was used for depletion of Sf9 cells. We used a pCI-neo vector (Promega) to express HA from the X31 strain of influenza virus (A/Aichi/1968/H3N2) in HeLa cells (Wilson et al., 1981; Doms et al., 1985). To express HA in Sf9 cells, we used a pIB/V5-His plasmid (Invitrogen).

Rabbit polyclonal antibody P and mouse monoclonal antibodies N2, F1, and F2 have been characterized before (Doms et al., 1985; Copeland et al., 1986; Braakman et al., 1991). P immunoprecipitates all forms of HA: HA folding intermediates, monomers, trimers, aggregates, as well as acid- or SDS-treated and other unfolded and misfolded forms of HA. N2 is specific for trimeric X31 HA0, whereas F1 and F2 were made against non-denatured HA2, and recognize specific and different folding intermediates of HA (Braakman et al., 1991, 1992; Maggioni et al., 2005). The mouse monoclonal antibodies HC3, HC19, and HC100, have been mapped to epitopes A, B, and E, respectively, all in the top domain of HA0, and were described before (Daniels et al., 1984; Daniels et al., 1987; Wiley and Skehel, 1987; Hurtley et al., 1989; Braakman et al., 1992).

### 2.2. Pulse-chase folding assays

HeLa cells were transfected with a mixture of pCI-neo-HA and lipofectin (Invitrogen), and Sf9 cells were transfected with a mixture of pIB/V5-His-HA and Cellfectin II (Invitrogen) both at a ratio of 1:2.5 (w/w), according to the manufacturer's instructions (2 µg DNA per 6-cm dish of cells).

Pulse-chase experiments were performed essentially as described (Braakman et al., 1991; Braakman and Hebert, 2001; Jansens and Braakman, 2003). As comparison of cell types is optimally done at similar expression levels, we analyzed HeLa cells 24 h after transfection and Sf9 cells after 48 h. HA concentration will only affect trimerization rate, but not HA folding, glycosylation, or trimerization competence per se (Braakman et al., 1991). Both HeLa cells and Sf9 cells were washed twice with HBSS (Invitrogen-BRL). HeLa cells were depleted of cysteine and methionine (DMEM, no methionine, no cysteine) and Sf9 cells only of methionine (Grace's Insect Medium, unsupplemented, no methionine) for 15–30 min to limit competition by unlabeled amino acids during the pulse. Cells then were pulse labeled with [<sup>35</sup>S]-methionine and cysteine (Easytag™ Express Protein Labeling Mix, Perkin Elmer) on a waterbath in the presence of 10 mM Hepes pH 7.4 to maintain medium pH outside the incubator. Pulse times were longer at lower temperature because translation rate then is strongly reduced (~4× slower from 30 °C to 20 °C, our unpublished observations) and we aimed to avoid overpopulation of ribosome-attached nascent chains of HA in the radiolabeled cohort (for this and more details see Braakman et al., 1991). To stop labeling and monitor protein folding, cells were 'chased' for various time periods in full medium without FCS containing excess unlabeled methionine (plus cysteine for HeLa cells). Cells were

transferred to ice and washed twice with ice-cold HBSS, containing 20 mM N-ethylmaleimide (NEM) to prevent isomerization and formation of additional disulfide bonds in the protein. Cells were lysed with 0.5% (v/v) Triton X-100 in ice-cold MNT (20 mM MES, 100 mM NaCl, 30 mM Tris-HCl pH 7.5) containing 20 mM NEM and protease inhibitor cocktail (10 µg/ml each of chymostatin, leupeptin, antipain, and pepstatin, 1 mM PMSF, and 1 mM EDTA). Cell lysates were centrifuged for 10 min at 15,000 × g at 4 °C to remove nuclei.

### 2.3. Immunoprecipitation and SDS-PAGE

Procedures were as described before (Braakman et al., 1991). Goat anti-mouse IgG (1 µg per sample) was incubated for 1 h at 4 °C with Protein A Sepharose 4B fast-flow beads (50 µl of a 10% suspension per sample, Amersham Pharmacia Biotech AB). The primary monoclonal antibodies were added (100 µl of tissue culture supernatant containing F1, F2, A, B, E, or N2) and incubation was continued for 1 h. Polyclonal antibodies (3 µl of the anti-X31 antiserum) were incubated directly with Protein A Sepharose 4B fast-flow beads for 1 h. Lysates were added to the mixture and incubated at 4 °C for 1 h. The immunoprecipitates with the polyclonal antibodies were washed twice for 10 min with 1 ml of wash buffer (0.05% Triton X-100, 0.1% SDS, 0.3 M NaCl, 10 mM Tris-HCl pH 8.6) at room temperature; the F1, F2, A, B, E complexes were washed twice with 1 ml of wash buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.0) for HeLa samples and with 1 ml of wash buffer (0.05% Triton X-100, 0.05% SDS, 0.3 M NaCl, 10 mM Tris-HCl pH 8.6) for Sf9 cells; the N2 complexes were washed with 1 ml of wash buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris-HCl pH 8.0). The washed pellets were resuspended in 20 µl 10 mM Tris-HCl pH 6.8 and sample buffer was added to a final concentration of 200 mM Tris-HCl pH 6.8, 3% SDS, 10% glycerol, 0.004% bromophenol blue, and 1 mM EDTA. Samples were incubated at 95 °C for 5 min and analyzed by reducing or nonreducing 7.5% SDS-PAGE. The same amount of protein was loaded in each lane of a gel, based on the number of cells per dish, and confirmed by the labeling of total detergent cell lysates. Gels were stained with Coomassie Brilliant Blue R 250, dried, and exposed to Biomax MR films (Eastman Kodak, Rochester, NY) for display. Different exposure times were chosen for display to ensure visibility of all bands.

### 2.4. Endo H and PNGase F treatment

For Endo H digestion (Copeland et al., 1986), immune complexes were resuspended in 0.2% SDS in 100 mM sodium acetate (pH 5.5) and heated for 5 min at 95 °C. An equal volume of 100 mM sodium acetate (pH 5.5) then was added. Endo H (0.2 U) was added to 20 µl of each sample and incubated for 1 h at 37 °C.

For PNGase F digestion, immunoprecipitates were resuspended in 0.2% SDS and 25 mM EDTA in 50 mM Na<sub>2</sub>HPO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> pH 6.8, and heated for 5 min at 95 °C. Samples then were cooled on ice and an equal volume of 2% Triton X-100 and 25 mM EDTA in 50 mM Na<sub>2</sub>HPO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> pH 6.8, was added. Samples were incubated with 0.2 U of PNGase F in 20 µl for 1 h at 37 °C.

### 2.5. Trypsin assay for cell surface arrival of HA

For detection of HA at the cell surface, intact cells were trypsinized at the end of the chase period using TPCK-trypsin at 100 µg/ml in PBS for 30 min at 0 °C (Copeland et al., 1986). Trypsinization was terminated by two 5-min washes of cells in soybean trypsin inhibitor (100 µg/ml in PBS) on ice before lysis of cells for immunoprecipitation as described above.

### 3. Results

#### 3.1. HA folding is more efficient in Sf9 cells than in mammalian cells

When studied with the pulse-chase folding assay in mammalian cells, pulse-labeled newly synthesized HA runs as a single band in a reducing SDS-PAGE gel (Fig. 1B, R) and as three bands in a nonreducing gel: the native, fully oxidized HA (NT) and two folding intermediates, IT1 and IT2 (Fig. 1B, NR) (Braakman et al., 1991). The two folding intermediates have lower electrophoretic mobilities than NT because they still lack some of the six disulfide bonds, which render them less compact than NT. During the chase, during the ageing of the protein, HA folds and NT increases at the expense of folding intermediates IT1 and IT2. Whereas the first two disulfide bonds always form during translation (Daniels et al., 2003), most of HA's folding occurs post-translationally (Braakman et al., 1991; Chen et al., 1995).

We examined HA folding in Sf9 cells using the same pulse-chase assay (Fig. 1C). Because culture temperature of Sf9 cells is 28 °C and translation rate is much lower at this temperature (Braakman et al., 1991), we increased labeling time to 10 min. The reduced HAs from pulse labeled HeLa and Sf9 cells ran with similar electrophoretic mobilities, indicating similar glycosylation. In the nonreducing gel we detected mostly a native-like form NT, which was present to a large extent already at the end of the pulse. Much of HA appeared to have reached a Golgi-like form already at the end of the pulse, which suggests faster and more efficient HA folding and transport to

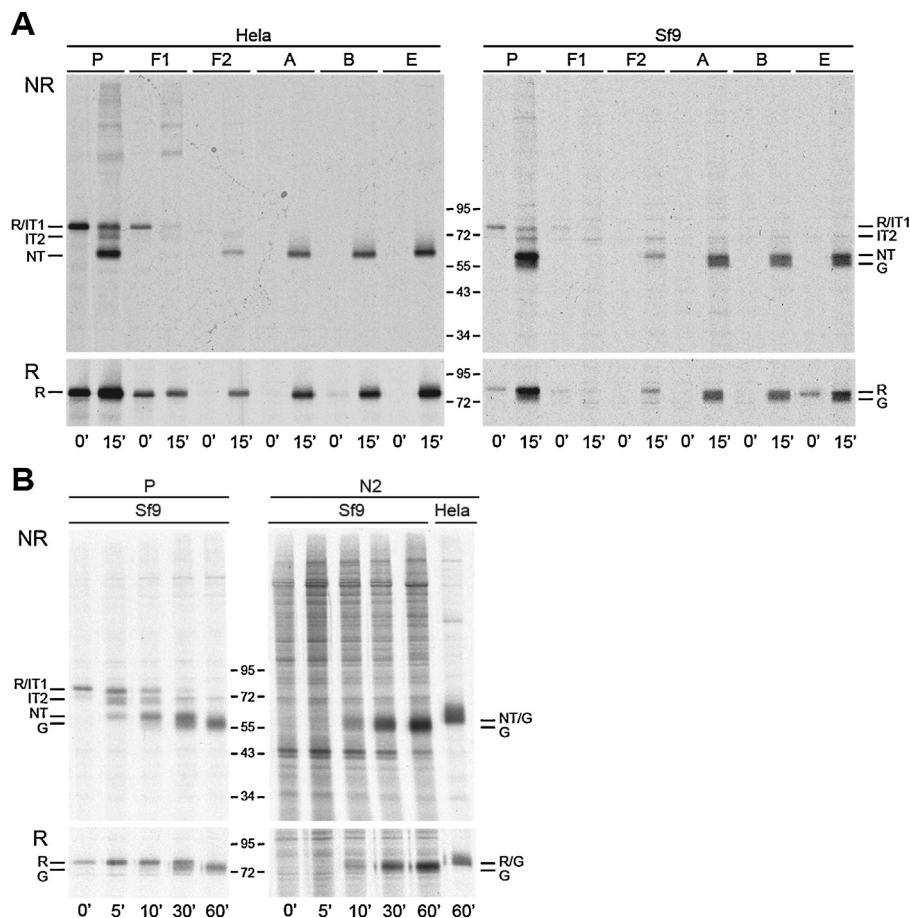
the Golgi complex in Sf9 cells than in HeLa cells. We did also detect IT1-like and IT2-like intermediates at 0-min chase time, which disappeared rapidly during the chase. Whereas in mammalian cells IT1 was more persistent, the persistent folding intermediate in insect cells was IT2 (Fig. 1C, NR).

Because HA folding was fast in the Sf9 cells, we next prevented co-translational disulfide bond formation in HA by adding 5 mM DTT to the pulse medium (Braakman et al., 1992). This delays oxidative HA folding until after synthesis, when DTT is absent from the chase medium. Now the two HA folding intermediates were clearly visible: both IT1 and IT2, next to NT and a Golgi-like form G (Fig. 1D, NR). The early appearance of G again indicated fast ER-to-Golgi transport of HA in the Sf9 cells, despite the enforced delay in oxidative folding. Again, we found IT2 to stay longer in Sf9 cells than IT1, which was the reverse in HeLa cells. An additional band appeared at 0-min and 5-min chase times, which may be an additional HA folding intermediate or an HA nascent chain (Fig. 1D, dot). A background band in Sf9 cells overlapped with IT2, and was also present in the reducing gels below the HA Golgi form (Fig. 1C and D, R).

Whereas the folding intermediates of HA were similar in HeLa and Sf9 cells, their ratios were clearly different, suggesting different kinetics for the sequential HA folding steps in insect cells.

#### 3.2. HA folding intermediates display the expected epitopes

Although the HA folding intermediates in Sf9 had the expected electrophoretic mobilities, they may be different from the



**Fig. 2.** HA folding intermediates have similar conformations in Sf9 cells and HeLa cells. Sf9 and HeLa cells expressing HA were analyzed as in Fig. 1D and B, respectively, but with 5 mM DTT in the pulse medium. (A) Immunoprecipitation was done in parallel with polyclonal P and conformational antibodies. (B) Immunoprecipitation was done in parallel with P and a trimer-specific HA antibody (N2).



intermediates found in mammalian cells. To test this, we used a panel of conformation-specific antibodies. As shown in the schematic structure of HA (Fig. 1A), F1 and F2 recognize epitopes in the stem domain that are present in early and late folding intermediates, respectively (Braakman et al., 1991, 1992). In mammalian cells, F1 recognizes only the relatively unfolded forms of HA: it precipitates reduced HA, folding intermediate IT1 and a fraction of folding intermediate IT2, but not the native NT form (Braakman et al., 1991, 1992). In contrast, F2 precipitates IT2 and early NT, but not IT1 or mature trimeric HA (Braakman et al., 1991; Chen et al., 1995; Tatu et al., 1995). The monoclonal antibodies HC3, HC19, and HC100 recognize epitopes A, B, and E in the top domain, which are formed in most HA chains already during synthesis and in others immediately after synthesis, indicating heterogeneity in folding rates. The top domain epitopes persist throughout HA's lifetime (Braakman et al., 1992; Maggioni et al., 2005).

In Sf9 cells, the results from the immunoprecipitations with the five antibodies showed that each antibody reacted with the different forms of HA as expected and comparable to the HeLa cells: the A, B, and E epitopes were present after 15 min of chase, whereas the F1 antibody recognized R and IT1. F2 did not detect IT1 but did immunoprecipitate IT2 and NT. The lack of F2 recognition for HA trimers explained the lower signal for NT compared to A, B, and E (Fig. 2A). HA did precipitate with the trimer-specific antibody N2, which indicated correct trimerization of HA after its folding (Fig. 2B). In contrast to HeLa cells, where N2 recognizes both ER and Golgi forms, in Sf9 cells it only recognized the Golgi form of HA, suggesting that HA left the ER immediately upon trimerization. Table 1 showed HA intermediates recognition by different antibodies in both HeLa cells and Sf9 cells. Taken together, the

**Table 1**

Recognition of various forms of HA during immunoprecipitation by different antibodies.

HA forms	P <sup>a</sup>	F1 <sup>b</sup>	F2 <sup>b</sup>	A <sup>b</sup>	B <sup>b</sup>	E <sup>b</sup>
R	++	++	—	—	—	—
IT1	++	++	—	+	+	±
IT2	++	+	++	++	++	++
NT	++	—	+	++	++	++
G	++	—	—	++	++	++

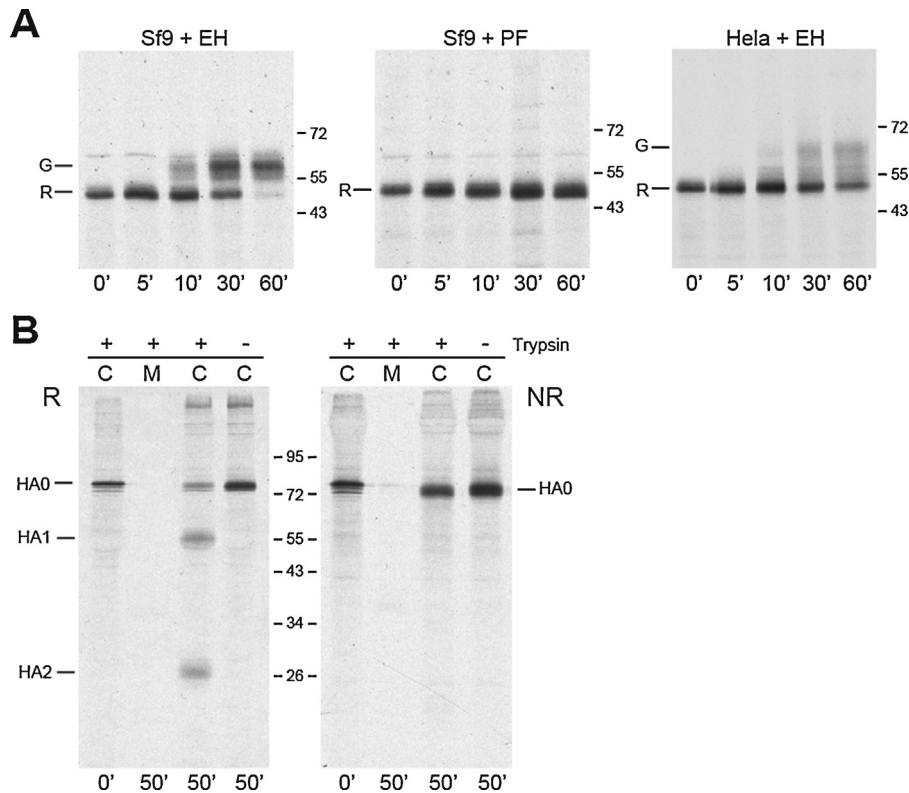
<sup>a</sup> Polyclonal antibody.

<sup>b</sup> Monoclonal antibody.

results show that in Sf9 cells, HA folded into its proper, native conformation with high efficiency.

### 3.3. HA is transported to the Golgi complex faster in Sf9 cell

Although a Golgi-like form of HA appeared very early in Sf9 cells, we confirmed transport using Endo H. This enzyme cuts oligomannose-type glycan chains but not complex glycan chains that form in the Golgi complex. We treated HA in both Sf9 and HeLa immunoprecipitates with Endo H. HA acquired its Endo H-resistant form much earlier in Sf9 cells than in HeLa cells, indicating faster transport to the Golgi complex (Fig. 3A). The undigested (Fig. 2B) and Endo H-resistant forms of HA in Sf9 cells ran lower than in HeLa cells, which indicated different glycan modifications in Sf9 cells and HeLa cells. PNGase F was used as a control because it removes all N-linked glycan chains, as seen in Fig. 3A.



**Fig. 3.** HA is transported to the Golgi complex and cell surface correctly in Sf9 cells. (A) Sf9 and HeLa cells expressing HA were analyzed as in Fig. 2B. After immunoprecipitation the washed immunoprecipitates were deglycosylated with Endo H (EH) or PNGase F (PF) as described in Section 2. After treatment, samples were analyzed by reducing 7.5% SDS-PAGE. (B) Sf9 cells expressing HA were [<sup>35</sup>S]-radiolabeled in the presence of 5 mM DTT for 10 min, followed by a 50 min chase at 28 °C. Cells were treated or not with trypsin as described in Section 2. Cells were lysed and lysates (C) and medium (M) were used for immunoprecipitation and further analyzed as in Fig. 1 using reducing 12% SDS-PAGE.

### 3.4. HA trimers arrive correctly at the Sf9 cell surface

In the natural host of influenza virus, HA will reside as mature trimers on the cell surface after infection. For a productive infection, HA0 (which denotes uncleaved HA) needs to be cleaved into HA1 and HA2 by a trypsin-like protease that is absent from most laboratory cell lines. Except for this activating cleavage, mature trimeric HA0 is resistant to trypsin digestion. HA0 monomers in contrast are very sensitive to trypsin and other proteases. Protease resistance therefore is an exclusive characteristic of HA trimers in their neutral conformation (Copeland et al., 1986).

As an independent method of assessing both conformation and cell-surface localization of HA0, we therefore subjected intact HA0-expressing Sf9 cells to digestion with trypsin. This approach has been used extensively on a range of cell lines and has shown that export from the ER is the limiting step for HA transport to the cell surface in mammalian cells, in other words: HA that exits the ER and reaches the Golgi complex, where its glycans are modified into endo H resistance will continue to travel to the cell surface (Copeland et al., 1986). After 50 min of chase about 50% of labeled HA0 had acquired trypsin resistance and reached the Sf9 cell surface, as it was cleaved into HA1 and HA2 by extracellular trypsin (Fig. 3B). Control samples in which trypsin was omitted showed no cleavage. The sample taken immediately after pulse labeling showed no digestion because HA had not been transported to the insect cell surface yet. To check whether HA1 and HA2 were connected by their native disulfide bond, we collected medium from the sample that had been subjected to trypsin digestion after a 50-min chase. HA1 was not detected in this medium, indicating that this subunit was still connected to HA2 via the native disulfide bond, as in mammalian cells (Fig. 3B). Our combined data show that HA in Sf9 cells arrived on the cell surface as a trimer with an intact interdomain disulfide bond. The resistance to trypsin proved it was folded and mature.

## 4. Discussion

We studied the folding process of the hemagglutinin from influenza virus, a mammalian virus, in Sf9 insect cells, and found that HA followed a similar folding pathway as in mammalian cells, but folded at much higher rate without much aggregation. During folding HA populated/was enriched in the same folding intermediates in insect cells as in mammalian cells. After monomer folding HA assembled into trimers and was transported to the cell surface correctly in Sf9 cells.

Glycosylation and disulfide bond formation are important for and occur during HA folding. Core glycosylation and glycan modifications affect protein folding in the ER, but this is completely conserved between mammals and insects. It does vary strongly in the Golgi complex, at a stage where folding and oligomerization of HA have been completed. In its folding pathway HA always populates two prominent disulfide-linked folding intermediates: IT1 and IT2, which fold into the native form NT (Braakman et al., 1991). Folding intermediates and pathways are defined by disulfide bonds and conformational epitopes and not by differences in glycan composition. In IT1 the first two disulfide bonds in the top domain have formed (Fig. 1A), in IT2 disulfide bonds 3 and 4 in the hinge region, and in NT all six disulfide bonds have formed (Hebert et al., 1997; Daniels et al., 2003; Maggioni et al., 2005). Our data now show that even in insect cells HA followed the same folding pathway. This suggests that HA's folding path is determined mainly by the protein itself, as it folds in the same way despite the very different cellular backgrounds. One deviation occurred, however, in the form of an additional minor HA folding intermediate between IT1 and IT2 at the 0-min chase time in Sf9 cells. Early HA folding in Sf9

cells hence may involve an alternative step. Interestingly, we found a similar band when we studied HA folding from the reduced form in a diluted detergent mammalian cell lysate where the ER is no longer intact (data not shown). This suggested that whereas insect cells may lack the identical folding assistance the intact mammalian ER provides, they do provide an alternative to help HA fold from its reduced form into the native conformation.

Our conclusions are consistent with previous studies, which have shown that kinetics and yields are highly diverse between HeLa, CHO, CV1, NIH 3T3, and HT1080 cells, but that the HA folding pathway is the same in every cell line (Copeland et al., 1988; Braakman et al., 1991; Segal et al., 1992; Braakman lab, unpublished observations). We here showed the invertebrate (insect)-derived Sf9 cells to perform better than any of the mammalian cells in rate and yield of HA folding. Folding of HA in mammalian cells requires calnexin and calreticulin (Hammond et al., 1994; Chen et al., 1995; Molinari et al., 2004). Insect cells hence must have a good folding and quality control system—similar to or replacing calnexin and calreticulin—that efficiently supports influenza virus HA folding. In the *D. melanogaster* genome we found 4 homologs of calnexin and 1 of calreticulin (Hoskins et al., 2007). Unfortunately the *Spodoptera* genome, from which Sf9 cells originate, has not been sequenced yet and homologs are not known.

In addition to the overall increase in folding rate in Sf9 cells, kinetics of individual folding steps changed. In mammalian cells IT1 was the long-lived intermediate of HA while IT2 disappeared fast; this was opposite in Sf9 cells. Formation of the first few disulfide bonds is a bottleneck of HA folding in mammalian cells (Maggioni et al., 2005), which may explain the long existence of IT1 during HA folding in HeLa cells. HA apparently passes this folding bottleneck easily in Sf9 cells. The persistence of IT2 in Sf9 cells implies that folding from IT2 to NT was slower than folding from IT1 to IT2. Because HA intermediates are always the same, the ER-resident folding factors in Sf9 cells may have acted differently than in HeLa cells. Specific insect chaperones and folding enzymes (perhaps the insect calnexin/calreticulin homologs) may help HA pass its IT1 bottleneck, and the same or other folding factors may enhance folding from IT2 to NT. Perhaps the optimal folding environment for HA (and any other protein for that matter) would be a mixture of insect and mammalian chaperones, an optimal set for each folding step.

The highly efficient HA folding we detected in Sf9 cells implicates insect cells as a good expression system for producing glycoproteins. The current HA-based subunit influenza vaccine Flublok contains three full-length recombinant HA proteins to help protect against two influenza virus A strains, H1N1 and H3N2, and one influenza virus B strain (Cox and Hashimoto, 2011). We examined HA from the H3N2 strain X31, but the complete conservation of disulfide bonding in all sequenced influenza virus HA antigens alludes to conservation of their folding pathways as well. Indeed, purified H1 HA displayed the same trypsin resistance we exploited in our pulse-chase assays (Wang et al., 2006). Our observation that X31 HA folds well in insect cells suggests that the vaccine products indeed have folded into correct HA structures and may explain the adequate immune response upon vaccination (Holtz et al., 2003; Cox and Hashimoto, 2011).

Our findings indicate that the insect cell ER supports HA folding efficiently although the exact mechanism may differ from that of mammalian cells. Humanized insect cells may be good for a protein that needs chaperones absent in insect cells or complex glycosylation (Harrison and Jarvis, 2006), but it is not required for folding of HA. We conclude that the insect cell system offers an excellent alternative for the production of influenza virus vaccines in chick embryos or mammalian cells, with anticipated high yields and quality. Moreover, our assays can establish for each biotechnologically interesting protein whether or not insect cells need to be humanized for efficacious protein production.

## Competing interest

The authors have no competing interests to report.

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