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

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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, it is likely to occur in a vectorial fashion from the N to C terminus of the growing polypeptide chain (4, 5).

In this study, we describe an approach that allows analysis of protein folding inside the unperturbed endoplasmic reticulum (ER). 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% CO2 atmosphere in a humidified incubator. They were split at a density of 1.0 × 106 cells/ml into 0.1 × 106 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 trimmerized 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 35S-labeled cysteine and methionine mixture (Prolin) 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 × 106 cells/ml, 1.0 × 106 cells/ml per time point were collected by centrifugation for 5 min at 200 × g in a Beckman GS-15 centrifuge at 4 °C, once washed with PBS, and resuspended in RPMI 1640 medium with 20 μM HEPS (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 μM per 1.0 × 106 cells/ml) for 30 min. Subsequently, the pulse was started by adding 500 μCi of 35S-labeled cysteine and methionine to 1.0 × 106 cells/ml. 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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The abbreviations used are: ER, endoplasmic reticulum; HA, hemagglutinin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propansulfonic acid; NEM, N-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; Endo H, endoglycosidase H; PBS, phosphate-buffered saline.
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 (15–20 mM NEM to alkylate any remaining free sulfhydryl groups). 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% bромphenol 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.

**Results**

**Influenza HA Folds and Is Transported to the Golgi Complex**

*In Hybridoma Cells*—Analysis of N-alkylated folding intermediates extracted from cultured cells has shown that folding of HA (a type 1 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 that do not interact with HA. 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).

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 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 35S-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. Soon after reaching the fully oxidized form (NT), HA trimersizes and is transported to the Golgi complex where the N-linked glycosaminoglycan 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.

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2 I. Braakman and A. Helenius, manuscript in preparation.
Antibodies Block Folding of Antigens in the ER

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. 2. Abbreviations are defined in the legend to Fig. 1. A, lysates were immunoprecipitated with anti-influenza antisemur 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 antisemur 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 an epitope in the stem domain of HA (7). Immunoprecipitation of P5D4 cells (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.

we used the N2 hybridoma cells that produce antibodies specific for fully folded HA trimers (14). As shown in Fig. 5A, 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 remained 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).

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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 antisemur (Fig. 2B). 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. 5A, 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
bound to the antibodies (Fig. 5B). It has previously been dem-

onstrated that the majority of the HA subunits trimerize al-
ready 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 forma-
tion but by misfolding of HA. Thus, association with an anti-
body 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 hybri-
doma cell as a “reaction vessel” in which antigens and antibod-
ies were mixed under unperturbed, physiological conditions.
The antigens in this case were nascent and newly synthesized
HA glycoproteins. Because HA was continuously being trans-
lated and translocated into the ER, it was presented to the
antibodies in all the different conformations that folding chains
normally display within the authentic, luminal ER
environment.

On the basis of prior immunoprecipitation data, the hybri-
doma 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 spe-
cifically with early intermediates of HA were found to disturb
folding and intracellular transport. The antibodies to the ma-
ture 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 mol-
ecule 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 for-
mation 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 immunoprecipi-
tation 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 compart-
ments using specifically designed vectors (21, 22). In one study,
the ER-targeted single-chain antibodies were shown to inter-
fer 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
carried 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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**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.

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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 antibod-
ies by reducing 7.5% SDS-PAGE followed by fluorography. mAb, mono-
clonal antibody.

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**Fig. 3.** Antibodies Block Folding of Antigens in the ER

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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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