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Protein Disulfide Isomerase Acts as a Redox-Dependent Chaperone to Unfold Cholera Toxin

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Summary

Cholera toxin is assembled from two subunits in the periplasm of *Vibrio cholerae* and disassembled in the analogous compartment of target cells, the lumen of the endoplasmic reticulum (ER), before a fragment of it, the A1 chain, is transported into the cytosol. We show that protein disulfide isomerase (PDI) in the ER lumen functions to disassemble and unfold the toxin once its A chain has been cleaved. PDI acts as a redox-driven chaperone; in the reduced state, it binds to the A chain and in the oxidized state it releases it. Our results explain the pathway of cholera toxin, suggest a role for PDI in retrograde protein transport into the cytosol, and indicate that PDI can act as a novel type of chaperone, whose binding and release of substrates is regulated by a redox, rather than an ATPase, cycle.

Introduction

Cholera toxin is produced by the bacterium *Vibrio cholerae* and acts on intestinal epithelial cells in mammals to induce massive salt and water secretion, resulting in diarrhea (Sears and Kaper, 1996). The toxin consists of A and B subunits, but the active component is only a fragment of the A chain, called A1, that is released from the rest of the protein and transported into the cytosol of target cells. Once in the cytosol, the A1 chain acts as an enzyme, catalyzing the ADP ribosylation of the heterotrimeric Gαs protein. This modification leads ultimately to the opening of chloride channels causing massive Cl^- and water secretion. While the effects of the A1 chain in the cytosol are reasonably well understood, little is known about how it is dissociated from the rest of the toxin molecule, and how it reaches the cytosol.

The assembly of cholera toxin from its A and B subunits takes place after transport of the individual chains into the periplasm of the *V. cholerae* (reviewed by Hirst, 1995). In this compartment, the subunits fold, form disulfide bridges, and associate with one another. In the final structure, five molecules of the B subunit form a ring surrounding the C-terminal part of a single A subunit molecule (Zhang et al., 1995). The holotoxin is secreted across the outer membrane of the *V. cholerae* where the A subunit is clipped ("nicked") at position R192 by a serine-like protease that is also secreted by the bacterium (Mekalanos et al., 1979). The resulting A1 and A2 fragments are connected by a disulfide bridge and remain tightly bound to the ring of B subunits, both through the C-terminal A2 fragment in the center of its pore and interactions of the N-terminal A1 fragment.

When the nicked cholera toxin encounters a mammalian cell, it first binds to the surface through an interaction of the B pentamer with the ganglioside GM1. It then travels backward through the secretory pathway to the Golgi apparatus where it encounters the KDEL receptor, which is normally responsible for retrieving lumenal proteins of the endoplasmic reticulum (ER) that have escaped their resident compartment. The A2 chain has a KDEL sequence at its C terminus which, at least in part, is responsible for retrograde transport of the toxin to the ER (Lencer et al., 1995). Once in the ER, the A1 chain must be transported across the membrane into the cytosol. Recent evidence suggests that it may be transported through the Sec61p channel (Schmitz et al., 2000). Although this protein-conducting channel has been originally identified in the transport of secretory and other proteins from the cytosol into the ER lumen, there is growing evidence that it may also be used in the reverse direction. Examples include the export of misfolded proteins from the ER for cytosolic destruction by the proteasome (reviewed by Kopito, 1997) and the degradation of MHC class I molecules in cytomegalovirus infected cells (Wiertz et al., 1996).

The release of the A1 chain from the other parts of the toxin molecule must be an active process because even the nicked holotoxin is a very stable molecule (Mekalanos et al., 1979). Most data, both for cholera toxin and related toxins, indicate that the release of the A1 chain occurs in the ER (Lencer et al., 1995; Orlandi, 1997). Although both the ER of eukaryotic cells and the periplasm of bacteria are compartments in which proteins fold, assemble, and form disulfide bridges, the toxin in the ER must undergo events that are the exact opposite to the ones it experiences during its biosynthesis in the periplasm: the subunits must disassemble, the disulfide bridges must be reduced, and the A1 chain must probably be unfolded to be transported through the Sec61p channel. How the disassembly and unfolding of the toxin would occur is unclear. The ER contains lumenal chaperones normally involved in the folding of proteins such as BiP, a member of the Hsp70 family of ATPases, Hsp94, protein disulfide isomerase (PDI), calnexin/calreticulin, and others. However, none of these chaperones has been demonstrated to unfold a protein. In fact, the only known "unfoldases" of native proteins are Hsp104 (Glover and Lindquist, 1998) and its relative ClpA in bacteria (Weber-Ban et al., 1999), which do not have known homologs in the ER lumen.

Here we provide explanations for why cholera toxin behaves differently in the periplasm of bacteria and in

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the ER of target cells, and how it is unfolded and disassembled in the ER. Our data show that the toxin needs to be nicked in the A chain to be unfolded, and that the protein disulfide isomerase (PDI) in the ER is largely responsible for disassembly and unfolding. In this reaction, PDI does not act as an oxido-reductase, but rather as a redox-dependent chaperone. In the reduced state, PDI binds to the toxin and unfolds it, while in the oxidized state, the substrate is released. Our data suggest a role for PDI in retrograde protein transport across the ER membrane and indicate that PDI belongs to a novel class of chaperones; this class is regulated by a redox cycle and not, like conventional chaperones, by an ATPase cycle.

Results

Unfolding of Cholera Toxin by an Activity in the ER Lumen

We designed an assay that mimics the postulated disassembly of the toxin in vivo: after arrival in the ER, the B subunits should remain bound to the ganglioside GM1 in the membrane, while the A1 fragment of the toxin should be dissociated. In our assay, membrane bound ganglioside is replaced by GM1 covalently bound to polystyrene beads. Upon incubation with ER proteins, the B subunits are expected to stay with the beads and the A1 chain to be released.

The A and B subunits of the toxin were expressed in *V. cholerae* and the holotoxin was purified (Rodighiero et al., 1999). Due to the presence of high levels of secreted proteases in the growth medium of *V. cholerae*, most of the toxin molecules (about 90%) had their A subunit cleaved into the A1 and A2 fragments. The purified, nicked holotoxin was bound to GM1-coated beads and incubated under different conditions. After sedimentation of the beads, the bound material and the supernatant were analyzed by nonreducing SDS-PAGE, followed by immunoblotting with an antibody directed against the A and B subunits (it reacts preferentially with the A subunit). When the beads were incubated with the control protein bovine serum albumin (BSA) in a buffer containing 1 mM reduced glutathione (GSH), both subunits were recovered in the bound fraction (Figure 1A, lane 1). The A subunit appeared as two bands. The upper band (A#) corresponds to the fraction of A subunit that is proteolytically nicked at Arg-192, but whose two fragments, A1 and A2, remain held together by the disulfide bridge Cys-187–Cys-199. The lower band is the A1 subunit (the A2 subunit is very small and does not react with the antibody). With the GSH concentration chosen, about half of the A# subunit was reduced. Since the A1 subunit was recovered with the B subunit on the ganglioside beads, the two polypeptides must be associated with one another by additional, noncovalent interactions, as previously described (Mekalanos et al., 1979). To test whether the holotoxin is dissociated by ER proteins, we prepared a lumenal extract from canine microsomes and incubated it with the toxin beads. A significant fraction of the A1 subunit was found to be released into the supernatant after collection of the beads (lanes 4 and 6). The efficiency of the release reaction was unaffected by the presence or absence of ATP

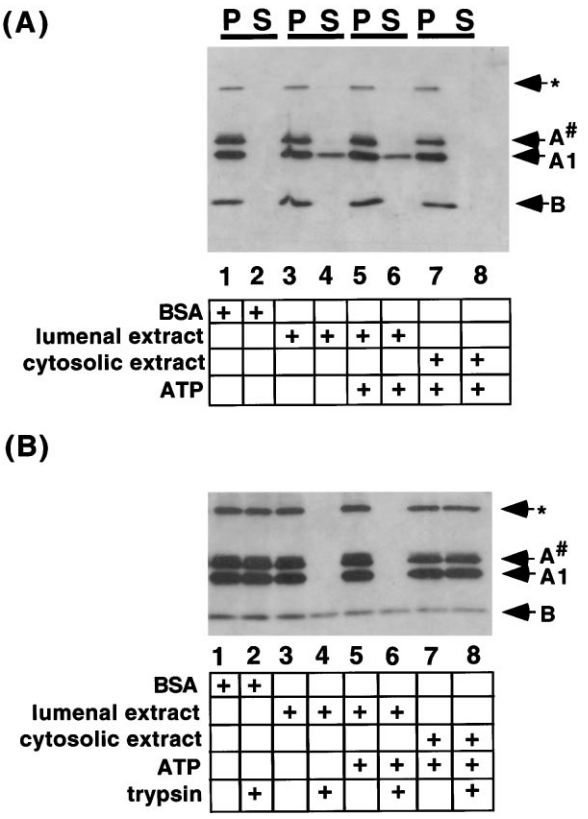

Figure 1. Dissociation and Unfolding of Cholera Toxin by an ER Lumenal Activity

(A) Beads covalently coated with the ganglioside GM1 were incubated with purified holotoxin (25 nM) in the presence of 1 mM GSH. BSA, ER lumenal extract, or cytosolic extract were added as indicated with or without 2 mM ATP. After sedimenting the beads, the pellet (P) and supernatant (S) were analyzed by nonreducing SDS-PAGE and immunoblotting with a toxin antibody. A# represents the proteolytically cleaved, disulfide bonded A subunit, A1 is the reduced fragment of the A subunit, and B is the B subunit. * indicates an unidentified band.

(B) Purified holotoxin (25 nM) was incubated in 1 mM GSH with either BSA, ER lumenal extract, or cytosolic extract with or without ATP. Where indicated, trypsin (100 μg/ml) was added. Samples were analyzed as in (A).

(lane 4 versus 6). Cytosolic proteins had no effect (lane 8 versus 7). We conclude that there is a specific activity in the ER lumen that affects the release of the A1 subunit from the rest of the toxin. In contrast to other reactions in which protein complexes are disassembled, no ATP seems to be required (Glover and Lindquist, 1998; Weber-Ban et al., 1999).

Next, we investigated whether the release of the A1 subunit was accompanied by its unfolding. To this end, we developed a protease protection assay. Addition of high concentrations of trypsin to the folded holotoxin left all polypeptide species intact (Figure 1B, lane 2 versus 1). However, after the addition of an extract derived from the ER lumen, the A and A1 subunits were degraded whereas the B subunit remained unaffected (lanes 4 and 6). Reduction of the disulfide bridge in the A subunit was insufficient to cause trypsin sensitivity (lane 2 versus 1). As in the release assay, the reaction was ATP indepen-

dent (lane 4 versus 6) and did not occur with cytosolic proteins (lane 8 versus 7). The fact that all the A1 chains were rendered trypsin sensitive but only some were released from the beads may perhaps be due to some nonspecific interactions between the unfolded A1 chains and the hydrophobic polystyrene beads. Taken together, these results indicate that the ER activity unfolds the entire A subunit; release of the A1 subunit from the rest of the toxin would then occur if the disulfide bridge to the A2 subunit is broken.

Unfolding Is Largely Caused by PDI

To identify the unfolding activity in the ER lumen, we bound the proteins of the lumenal extract to a Q-Sepharose column. Upon elution with a salt gradient, protein fractions were analyzed for their ability to confer trypsin sensitivity to the A and A1 subunits. Addition of fractions 9 or 10 resulted in complete degradation of these toxin polypeptides (Figure 2A, lanes 18 and 20), similar to the effect observed with the unfractionated extract (lane 4). Fractions 4 and 5 stimulated trypsin degradation of the A1 subunit, but left the A subunit largely intact (lanes 8 and 10). We concentrated on the activity in fractions 9 and 10.

Fractions 9 and 10 contained one major band of approximately 60 kDa (Figure 2B, lanes 8 and 9). This band was excised and identified as protein disulfide isomerase (PDI) by mass spectroscopy. Immunoblotting with an antibody directed against PDI confirmed this identification (Figure 2C, lanes 8 and 9). In addition, further purification of the material in fraction 9 on a S-Sepharose column demonstrated perfect cofractionation of PDI and the unfolding activity (Figure 2D). To confirm that the major unfolding activity in the ER lumen can be attributed to PDI, we immunodepleted PDI from the extract (Figure 2E, left panel, lane 2 versus 1). While mock-depleted extract confered trypsin sensitivity to the toxin A and A1 subunits, the PDI-depleted extract was inactive (Figure 2E, right panel, lanes 6 versus 14). Interestingly, the activity of the extract was very sensitive to dilution (lane 8). PDI was also the active component in the release assay: the purified protein released the A1 subunit from toxin bound beads (Figure 2E, lane 4 versus 2 and 6). We conclude that the major unfolding and disassembly activity in the ER lumen can be attributed to PDI. It should be noted that in both the unfolding and release assays, PDI was in an approximately 40-fold molar excess over the toxin.

PDI is a member of a family of oxidoreductases that contain at least one thioredoxin domain (reviewed by Noiva, 1999). PDI contains two such domains with a characteristic Cys-XX-Cys motif, and a peptide binding region. To test whether unfolding is a general property of the PDI family, we used DsbA, a bacterial homolog with a single thioredoxin domain. DsbA did not unfold the toxin (data not shown). Also, this enzyme did not reduce the native A subunit, although it was perfectly active following denaturation of the A subunit in urea (Figure 2G, lanes 3 and 6). In contrast, PDI could reduce even the native A subunit (lane 2). One possible interpretation is that PDI has an unfolding property that DsbA lacks. It would then appear that the toxin must be first unfolded before an oxidoreductase can reduce the disulfide bridge, likely because the Cys-187–Cys-199 bond is partially buried within the folded protein (Zhang et al., 1995).

Unfolding by PDI Is Dependent on Its Redox State

Since PDI is an oxidoreductase, we tested whether the unfolding activity of PDI is dependent on the redox potential. When purified PDI was tested under reducing conditions in the presence of GSH, it rendered the A and A1 subunits trypsin sensitive (Figure 3A, lane 8 versus 7). In contrast, under oxidizing conditions in the presence of GSSG, PDI had no effect (lane 6 versus 5). Control experiments with BSA showed that the toxin remains protease resistant under both reducing and oxidizing conditions (lanes 4 versus 3 and 2 versus 1). A mixed disulfide bond between PDI and the A1 chain could not be detected in the nonreducing SDS gels (not shown). PDI had the same redox-dependent unfolding activity when the isolated A subunit, rather than the holotoxin, was used as a substrate; again it rendered the A1 subunit sensitive to trypsin digestion under reducing, but not oxidizing, conditions (Figure 3B, lane 8 versus 7 and 6 versus 5). These data are consistent with the possibility that PDI in its reduced state binds and unfolds the toxin.

To test directly whether PDI exhibits redox-dependent binding to the toxin, we developed a cross-linking assay. Purified PDI was incubated with the A subunit in either oxidizing (GSSG) or reducing (GSH) conditions. A carbodiimide was added to induce cross-links between carboxyl and amino groups of the two proteins. A cross-linked product of the expected size was observed only under reducing conditions (Figure 3C, lane 8) and not in any of the control experiments (lanes 1–7). The interaction between PDI and the toxin was specific for the A subunit and was not seen with isolated B subunit (data not shown), in agreement with the previous data demonstrating that PDI only unfolds the A subunit.

Next we investigated whether the interaction of PDI with the A subunit is reversible. The two proteins were first incubated under reducing conditions (1 mM GSH) to stimulate binding. GSSG was then added at different concentrations and cross-links were induced. In the presence of 1 mM GSSG, cross-links could still be seen (Figure 3D, lane 8), whereas they disappeared in the presence of 30 or 50 mM GSSG (lanes 9 and 10). These results show that the interaction between reduced PDI and the A subunit can be reversed by oxidation, but they also indicate that unphysiologically high GSSG concentrations are required. Similar results were obtained when GSH and GSSG at different concentrations were added at the same time; again cross-links were seen at low, but not high, GSSG concentrations (lanes 5–7). The toxin itself was mostly in its oxidized state at high GSSG/GSH ratios (lanes 6 and 7), whereas it remained largely reduced when the same high GSSG concentration was added subsequent to GSH (lanes 9 and 10). These data show that the interaction between PDI and toxin is determined by the redox state of PDI, and not of its partner. Furthermore, the results indicate that the disulfide bridge in the nicked A subunit, once reduced, is inefficiently reformed by the addition of GSSG.

Protease protection experiments provided evidence that the A and A1 subunits are kept unfolded by being

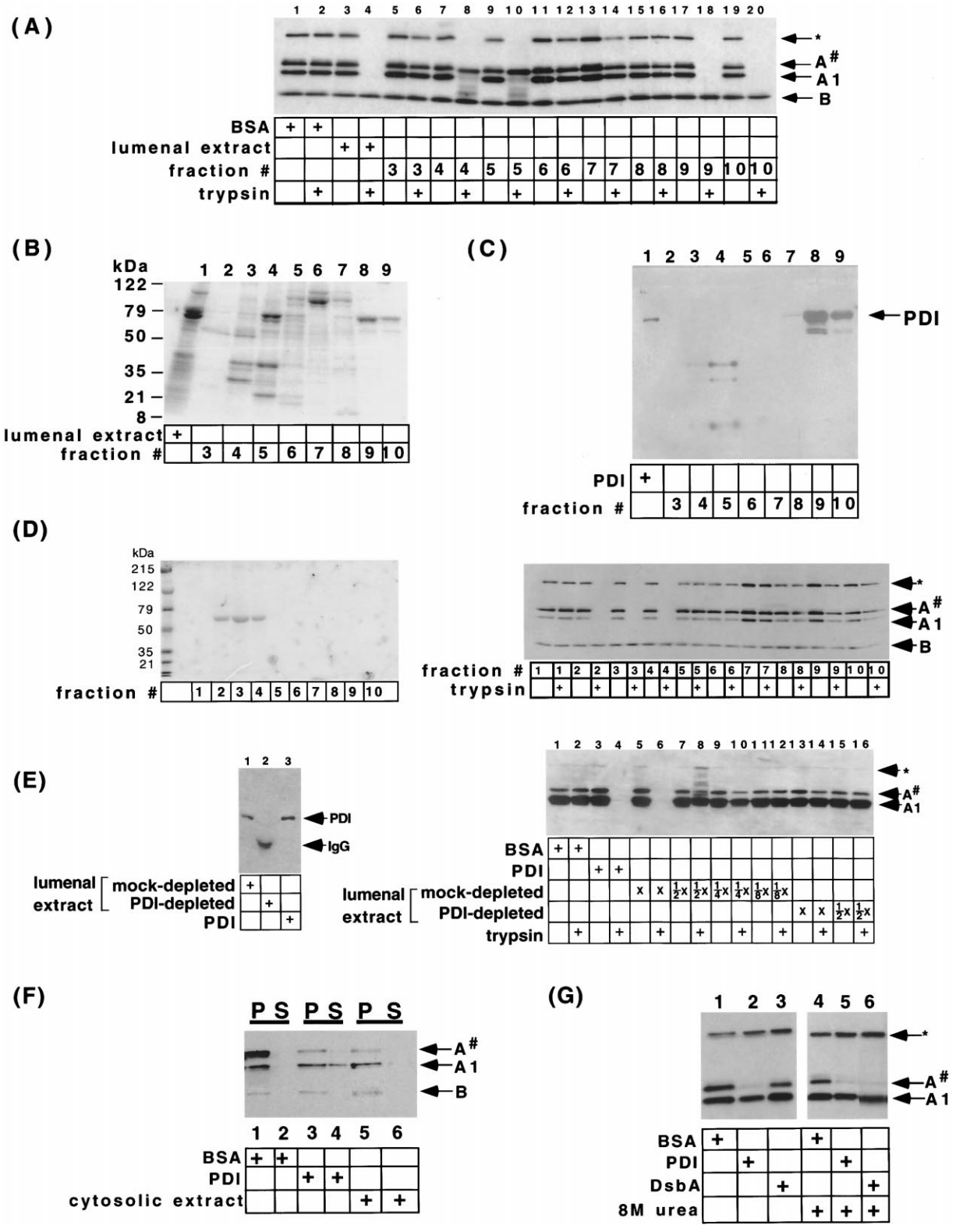

Figure 2. PDI Is Responsible for the Major Toxin Unfolding Activity in the ER

(A) ER lumenal protein was bound to a Q-Sepharose column and eluted with a continuous salt gradient. Fractions were incubated with holotoxin (25 nM) in the presence of 1 mM GSH, and trypsin (100 μg/ml) was added where indicated. All samples were analyzed by nonreducing SDS-PAGE and immunoblotting as in Figure 1.

(B) Fractions in (A) were analyzed by nonreducing SDS-PAGE followed by staining with Coomassie blue.

(C) Fractions in (A) were analyzed by nonreducing SDS-PAGE followed by immunoblotting with an antibody directed against mammalian PDI.

bound to the reduced form of PDI. As shown in Figure 3E, lane 1 versus 2, the A and A1 subunits were degraded by trypsin when PDI was present under reducing conditions. Both chains also remained sensitive to trypsin after addition of a low concentration of GSSG to mimic the in vivo redox state of the ER (Hwang et al., 1992) (lane 5 versus 6). However, when high concentrations of GSSG were added, the A and A1 subunits reached a protease-protected state (lane 4 versus 3). These data also show that the A and A1 chains can refold once they are released from the oxidized form of PDI.

To confirm that the redox state of PDI determines its unfolding activity, we modified all cysteines of PDI with iodoacetamide, thus blocking any reformation of disulfide bridges under oxidizing conditions. When tested in the presence of GSH, the modified enzyme was still able to render the A subunit sensitive to trypsin digestion (Figure 3F, lane 6 versus 2). However, while addition of GSSG abolished the unfolding activity of the unmodified PDI, it had little effect on the modified enzyme (lanes 4 versus 8). Taken together, the results show that with the cysteines reduced, PDI is in a state where it can bind and unfold the A subunit, whereas upon formation of disulfide bridges, it is converted into a low binding state.

A conformational change in PDI could be demonstrated directly by protease protection experiments. Under oxidizing conditions, PDI was significantly more sensitive to trypsin or chymotrypsin than under reducing conditions (Figure 3G, lanes 3 and 7 versus 4 and 8). These results are consistent with a model in which PDI would change from an open and proteolytically accessible conformation in the oxidized state into a closed and proteolytically more resistant conformation in the reduced state.

Yeast PDI behaved in a similar way as the mammalian enzyme, despite their low sequence similarity (less than 25% identical amino acids). Purified yeast PDI was unable to unfold the A subunit under oxidizing conditions (Figure 4A, lane 6 versus 5), but was active under reducing conditions (lane 8 versus 7). Similarly, it gave cross-links to the A subunit only under reducing conditions (Figure 4B, lane 4 versus 3). Since yeast PDI would never encounter cholera toxin, yet is able to unfold it, these results suggest that PDI in both yeast and mammals can act on other polypeptide substrates.

To test this possibility, we used unfolded calmodulin, a protein that lacks cysteines, to exclude any influence of the redox conditions on the substrate. The interaction of urea-denatured calmodulin with mammalian PDI was probed again with the bifunctional carbodiimide cross-linker. A prominent cross-linked product was observed under reducing conditions, while it was much weaker under oxidizing conditions (Figure 4C, lane 8 versus 7). Taken together, PDI appears to be a general polypeptide binding protein whose interaction is regulated by its redox state.

Unfolding of Cholera Toxin Requires Proteolytic Cleavage of the A Subunit

Cholera toxin is normally cleaved within the Cys-187–Cys-199 loop of the A subunit after its secretion from bacteria (Mekalanos et al., 1979). We, therefore, tested whether cleavage is required for unfolding by PDI. We expressed a mutant version of cholera toxin in *E. coli* that contained an inactivating mutation of the trypsin nicking site in the Cys-187–Cys-199 loop (R192G). The mutant protein was purified and its biological activity compared with that of the wild-type protein. Figure 5A shows the time course of toxin-induced Cl^- secretion when applied to intact human intestinal T84 cell monolayers. After a 40 min lag phase, corresponding to the time required for trafficking of the toxin from its site of binding on the apical membrane to its site of action inside the cell, wild-type toxin induced a maximal Cl^- secretory response (circles). In contrast, the R192G mutant exhibited strongly attenuated activity (triangles). These data confirm previous studies indicating that cleavage of the A subunit into the A1 and A2 subunits is required for efficient toxin action (Mekalanos et al., 1979; Lencer et al., 1997).

Next we tested the unfolding of the R192G mutant in vitro. SDS-PAGE confirmed that the mutant A subunit was indeed uncleaved; even under reducing conditions and in the presence of PDI only, one band corresponding to the intact A subunit was seen (Figure 5B, lane 7), whereas the wild-type protein nicked by *V. cholerae* proteases gave rise to two bands under the same conditions (lane 3). When tested for unfolding by PDI, the mutant A subunit was totally resistant to trypsin digestion, in contrast to the wild-type protein (Figure 5B, lane 8 versus 4). Thus, cleavage of the A subunit is required for unfolding by PDI. In fact, PDI did not even interact with uncleaved A subunit since a cross-linked product between the two proteins could not be observed under reducing conditions (Figure 5C, lane 2 versus 4). These results suggest that cleavage of the A subunit is required for it to be recognized and unfolded by PDI.

The Redox Potential of the ER Determines Toxin Activity In Vivo

Based on our in vitro results indicating that PDI unfolds the toxin in the presence of reducing agents, we asked

(D) Fraction 9 of (A) was bound to an S-Sepharose column. Elution was performed with a continuous salt gradient. The fractions were analyzed by staining with Coomassie blue (left panel) and for their ability to render the holotoxin trypsin sensitive (right panel).

(E) ER lumenal extract was immunodepleted of PDI or mock-depleted. Immunoblotting with a PDI antibody was used to confirm the depletion (left panel). Depleted and mock-depleted extract were incubated with isolated A subunit of cholera toxin (70 nM) in the presence of 1 mM GSH and treated with 100 μg/ml trypsin (right panel). Some controls were performed with BSA and others with purified mammalian PDI, added at a concentration equivalent to that in the undiluted, mock-depleted extract (3.5 μM). Samples were analyzed as in (A). X gives the dilution of the extract.

(F) GM1-coated beads were incubated with 25 nM holotoxin in the presence of 1 mM GSH. BSA, purified mammalian PDI (3.5 μM), or cytosolic extract were added. After sedimentation, the pellet (P) and supernatant (S) were analyzed by nonreducing SDS-PAGE and immunoblotting.

(G) Native or denatured (8 M urea) A subunit (70 nM) was incubated with BSA, purified mammalian PDI (3.5 μM), or purified DsbA (4 μM) in the presence of 1 mM GSH. Samples were analyzed by nonreducing SDS-PAGE and immunoblotting.

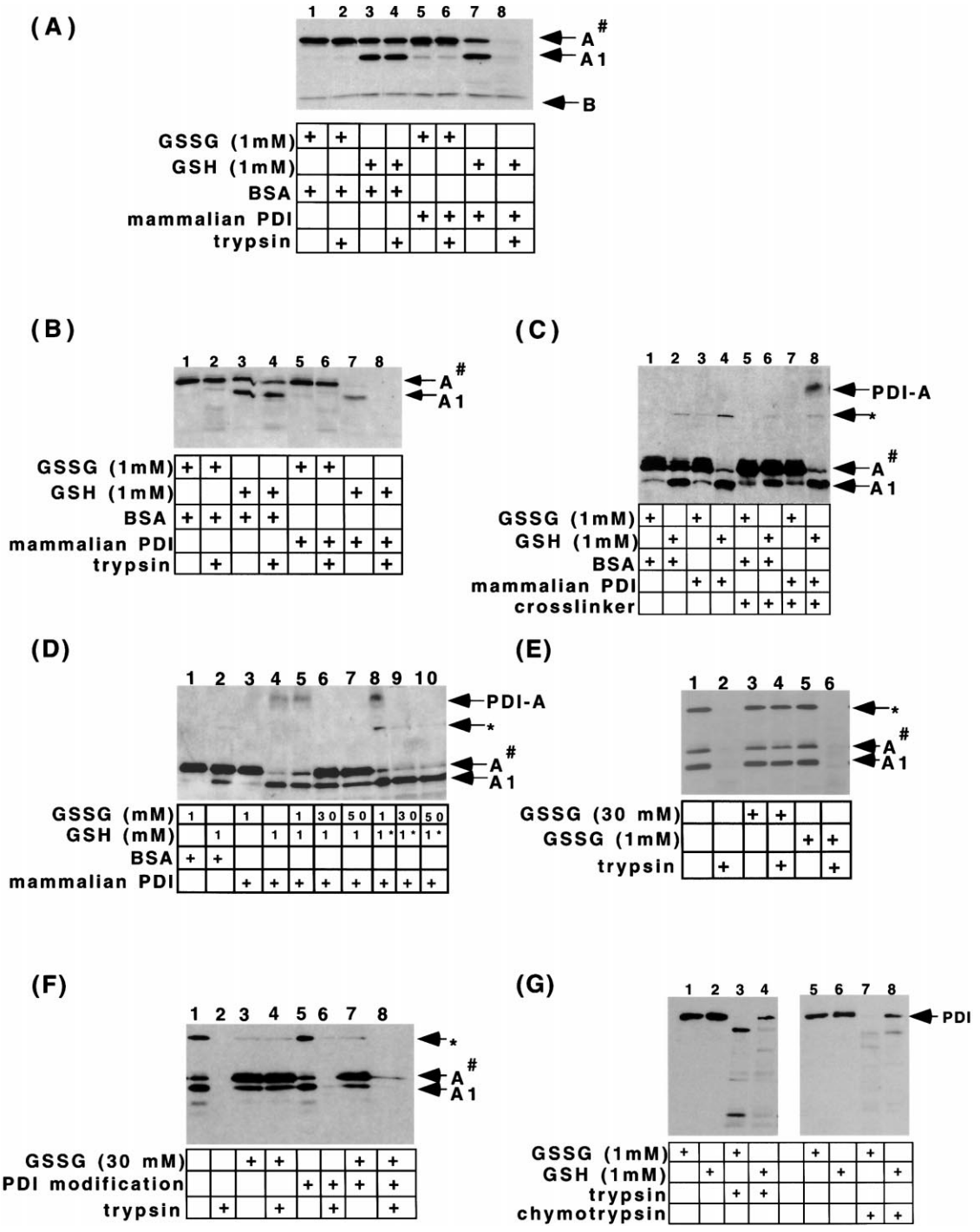

Figure 3. Unfolding by PDI Depends on Its Redox State

(A) Purified holotoxin (25 nM) was incubated with either GSSG (1 mM) or GSH (1 mM) in the presence of BSA or purified mammlian PDI (3.5 μM). Trypsin (100 μg/ml) was added where indicated. All samples were analyzed by nonreducing SDS-PAGE and immunoblotting as in Figure 1.

(B) Isolated A-subunit (70 nM) was incubated with either 1 mM GSSG or 1 mM GSH in the presence of BSA or purified mammalian PDI (3.5 μM). Samples were analyzed as in (A).

(C) Isolated A subunit (70 nM) was incubated with either 1 mM GSSG or 1 mM GSH in the presence of BSA or purified mammalian PDI (3.5 μM). A carbodiimide cross-linker (EDAC) was added, where indicated, to induce cross-links. Samples were analyzed as in (A). PDI-A represents a cross-linked product between PDI and the A subunit.

(D) Isolated A subunit (70 nM) was incubated with purified PDI (3.5 μM) in the presence of 1 mM GSH. Increasing concentrations of GSSG were added either simultaneously (lanes 5, 6, and 7) or subsequently (lanes 8, 9, and 10). 1* represents samples in which 1 mM GSH was added prior to the addition of GSSG. The cross-linker was then added to induce cross-links. Samples were analyzed as in (A).

(E) Isolated A subunit (70 nM) was incubated with PDI (3.5 μM) in the presence of 1 mM GSH with or without the subsequent addition of 1 mM or 30 mM GSSG. Trypsin (100 μg/ml) was added where indicated. Samples were analyzed as in (A).

if this step might be rate limiting in vivo; if so, addition of a reductant to intact cells might increase the biological activity of the toxin. Indeed, the addition of 5 mM dithiothreitol (DTT), a reducing agent known to permeate into the ER of intact cells (Braakman et al., 1992), resulted in a more rapid onset and larger Cl^- secretory response in T84 intestinal epithelial cells (Figure 6A, open circles) when compared to a control in the absence of DTT (filled circles). The signal dropped after some time, likely because the intactness of the monolayer of cells was compromised. This is supported by an experiment in which forskolin, a known stimulator of protein kinase A, was added at the end of the time course; it elicited a much weaker signal when DTT was present (Figure 6A). In contrast to DTT, the addition of the oxidant diamide completely inhibited any induction of Cl^- current by the toxin (diamonds). Diamide did not inhibit the secretory response induced by forskolin added at the end of the experiment. These data are consistent with the idea that shifting the environment in the ER to more reducing conditions accelerates the rate-limiting step in the pathway used by the toxin.

To show that the DTT-induced increase in toxin action was not due to a direct effect on the reduction of the toxin itself, we generated and purified a toxin mutant containing an A subunit lacking cysteines (C187S, C199S). This mutant was less stable than the wild-type protein, as evidenced by its sensitivity to trypsin in the absence of PDI (data not shown). It elicited an attenuated Cl^- secretory response when applied to T84 cells under normal conditions (Figure 6B, filled circles). Nonetheless, when DTT was added at 60 min, a stimulation of the Cl^- secretory response was observed (open circles), similar to the results with wild-type toxin. DTT had no detectable effect on monolayers not exposed to cholera toxin (open squares). Identical results were obtained using toxin variants containing only a single serine substitution at either Cys-187 or Cys-199 (data not shown). Thus, the effect of the reductant DTT must be on a component of the cell and not on the toxin itself, consistent with the postulated role of PDI.

Discussion

Our results provide insight into the pathway of cholera toxin from its synthesis in bacteria up to its arrival in the lumen of the ER in mammalian cells, where its A1 fragment is released from the rest of the toxin and unfolded in preparation for its translocation into the cytosol. Using a biochemical fractionation approach that made no assumptions about the nature of the unfolding activity in the ER, we found that PDI is responsible for the major activity and that it is acting as a redox-driven chaperone. These results have implications for the specific pathway of cholera toxin, for the role of PDI in retrograde transport of proteins across the ER membrane, and for the folding of proteins in general. These points will be discussed separately.

Unfolding of Cholera Toxin

Our results explain why cholera toxin is assembled and folded in the periplasm of bacteria and disassembled and unfolded in the ER, even though both compartments normally support the folding of polypeptides. One decisive factor is that the A subunit needs to be cleaved into the A1 and A2 fragments for unfolding to occur. The periplasm of bacteria seems to lack proteases that cleave the A chain, and in this compartment, the two subunits can therefore assemble and fold into the native holotoxin. Before the toxin can affect mammalian cells, it needs to be nicked, mostly by proteases secreted by *V. cholerae* or present in the intestinal lumen of mammals. The cleaved form of the A subunit must have some features that allow PDI to recognize it, likely some hydrophobic segments that are transiently exposed by thermal fluctuation of the polypeptide chains. Another possible difference between the periplasm of bacteria and the ER of eukaryotes is that a PDI-like unfolding activity may not exist in prokaryotes. Indeed, DsbA, one of the homologs of PDI in *E. coli*, did not have unfolding activity in our in vitro experiments. Some redox-dependent peptide binding of DsbA or other members of the thioredoxin family seems possible, however, given that thioredoxin itself binds T7 DNA polymerase in the reduced, but not oxidized state (Adler and Modrich, 1983).

Our data support the idea that the toxin travels as an intact, though nicked, molecule from the surface of mammalian cells to the ER. A previous report claimed that the A and B subunits are separated in the Golgi and that only the A subunit makes it all the way to the ER (Bastiaens et al., 1996). Although our data are consistent with this model because PDI can unfold the isolated A chain, recent experiments employing a mutant B chain containing an N-glycosylation site provided direct evidence that the B subunit reaches the ER (Y. Fujinaga and W. Lencer, unpublished data). In addition, immunoelectron microscopy supports this conclusion (Thyberg, 2000). We propose that the ring of B subunits binds to the ganglioside GM1 at the cell surface, and that this interaction remains intact all the way to the ER. Upon reaching the Golgi, the A2 subunit located in its central pore would interact through its C-terminal KDEL sequence with the multispanning KDEL receptor and allow retrograde transport of the holotoxin into the ER. A similar model has been proposed for related toxins (Hazes and Read,1997).

Once in the ER, PDI binds to the A1 chain and causes it to unfold, but it leaves the ring of B chains intact. Our experiments suggest that reduction of the disulfide bridge between the A1 and A2 fragments occurs in a subsequent step. The A1 chain is then released and

(F) Purified mammalian PDI (3.5 μM) was incubated with 1 mM GSH, modified with iodoacetamide, and dialyzed (PDI modification). Modified PDI, and as a control unmodified PDI, were then incubated with 30 mM GSSG and isolated A subunit (70 nM), followed by incubation with trypsin (100 μg/ml). Samples were analyzed as in (A).

(G) Purified mammalian PDI (3.5 μM) was incubated in either 1 mM GSH or 1 mM GSSG with trypsin (100 μg/ml) or chymotrypsin (500 μg/ml). Samples were analyzed by nonreducing SDS-PAGE followed by immunoblotting with an antibody against PDI.

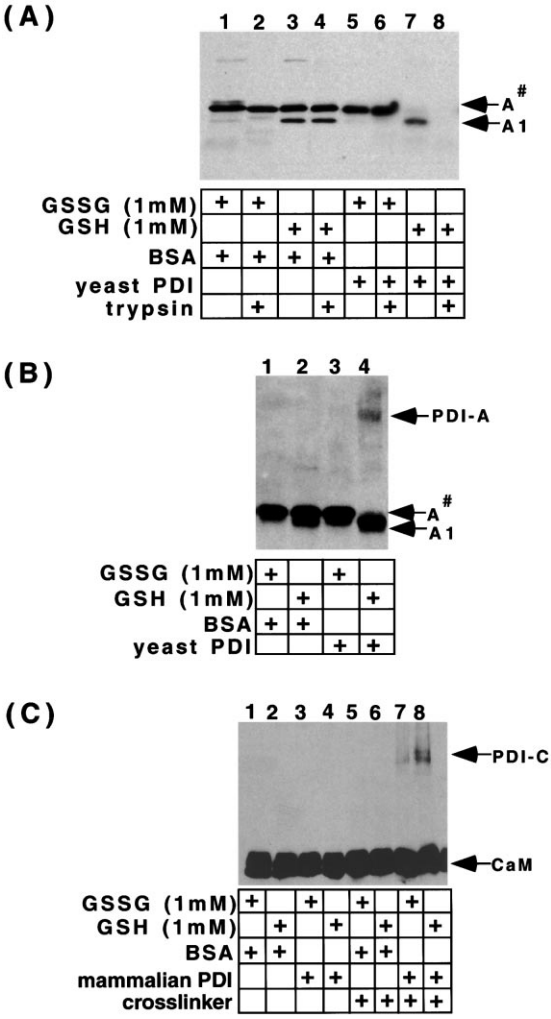

Figure 4. PDI Is a Redox-Dependent Chaperone

(A) Isolated A subunit (70 nM) was incubated with 1 mM GSSG or 1 mM GSH in the presence of either BSA or purified yeast PDI (3.5 μM). Trypsin (100 μg/ml) was added where indicated. The samples were analyzed by nonreducing SDS-PAGE and immunoblotting with a toxin antibody.

(B) Isolated A subunit was incubated with 1 mM GSSG or 1 mM GSH in the presence of either BSA or yeast PDI (3.5 μM). Cross-linking was performed where indicated. Samples were analyzed as in (A).

(C) Purified human calmodulin (1 μM) was incubated in 1 mM GSSG or 1 mM GSH with either BSA or purified mammalian PDI (3.5 μM). Cross-linking was performed where indicated. Samples were analyzed by nonreducing SDS-PAGE followed by immunoblotting with an antibody directed against mouse calmodulin. CaM represents calmodulin, and PDI-CaM a cross-linked product between calmodulin and PDI.

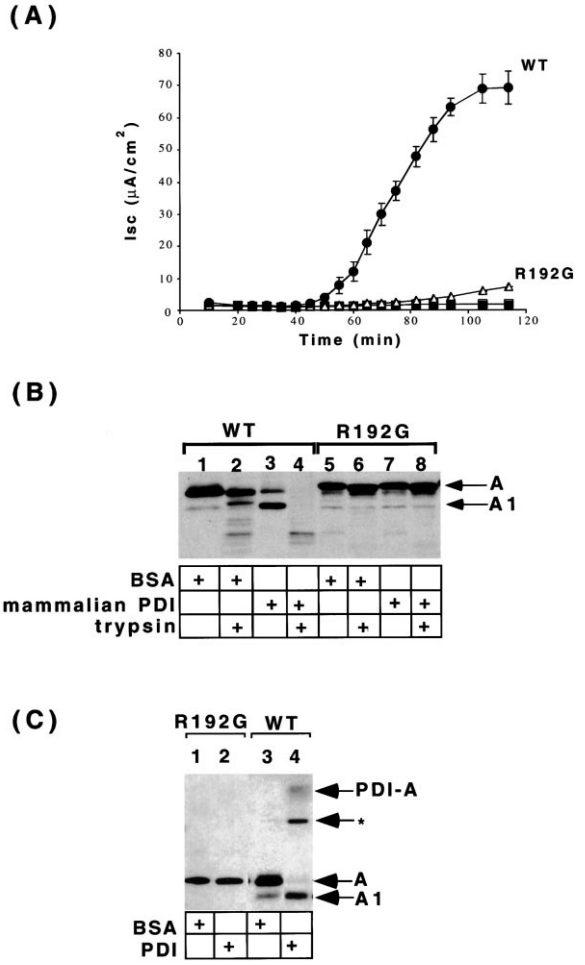

Figure 5. Proteolytic Cleavage of the A Subunit Is Required for Its Unfolding

(A) Cl^--secretion from a monolayer of T84 epithelial cells was measured as a short circuit current (Isc) over time. The cells were incubated either in buffer alone (black square) or in the presence of 5 nM wild-type (WT) holotoxin (black circle), or 5 nM noncleavable R192G mutant (open triangle). The data shown are a single experiment representative of six independent studies. Data points show the mean ± S.D. of two independent monolayers.

(B) BSA or purified mammalian PDI (3.5 μM) were incubated with wild-type A subunit (70 nM) or with the noncleavable A subunit mutant R192G (70 nM) in the presence of 1 mM GSH. Trypsin (100 μg/ml) was added where indicated. Samples were analyzed by nonreducing SDS-PAGE and immunoblotting with a toxin antibody. A represents the proteolytically cleaved, disulfide bonded A subunit for lanes 1–4 or the noncleaved A subunit for lanes 5–8.

(C) BSA or purified mammalian PDI (3.5 μM) were incubated with wild-type A subunit (70 nM) or with the noncleavable A subunit mutant R192G (70 nM) in the presence of 1 mM GSH. Cross-linker was added and the samples were analyzed as in (B).

transported into the cytosol, while the A2 chain likely remains bound inside the intact B-ring.

A Role for PDI in Retrograde Transport?

It is uncertain whether the A1 fragment of cholera toxin is translocated in an unfolded conformation across the ER membrane. However, the Sec61p channel transports only unfolded or loosely folded proteins into the lumen of the ER, and it is therefore possible that it would do so also in the retrograde direction. Unfolded A1 chain may be generated by PDI close to the Sec61p channel, even though the enzyme is present at high concentrations throughout the ER. Since PDI needs to be in the reduced state to unfold the toxin, and since the oxidized form is predominant in the ER lumen (Frand and Kaiser, 1999), one could imagine that the reduced form would be generated locally by a PDI reductase that is associ-

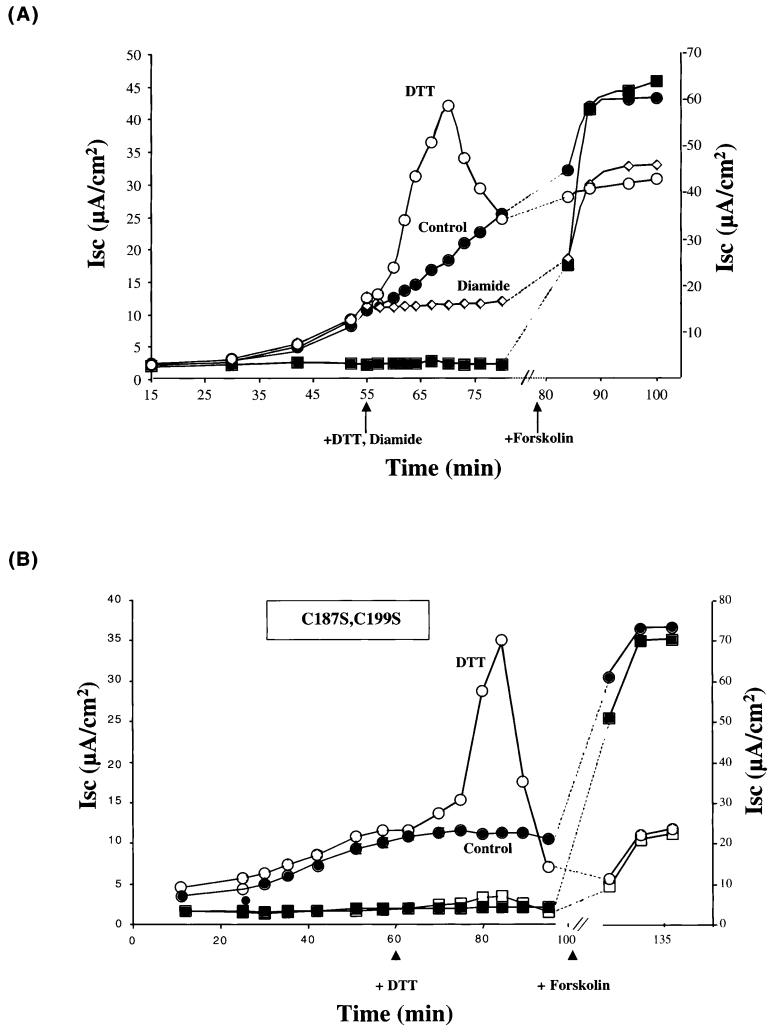

Figure 6. The In Vivo Activity of Cholera Toxin Depends on the Redox Potential

(A) Cl^- secretion from a monolayer of T84 epithelial cells was measured as a function of time after addition of buffer alone (black square) or of buffer containing 20 nM wild-type (WT) holotoxin (black circle, open circle, diamond). DTT (5 mM final, open circle) or diamide (0.5 mM final, diamond) were added as indicated (first arrow). At the end of the time course, forskolin (10 μM) was added (second arrow). The data shown are a single experiment representative of six independent studies. The standard deviations were similar to those shown in Figure 5A.

(B) A similar experiment as in (A) was performed with buffer alone (black square, open square) or with the C187S,C199S mutant holotoxin (1 nM black circle, open circle). DTT (5 mM, open circle, open square) was added as indicated (first arrow).

ated with the channel. Release of the unfolded A1 chain from PDI could occur by a PDI oxidase that need not be localized to the channel. Alternatively, the oxidase, and not the reductase, may be associated with the channel, resulting in localized release of the A1 chain from PDI. It is, of course, also conceivable that the unfolded A1 subunit is transferred from PDI to a downstream chaperone prior to being translocated through the channel. In any case, since the A1 chain can spontaneously refold after release from PDI into buffer, there may be a mechanism that prevents folding before the protein has reached the cytosol.

An increase in the concentration of reduced PDI would be expected to result in more unfolded A1 being offered to the Sec61p channel and could thus explain why more toxicity was observed upon addition of a reducing reagent to intact cells. According to our model, extremely reducing conditions in the ER would also be expected to be inhibitory because they would prevent the release of PDI from its substrate. However, we determined that under our experimental conditions, only a fraction of PDI became reduced, while most of it remained oxidized (data not shown). Interestingly, several proteins in the ER are rapidly degraded when reducing reagents are added (Young et al., 1993; Wilson et al., 1995; Wainwright and Field, 1997; Courageot et al., 1999), which may perhaps again be explained by reduced PDI stimulating their retrograde translocation.

Other data also support the view that PDI and related enzymes play a role in the initiation of retrograde transport across the ER membrane. Mutants of PDI, in which the putative peptide binding domain was perturbed, led to defects in the retrograde transport of unfolded proteins from the ER into the cytosol (Gillece et al., 1999). More recently, it was found that overexpression of Eug1p, a homolog of PDI in yeast, suppressed the defect in retrograde transport seen with certain Sec61p mutants (R. Schekman, personal communication). It is also interesting that US11, a protein triggering retrograde transport of MHC class I molecules in cytomegalovirus infected cells, has a Cys-XX-Cys motif similar to that in thioredoxin.

PDI: a Member of a Novel Class of Chaperones

Our results demonstrate that PDI binds polypeptide substrates in the reduced, but not oxidized state. The redox state affects PDI, rather than the substrate, and results in a conformational change, consistent with the idea

that it would be more compact in the reduced state. We therefore propose that PDI is a chaperone that changes its affinity for substrates during cycles of oxidation and reduction of its disulfide bridges. These cycles were previously thought to be linked exclusively with the oxidation or reduction of cysteines in substrates.

PDI has been regarded as a chaperone before because it could prevent aggregation of proteins and bind peptides in vitro (Song and Wang, 1995; Gilbert, 1997; Klappa et al., 1997). Our results demonstrate that the peptide binding affinity is regulated by the redox state of PDI. Although PDI functioned as an "unfoldase" in our experiments, it is probably different from real unfoldases of the Hsp104/ClpA family of ATPases. The latter form rings around a polypeptide substrate and "walk" along them, leaving behind an unfolded polypeptide chain. PDI, on the other hand, may simply bind to an unfolded polypeptide domain, rather than causing a folded protein to unfold.

How the chaperone function of PDI is coordinated with its previously characterized oxidoreductase activity is unclear. PDI can act both as an oxidase to form disulfide bridges in newly synthesized proteins as they emerge into the lumen of the ER, and as an isomerase to reshuffle wrongly formed disulfide bridges (reviewed by Noiva, 1999). At least in the case of the isomerase activity, the enzyme would benefit from its chaperone function because it must recognize that the substrate is misfolded. As in the case of cholera toxin, PDI may first bind in its reduced state to the substrate and then affect the reduction of misformed disulfide bridges. When it acts as an oxidase, PDI in its oxidized form could bind to the reduced polypeptide substrate with low affinity. It would then oxidize the cysteines of the substrate and become reduced itself, thus being converted into the more strongly binding conformation. The interaction energy could be used to drive oxidation of the substrate. Reoxidation of PDI would occur by its upstream partner enzyme Ero1p and would result in the release of the substrate (Pollard et al., 1998; Frand and Kaiser, 1999).

The redox-regulated binding cycle of PDI is remarkably similar to the ATP binding cycle of conventional chaperones (Figure 7). In the case of Hsp-70, for example, the substrate initially interacts with the ATP form of Hsp-70, which has an open peptide binding pocket (for review, see Bukau and Horwich, 1998). This interaction is weak and allows the peptide to enter and leave the binding site rapidly. Following ATP hydrolysis, the peptide binding pocket closes and the substrate is bound much more tightly. Release occurs upon nucleotide exchange. The postulated redox-driven cycle of PDI is similar. PDI could bind a substrate with an open binding pocket in its oxidized state, and clamp around it upon reduction. Substrate release would require reoxidation of PDI. For both classes of chaperones, it is the abundant form of the chaperone that has a weak interaction with the substrate: the ATP form in the case of Hsp-70s and other ATP-dependent chaperones, and the oxidized form in the case of PDI in the ER lumen (Frand and Kaiser, 1999). The transitions between the ATP and ADP forms of Hsp-70s are often affected by cofactors and analogous cofactors may also exist for PDI.

PDI does not appear to be the only redox-regulated

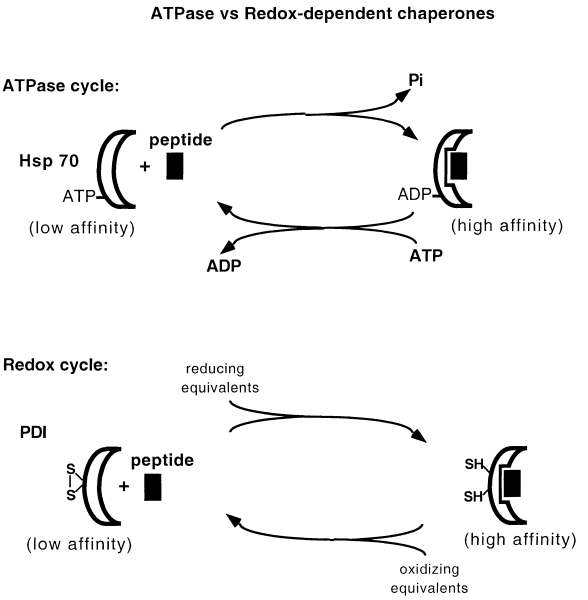

Figure 7. Similarities between Redox- and ATPase-Dependent Chaperones

Shown are the binding cycles for Hsp-70, a representative of the ATP-dependent chaperones, and for the redox-dependent PDI. The cycle of Hsp-33, a redox-dependent chaperone in the cytosol, is postulated to be reversed, with the oxidized form being tight binding. For details see text.

chaperone. A cytosolic protein with a thioredoxin-containing motif, Hsp-33, has been identified as a chaperone whose substrate interaction is affected by its redox state (Jakob et al., 1999). In this case, however, it is the oxidized, rather than the reduced form that strongly binds to substrates. Since reducing conditions prevail in the cytosol, these results are in agreement with our hypothesis that it is always the more abundant form of a chaperone that makes weak contact with a substrate. Hsp-33 was thought to function primarily under oxidative stress to rescue misfolded proteins, but it is a constitutive protein and a significant percentage of it is in the oxidized state even under resting conditions (Jakob et al., 1999). We therefore propose that it belongs together with PDI to a novel family of chaperones whose binding cycle is regulated by its redox state.

The emerging picture is that all chaperones may undergo binding cycles driven by energy, be it in the form of ATP or oxidation equivalents. Why is energy needed? A likely explanation is that the system must be in a nonequilibrium state to allow cycles of binding and release as well as regulation of these processes. Under equilibrium conditions, the binding constant between a chaperone and a substrate could in principle be high enough to lower the concentration of the unbound polypeptide sufficiently to prevent its aggregation. However, efficient productive folding would not be possible unless there was a mechanism to actively release the substrate from the chaperone. In reality, most chaperones must recognize substrates of widely different amino acid sequence, and the equilibrium binding constants are therefore moderate. To combine a fast on-rate with a low off-rate, the initial chaperone-substrate complex must

undergo a conformational change. This mechanism allows a chaperone to act like a "mouse trap," clamping around peptides that do not perfectly fit into its binding pocket (Misselwitz et al., 1998). The conformational change from high to low off-rates requires external energy. Thus, a nonequilibrium situation is likely a general feature of chaperone function.

Experimental Procedures

Materials

The A subunit of cholera toxin and human brain calmodulin were purchased from Calbiochem, mammalian PDI from Takara Biomedicals, and antibody against mouse calmodulin from Upstate Biotechnology. Purified bacterial DsbA and antibodies against mammalian PDI were gifts from Dr. James Bardwell (University of Michigan, MI) and Dr. Hidde Ploegh (Harvard Medical School, MA), respectively. Cholera toxin antibodies directed against the A and B subunits were described previously (Lencer et al., 1997).

Mutagenesis of Cholera Toxin A Subunit

The plasmid pATA14 encoding wild-type holotoxin (Rodighiero et al., 1999) was used as a template for oligonucleotide-directed mutagenesis resulting in the following plasmids: pRC37 containing mutant A subunit R192G, pRC38 encoding mutant A subunit C187S, pRC39 containing mutant A subunit C199S, and pRC40 encoding the double mutant C187S, C199S.

Purification of Wild-Type and Mutant Holotoxin

Wild-type holotoxin was expressed and purified from *Vibrio cholerae* as previously described (Rodighiero et al., 1999). For the purification of mutant toxins, periplasmic extracts were filtered through a 0.8 μ pore filter and dialyzed overnight against two changes of Hanks balanced salt solution. The concentration of the toxin subunits in the periplasmic extracts was determined with an enzyme-linked immunosorbent assay as reported previously (Lencer et al., 1997).

Purification of Yeast PDI

A fusion protein between glutathione-S-transferase and yeast PDI (GST-PDI) was expressed in *E. coli*. (the encoding plasmid is a gift from Dr. William Lennarz, SUNY Stony Brook). The cells were homogenized in PBS containing 0.1% Triton X-100 and the extract was incubated with glutathione-Sepharose beads (Pharmacia) for 12 hr at 4°C. After washing the beads extensively with PBS, the protease factor Xa (Promega) was added to cleave the linkage between GST and PDI. The beads were sedimented and the supernatant used for experiments.

Fractionation of ER Lumenal Extract from Canine Pancreas

An ER lumenal extract was prepared by adding 0.2% digitonin to a suspension of 2.2 equivalents/μl canine microsomes followed by centrifugation (for definition of equivalents, see Walter et al., 1981). About 150 mg of the extract was bound to a 10 ml Q-Sepharose column in 50 mM HEPES, pH 7.2, 250 mM sucrose, 2 mM $MgCl_2$. Bound material was eluted with a linear 0 to 1 M potassium acetate gradient. Fractions of 1 ml were collected, dialyzed, and analyzed for both protein content and ability to render the A and A1 subunits trypsin sensitive. Fraction 9 was subjected to further fractionation on a 1 ml SP-Sepharose column. The material was bound in 10 mM sodium acetate, pH 4.5, 50 mM NaCl, 250 mM sucrose, and 2 mM $MgCl_2$ and eluted at pH 6.0 with a linear 0.1 to 1 M sodium acetate gradient. Fractions of 0.5 ml were collected, dialyzed, and analyzed as above.

Release Assay

Polystyrene beads containing surface carboxyl groups (Polysciences) were coated with lyso-monosialoganglioside GM1 (Matreya) by following the manufacturer's instructions. 10 μl of GM1-coated beads (50% bead volume) were washed with 50 mM HEPES, pH 7.2, 250 mM sucrose, 50 mM potassium acetate, 2 mM $MgCl_2$, and incubated with wild-type holotoxin (25 nM) for 20 min at 22°C. After washing, BSA (5 μM), ER lumenal extract (2 mg/ml), cytosolic extract (2 mg/ml), purified mammalian PDI (3.5 μM), or yeast PDI (3.5 μM) were added in the presence of GSH (1 mM) and incubated for 30 min at 22°C. The beads were sedimented and the supernatant was removed and both fractions were analyzed by nonreducing SDS-PAGE and immunoblotting with a toxin antibody.

Trypsin Sensitivity Assay

Purified holotoxin (25 nM) or isolated A subunit (70 nM) were incubated with either BSA, ER lumenal extract (2 mg/ml), cytosolic extract (2 mg/ml), purified mammalian or yeast PDI (3.5 μM) in the presence of GSH (1 mM) or GSSG (1 mM) for 30 min at 30° C. Trypsin (0.1 mg/ml) was added for 30 min at 4°C and the reaction was stopped by the addition of 1 mM L1-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride (TLCK) for 10 min at 4°C. Samples were analyzed by nonreducing SDS-PAGE followed by immunoblotting with a toxin antibody.

Immunodepletion of PDI

100 μl lumenal extract was incubated with 10 μl PDI antibodies overnight at 4°C, followed by addition of 20 μl protein A Sepharose beads. Mock-depletion was performed without antibodies.

Cross-Linking Assay

Isolated A subunit (70 nM) was incubated with either BSA or 3.5 μM purified mammalian or yeast PDI in the presence of 1 mM GSH or 1mM GSSG for 30 min at 30°C. In some experiments, increasing concentrations of GSSG were subsequently added for an additional 15 min at 30°C. Five mM of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) was added for 15 min at 22°C to generate cross-links. After addition of 80 mM glycine, the samples were analyzed by nonreducing SDS-PAGE and immunoblotting.

Electrophysiology

Measurements of short circuit currents (Isc) and resistance (R) were performed on confluent monolayers of human intestinal T84 cells grown on 0.33 cm^2 filters, as previously described (Lencer et al., 1997).

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