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Membrane glycoprotein folding, oligomerization and intracellular transport: effects of dithiothreitol in living cells

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Using influenza hemagglutinin (HA0) and vesicular stomatitis virus G protein as model proteins, we have analyzed the effects of dithiothreitol (DTT) on conformational maturation and transport of glycoproteins in the secretory pathway of living cells. While DTT caused reduction of folding intermediates and misfolded proteins in the endoplasmic reticulum (ER), it did not affect molecules that had already acquired a mature trimeric conformation, whether present in the ER or elsewhere. The conversion to DTT resistance was therefore a pre-Golgi event. Reduction of folding intermediates was dependent on the intactness of the ER and on metabolic energy, suggesting cooperativity between DTT and ER folding factors. DTT did not inhibit most cellular functions, including ATP synthesis and protein transport within the secretory pathway. The results established DTT as an effective tool for analyzing the folding and compartmental distribution of proteins with disulfide bonds.

Key words: disulfide bonds/influenza hemagglutinin/intracellular transport/oligomerization/protein folding

Introduction

In previous studies, we have shown that addition of dithiothreitol (DTT) to the medium of live cells inhibits oxidation and folding of proteins in the ER (Braakman *et al.*, 1992a,b; de Silva *et al.*, 1993). It prevents disulfide bond formation in newly synthesized proteins and induces reduction of partially oxidized folding intermediates. It does not, however, appreciably affect translation, translocation or early covalent modifications such as N-linked glycosylation and signal sequence removal. The effects of DTT on disulfides are reversible: when the reducing agent is removed, proteins begin to fold post-translationally. Soon they emerge as fully folded, functional proteins. These DTT effects have proven useful for analyzing various cellular aspects of protein folding (Braakman *et al.*, 1992a,b; de Silva *et al.*, 1993; Helenius *et al.*, 1992, 1993; Lodish *et al.*, 1992).

Analysis of the disulfide bonds in proteins with known crystal structure shows that >70% are solvent inaccessible (Thornton, 1981; Srinivasan *et al.*, 1990). Therefore, the disulfide bonds in many mature, folded proteins are not sensitive to moderate concentrations of reducing agents unless the proteins are denatured (Thornton, 1981). This is also true for the influenza hemagglutinin precursor (HA0), which we use in our folding studies as a model protein. While folding intermediates of HA0 are readily reduced by DTT, the mature HA0 is not (Braakman *et al.*, 1992a,b; Godley *et al.*, 1992). HA0 and other proteins with disulfide bonds (Lodish *et al.*, 1992) evidently undergo a conversion from DTT sensitivity to DTT resistance during their conformational maturation in the cell.

In this paper we determine when, where and why influenza HA0 and vesicular stomatitis virus (VSV) G protein convert from a DTT-sensitive to a DTT-resistant form. The answers obtained provide useful information about the mechanisms of protein folding and about the unique properties of the ER lumen as folding environment. They also show that DTT is a powerful reagent not only for folding studies but also for following the movement of proteins from the ER into the secretory pathway.

Results

VSV G protein and HA0 acquire DTT resistance early in the secretory pathway

Influenza HA0 (84 kDa) and VSV G protein (67 kDa) are type I membrane glycoproteins with large ectodomains that fold inside the ER lumen (see Wiley and Skehel, 1987; Machamer *et al.*, 1990; de Silva *et al.*, 1993). Folding and intrachain disulfide bond formation begin co-translationally and continue post-translationally for a few minutes (Machamer *et al.*, 1990; Braakman *et al.*, 1991, 1992a; de Silva *et al.*, 1993). Mature HA0 and VSV G protein both have six intrachain disulfide bonds, the formation of which is a prerequisite for correct folding (Braakman *et al.*, 1992a; de Silva *et al.*, 1993). The folded and oxidized subunits assemble into non-covalent homotrimers with a half-time of ~7-10 min after synthesis. They thereby acquire transport competence, and move to the Golgi complex where the N-linked carbohydrates undergo trimming and terminal glycosylation (Copeland *et al.*, 1986, 1988; Gething *et al.*, 1986).

To determine at which stage in this maturation and intracellular transport pathway newly synthesized VSV G protein and HA0 become DTT-resistant, cells expressing G protein or HA0 were pulse-labeled for 2 min with [^{35}S]cysteine and [^{35}S]methionine, and chased for different times up to 30 min. After each chase time, DTT was added to the medium for an additional 5 min. To block further disulfide bond formation, the cells were then alkylated with *N*-ethylmaleimide (NEM) (Braakman *et al.*, 1991). They were detergent-lysed and the lysates subjected to immunoprecipitation with antibodies against the VSV G protein or against the influenza virus (Braakman *et al.*, 1991; de Silva *et al.*, 1993). The immunoprecipitates were analyzed in non-reduced and reduced form by SDS-PAGE, and the labeled bands visualized by fluorography.

With G protein (Figure 1), we observed that DTT-resistant forms began to form immediately after the pulse. Lanes 1-5 show the folding status of the G protein in non-reducing and

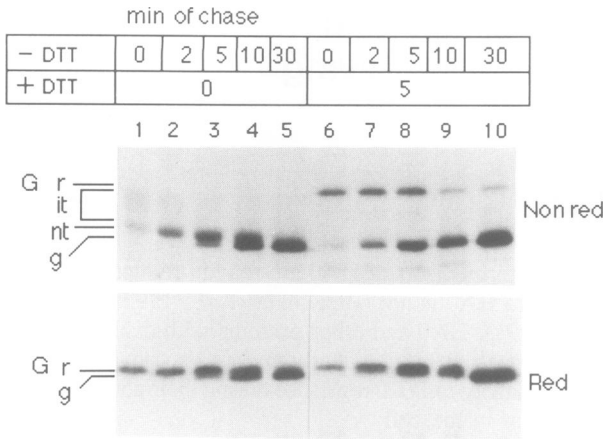

Fig. 1. VSV G protein becomes DTT-resistant during its maturation. VSV-infected CHO15B cells were pulse-labeled for 2 min and chased without DTT in duplicates for the indicated times. One of the dishes in each pair was chased for an additional 5 min with medium containing 5 mM DTT. The cells were lysed and the lysates were immunoprecipitated with a polyclonal antibody against VSV G protein, as described in Materials and methods. The immunoprecipitates were analyzed in non-reduced (top panel) and reduced forms (bottom panel) by 7.5% SDS−PAGE. The symbols refer to the different forms of the VSV G protein in the cell: r, reduced form; it, folding intermediates; nt, oxidized pregolgi form; g, golgi form.

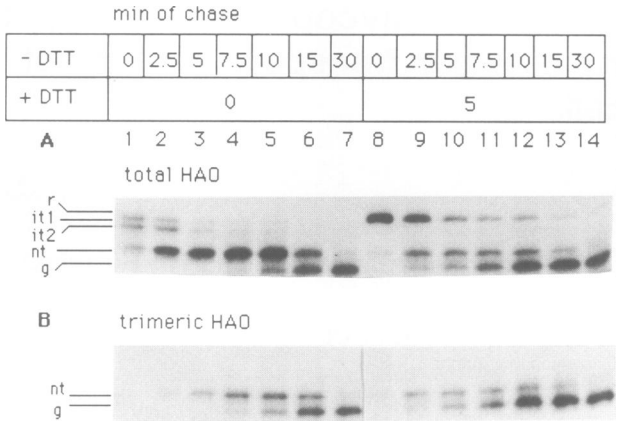

Fig. 2. HA0 trimers, but not the folding intermediates, are DTT-resistant. Influenza-infected CHO15B cells were pulse-labeled for 2 min and chased in duplicates as indicated in the Figure. As in Figure 1, one of each pair of dishes was chased for an additional 5 min with medium containing 5 mM DTT. The cells were lysed as indicated in the methods. The lysates were immunoprecipitated with both (**A**) a polyclonal antiserum that recognizes all conformational forms of HA0 and (**B**) a trimer-specific monoclonal antibody (N2). The samples were analyzed by 7.5% SDS−PAGE in non-reduced forms. The symbols refer to the various forms of HA0 in the cell: r, reduced form; it1 and it2, folding intermediates; nt, oxidized pregolgi form; g, golgi form.

reducing gels at different times of chase before the addition of DTT. As previously reported (Machamer *et al.*, 1990; de Silva *et al.*, 1993), the protein could be seen to undergo progressive oxidation and carbohydrate modifications, resulting in increasing electrophoretic mobility in non-reducing gel (lanes 1−5). After 5 min of chase (lane 3), some of the labeled G proteins began to reach the Golgi complex judging by their faster gel mobility both in the reduced and the non-reduced samples (labeled g). In the CHO15B cells used in this experiment, trimming takes place in the Golgi, but terminal glycosylation is blocked (Balch *et al.*, 1986) leaving the $Man_5GlcNAc_2Asn$ as the final form of the N-linked side chain.

When the cells were incubated after each chase time for an additional 5 min with 5 mM DTT-containing medium, some of the G protein was reduced. Reduction was seen as the loss of faster migrating G protein bands and the appearance of a more slowly migrating band marked 'r', which corresponded to fully reduced G protein (Figure 1, lanes 6−10). All partially oxidized folding intermediates (it) and some of the oxidized G protein present early in the chase (nt) were reduced in this way (lanes 6 and 7). By 10 min, a major part of the G protein in the nt band had become DTT-resistant. Whereas a small amount of reduced G protein was, however, still generated after 30 min chase, it was clear that the majority had become resistant to DTT with a half-time of ~5 min. Trimmed forms of G protein never displayed DTT sensitivity.

From these results we concluded that while partially oxidized folding intermediates of G protein and early oxidized forms were sensitive to DTT, late forms of nt as well as Golgi forms were resistant. Evidently, G protein converted to DTT-resistant forms before reaching the Golgi complex.

Similar results were obtained for HA0 (Figure 2). Figure 2A (lanes 1−7) shows the folding status before treatment with DTT. As observed for G protein, the progression from slowly to more rapidly migrating HA0 forms is seen in control cells as the protein folds, acquires disulfide bonds, trimerizes and gets transported to the Golgi complex. The two partially oxidized folding intermediates consistently observed with HA0 as distinct bands are labeled 'it1' and 'it2'. The band labeled 'nt' corresponds to oxidized, untrimmed HA0, and band 'g' to the trimmed Golgi forms [see Braakman *et al.* (1991) for these assignments].

When DTT was added, different amounts of fully reduced HA0 (r) appeared (Figure 2A, lanes 8−13) depending on the length of the preceding chase time. Immediately after the pulse (lane 8), almost all the labeled HA0 was DTT-sensitive except for a small amount of resistant nt. The amount of r decreased with a half-time of ~2−5 min and disappeared entirely after 15−30 min. As for G protein, all the DTT-sensitive forms were untrimmed. These findings indicated that similar to G protein, HA0 is DTT-sensitive during early stages of folding and conformational maturation, but acquires DTT resistance prior to arrival in the Golgi complex. Although the experiments documented here employed only 5 min DTT treatment, we have shown that the results are identical over 1−30 min range of incubations (see below, Figure 5).

Note that trimming of the DTT-resistant forms of HA0 continued during the chase in the presence of DTT (cf. Figure 2A, lanes 2−5 with lanes 9−12, respectively). We will return to this observation later.

HA0 trimers are DTT-resistant in the ER

Since the time-course of HA0 conversion to DTT resistance was similar to that previously observed for trimer formation (see Copeland *et al.*, 1986; Gething *et al.*, 1986), we precipitated the lysates from DTT-treated and control cells (Figure 2A) with a trimer-specific monoclonal antibody to HA0. Both trimmed and untrimmed trimeric forms of HA0 are precipitated by this antibody (N2; Copeland *et al.*, 1988).

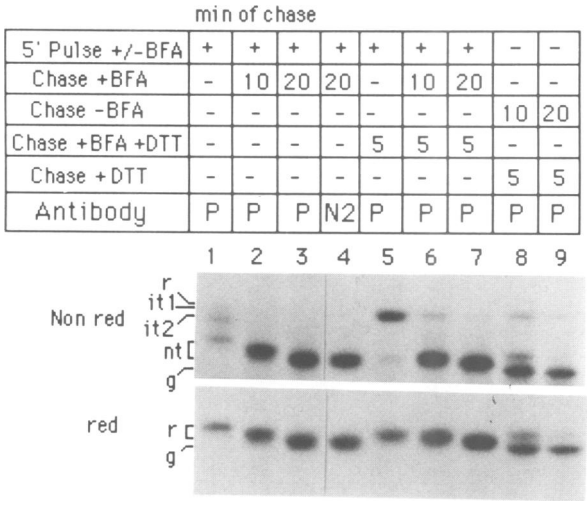

Fig. 3. HA0 trimerizes and becomes DTT-resistant in the presence of Brefeldin A. Influenza-infected CHO15B cells were preincubated with medium containing 1 μg/ml BFA for 15 min (lanes 1–7). The cells were then pulse-labeled with BFA and chased either with BFA alone or with BFA and 5 mM DTT as indicated. At the end of the chase the cells were lysed and immunoprecipitated either with the polyclonal antiserum (P) or with the trimer specific monoclonal antibody (N2, lane 4) as in Figure 2. The control cells (lanes 8 and 9) were not exposed to BFA.

The results in Figure 2B (lanes 8–14) showed that trimers were DTT-resistant, whether trimmed or untrimmed. We concluded that the conversion from DTT sensitivity to DTT resistance coincided with, or preceded, trimer formation.

To confirm that HA0 trimers in the ER were DTT-resistant, we used Brefeldin A (BFA), a drug that effectively inhibits ER to Golgi transport by fusing the Golgi elements with the ER (see Klausner *et al.*, 1992). It does not inhibit protein synthesis or translocation. HA0-expressing cells were pretreated with BFA for 15 min and then pulse-labeled and chased in media containing BFA. As shown in Figure 3 (lanes 1–3), the pattern of the HA0 bands at different times of chase was normal except that the electrophoretic mobility of nt increased somewhat, presumably because of gradual trimming occurring in the mixed ER-Golgi compartment. When, after 20 min chase, the lysates were immunoprecipitated with the trimer-specific N2 monoclonal, virtually all of the HA0 was found to be trimeric (Figure 3, lane 4). These results showed that BFA treatment does not interfere with HA0 folding and trimer formation. The Golgi enzymes present in the combined ER-Golgi compartment thus have no detectable effect on the conformational maturation process.

When DTT was added to such BFA-treated cells, folding intermediates it1 and it2 were reduced as in control cells (Figure 3, lane 5). The major part of nt, most likely all trimeric, was DTT-resistant (lanes 6 and 7). This result showed that trimers represent a conformational form of HA0 that is intrinsically resistant to DTT, whether exposed to the reducing agent in the ER or in subsequent compartments of the secretory pathway.

DTT effects on the folding intermediates depend on the intactness of the ER

In contrast to trimeric HA0, the folding intermediates were clearly DTT-sensitive. Was this only because they were incompletely folded, or was it also because the conditions in the ER made their disulfide bonds accessible to reducing agents? To answer this question, we analyzed the DTT sensitivity of it1, it2 and nt *in vitro*.

We pulse-labeled influenza-infected cells for 3 min, whereafter the cells were detergent-lysed in a buffer containing 5, 10 or 20 mM DTT. Under these conditions, the ER was solubilized and the ER factors diluted at least 10^4-fold. After incubation with the reducing agent at 37°C for 5 min, the lysates were alkylated and the effects of DTT treatment analyzed on non-reducing SDS–PAGE. The results (Figure 4A, lanes 2–4) showed that it1 and it2 were not reduced to r. A fraction of nt was reduced to it2, but a large part remained unchanged, even in 20 mM DTT. Only if the samples were boiled to denature the protein, full reduction was achieved (lane 5).

These results indicated that the intrachain disulfides in these folding intermediates were not inherently accessible to DTT. The effects of DTT in living cells were much more complete than *in vitro*, suggesting that conditions in the intact ER were somehow important for facilitating an efficient reduction process.

Reduction in the ER requires ATP

One of the unique properties of the ER lumen that could explain the enhanced effect of DTT, is the presence of lumenal folding enzymes and chaperones. Many of these are known to interact with newly synthesized proteins and thought to provide assistance during the folding process (see Rothman, 1989; Ellis and Van der Vies, 1991; Gething and Sambrook, 1992). The best characterized are protein disulfide isomerase (PDI), a redox enzyme, and BiP/GRP78, an ATP-dependent molecular chaperone. To test whether DTT's action was potentiated by ER factors, we analyzed the effects of DTT under ATP depletion conditions. ATP is needed for the function of some of the chaperones (Rothman, 1989), and we have previously shown that HA0 folding is impaired in the absence of metabolic energy (Braakman *et al.*, 1992b).

HA0-expressing cells were pulse-labeled and then incubated with DTT under ATP-depleting conditions (Braakman *et al.*, 1992b). As shown in Figure 4B, ATP depletion alone resulted in the loss of it1 and it2 and the formation of disulfide-bonded aggregates of HA0 ('aggregates' in Figure 4B, lane 2) (see Braakman *et al.*, 1992b). Addition of DTT to these cells under ATP depletion conditions resulted in the disappearance of the cross-linked aggregates and the reappearance of a band that migrates at a rate equivalent to that of r or it1 (r and it1 are difficult to distinguish) and a partially oxidized form of HA0 with mobility somewhat faster than it2 (Figure 4B, lane 3). When after 10 min, regular medium was added and the ATP levels in the cell were restored (lane 4), two effects were noted. First, the it1- and it2-like forms were now reduced, judging by the formation of the reduced r form. Secondly, most of the nt that was DTT-resistant, moved to the Golgi complex (an effect that will be discussed below).

Thus, DTT was capable of reducing intermolecular disulfide bonds that hold aggregates of misfolded HA0 together under energy depletion conditions. But, unlike its effect in energized cells, it could not reduce the intramolecular disulfide bonds in the partially oxidized intermediates. The inhibition was fully reversible when ATP

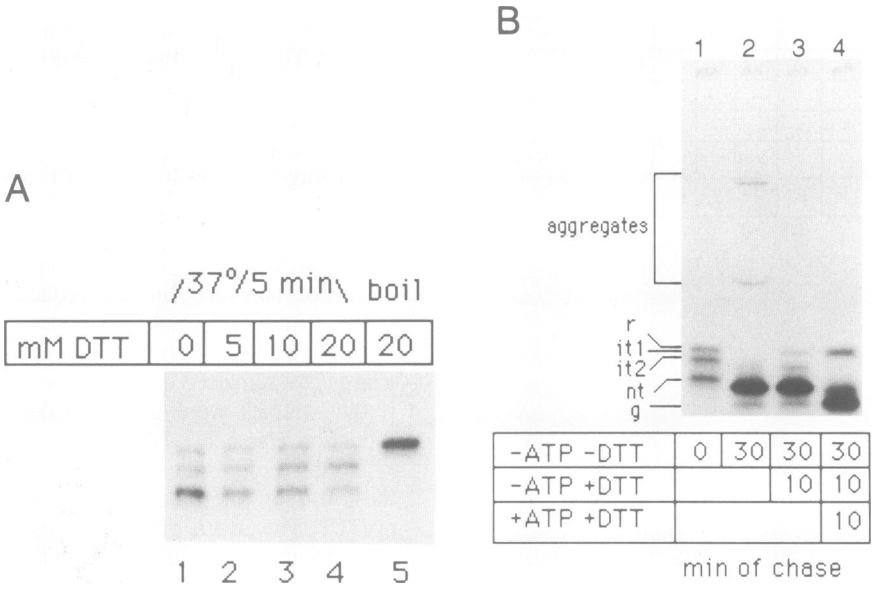

Fig. 4. The ER environment and ATP are needed for reduction of HA0 by DTT. (**A**) Influenza-infected CHO15B cells were pulse-labeled for 3 min, alkylated with 1 mM NEM and lysed with 0.5% Triton X-100 in MNT containing 5, 10 or 20 mM DTT. The lysates were incubated at 37°C as indicated and alkylated with 50 mM NEM. The lysates were immunoprecipitated and analyzed by 7.5% SDS–PAGE as in Figure 2A. (**B**) Influenza-infected CHO15B cells were pulse-labeled for 2 min (lane 1) and chased for 30 min under ATP depletion condition (lane 2). This was followed by a 10 min incubation in 5 mM DTT under ATP depletion (lane 3) and subsequent restoration of ATP levels in the presence of DTT (lane 4). The cells were lysed, immunoprecipitated and analyzed by 7.5% SDS–PAGE without reduction as in Figure 2A.

synthesis was restored; DTT reacquired its full reducing power in the ER. We concluded that metabolic energy was required for DTT-mediated reduction of it1- and it2-like forms, but not for reduction of aberrant interchain disulfide bonds. This may suggest that BiP/GRP78 or some other ATP-dependent chaperone, is responsible for making hidden disulfides more accessible to reduction.

In addition to reducing the interchain disulfide bonds in aggregates formed under ATP depletion conditions, DTT can also reduce the covalent bonds in misfolded aggregates formed in the spontaneous side pathways (Hurtley *et al.*, 1989), aggregates that arise during synthesis of non-glycosylated HA0 in the presence of tunicamycin and also the cross-linked, misfolded aggregates appearing after treatment with diamide (data not shown). So, DTT does not only reduce incompletely folded proteins, but also a variety of permanently and transiently misfolded forms that contain aberrant interchain disulfide bonds.

It is noteworthy that the nt band displayed a shift in mobility between lanes 1 and 2 (Figure 4B). We have seen this shift regularly ~1–5 min after chain completion and believe that it reflects partial mannose trimming of N-linked sugars taking place in the ER (Kornfeld and Kornfeld, 1985). Alternatively, it could represent the formation of one or more of the smaller disulfide loops, for which we do not yet have assays.

ER to Golgi transport functions in the presence of DTT

Finally, we were interested in analyzing whether DTT had an inhibitory effect on the secretory pathway in addition to its effects on folding. We first measured whether incubation of cells in the presence of 5 mM DTT affected the ATP level in CHO15B cells, and found that over a time period of 1 h the ATP levels stayed constant (not shown).

The transport of glycoproteins from the ER to the Golgi

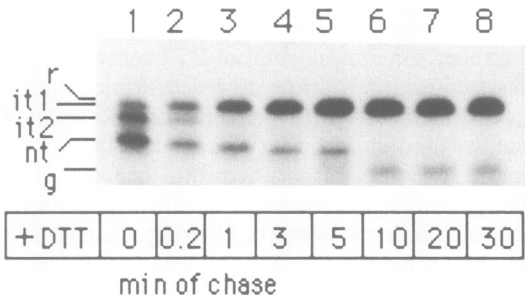

Fig. 5. Transport from ER to Golgi is intact in the presence of DTT. Influenza-infected CHO15B cells were pulse-labeled for 3 min (lane 1) and chased for the indicated times with medium containing 5 mM DTT (lanes 2–8). The cells were lysed, immunoprecipitated and analyzed in non-reduced form by 7.5% SDS–PAGE as in Figure 2A.

was followed in CHO15B cells by monitoring the trimming of high mannose sugars in the *cis*-Golgi compartment. Since the reduced form of HA0 in the ER is mostly unfolded, and therefore transport-incompetent, we had to follow the transport of trimers, which are DTT-resistant. We monitored the fate of the population of DTT-resistant nt already present after a short pulse. After a 3 min pulse without reducing agents (Figure 5, lane 1), cells were chased from 1 to 30 min with DTT, in parallel dishes (lanes 2–8).

As seen earlier, after a 3 min pulse without reducing agent the two folding intermediates (it1, it2) and the fully oxidized form of HA0 (nt) were present (lane 1). Cells briefly exposed to DTT showed a major band of reduced HA0 (r), some folding intermediates and some nt (Figure 5, lane 2). Already after 1 min in DTT, all the it1 and it2 were reduced to r, whereas some of the nt band remained unchanged. On extending the DTT chase up to 10 min, the DTT-resistant nt band was replaced by the corresponding trimmed form g, indicating that the DTT-resistant HA0 had moved from

the ER to the Golgi complex. After 30 min all the nt had become trimmed (Figure 5, lane 8). The increase in label seen at longer chase times can be attributed to the completion of nascent chains during this period, because cycloheximide was not included in the chase medium in this experiment.

We concluded that DTT did not interfere with the transport machinery responsible for ER to Golgi transport. Thus the reason that the reduced HA0 was not transported was not because the transport machinery was not functional but because the HA0 did not have a transport competent conformation.

Transport from the ER to the plasma membrane is not inhibited by DTT

To determine whether transport of HA0 all the way to the plasma membrane can take place in the presence of DTT, we made use of a surface trypsinization assay developed by Matlin and Simons (1983). Following a 5 min pulse with labeled amino acids, the HA0-expressing cells were chased at 15°C for 30 min, during which time the HA0 continues to fold and trimerize but does not reach the Golgi, as seen by the absence of trimming (Copeland *et al.*, 1988). It is trapped in the so-called 15°C compartment, i.e. the intermediate compartment between the ER and the *cis*-Golgi (Saraste and Kuismanen, 1984). DTT was then added and the temperature raised to 37°C to allow transport of HA0 out of the pre-Golgi compartment. The cells were trypsinized in the cold to cleave HA0 that had reached the surface into its subunits HA1 and HA2, and lysates were immunoprecipitated with the polyclonal anti-HA0 antiserum. SDS–PAGE analysis of the reduced samples was carried out to detect the presence of HA2, the most easily detectable cleavage product.

As seen in Figure 6, surface trypsinization resulted in the generation of HA2, whether the 37°C chase was in the presence (lane 7) or absence (lane 5) of DTT. This indicated that transport from the pre-Golgi compartment to the cell surface took place in the presence of DTT. It should be noted that the HA2 in DTT-treated cells runs slightly slower than the HA2 in control cells. This could mean that trimming of N-linked carbohydrates is either slower or incomplete in the presence of DTT.

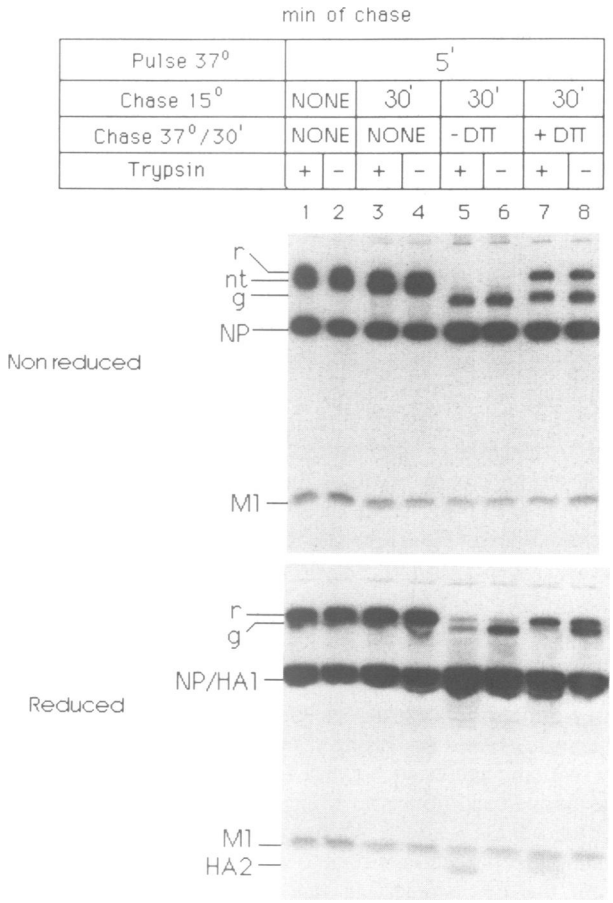

Fig. 6. Transport of HA0 to the plasma membrane occurs in the presence of DTT. Influenza-infected CHO15B cells were pulsed and chased in duplicates as indicated. After stopping the chase half of the dishes were treated with 100 μg/ml trypsin on ice for 10 min and the trypsin was inactivated with 500 μg/ml Soybean Trypsin Inhibitor (STI), as indicated in Materials and methods. The cells were lysed and immunoprecipitated as in Figure 2A. The immunoprecipitates were analyzed in non-reduced (top panel) and reduced (bottom panel) forms by 12.5% SDS–PAGE. The symbols refer to the cross-reacting viral proteins brought down during immunoprecipitation: NP, nuclear protein; M, matrix protein.

Discussion

DTT is a non-specific agent and therefore likely to cause many effects on cells. Yet, our results show that it can be used as a reversible and relatively specific inhibitor of protein folding in the ER, at least during short-term incubations. While folding intermediates and newly synthesized proteins are reduced, protein synthesis, translocation and glycosylation continue (Braakman *et al.*, 1992a). The secretory pathway remains functional and the level of ATP stays essentially unchanged. Taken together, our findings establish DTT as a useful tool for analyzing early events in the maturation of proteins in the ER and their transport in the secretory pathway.

Normally, the conditions in the ER lumen are more oxidizing than in the cytosol. Recent experiments have suggested that the lumen is buffered by glutathione (Hwang *et al.*, 1992). GSSG and GSH are present in a ratio of ~1:3, which is 10-fold higher than in the cytosol. The system responsible for maintaining the redox gradient over the ER membrane is not known, but our results show that it can be easily overcome by DTT. Apparently, DTT is sufficiently membrane permeable in its reduced form to reach the ER almost instantly after addition to the medium. We have found that weaker disulfide reducing agents, such as 2-mercaptoethanol, have little or no effect on the disulfide bonds of HA0 (Braakman *et al.*, 1992a). However, 2-mercaptoethanol has been shown by others to affect secretion of unpolymerized IgM in lymphocytes, indicating that it does enter the cell and can affect regulatory mechanisms in the ER (Alberini *et al.*, 1990; Sitia *et al.*, 1990).

The reasons why the effects of DTT have turned out to be milder than could be anticipated, are probably twofold. First, the vast majority of cytoplasmic proteins is devoid of disulfide bonds. They are designed for the reducing environment prevailing in the cytosol, which does not support the oxidation of cysteines (Gilbert, 1990). Secondly, most mature folded proteins present in the cell that do have disulfide bonds (located in the plasma membrane, the lumenal spaces, and the membranes of the vacuolar

organelles), are insensitive to reducing agents unless denatured. As a consequence, the short-term effects of DTT appear to be limited to newly synthesized proteins and folding intermediates in the ER. The ER is the site of folding and maturation of all the proteins rich in disulfide bonds.

The time window during which pulse-labeled HA0 and G protein were found to be sensitive to DTT was relatively short. Both for HA0 and G protein, the majority became DTT-resistant within 5–15 min after synthesis. The conversion to the resistant forms took place before the proteins were trimmed, coinciding or slightly preceding the formation of stable trimers. Preliminary results using velocity gradients to separate monomers and trimers suggest that the conversion to DTT resistance for some HA0 molecules may indeed, somewhat precede the trimerization event (U.Tatu and A.Helenius, unpublished). Further studies are in progress on this observation. In any event, the conversion from the DTT-sensitive to the DTT-resistant conformations occurs either in the ER or in the intermediate compartment between the ER and the Golgi complex (Saraste and Kuismanen, 1984; Schweizer *et al.*, 1990).

That folding intermediates are particularly sensitive to DTT is explained in part by their incomplete folding status. Folding intermediates are known to have a less condensed three-dimensional structure than fully folded proteins (Jaenicke, 1987), providing easier access to disulfide bonds by reducing agents such as DTT. Our results indicated that the unique conditions prevailing in the ER were also crucial for the full DTT effect. We found that intermediates it1, it2 and nt were, in fact, resistant to 20 mM DTT when removed from the ER, suggesting that their disulfides were not inherently exposed to the reducing agent. We also observed that the potency of DTT in intact cells depended on metabolic energy. Taken together, these observations indicated that factors dependent on metabolic energy inside the intact ER lumen somehow promoted the reducing effect of DTT on the folding intermediates, but had no effect on the mature, folded trimers.

As a compartment devoted to protein folding and assembly, the ER lumen contains a number of folding factors and chaperones at high concentrations (see Rothman, 1989; Ellis and Van der Vies, 1991; Gething and Sambrook, 1992). These include BiP/GRP78, an ATP-driven chaperone, and protein disulfide isomerase (PDI), a redox enzyme. The folding machinery probably provides continuous conformational 'massage' to proteins in different stages of folding, thus enhancing their chances of finding their correct conformations and preventing aggregation. We think that the combined effect of these factors may facilitate exposure of the buried disulfide bonds in proteins that are still incompletely folded and make them accessible to DTT. Since PDI under reducing conditions can act as a reductase (Creighton *et al.*, 1980), we cannot exclude the possibility that some of the reduction observed is actually PDI-catalyzed. In this scenario, DTT's role could be indirect; it could serve to reduce the active site cysteines in PDI. In contrast to the folding intermediates, the trimeric form of HA0 was not reduced after addition of DTT to cells, whether it was present in the ER or elsewhere. It probably represented a stable, fully folded protein, no longer a substrate for the folding machinery.

DTT-induced effects on folding are likely to be limited to proteins that require disulfide bonds for the folding process. Since disulfides and cysteine-rich domains are very common and often important for the folding and stability of membrane glycoproteins and secretory proteins, the proteins sensitive to DTT will probably include the majority of proteins made in the ER. The proteins for which we have so far demonstrated reversible DTT effects include influenza HA0, VSV G protein, SFV spike glycoproteins and the LDL receptor (Braakman *et al.*, 1992a,b; de Silva *et al.*, 1993; K.Matter and I.Mellman, unpublished observations). DTT reduction of newly synthesized asialoglycoprotein receptor and serum albumin in hepatoma cells has also been reported recently (Lodish *et al.*, 1992). The transport of proteins which do not have disulfide bonds or where such bonds are not necessary for folding into transport competent conformations, are unffected by DTT. This has been recently demonstrated for α_1-antitrypsin in HepG2 cells (H.Lodish, personal communication), for Secretogranin (Sg II) in neuroendocrine cells (Chanat *et al.*, 1993) and for M protein of the mouse Hepatitis virus (D.J.Opselter and P.Rottier, personal communication). All of these proteins are devoid of disulfide bonds. Moreover, it has been shown that glycosphingolipids continue to be transported from the golgi complex to the plasma membrane in the presence of DTT (G.Van Meer, personal communication).

For analyzing the folding and transport of proteins with critical disulfide bonds, the use of DTT provides a valuable new approach. We have shown that DTT causes the arrest and accumulation of the newly synthesized proteins in pre-Golgi compartments in unfolded forms. Their folding can then be initiated by DTT removal and their fate followed as they synchronously undergo conformational maturation and intracellular transport. Furthermore, DTT can be used to monitor the rate and efficiency by which proteins move from the ER to the Golgi complex. Our data show that molecules that are reduced after addition of DTT to cells constitute a collection of folding intermediates and incorrectly folded forms of proteins in the ER, whereas molecules that are unaffected correspond to the transport-competent or already transported molecules.

Materials and methods

Cells and viruses

CHO15B were propagated as previously described. This is a mutant cell line defective in the *N*-acetylglucosamine transferase 1 (Balch *et al.*, 1986), which is a medial Golgi enzyme that transfers *N*-acetylglucosamine to the $Man_5GlcNAc_2$ form of N-linked sugars. This defect allows a distinction between the pre-Golgi and the Golgi forms of HA0 through a mobility difference in SDS–PAGE. CV-1 (African green monkey kidney) cells were grown as described by Doxsey *et al.* (1985). The allantoic fluid of 11 day-old chicken embryos infected with the X31 strain of the influenza virus was used for routine infections of CHO15B cells. Infected cells were used between 4 and 6 h post-infection for the pulse–chase expriments. The infections were carried out as described by Copeland *et al.* (1986). For VSV experiments, infections were carried out with Indiana serotype VSV wild-type as described by de Silva *et al.* (1990).

Antibodies

The polyclonal anti-HA0 antiserum and the monoclonal antibody N2 used have been described previously by Braakman *et al.* (1991) and Copeland *et al.* (1986). The polyclonal anti-HA0 antiserum, which was raised against the whole virus, binds to all conformational forms of HA0 in the cell. It also reacts with the influenza nucleoprotein (NP, 56 kDa) and matrix protein (M, 27 kDa). The monoclonal antibody N2 binds specifically to the B epitope in the top of the trimeric HA0 (Wiley *et al.*, 1981). The polyclonal antiserum against VSV G protein was obtained from Dr Mike Whitt. Immunoprecipitations were carried out as described by Copeland *et al.* (1988).

Metabolic labeling and the pulse–chase protocol

The folding of HA0 was followed as described by Braakman *et al.* (1991). Briefly, CHO15B cells were infected with the X31 strain of influenza virus in RPMI medium, 0.2% BSA, 10 mM HEPES pH 6.8 and rocked at 37°C. After 1 h, 4.5 ml of α-MEM containing 8% FCS was added to the dishes and they were incubated at 37°C. Approximately 4–6 h post-infection the dishes were washed twice with PBS and 2.5 ml of methionine and cysteine free medium was added to starve the cells of these amino acids. After 15–30 min of starvation each dish was pulse-labeled with 0.1 mCi Trans[^{35}S]label at 37°C. The pulse medium was removed, and after a quick wash with the chase medium, the cells were chased at 37°C in α-MEM with 8% FCS for different time intervals. The chase was stopped by aspirating the medium and adding ice-cold PBS containing 20 mM NEM to block the free sulfhydryl groups in the protein and prevent any further disulfide formation. The cells were then lysed with cold MNT (20 mM MES, 100 mM NaCl and 30 mM Tris–HCl pH 7.5) containing 0.5% Triton X-100, 10 μg/ml each of chymostatin, leupeptin, antipain and pepstatin, 1 mM PMSF and 1mM EDTA. The cell lysates were centrifuged at 7000 *g* for 10 min in the cold to pellet the nuclei. The supernatant was used for immunoprecipitation. When indicated, some dishes were further chased with medium containing 5 mM DTT. Tunicamycin (Calbiochem, La Jolla, CA) was used for generating non-glycosylated HA0 as described by Hurtley *et al.* (1989). It was added to the medium at a concentration of 5 μg/ml from a 5 mg/ml stock in DMSO. Pretreatment with tunicamycin was carried out 40–60 min before metabolic labeling. Where indicated, BFA (Epicentre technologies, Madison, WI) was used at a concentration of 1 μg/ml from a 1 mg/ml stock in ethanol.

Immunoprecipitation

Fixed *Staphylococcus aureus* cells and antibody complexes were incubated with the cell lysates for 1 h at 4°C on a rotator. The complexes were washed twice with 0.05% Triton X-100, 0.1% SDS, 0.3 M NaCl, 10 mM Tris–HCl pH 8.6 and resuspended in 10 mM Tris and 1 mM EDTA, pH 6.8. The immunoprecipitates were heated to 95°C for 5 min in the sample buffer (200 mM Tris, pH 6.8, 3% SDS, 10% glycerol, 1 mM EDTA and 0.004% bromophenol blue) with or without 20 mM DTT and analysed by SDS–PAGE.

Cell surface trypsinization

Surface arrival of HA0 was tested by treating the intact cells with 0.5 ml of trypsin (100 μg/ml in PBS) for 10 min, which cleaves HA0 into its subunits HA1 and HA2. This was followed by inactivation of the enzyme by treatment with 1 ml of 500 μg/ml soybean trypsin inhibitor in PBS for 15 min, at 4°C on a rocker. The trypsin-treated cells were lysed, immunoprecipitated with the polyclonal anti-HA0 antiserum and analyzed by reducing and non-reducing SDS–PAGE. The presence of the HA2 band in the trypsin-treated cells was taken as an indication of the surface arrival of HA0 (Matlin and Simon, 1983).

ATP depletion

Cells were depleted of ATP by incubation in PBS containing 10 mM sodium azide (Braakman *et al.*, 1992b). The extent of ATP depletion of cells was determined by measuring the ATP concentrations in the detergent cell lysates (see Lundin and Thore, 1975, and references therein). The lysates were mixed with luciferase to 0.8 μg/ml and luciferin to 100 μg/ml final concentrations (both from Boerhinger Mannheim, Indianapolis, IN). Photons emitted were counted immediately after mixing in the ^{3}H channel of a Beckman scintillation counter. The square roots of the counts emitted were proportional to the amount of ATP in the reaction mixture.

Reduction in the cell lysate

The influenza-infected CHO15B cells were pulse-labeled with Trans[^{35}S]label as described above. The pulse was stopped with ice-cold PBS containing 1 mM NEM to block the free sulfhydryl groups in proteins. The cells were lysed with 0.5% Triton X-100 MNT containing the protease inhibitors with or without 5, 10 or 20 mM DTT and the lysates were incubated at 37°C for 5 min. The lysates were treated with 50 mM NEM to quench the excess DTT and the newly generated free thiols in the proteins. Immunoprecipitations were carried out using the rabbit polyclonal antiserum raised against the whole virus as described above. The immunoprecipitates were analyzed by non-reducing SDS–PAGE.

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