Protein Unfolding Is Not a Prerequisite for Endoplasmic Reticulum-to-Cytosol Dislocation*

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We examined the effects of protein folding on endoplasmic reticulum (ER)-cytosol transport (dislocation) by exploiting the well-characterized dihydrofolate reductase (DHFR) domain. DHFR retains the capacity to bind folate analogues in the lumen of microsomes and in the ER of intact cells, upon which it acquires a conformation resistant to proteinase K digestion. Here we show that a Class I major histocompatibility complex heavy chain fused to DHFR is still recognized by the human cytomegalovirus-encoded glycoproteins US2 and US11, resulting in dislocation of the fusion protein from the ER in vitro and in vivo. A folded state of the DHFR domain does not impair dislocation of Class I MHC heavy chains in vitro or in living cells. In fact, a slight acceleration of the dislocation of DHFR heavy chain fusion was observed in vitro in the presence of a folate analogue. These results suggest that one or more of the channels used for dislocation can accommodate polypeptides that contain a tightly folded domain of considerable size. Our data raise the possibility that the Sec61 channel can be modified to accommodate a folded DHFR domain for dislocation, but not for translocation into the ER, or that a channel altogether distinct from Sec61 is used for dislocation.

Nascent polypeptides destined for secretion or membrane insertion enter the ER via a protein channel referred to as the translocon (1). N-Linked oligosaccharides are added cotranslationally, and the formation of disulfide bonds may be initiated on the nascent polypeptide. Folding of nascent chains is assisted by an elaborate system of chaperones that includes calnexin, calreticulin, and oxidoreductases such as PDI and ERP57. To exit the ER along the secretory pathway, proteins that contain a tightly folded domain of considerable size. Our data raise the possibility that the Sec61 channel can be modified to accommodate a folded DHFR domain for dislocation, but not for translocation into the ER, or that a channel altogether distinct from Sec61 is used for dislocation.


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The abbreviations used are: ER, endoplasmic reticulum; MHC, major histocompatibility complex; HC, class I MHC heavy chain; DHFR, dihydrofolate reductase; MTX, methotrexate; TMX, trimetrexate; EGFP, enhanced green fluorescent protein; ppcDHFR, hybrid between preprocecropin A and DHFR; RRL, rabbit reticulocyte lysate; EndoH, endo glucosidase H; PBS, phosphate-buffered saline; ZLLL, N-benzylxoy carbonyl-Leu-Leu-Leu-allylhyde; ZL_vS, N-benzylxoy carbonyl-Leu-Leu-Leu-vinyl sulfone.

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lationally into the ER and acquire their tertiary structure inside the ER lumen. The translocation channel itself is believed capable of accommodating polypeptide segments in an α-helical configuration. However, completion of folding is thought to take place in the ER lumen (15). Consistent with these findings, the translocation of a hybrid between the presecretory protein, preprosecoprin A, and DHFR (ppcecDHFR) into mammalian microsomes is signal peptide-dependent and will proceed post-translationally. Although membrane insertion and signal peptide removal of the preprosecoprin moiety did not require unfolding of the DHFR domain, completion of translocation required unfolded DHFR, because translocation was blocked by inclusion of MTX. When MTX is present, and hence the DHFR portion of the ppcecDHFR is compactly folded, ppcecDHFR is susceptible to proteinase K digestion when translated in the presence of microsomes. It was concluded that folded MTX-occupied DHFR is unable to fit into the Sec61 aqueous pore to allow import into the ER (16).

Here we show that a DHFR-HC fusion protein is recognized by US2 and US11 for ER dislocation in vitro and in vivo. DHFR retains the capacity to bind folate analogues in the lumen of microsomes and in the ER of intact cells. Folding of the DHFR domain does not impair dislocation of Class I MHC heavy chains in vitro or in living cells. These results suggest that the channel(s) used for dislocation can accommodate polypeptides that contain a tightly folded domain of considerable size. Our data raise the possibility that the Sec61 channel can be modified to accommodate a folded DHFR domain for dislocation, but not for translocation into the ER, or that a channel distinct from Sec61 is used for dislocation.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—U373-MG astrocytoma cells (control), and US2 transfectants (US2+) were maintained as described previously (17). DHFR-HC (U373DHFR-HC) cells and US2-DHFR-HC (US2DHFR-HC) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 0.5 mg/ml G418 (Invitrogen, Frederick, MD). The DHFR-HC was transfected into control and US2 transfectants (US2+) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 0.5 mg/ml G418 (Invitrogen, Frederick, MD). The DHFR-HC was transfected into control and US2+ cells using LipofectAMINE (Invitrogen, Frederick, MD) and the standard protocol provided by the manufacturer.

In Vitro Transcription, Translation, and Degradation Assay—pCDNA3.1-DHFR-HC was linearized with Sgfi for in vitro transcription. Transcription and translation were performed as described previously (18). In vitro degradation assays were carried out by translating the indicated mRNAs in rabbit reticulocyte lysates (RLRs) in the presence of microsomes as described (18). Microsomes were spun down (20,000 × g, 15 min) and resuspended in 10 μl of homogenization buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.25 M sucrose) containing 1 mg/ml RNase (Roche Molecular Biochemicals) with or without 0.1 mM TMX and incubated for 30 min at 30 °C. 15 μl of Flexi RRL in the presence or absence of TMX containing 50 μM of the proteasome inhibitor ZLLL (MG132) were added, and aliquots were withdrawn at the indicated time points. A microsomal pellet was obtained by centrifugation (20,000 × g, 15 min). Reducing sample buffer was added to the pellets, which was analyzed using SDS-PAGE.

In Vitro TMX Binding Experiments—DHFR-HC mRNA was translated in the presence of control microsomes. Microsomes were resuspended in 10 μl of homogenization buffer and incubated for 30 min at 30 °C with or without TMX. To measure the extent to which carryover of TMX with the pellet fraction occurred, unlabeled microsomes were treated in a similar fashion. Microsomes were spun down and washed once with 1 ml of cold homogenization buffer. Microsome pellets were resuspended, combined, and lysed together in homogenization buffer containing 1% Triton X-100 with or without proteinase K, as indicated.

Metabolic Labeling of Cells, Pulse-chase Analysis, Immunoprecipitation, and EndoH Digestion—Cells were detached by trypsin treatment, followed by starvation in methionine/cysteine-free Dulbecco’s modified Eagle’s medium for 1 h. Cells were metabolically labeled with 500 μCi/ml of [35S]methionine/cysteine (1200 Ci/mmol, PerkinElmer Life Sciences, Boston, MA) at 37 °C for the times indicated. Pulse-chase experiments, cell lysis, and immunoprecipitation were performed as described previously (19). The immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. Folate analogues were added during the starvation, pulse, and chase periods. TMX (kind gift from Medimmune, Gaithersburg, MD) was used at 0.1 mM, and TMX (Aldrich, WI) was used at 10 μM. EndoH (New England BioLabs, Beverly, MA) digestions were performed according to the manufacturer’s instructions.

Subcellular Fractionation—Subcellular fractionation of metabolically labeled US2DHFR-HC cells was performed as described previously (17).

In Vivo TMX Binding Experiments—One million US2DHFR-HC cells were pulse-labeled for 15 min at 37 °C with or without TMX. To measure the extent to which TMX binding to DHFR occurred post-lysis rather than in the ER, the same number of unlabeled U373 cells was exposed to TMX in the same manner. These cells were washed twice with cold PBS, and the unlabeled cells were resuspended and combined with pulse-labeled cells. The mixed unlabeled and pulse-labeled cells were spun down and lysed together in 1 ml of Nonidet P-40 0.5% lysis buffer. DHFR-HC was immunoprecipitated, and resistance to proteinase K was assessed, as indicated. Carryover of TMX from the unlabeled cells should then be able to stabilize the 21-kDa labeled DHFR-HC not exposed to TMX in the course of metabolic labeling. To achieve maximal binding of TMX to the DHFR moiety, 0.1 mM TMX was added directly to lysates where indicated. DHFR-HC was immunoprecipitated and one-half of the sample was kept on ice, while the other half of each sample was treated with proteinase K as described below. TMX binding to DHFR-HC was calculated as the ratio between the 21-kDa digestion product and the intact DHFR-HC. The maximal binding control, in which TMX was added to lysates, was used to normalize for percentage of binding.

Proteinase K Digestion—Microsomes were lysed in 30 μl of homogenization buffer containing 1% Triton X-100. Proteinase K was added to a final concentration of 100 μg/ml. Digestion was performed on ice for 30 min. Phenylmethylsulfonyl fluoride (2 mM final concentration) was added to inactivate proteinase K. Reducing sample buffer was added, and samples were boiled and immediately loaded onto the gel. Digestion of αHC immunoprecipitates was done in a similar fashion. Immunoprecipitates were washed twice with NETI buffer (Nonidet P-40 0.5%, NaCl 150 mM, EDTA 5 mM, Tris (pH 7.4) 50 mM), 30 μl of homogenization buffer containing 1% Triton X-100 was added, and proteinase K was added to a final concentration of 100 μg/ml at room temperature for 45 min. Phenylmethylsulfonyl fluoride (2 mM) was added, and an equal volume of 2-fold concentrated reducing sample buffer was added. Samples were boiled and immediately loaded onto the gel.

RESULTS

In Vivo Translated DHFR-HC Retains Folding Capacity in Microsomes—We generated a polypeptide composed of the H-2Kb signal sequence, followed by DHFR fused in-frame to the N terminus of HLA-A2 heavy chain. We first verified that DHFR, a cytosolic enzyme, can fold properly in the lumen of the ER, where both redox potential and calcium concentrations differ significantly from those in the cytosol. The H-2Kb-DHFR construct was translated in vitro in the presence of microsomes derived from U373 cells (Fig. 1). Following isolation of the microsomal fraction by centrifugation, we added trimetrexate (TMX), a membrane-permeable folate analogue, to the microsomes. Microsomes were then extensively washed to eliminate traces of unbound drug, lysed in 1% Triton X-100, and subjected to proteinase K digestion. The generation of a 21-kDa DHFR fragment resistant to proteinase K digestion is indicative of binding of the folate analogue and stabilization of the tightly folded conformation of DHFR. Inclusion of TMX renders the DHFR portion of the DHFR-HC fusion resistant to proteinase K digestion, as judged by the appearance of a 21- kDa polypeptide only when microsomes were preincubated with TMX (Fig. 1, lanes 2 and 3). To exclude the possibility of binding of TMX to DHFR after lysis, rather than in intact
microsomes, we incubated a separate preparation of microsomes with TMX, washed the pellet, and lysed these microsomes together with DHFR-HC-containing microsomes that had not been exposed to TMX. Digestion of the DHFR-HC fusion by proteinase K was complete in this case (Fig. 1, lane 4), excluding the possibility of carryover of the drug. Hence, DHFR-HC inserted in the ER folds properly and acquires a tight conformation when bound to a folate analogue.

**DHFR-HC Dislocates in Vitro in a US2- or US11-dependent Manner**—We next examined the ability of DHFR-HC to undergo US2/US11-mediated degradation. In previous work we established an *in vitro* system that recapitulates US2/US11-mediated degradation. The hallmark of this *in vitro* reaction is the disappearance of glycosylated HLA-A2 HC from the pellet fraction in a US2- or US11-dependent manner. Over time, we observe the appearance of a soluble deglycosylated intermediate released from the microsomal fraction, when incubation is carried out in the presence of the proteasome inhibitor ZLLL (18). We applied the same *in vitro* system to DHFR-HC. DHFR-HC mRNA was translated in the presence of microsomes derived from control or US2- or US11-expressing cells (Fig. 2A). In all three types of microsomes some of the glycosylated DHFR-HC was detected in the supernatants, probably due to inefficient separation of the pellet and supernatant fractions by the conditions of centrifugation used. Therefore, as an indicator of dislocation we considered both the loss of glycosylated DHFR-HC from the pellet fraction and the appearance of the deglycosylated intermediate over time in the supernatant fraction. We did not observe the appearance of a deglycosylated intermediate in the supernatants of U373-derived microsomes (Fig. 2A, Supernatants, lanes 1 and 2). However, when DHFR-HC was translated into microsomes derived from US2+ and US11+ cells, a polypeptide with higher mobility appeared at the 60-min chase point. This polypeptide corresponds to deglycosylated DHFR-HC and is indicative of a US2/US11-dependent dislocation reaction (Fig. 2A, Supernatants, lanes 5, 6, 9, and 10). As for HLA-A2 HC (18), US2+ microsomes support better the dislocation of DHFR-HC than US11+ microsomes, as measured by disappearance from the microsome fraction (Fig. 2B) and accumulation of the deglycosylated intermediate in the supernatants (Fig. 2A, Supernatants, lanes 6 and 8 versus lanes 10 and 12).

We then measured whether formation of a tightly folded domain inside the ER lumen would impede dislocation. Microsomes were incubated at 30°C with TMX 30 min prior to the chase to ensure efficient binding of the drug to the DHFR-HC protein. We did not detect inhibition of dislocation of DHFR-HC from microsomes treated with TMX. In fact, we consistently observed a slight but reproducible increase in the amount of deglycosylated intermediate released from US2+ microsomes but not for US11+ microsomes (Fig. 2A, Supernatants, lane 8 versus 6). We examined a more detailed time course for dislocation of DHFR-HC from US2+ microsomes. At all chase points, the deglycosylated intermediate was more abundant for microsomes treated with TMX when compared with controls (Fig. 2C, lanes 6 versus 2 and 7 versus 3). We conclude that the compactly folded DHFR moiety does not impede dislocation of the DHFR-HC fusion.

**DHFR-HC Is Stable in U373 Cells and Largely Retained within the ER**—Unassembled class I MHC heavy chains are recognized by the quality control machinery and are subject to dislocation followed by proteasome-dependent degradation (20, 21). It was therefore necessary to verify that DHFR-HC does not dislocate spontaneously in control cells. U373 cells were transfected with DHFR-HC, and the stability of the fusion protein was monitored by pulse-chase analysis. We also assessed intracellular transport of the fusion protein by performing EndoH digestion. DHFR-HC was stable in control cells even after 3 h of chase (Fig. 3, lanes 1, 3, and 5). While endogenous class I MHC acquired EndoH resistance at 90 min. of chase, the DHFR-HC fusion remains EndoH sensitive even after 3 h of chase (Fig. 3, lanes 2, 4, and 6). Inclusion of TMX affected neither stability nor EndoH sensitivity of the protein (Fig. 3, lanes 7–12). We conclude that the DHFR-HC is relatively stable and fails to leave the ER and that the stability of DHFR-HC is not affected by DHFR ligands.

**TMX Binds DHFR-HC in the ER Lumen**—To demonstrate binding of TMX to the DHFR domain of DHFR-HC fusion inside the ER lumen, US2*DHFR-HC* cells were pulsed for 15 min. in the presence or in the absence of TMX followed by immunoprecipitation and treatment with proteinase K. Under these conditions, radiolabeled DHFR-HC corresponds to ER-disposed protein. To control for binding of TMX to the DHFR moiety post-lysis and to assess the efficiency of TMX binding, we performed the experiment as outlined in Fig. 4A. Pulse-labeled cells not exposed to TMX were washed and mixed with the same number of unlabeled cells incubated with TMX. If binding of TMX to the DHFR-HC fusion would occur post-lysis by carryover of the drug, one should expect to see stabilization of labeled fusion protein obtained from cells not exposed to TMX (Fig. 4A, lanes 1 and 2). As a negative control, 35S-labeled US2*DHFR-HC* cells were mixed with unlabeled cells not exposed to TMX (Fig. 4A, lanes 3 and 4). Upon proteinase K digestion of anti-heavy chain immunoprecipitates exposed post-lysis to TMX, a 21-kDa band appeared, indicating some carryover of TMX (Fig. 4B, lane 2). In addition, we included a control for maximal binding of TMX to the DHFR moiety (Fig. 4A, lanes 7 and 12).
and 8). US2\textsubscript{DHFR-HC} cells were pulse-labeled in the presence of TMX, washed, and lysed. We then added TMX directly to the lysates to obtain maximal exposure of the DHFR domain to TMX. Under these conditions DHFR was stabilized significantly better (Fig. 4B, lane 8). We quantified the binding of each condition by calculating the ratio of the 21-kDa digestion product over the intact DHFR-HC. The ratio for maximal binding, in which TMX was added to lysates, was defined as 100% binding. Binding of TMX to DHFR-HC was 86.5% of the maximal binding observed in a detergent lysate and at least 3-fold higher than binding post-lysis (Fig. 4C). These results indicate that binding of TMX to the DHFR domain of DHFR-HC fusion occurs pre-lysis inside the ER lumen.

Rate of Dislocation of DHFR-HC in Cells Is Not Affected by DHFR Folding—We performed pulse-chase experiments to monitor the dislocation of DHFR-HC coexpressed with US2. We saw that pulse-labeled DHFR-HC expressed in US2\textsubscript{DHFR-HC} disappeared completely within 1 h of chase (not shown). In the presence of proteasome inhibitor, glycosylated DHFR-HC was converted to the deglycosylated intermediate (Fig. 5B). To test whether TMX affects the dislocation rate in live cells, US2\textsubscript{DHFR-HC} cells were pulse-labeled for 5 min and chased for the indicated times in the absence (Fig. 5A) or presence (Fig. 5B) of proteasome inhibitors. In the absence of proteasome inhibitors, DHFR-HC was mostly degraded after 20 min of chase (Fig. 5A, lanes 1–3). In the presence of the proteasome inhibitor ZZ\textsubscript{4}VS, we observed the conversion of DHFR-HC to the deglycosylated intermediate (Fig. 5B, lanes 10–12). As a control, we treated cells with MTX, for which its hydrophilicity should exclude it from the ER, to account for possible effects downstream of dislocation, which might include the inability of folded DHFR to be targeted for proteasomal degradation as efficiently as DHFR not exposed to MTX (22). We did not observe any effect of TMX or MTX on the dislocation rate of DHFR-HC, regardless of the presence of proteasome inhibitors (Fig. 5A, lanes 4–9 and Fig. 5B, lanes 13–18).
extremely rapid unfolding occurred at 37 °C, then perhaps a chase at room temperature might slow down the process. Even at room temperature, DHFR-HC was dislocated at a rate similar to that seen for the endogenous HC. We did not detect any effect on dislocation upon inclusion of TMX (not shown). These data are consistent with the hypothesis that complete unfolding is not required for dislocation.

In keeping with our earlier observations, inclusion of TMX did not change the subcellular localization of the DHFR-HC fusion. The deglycosylated fusion product fractionated with the cytosolic compartment, irrespective of the folded state of the DHFR moiety (Fig. 5C, lane 8 versus 6). We conclude that the DHFR-HC fusion behaves like the endogenous Class I heavy chains as far as US2-mediated dislocation is concerned. Importantly, the folded state of the DHFR domain does not interfere with ER dislocation.

**DHFR-HC Is Dislocated in a Folded State**—To exclude the possibility that rapid unfolding in the ER precedes dislocation despite the binding of TMX, we examined the resistance of the DHFR-HC fusion protein to proteinase K when chased in the presence or absence of TMX. US2<sub>DHFR-HC</sub> cells were pulse-labeled for 15 min in the presence of TMX, washed twice with PBS, and chased for 60 min to ensure complete conversion of glycosylated DHFR-HC to the deglycosylated intermediate, a reaction that occurs in the cytoplasm. Following TMX removal, cells were chased in the presence (Fig. 6A, lanes 11 and 12) or absence (lanes 9 and 10) of TMX. DHFR-HC was immunoprecipitated and digested with proteinase K. To achieve maximal binding of TMX to the DHFR moiety, TMX was included throughout the pulse and chase as well as added into the lysis buffer (Fig. 6A, lanes 13 and 14). This level of binding was defined as 100% binding. Resistance of DHFR-HC to proteinase
Unfolding Is Not Required for Dislocation

FIG. 5. US2-mediated dislocation of DHFR-HC in live cells is not affected by DHFR folding. US2\textsubscript{DHFR-HC} cells were pulse-labeled with \[^{35}\text{S}\]methionine for 5 min and chased up to 20 min in the absence (A) or in the presence (B) of the proteasome inhibitor, ZL\textsubscript{3VS}. TMX (lanes 4–6 and 13–15) or MTX (lanes 7–9 and 16–18) were added 1 h prior to metabolic labeling throughout the chase. Cells were lysed in 1% SDS, diluted to 0.07% SDS with Nonidet P-40 lysis mix, and then followed by immunoprecipitation with anti-class I heavy chain serum. The immunoprecipitates were analyzed by SDS-PAGE (12%). C, US2\textsubscript{DHFR-HC} cells were pulse-labeled with \[^{35}\text{S}\]methionine for 15 min and chased for 60 min in the presence of the proteasome inhibitor, ZL\textsubscript{3VS}. TMX (lanes 3, 4, 7, and 8) was added 1 h prior to metabolic labeling throughout the chase. Cells were homogenized and subjected to fractionation as described under “Experimental Procedures.” The DHFR-HC and endogenous HC were recovered from the 100-kg pellet (p) and the 100-kg supernatant (s) by immunoprecipitation with anti-class I heavy chain serum. The immunoprecipitates were analyzed by SDS-PAGE (12%).

FIG. 6. DHFR-HC dislocates as a folded protein. A, US2\textsubscript{DHFR-HC} cells were pulse-labeled with \[^{35}\text{S}\]methionine for 15 min in the absence (lanes 1–6) or in the presence (lanes 7–14) of ZL\textsubscript{3VS}. Cells were washed twice with cold PBS and chased in the presence (lanes 11–14) or in the absence (lanes 7–10) of TMX. Cells were lysed in Nonidet P-40 lysis buffer. TMX was added directly to lysates (lanes 5 and 6, and 13 and 14). Lysates were immunoprecipitated with anti-class I heavy chain serum (aHC). Half of the immunoprecipitates were digested with proteinase K, and samples were analyzed by SDS-PAGE (12%). B, autoradiograms were quantitated with ALPHAIMAGER software (Alpha Innotech, San Leandro, CA). Binding of TMX to DHFR-HC was calculated from the ratio of the band intensity of proteinase K-digested DHFR-HC (lanes 2, 4, 6, 8, 10, 12, and 14) relative to intact DHFR-HC (upper band, lanes 1, 3, 5, 7, 9, 11, and 13), respectively. Binding was plotted as percentages of the ratio derived for maximal binding (21-kDa fragment in lane 6 is relative to DHFR-HC in lane 5 for pulse and 21-kDa fragment in lane 14 is relative to DHFR-HC in lane 13 for chase).
K when TMX was present during the pulse and chase was 72% of maximal binding (Fig. 6B), 62% when TMX was present only during the pulse period, and removed by washing prior to the 60-min chase. We also measured the binding of TMX to DHFR-HC during the pulse. US2 was stably transfected into control and US2 cells were pulse-labeled for 15 min in the presence of TMX. Cells were washed twice with PBS, lysed, and subjected to immunoprecipitation and proteinase K digestion (Fig. 6A, lanes 3 and 4). Addition of TMX during the pulse stabilized DHFR-HC to 75% of level seen when TMX was added to the lysate (Fig. 6A, lanes 5 and 6, and Fig. 6B). Neither ER-resident DHFR-HC nor the deglycosylated intermediate were stabilized when TMX was omitted (Fig. 6A, lanes 1 and 2, and 7 and 8). The similar levels of stabilization of DHFR-HC conferred by TMX in all three conditions indicates that binding of TMX to the DHFR domain of DHFR-HC fusion during the pulse accounts for most of the binding observed at the end of the chase period when TMX is removed by washing. Therefore, TMX binds to DHFR in the ER and interacts stably with DHFR-HC in the course of dislocation.

**DISCUSSION**

The US2-mediated dislocation of Class I MHC heavy chains from the ER involves a viral product that interacts with the correctly folded protein, as inferred from the crystal structure of the US2-HC/β2m complex (23). This interaction diverts the HC and US2 itself with remarkably rapid kinetics (half-life of 3–5 min) back to the cytosol for degradation by the proteasome. When the proteasome is blocked, the HC accumulates in the cytosol as a deglycosylated intermediate (24, 25). The ability of the class I molecule to fold and interact with US2 and the extraordinarily rapid rate of dislocation thus make the US2-Class I MHC interaction a suitable model to study spatial and conformational constraints on dislocation.

In the course of dislocation the Class I HC undergoes disulfide bond reduction prior to deglycosylation, suggesting an unfolding step to assure smooth threading of the substrate through the translocon and the proteasome (17). We have shown that an EGFP-HC fusion is also a substrate for US2- and US11-dependent dislocation (7). The results of these experiments suggest the possibility that folded, fluorescent EGFP can be transported across the ER membrane. The EGFP-HC model, however, does not allow control over the folded state of EGFP. It is difficult to exclude the formal possibility that partial unfolding of the EGFP-HC fusion is required for dislocation. Partial unfolding might be followed by rapid refolding, thus accounting for fluorescent EGFP-HC species in the cytosol.

To address the necessity of unfolding for proteins to be dislocated from the ER, we chose DHFR as a fusion partner to Class I HC. In the presence of folate analogues DHFR acquires a tightly folded, highly protease-resistant conformation. Thus, the inclusion or omission of these small molecules allows control over the stability of the DHFR moiety. Furthermore, DHFR fusions have been used extensively to address questions pertaining to protein translocation. The best studied examples concern proteins destined for import into mitochondria and peroxisomes. DHFR fused to a mitochondrial signal peptide fails to be imported when the compactly folded state is imposed on DHFR by inclusion of folate analogues (9). Curiously, DHFR targeted to the TAT translocation system of chloroplasts is still translocated across the thylakoid despite the presence of MTX (26). This supports the notion that the machinery responsible for TAT-mediated translocation apparently can transport proteins in a fully folded state. Therefore, assaying DHFR transport in the presence or absence of folate analogues allows an assessment of how folding affects the translocation of a DHFR-containing reporter substrate.

The DHFR moiety, however, has not been used as a fusion partner to study export from a membrane-delimited compartment, mainly owing to limitations of membrane permeability of folate analogues such as MTX. Therefore we used TMX, a lipophilic DHFR inhibitor in use as an anti-protozoan drug (27), to impose tight folding of an ER-localized DHFR domain. The affinity of TMX for murine DHFR is $1–10 \mu M$. We used a concentration of 100 $\mu M$ to ensure efficient (over 90%) binding. At this concentration, TMX interacted with and induced stable binding of DHFR-HC expressed in microsomes, as assessed by resistance to proteinase K (Fig. 1).

First we tested the effects of folate analogues on the dislocation of DHFR-HC in vitro, using an assay that recapitulates US2- and US11-mediated dislocation. We expected that if unfolding of the dislocation substrate is required, enforcing a folded conformation of the DHFR domain should slow down dislocation or even abrogate it, as shown for import of DHFR fusions into mitochondria. In fact, for US2+ microsomes but not for US11+ microsomes we observed, if anything, an enhancement of dislocation, as judged by greater accumulation of the diagnostic soluble deglycosylated intermediates at the early time points of chase (Fig. 2C). These data suggest potentiation rather than inhibition of dislocation by the presence of a tightly folded DHFR moiety. Because acceleration was seen only in US2+ and not in US11+ microsomes, it is unlikely that this effect is due to events downstream of dislocation, such as activation of peptide N-glycanase or other factors involved in the generation of the soluble intermediate. This selectivity, observed when DHFR ligands are included for US2-mediated dislocation, could therefore be attributed to the different mechanisms utilized by US2 and US11 to degrade class I HC. For example, US2 dislocates the HC in a manner that is rather independent of the HC cytosolic tail sequence, whereas US11-mediated dislocation requires a complete HC cytoplasmic tail (18). Furthermore, a tailless US2 is incapable of dislocating HC, whereas tailless US11 retains the capacity to dislocate the HC (18). Therefore, it is conceivable that folding of the DHFR domain in the ER lumen facilitates the interaction with US2 either directly or indirectly. Nonetheless, for neither US2- nor US11-mediated dislocation does the folding of the luminal DHFR domain inhibit the transfer of the DHFR-HC fusion protein from the membrane fraction to the soluble fraction.

Next, we verified these results in live cells. The DHFR-HC was stably transfected into control and US2+ astrocytoma cells. We found that DHFR-HC is highly stable in control U373 cells and is retained largely in the ER (Fig. 3). In vivo binding of TMX to DHFR in the ER was assessed by measuring proteinase K resistance and found to be as efficient as binding to solubilized DHFR-HC (Fig. 4). Control experiments excluded carryover and post-lysate binding of the drug as an explanation for the observed proteinase K resistance. In US2+ cells, the DHFR-HC was dislocated rapidly, with kinetics similar to those seen for endogenous HC (Fig. 5). We could not show a significant acceleration of dislocation upon DHFR folding, as we observed in vitro. This observation is most likely attributable to the more rapid kinetics of dislocation in vivo. Regardless, both in TMX- or MTX-treated cells, dislocation occurs at the same rate. To establish that dislocation had in fact occurred in the manner seen for the endogenous HC, we also verified the transport of DHFR-HC from the ER membrane to the cytoplasm by subcellular fractionation. Following a 15-min pulse label, glycosylated DHFR-HC and glycosylated endogenous heavy chain were retrieved from the pellet fraction. After a 60-min chase in the presence of proteasome inhibitor, the deglycosylated DHFR-HC fusion protein accumulated mainly in the cytosolic fraction, regardless of the inclusion of TMX (Fig.
5C), and similar to the behavior of endogenous heavy chains. Therefore, tight folding of the DHFR moiety did not impose any constraints on dislocation. To exclude the remote possibility of rapid unfolding of the DHFR moiety, an unlikely event in view of the demonstrated binding of a folate analogue, and dislocation as an unfolded polypeptide, we compared the degree of binding of TMX to DHFR when cells are pulse-labeled in the presence of TMX and chased in the presence or absence TMX. The stabilization of DHFR in both cases was similar and comparable to the binding of TMX to DHFR during the chase period (Fig. 6). We conclude, therefore, that the vast majority of DHFR-HC molecules dislocate as a protein that retains TMX binding.

Proteins can be translocated across membranes in a fully folded state, while the membrane compartment in question maintains a gradient of low mw solutes. Perhaps the most striking example for this phenomenon is the peroxisomal import of colloidal gold particles conjugated to proteins bearing the peroxisomal targeting signal (13). Therefore, dislocation from the ER into the cytoplasm may be regulated in a way that prevents leakage of other ER contents into the cytosol, yet can accommodate folded proteins.

Several lines of biochemical and genetic evidence support the involvement of the Sec61 translocon as the channel through which proteins are exported back to cytosol. In yeast, degradation of misfolded ER proteins is tightly coupled to the unfolded protein response. In a genetic screen that exploited this coupling, several alleles of Sec61 were reported to significantly inhibit the dislocation of CPY*, a misfolded ER protein, with minimal disturbance of ER import (28). These alleles, however, were found to support ER degradation of Ubc6 (29) and the mouse class I MHC allele H-2Kβ.2 These findings suggest the existence of alternative routes for dislocation that may not involve Sec61. An alternative mechanism for ER dislocation might involve multiquitination of the substrate and extraction from the lipid bilayer in an ATP-dependent manner. Although this might be a plausible mechanism for Ubc6, which is tail-anchored to the ER membrane with most of the protein facing the cytoplasm, it is more difficult to envisage such a mechanism for a classic type I membrane proteins like class I MHC products. Moreover, K → R mutations that remove all lysines from the class I cytosolic tail do not prevent US11-mediated dislocation of HC, whereas these mutants obviously cannot be ubiquitinated in their cytoplasmic tail (6).

Biochemical studies demonstrate that dislocated HC associates with Sec61β in US2+ cells or by dithiothreitol treatment in control cells (24). In addition, elegant cross-linking experiments show that Sec61α interacts directly with ApoB100 destined for ER degradation. In fact, ApoB100 lingers as a nascent protein until assembly with lipids is completed, and degradation is initiated on the nascent chain (30). This model is different from what we propose for US2-mediated degradation. US2 recognizes fully folded class I MHC as indicated by coimmunoprecipitation of US2 and Class I heavy chains with W6/32, an antibody that recognizes fully assembled MHC (24). Furthermore, Daudi-derived microsomes, which lack the MHC light chain β2m, do not support dislocation in vitro. Dislocation ensues, however, when microsomes are reconstituted with β2m.3 These findings suggest that dislocation of Class I heavy chains is initiated by US2 for Class I molecules that must have left the translocon already. Either the translocon is re-engaged subsequently, or an altogether different channel is used for dislocation. The mechanism by which Class I is recruited to such channels remains to be identified.

The example of the mechanosensitive channel MscL shows how adjustment of the packing angle of the transmembrane helical segments can result in a change from an almost completely sealed pore to a channel with a pore size of over 25 Å (31). This type of rearrangement might be used more generally to control permeability across a biological membrane, such as the ER. The maximum pore size seen for the modestly sized MscL channel might be significantly smaller than that for the multidistinct assembly such as Sec61, for which changes in pore size could be even more dramatic. A recent study by Helenius and coworkers (32) demonstrated that nascent polypeptides can fold partially within the cavity of the translocon pore when forced to fold in close proximity to surface of the ER membrane. Conformational changes of this type might reconcile our findings with the prevailing view that proteins are dislocated through the Sec61 translocon, or through any other channel, in a largely unfolded state. Although tightly folded DHFR cannot be inserted into the ER lumen through the Sec61 translocon, the spatial restrictions for dislocation from the ER might be different. Fluorescence quenching experiments indicate a diameter for the Sec61 pore of up to 60 Å during active protein translocation (33). A dilated pore of that size is sufficient to accommodate a fully folded DHFR moiety, the smallest cross-section of which is estimated to be 40 Å. The translocon, upon engagement of a dislocation substrate, may acquire a dilated conformation through which even folded proteins can be efficiently dislocated. Accordingly, we propose that proteins can dislocate in a partially folded state with the retention of considerable tertiary structure. This obviates the need for complete unfolding in the ER prior to dislocation and might explain how toxins that travel from the ER to the cytoplasm retain their toxicity (34, 35). Because folded proteins can apparently be exported from, but not imported into the ER, the dislocation process cannot be described as ER translocation in reverse, but must require a distinct conformation of the known translocon or different channels altogether.

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