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Expression of Antibody Interferes with Disulfide Bond Formation and Intracellular Transport of Antigen in the Secretory Pathway*

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To determine whether antibodies would interfere with the folding of glycoprotein antigens in the endoplasmic reticulum lumen of living cells, hybridoma cells producing monoclonal anti-hemagglutinin (HA) antibodies were infected with influenza virus. The fate of the newly synthesized HA was determined using an established pulse-chase approach. When the monoclonal antibodies were against epitopes present on early folding intermediates, folding and intracellular transport of HA to the Golgi complex were severely disturbed. On the other hand, when the antibodies were specific for the native HA trimers, immune complexes were formed, but folding or transport of HA was not affected. The use of antibodies in this way provided *in situ* information about the protein folding process inside the endoplasmic reticulum lumen of cells without external perturbation of the folding chains or the folding compartment.

The process of protein folding in the living cell is a more complicated process than previously recognized. It is dependent on a milieu that differs between cellular compartments. It is assisted by a panel of molecular chaperones and folding factors and affected by a variety of processes such as targeting, membrane translocation, covalent modification, and degradation (1–3). Moreover, because folding of proteins begins already at the level of the growing nascent chain, it is likely to occur in a vectorial fashion from the N to C terminus of the growing polypeptide chain (4, 5).

Whereas the classical *in vitro* refolding approach used by Anfinsen and Scheraga (6) has provided much basic information about the biophysical principles behind the folding of polypeptides, it does not allow for conditions prevailing in the cell to be adequately reproduced. To study protein folding in cells, we and others have therefore made extensive use of a pulse-chase approach in which the folding of a radioactively labeled cohort of proteins is followed in tissue culture cells (7, 8). Although it reflects the folding process inside the live cell, this technique is also not problem-free. Because the labeled, partially folded proteins must be extracted from the cells and biochemically analyzed, there is no guarantee that their conformation after cell lysis faithfully reflects the conformation they have inside the cell. Ideally, one should be able to monitor the conformation of the folding proteins *in situ*.

In this study, we describe an approach that allows analysis of protein folding inside the unperturbed endoplasmic reticulum (ER).[1] It is based on the use of conformation-dependent monoclonal antibodies. The antigen whose folding is to be monitored and antibodies against it are co-expressed in the same cell, and the effects of their interaction on antigen folding are analyzed by the pulse-chase approach. As shown here using influenza hemagglutinin (HA) as a model protein, it is possible to determine whether specific conformations occur during the folding process and whether antibody binding affects it.

EXPERIMENTAL PROCEDURES

Cell Lines and Virus—All hybridoma cell lines were grown in Iscove's Dulbecco's modified Eagle's medium with 10% fetal calf serum, 1% gentamycin, and 1% Glutamax I (Life Technologies, Inc.) at 37 °C and 5% CO_2 atmosphere in a humidified incubator. They were split at a density of 1.0×10^6 cells/ml into 0.1×10^6 cells/ml. The P5D4 hybridoma cells produce monoclonal antibodies against the cytosolic tail of vesicular stomatitis virus G-protein (9). The F1 hybridoma cells produce monoclonal antibodies against nascent chains and early folding intermediates of HA (10), and the N2 hybridoma cells produce antibodies that are specific for trimerized HA (11, 12).

The X31/A/Aichi/1968 strain of influenza virus was prepared as described previously (13). The rabbit polyclonal antiserum raised against X31 influenza virus immunoprecipitates all forms of HA, the viral nucleoprotein, and matrix protein (7, 14).

Reagents—The ^{35}S-labeled cysteine and methionine mixture (Promix) was purchased from Amersham Pharmacia Biotech. CHAPS was from Pierce, and Endo H was from New England Biolabs. Media and reagents for cell culture were obtained from Life Technologies, Inc. All other reagents were purchased from Sigma.

Viral Infection and Pulse-Chase Analysis—After hybridoma cells were grown to 1.0×10^6 cells/ml, 1.0×10^7 cells per time point were collected by centrifugation for 5 min at $200 \times g$ in a Beckman GS-15 centrifuge at 4 °C, washed once with PBS, and resuspended in RPMI 1640 medium with 20 mM HEPES (pH 6.8) and 0.2% bovine serum albumin. X31 influenza virus was added at a multiplicity of infection of 10 and bound to the cell surface for 1 h at room temperature on a rocker. Cells were collected as before and resuspended in normal growth medium and incubated for 14–18 h under normal growth conditions. The cells were then washed with PBS and starved in Cys/Met-free medium (300 μl per 1.0×10^7 cells) for 30 min. Subsequently, the pulse was started by adding 500 μCi of ^{35}S-labeled cysteine and methionine to 1.0×10^7 cells. The pulse was stopped and the chase started by adding 10 mM unlabeled cysteine and methionine and 2 mM cycloheximide to inhibit further translation (7). After the pulse or after additional periods of chase the cells were immediately lysed. When looking for secreted components in the cell supernatant, the pulse or chase was stopped by adding 700 μl of ice-cold PBS containing 20 mM *N*-ethylmaleimide (NEM), and the cells were separated from the supernatant by centrifugation at 1,500 rpm in an Eppendorf centrifuge at 4 °C.

Cell Lysis, Immunoprecipitation, and SDS-PAGE—After chase or

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[1] The abbreviations used are: ER, endoplasmic reticulum; HA, hemagglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; Endo H, endoglycosidase H; PBS, phosphate-buffered saline.

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directly after pulse, cells were lysed by adding an equal volume of 2× lysis buffer (4% CHAPS, 100 mM HEPES, 400 mM NaCl (pH 7.6)) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 20 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin) and 20 mM NEM to alkylate any remaining free sulfhydryl groups. When the cells were separated from their medium, they were lysed in 1× lysis buffer containing protease inhibitors and 20 mM NEM. A postnuclear supernatant was prepared by centrifuging the lysates at 16,000 × *g* for 5 min at 4 °C.

150 μl of the postnuclear supernatant was incubated with protein A-Sepharose CL-4B beads (15-μl bead volume) and rotated at 4 °C for at least 3 h in the presence or absence of 5 μl of anti-influenza antiserum. Immune complexes were pelleted at 2,500 × *g* for 2 min and washed with agitation three times for 5 min each. In the case of immunoprecipitation with protein A only, the immune complexes were washed with a wash buffer containing 0.5% CHAPS/HEPES-buffered saline (200 mM NaCl and 50 mM HEPES (pH 7.6)) and in the case of anti-influenza immunoprecipitation, with wash buffer containing 10 mM Tris-Cl (pH 6.8), 0.05% Triton X-100, and 0.1% SDS (7). The washed complexes were solubilized by the addition of nonreducing sample buffer (100 mM Tris-Cl (pH 6.8), 4% SDS, 0.2% bromphenol blue, and 20% glycerol) and heated for 5 min at 95 °C. For reducing conditions, 125 mM dithiothreitol was added to the samples. The samples were analyzed by 7.5% SDS-PAGE on 10.5-cm gels followed by fluorography.

Endoglycosidase H Digestion—Anti-influenza immunoprecipitates from pulse-chased, influenza-infected hybridoma cells were washed three times as above. The immune complexes were resuspended in 0.2% SDS in 100 mM NaOAc (pH 5.5) and heated for 5 min at 95 °C. An equal volume of 100 mM NaOAc (pH 5.5) was added and the samples were divided in two. To one half of the samples only buffer was added and to the other half, buffer plus 0.5 unit of Endo H (15). The samples were incubated for 16 h at 37 °C. Digestions were stopped by the addition of reducing sample buffer, heated for 5 min at 95 °C, and analyzed by 7.5% SDS-PAGE on 10.5-cm gels followed by fluorography.

RESULTS

Influenza HA Folds and Is Transported to the Golgi Complex in Hybridoma Cells—Analysis of NEM-alkylated folding intermediates extracted from cultured cells has shown that folding of HA (a type I membrane glycoprotein, 84 kDa) starts cotranslationally and continues posttranslationally in the lumen of the ER (7, 10). 10–30 min after chain completion, when the molecules have acquired six intrachain disulfide bonds, they assemble into homotrimers and are transported out of the ER to the Golgi complex (12, 14, 16).

To analyze the folding process, immunoprecipitations of detergent-extracted, pulse-labeled HA have been performed using conformation-specific antibodies, and mobility differences between differentially oxidized intermediates have been monitored by SDS-PAGE (17). The panel of antibodies used included monoclonals that react specifically with differentially oxidized early forms of HA, fully oxidized HA forms, and HA trimers. As indicated by the immunoprecipitation, some of the epitopes were expressed transiently during the folding process (17) and some were detected already on growing nascent chains (10).

To determine whether the epitopes that these antibodies react with are actually present on the molecules during folding inside the ER, HA was expressed in the hybridoma cells that produced the antibodies. Like HA, the antibodies fold and assemble inside the ER lumen and can react with their antigens within this compartment (18).

Control experiments were first performed using a hybridoma cell line that produces antibodies that do not interact with HA. The cell line was P5D4, a hybridoma line that produces antibodies against the cytosolic tail of vesicular stomatitis virus G-protein (9). The cells were infected with the X31 strain of influenza virus using a procedure previously described for Chinese hamster ovary cells (7). In the case of Chinese hamster ovary cells, optimal HA expression is achieved 5 h postinfection. However, in hybridoma cells the cytopathic effects started 24 h postinfection, and optimal expression of HA was reached

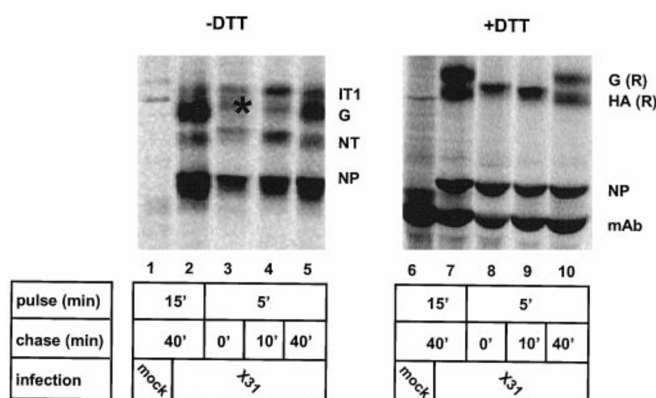

FIG. 1. **Folding of influenza HA in P5D4 hybridoma cells.** P5D4 hybridoma cells (their monoclonal antibodies (*mAb*) are specific for the cytosolic tail of vesicular stomatitis virus G-protein) were infected with X31 influenza virus. 18 h postinfection, the cells were pulse-labeled for 15 or 5 min with 500 μCi of ^{35}S-labeled cysteine and methionine and chased for 0–40 min in the presence of cycloheximide. Reduced and nonreduced anti-influenza immunoprecipitates of the lysates were analyzed by nonreducing and reducing 7.5% SDS-PAGE followed by fluorography. *Lanes 1* and *6* show mock-infected cells. *DTT*, dithiothreitol; *IT1*, first folding intermediate of HA; *G*, Golgi form of HA; *NT*, oxidized native form of HA; *NP*, nuclear protein of the virus. The *asterisk* in *lane 3* marks the second folding intermediate (IT2) of HA.

at 14–18 h after infection. As detected by indirect immunofluorescence, 90% of the cells were infected.

At 18 h postinfection, the P5D4 cells were pulse-labeled for 5 min with ^{35}S-labeled methionine and cysteine and chased for 0–40 min. As a control, infected and mock-infected cells were pulse-labeled for 15 min and chased for 40 min. After the pulse or after the chase, the cells were lysed with detergent in the presence of 20 mM NEM to alkylate the free sulfhydryl groups and prevent further disulfide bond formation (7). Postnuclear supernatants were immunoprecipitated using anti-influenza antibodies and analyzed by nonreducing and reducing SDS-PAGE.

Folding of HA proceeds via two easily recognized intermediates, IT1 and IT2, that differ in the number of intrachain disulfide bonds. IT1 contains one or more of the small disulfide loops but lacks both of the major loop-forming disulfides, 14–466 and 52–277. IT2 has disulfide 52–277, but lacks disulfide loop 14–466.[2] Soon after reaching the fully oxidized form (NT), HA trimerizes and is transported to the Golgi complex where the *N*-linked glycans undergo terminal glycosylation.

The results showed that after a 5-min pulse all three forms, IT1, IT2, and NT, were present (Fig. 1, *lane 3*). The *asterisk* marks IT2, which has a slightly lower mobility than the Golgi form (*lane 2* or *5*). The mobility of NT, the fastest moving of the HA bands, increased during the chase. This is an effect caused by the trimming of glucose and mannose residues from the six core oligosaccharide chains (19). The Golgi form only became visible at later chase times (Fig. 1, *lanes 3–5*). In nonreduced gels, it had a mobility almost similar to IT2 but was easily distinguished after reduction by a lower mobility, due to the extra sugars added during terminal glycosylation.

The changes in gel pattern showed that HA folded normally in hybridoma cells and that about half of it reached the Golgi compartment within 40 min, which corresponds to the rate of maturation seen in Chinese hamster ovary cells (7). Labeled antibody bands were also present in both the reduced (indicated by *mAb* in Fig. 1) and nonreduced gels (not shown). Thus, the production of antibodies did not interfere with the process

[2] I. Braakman and A. Helenius, manuscript in preparation.

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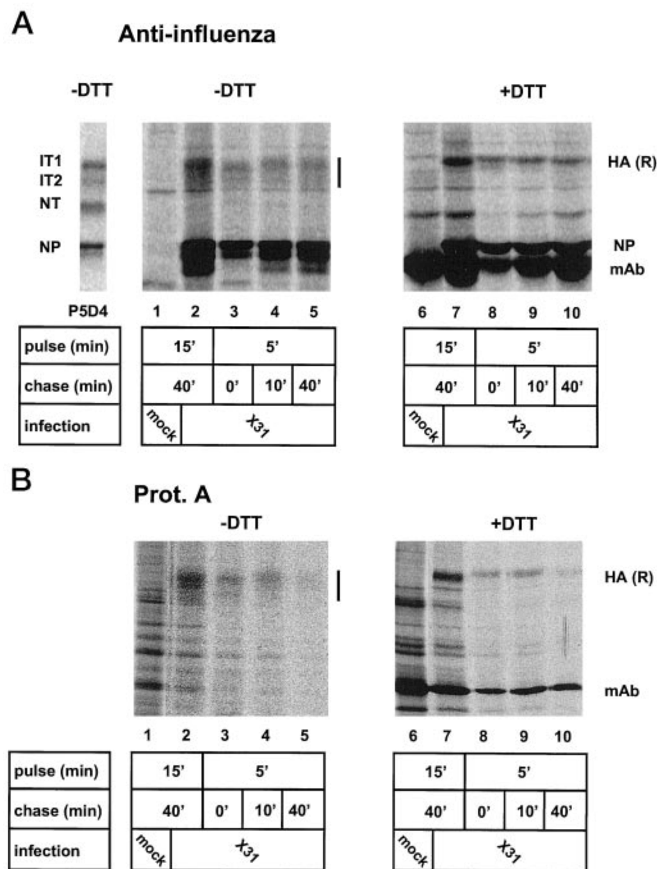

FIG. 2. **Folding of influenza HA in F1 hybridoma cells is disturbed.** F1 hybridoma cells (their monoclonal antibodies (*mAb*) are specific for nascent chains, *IT1* and *IT2*) were infected and pulse-chased as described in the legend to Fig. 1. Abbreviations are defined in the legend to Fig. 1. *A*, lysates were immunoprecipitated with anti-influenza antiserum and analyzed with nonreducing and reducing 7.5% SDS-PAGE. The *vertical line* marks the smear of folding intermediates under nonreducing conditions. An X31-infected P5D4 cell lysate (pulsed for 5 min) was immunoprecipitated with anti-influenza antiserum as a marker for the folding intermediates (*P5D4*). *B*, lysates were immunoprecipitated with protein A (*Prot. A*) beads only and analyzed by nonreducing and reducing 7.5% SDS-PAGE followed by fluorography. Again, the *vertical line* marks the smear of folding intermediates of HA. The relatively high amount of background (*A* and *B*) is due to the fact that the lysates could not be precleared with protein A beads.

of normal HA folding in the ER.

Antibodies against HA Interfere with Folding and Transport—To test whether synthesis of antibodies specifically targeted against HA would affect HA folding and transport, a hybridoma cell line, F1, was chosen. F1 antibodies react with an epitope in the stem domain of HA (7). Immunoprecipitation indicates that the F1 epitope is transient; it is expressed on full-length and nascent HA molecules that are present in the IT1 and IT2 forms but not at later stages of maturation (7, 10).

Fig. 2*A* shows that, in contrast to the P5D4 cell control (*lane* marked *P5D4*), the HA produced in F1 cells failed to fold properly. In nonreduced gels, the HA ran as a smear with a mobility slower or equal to that of IT2 (*lanes 2–5*). No NT was formed. Apparently, formation of the correct intrachain disulfide bonds was disturbed. Furthermore, in the reduced gel (Fig. 2*A*), no Golgi form appeared, indicating that the glycans did not undergo terminal glycosylation (*cf*. Fig. 2*A*, *lanes 7* and *10* with Fig. 1, *lanes 7* and *10*). This implied that the HA was trapped in the ER as a result of interaction with the antibody.

To confirm that the HA was retained in the ER in the F1 cells, its Endo H sensitivity was determined. Although the HA signal was weak in the F1 cells, it was apparent that it re-

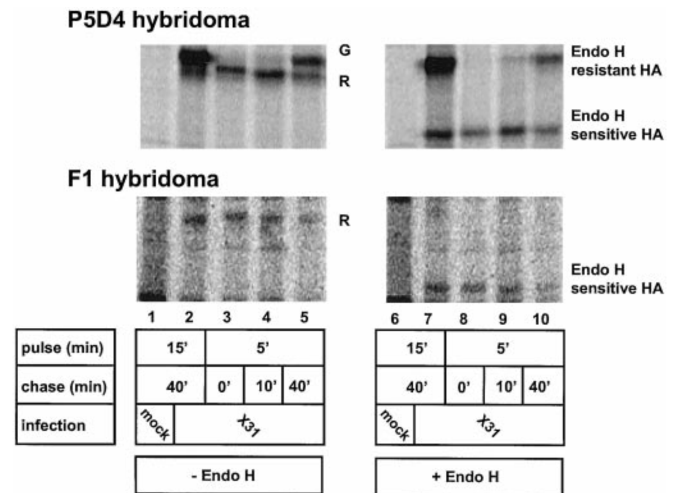

FIG. 3. **Endoglycosidase H digestion of HA from X31-infected P5D4 and F1 cells.** Anti-influenza immunoprecipitates from P5D4 and F1 hybridoma cells were obtained as described in the legends to Figs. 1 and 2. The precipitated proteins were divided in two; one half was digested with Endo H at 37 °C for 16 h and the other half was incubated without Endo H at 37 °C for 16 h. They were then analyzed by reducing 7.5% SDS-PAGE followed by fluorography. *G*, reduced Golgi form of HA; *R*, reduced non-Golgi form of HA. Low mobility HA is Endo H-resistant, and high mobility HA is still sensitive to Endo H.

mained Endo H-sensitive throughout the chase, in contrast to the HA in P5D4 cells (Fig. 3), half of which reached an Endo H-resistant form within 40 min. The uninhibited secretion of antibodies into the medium proved that the inhibition of intracellular transport of HA was not caused by a general defect in the secretory pathway (Fig. 4).

To show more directly that the F1 antibodies produced in the ER were the cause of the disturbed folding, we examined whether they were bound to the pulse-labeled HA. To bring down cellular immune complexes, we precipitated cell lysates with protein A-coated beads without added antibodies. The labeled HA was precipitated, revealing the same smeary pattern in nonreduced gels as seen with immunoprecipitation using the anti-influenza antiserum (Fig. 2*B*). During the chase, the amount of radiolabeled HA complexed to antibodies decreased (*cf. lane 5* or *10* with *4* or *9*, respectively). Although we do not know the reason for this decrease (it might be that the interaction between antibody and HA is transient), these results indicated that at least a fraction of the endogenous anti-HA antibodies were, indeed, bound to incompletely or incorrectly folded HA molecules.

We concluded that F1 antibodies were able to bind to the HA molecules inside the lumen of the ER and that the binding effectively interfered with normal folding and transport. Instead of trapping HA intermediates in the familiar IT1 and IT2 forms, the antibodies caused the formation of a more heterogeneous set of intermediates than normally seen in cell lysates. The antibodies were clearly able to interfere with proper maturation and transport of the newly synthesized HA.

Antibodies against HA Trimers Do Not Interfere with Transport of HA to the Golgi Complex—We then asked whether retention of HA in the ER could also be observed when antibodies were complexed to fully folded HA molecules. For this we used the N2 hybridoma cells that produce antibodies specific for fully folded HA trimers (14). As shown in Fig. 5*A*, HA folded normally to NT in N2 cells,and a large fraction was transported to the Golgi complex where it was terminally glycosylated. Precipitation with protein A-coated beads without added antibodies only brought down the terminally glycosylated Golgi intermediates, indicating that only late forms of HA

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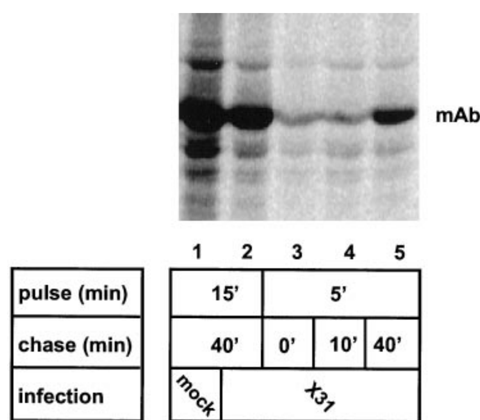

FIG. 4. **Antibody secretion by X31-infected F1 cells.** F1 cells were infected and pulse-chased as described in the previous figure legends. However, after the chase, the cells were resuspended in PBS containing 20 mM NEM. The cells were analyzed for their ability to secrete soluble antibodies by immunoprecipitating the cell supernatant with protein A beads. The immunoprecipitates were analyzed for radiolabeled antibodies by reducing 7.5% SDS-PAGE followed by fluorography. *mAb*, monoclonal antibody.

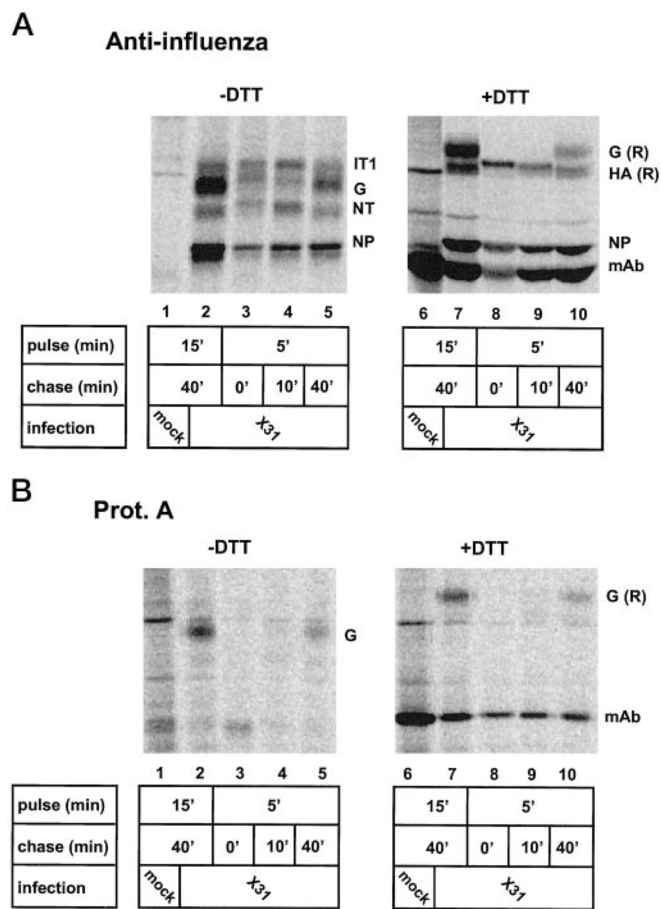

FIG. 5. **Folding and transport of HA in N2 hybridoma cells is not disturbed.** N2 hybridoma cells (their monoclonal antibodies (*mAb*) are specific HA trimers) were infected and pulse-chased as described in the previous figure legends. Cell lysates were immunoprecipitated with anti-influenza antiserum (*A*) or with protein A (*Prot. A*) beads only (*B*). The immunoprecipitates were analyzed by nonreducing and reducing 7.5% SDS-PAGE followed by fluorography. Abbreviations are defined in the legend to Fig. 1.

bound to the antibodies (Fig. 5*B*). It has previously been demonstrated that the majority of the HA subunits trimerize already in the ER (12). This suggests that HA trimers associated with N2 antibodies in the ER were able to move to the Golgi complex. The retention of HA in F1 hybridoma cells is therefore most likely not caused by the antibody-antigen complex formation but by misfolding of HA. Thus, association with an antibody as such may not be the cause of HA retention in the ER but rather the effect it has on the antigen's folding and maturation.

DISCUSSION

The approach taken was to use the ER lumen of the hybridoma cell as a "reaction vessel" in which antigens and antibodies were mixed under unperturbed, physiological conditions. The antigens in this case were nascent and newly synthesized HA glycoproteins. Because HA was continuously being translated and translocated into the ER, it was presented to the antibodies in all the different conformations that folding chains normally display within the authentic, lumenal ER environment.

On the basis of prior immunoprecipitation data, the hybridoma cells were chosen so that the antibodies were expected to react with nascent chains and early folding intermediates or with fully folded molecules. In both cases, immune complexes were formed. In the case of antibodies against nascent chains and early folding intermediates, it was clear that the immune complexes formed in the ER because no Golgi modifications could be observed in the HA. The antibodies that reacted specifically with early intermediates of HA were found to disturb folding and intracellular transport. The antibodies to the mature trimeric HA had no effect on folding or intracellular transport.

The effects of the antibodies were likely to be caused by antibody-induced misfolding. Attachment of a bulky IgG molecule to the F1 epitope on a growing nascent chain or newly synthesized full-length HA molecule would limit the freedom of the folding chain and prevent interactions with molecular chaperones and folding enzymes. This would result in the formation of incorrectly oxidized, non-native conformers retained in the ER by the quality control system. Alternatively, the presence of HA bands that have not been seen previously could mean that folding of HA does not have as distinct a set of intermediates as suggested by previous data.

Another conclusion that can be drawn from these results is that the conformational epitopes identified by immunoprecipitation using solubilized folding intermediates of HA were also present in the protein during *in situ* folding. Among the three cell lines tested, we did not find a single case in which a specific epitope would not cause antibody binding. This suggested that, at least as far as HA is concerned, the emerging picture of *in vivo* folding based on the pulse-chase approach is on the right track.

Expression of specific antibodies has been used in the past to block the formation of functional nuclear pores and to identify the location of antigens in the Golgi complex (18, 20). In these studies the hybridoma cells were not used directly, but the mRNA for the heavy and light chains of an antibody were microinjected into tissue culture cells. More recently, studies have been performed in which single-chain antibodies were expressed intracellularly and targeted to specific compartments using specifically designed vectors (21, 22). In one study, the ER-targeted single-chain antibodies were shown to interfere with the post-ER cleavage of human immunodeficiency virus, type I gp160, indicating that the antigen was retained in the ER (23). However, it is very likely that this retention was caused by the ER retention signal on the antibody and not by misfolding of the antigen. From the results described here, it is clear that antibodies can be successfully used to analyze pro-

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tein folding in the ER of living cells and for targeted interference with the maturation of specific antigens. In the future, one might be able to screen single-chain antibody libraries (24) expressed intracellularly for the identification of new factors (antigens) that affect folding of proteins in the ER.

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