Many proteins of the secretory pathway contain disulfide bonds that are essential for structure and function. In the endoplasmic reticulum (ER), Ero1α and Ero1β oxidize protein disulfide isomerase (PDI), which in turn transfers oxidative equivalents to newly synthesized cargo proteins. However, oxidation must be limited, as some reduced PDI is necessary for disulfide isomerization and ER-associated degradation. Here we show that in semipermeable cells, PDI is more oxidized, disulfide bonds are formed faster, and high molecular mass covalent protein aggregates accumulate in the absence of cytosol. Addition of reduced glutathione (GSH) reduces PDI and restores normal disulfide formation rates. A higher GSH concentration is needed to balance oxidative folding in semipermeable cells overexpressing Ero1α, indicating that cytosolic GSH and luminal Ero1α play antagonistic roles in controlling the ER redox. Moreover, the overexpression of Ero1α significantly increases the GSH content in HeLa cells. Our data demonstrate tight connections between ER and cytosol to guarantee redox exchange across compartments; a reducing cytosol is important to ensure disulfide isomerization in secretory proteins.

The cytosol and the endoplasmic reticulum (ER) are the main folding compartments of eukaryotic cells (1–3). The latter is the site of the production of proteins destined to the organelles of the central vacuolar system and to extracellular space. These molecules, collectively termed “secretory” proteins hereafter, are co-translationally translocated into the ER (4). Secretory proteins are designed to work in the extracellular environment, which differs substantially from the cytosol. Indeed their folding takes place in the ER, where the calcium concentration and the ratio between reduced glutathione (GSH) and oxidized glutathione (GSSG) resembles those that will be encountered in the extracellular environment. In the ER, secretory proteins undergo sequential processing steps, some of which are unique to this compartment (i.e. disulfide bond formation and N-linked glycosylation). These modifications are coupled to a tight quality control schedule that restricts transport to the Golgi apparatus to native conformers. Incompletely folded or assembled molecules are retained in the ER and eventually dislocated to the cytosol for proteasomal degradation (for reviews, see Refs. 1 and 5). In this respect, the ER can be viewed as a specialized and highly selective school wherein proteins are trained to acquire their functional native conformation.

A fundamental difference between cytosolic and secretory proteins is the abundance of disulfide bonds in the latter (6). Disulfides are often essential for folding and confer stability to secreted proteins (7). The abundance of ER resident oxidoreductases of the protein disulfide isomerase (PDI) family underscores their importance in the ER protein factory (6, 8). Disulfide bond formation requires that oxidizing conditions be maintained in this organelle. GSSG has long been considered the main source of oxidizing equivalents for the secretory pathway (9). However, yeast mutants deficient in glutathione synthetase can form disulfide bonds (10). Moreover, defects in Ero1p, the specific PDI oxidase, can be rescued by deletion of the GSH1 gene, suggesting that in yeast, GSH competes with newly synthesized proteins for the oxidizing equivalents provided by Ero1p (11). Higher eukaryotes have two Ero1-like genes, Ero1α and Ero1β (12, 13). Both can oxidize PDI and promote disulfide formation (14).

The notion that expression of Ero1β is induced by ER stress provides a mechanism whereby cells can adjust the oxidative power of the ER when their protein load becomes excessive. However, cells face an equally important problem, that is preventing excessive oxidation within the ER. Indeed, disulfides must be isomerized during folding (15) and reduced prior to dislocation of terminally misfolded molecules targeted to proteasomal degradation (16). Inefficient degradation can lead to the accumulation of misfolded proteins in the ER and cytotoxicity (for review, see Ref. 17), thus underscoring the importance of precisely regulating the ER redox state.

In this study, we investigated the role of cytosolic factors, glutathione in particular, in controlling oxidative folding in higher eukaryotes, where this abundant tripeptide plays an important role also in regulating apoptosis (reviewed in Ref. 18). Moreover, we studied whether and how cells can sense and respond to the hyper-oxidation in the ER induced by Ero1α over-expression.

Our results demonstrate that GSH acts as the main antagonist of Ero1α, limiting disulfide bond formation in the ER.
Interestingly, overexpressing Ero1α, but not an inactive mutant, induces a significant increase in the intracellular content of reduced glutathione, indicating the existence of intercompartmental compensatory pathways that maintain proper redox homeostasis.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Reagents—HeLa cells were obtained from American Type Culture Collection and maintained in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Rabbit anti-PDI and rabbit anti-ribophorin antibodies were kind gifts of Drs. A. Benham and I. Braakman (Utrecht University, The Netherlands) and Dr. E. Ivessa (Vienna University, Austria), respectively. In these conditions, all accessible thiol-cysteines should be bound to NEM. Lysates were then incubated for 15 min at 50 °C with 50 mM DTT to obtain reduction of disulfides and then precipitated with 10% trichloroacetic acid. The trichloroacetic acid-insoluble material was resuspended in alkylation buffer (80 mM Tris-Cl, pH 8.0, 2% SDS, 25 mM 5000 mPEG-maleimide) and incubated for at least 30 min at room temperature to achieve alkylation of reduced disulfides. The PDI redox state was then analyzed by standard SDS-PAGE and Western blotting protocols.

Ero1α Redox Isoforms—HeLa cells were transfected with myc-tagged Ero1α expression vector and treated as described for JcM oxidation assay. The ratio of Ero1α redox isoforms (Ox1 and Ox2, Ref. 20) was assessed by standard non-reducing SDS-PAGE and Western blotting protocols.

Glutathione Measurement—Intracellular glutathione was assayed upon formation of S-carboxymethyl derivatives of free thiols with iodoacetamide, followed by extraction and reversed-phase high performance liquid chromatography (HPLC) measurement with an UV detector. As described previously (14), both intra- and inter-chain disulfide bonds are formed upon DTT removal, resulting in the accumulation of species migrating with accelerated and retarded mobility, respectively, and in the disappearance of the band corresponding to reduced JcM chains.

RESULTS

Soluble Cytosolic Proteins Are Not Essential for Disulfide Bond Formation—To assess the importance of cytosolic factors in controlling disulfide bond formation in the ER, we analyzed oxidative protein folding in semipermeable (SP) cells. Because of the differences in cholesterol content, digitonin permeabilizes the plasma membrane to a much greater extent than the ER or other intracellular organelles (19). Indeed, two cytosolic proteins, endogenous thioredoxin (14.5 kDa) and a transfected red fluorescent protein (26 kDa), disappeared upon digitonin treatment, whereas two ER resident proteins, PDI and ER-yellow fluorescent protein (57 and 27 kDa, respectively), were not affected (Fig. 1). Having validated the system, we analyzed disulfide bond formation using a JcM, as described previously (14). Briefly, intact or SP cells expressing JcM were exposed to DTT to reduce disulfide bonds in ER proteins to synchronize their oxidation. After two washes in cold PBS, cells were incubated for different times in the absence of DTT. Oxidative folding of JcM was monitored by SDS-PAGE in non-reducing conditions. As described previously (14), both intra- and inter-chain disulfide bonds are formed upon DTT removal, resulting in the accumulation of species migrating with accelerated and retarded mobility, respectively, and in the disappearance of the band corresponding to reduced JcM chains. These processes display similar initial kinetics (14), although, as oxidation proceeds, most oxidized JcM is present as high molecular mass complexes. Moreover, the disappearance of reduced JcM is more representative of JcM oxidation, because isomerization processes might also be involved in the formation of oxidized species (monomers, dimers, and high molecular mass complexes). Therefore, for the sake of brevity, only monomeric JcM are shown in most figures, with one exception (see Fig. 4, in which aggregate formation is investigated).

The kinetics of JcM oxidation were strikingly faster in SP cells than in intact cells (Fig. 2, compare middle and upper panels, respectively). Re-adding cytosol to SP cells reduced the oxidative folding rate (Fig. 2, lower panel), restoring the kinetics observed in intact cells. Because in digitonin-treated cells...
The addition of 10 mM GSH to SP cells was sufficient to alter disulfide bond formation. After elimination of DTT, cells were incubated at 24°C. In view of the results obtained in yeast (11), we reasoned that the inhibitory effect exerted by the cytosol on disulfide formation could be mediated by reduced glutathione (GSH). The GSH concentration in mammalian cells ranges from 1 to 10 mM (24). Indeed, the addition of 10 mM GSH to SP cells was sufficient to alter the disulfide formation rate to that seen in intact cells. Conversely, 1 mM GSH displayed minor effects (Fig. 3A, third and fourth panels, respectively, and Fig. 3B). Adding 10 mM GSH to intact cells had marginal effects upon JcM oxidation (Fig. 3C, compare upper and middle left panels), indicating that either GSH could not cross the plasma membrane or that cytosolic GSH was enough to mask the effect of exogenous molecules.

The above findings indicate that GSH is effective in limiting JcM oxidation at physiological concentrations and support the idea that glutathione could be the main cytosolic component responsible for buffering disulfide bond formation in the ER. This effect is due to the reducing action of GSH, because the addition of 10 mM GSSG did not inhibit JcM oxidation (Fig. 3C). Glutathione Depletion in Living Cells Accelerates JcM Oxidation and Induces Aggregate Formation—If GSH were the main factor limiting ER oxidative folding, lowering its pool in intact cells should increase disulfide bond formation. To test this possibility, we treated cells overnight with an inhibitor of glutathione synthesis (BSO), and then with the GSH-inactivating drug DEM (see “Experimental Procedures” for details). Although less markedly than in digitonin-treated cells, the rate of disulfide formation was accelerated in BSO-DEM-treated cells (Fig. 4A). In contrast, virtually all of the JcM chains remained intact or SP cells (second panel). ER-yellow fluorescent protein was eliminated by digitonin treatment (compare A with C), whereas cytosolic red fluorescent protein was eliminated by digitonin treatment (compare B with D). E, immunoblot of intact and digitonin-treated (semi perm.) cell lysates. The cytosolic protein thioredoxin (TRX) disappeared after permeabilization, whereas ER resident PDI was unchanged.

The oxidation of JcM at 37°C was almost complete after only 2 min, the experiments were performed at 24°C to lower the oxidation rate.

The above data demonstrate that soluble cytosolic proteins are dispensable for the formation of disulfide bonds in JcM. On the contrary, the cytosol seems to contain factor(s) which are responsible for the decrease in the oxidation rate of the reporter protein.

GSH Addition Limits JcM Oxidation in SP Cells—In view of the results obtained in yeast (11), we reasoned that the inhibitory effects exerted by the cytosol on disulfide formation could be mediated by reduced glutathione (GSH). The GSH concentration in mammalian cells ranges from 1 to 10 mM (24). Indeed, the addition of 10 mM GSH to SP cells was sufficient to alter the disulfide formation rate to that seen in intact cells. Conversely, 1 mM GSH displayed minor effects (Fig. 3A, third and fourth panels, respectively, and Fig. 3B). Adding 10 mM GSH to intact cells had marginal effects upon JcM oxidation (Fig. 3C, compare upper and middle left panels), indicating that either GSH could not cross the plasma membrane or that cytosolic GSH was enough to mask the effect of exogenous molecules.

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treatment.

insoluble high molecular mass JcM aggregates. HeLa cells expressing JcM were treated with BSO and DEM (see “Experimental Procedures”) or nothing. After elimination of DTT, cell lysates were loaded as markers of detergent-soluble and -insoluble fractions were boiled 10 min in Laemmli buffer and analyzed by SDS-PAGE of maleimide-modified samples. As shown in Fig. 5B, alkylation of samples with mPEG-maleimide induces changes in PDI electrophoretic mobility according to the redox state of the molecule. A mobility shift consistent with a more oxidized state can be observed in SP cells (lane 2), whereas the addition of 10 mM GSH could restore the higher mobility of a more reduced PDI (lane 3).

The above findings suggest that cytosolic GSH influences the redox state of PDI, thus modulating its ability to form and isomerize disulfide bonds in cargo proteins.

**Glutathione and ER Redox Control**

![Image](image_url)

**Fig. 5.** Endogenous PDI was more oxidized in SP cells. A, native gels. Total lysates of HeLa cells were resolved by polyacrylamide gels under non-denaturing conditions and immunoblotted against PDI. Diamide and DTT-treated cells were loaded as markers of in vivo oxidized and reduced conditions, respectively (lanes 1 and 4). In vitro reduction was performed by adding 10 mM DTT to the cell lysate (lane 5). Lane 2, the shift in PDI mobility induced by cytosol elimination. Lane 3, endogenous PDI in intact cells. *B*, thiol-accessibility assay. mPEG-maleimide-alkylated samples from HeLa cells treated as indicated (see “Experimental Procedures”) were resolved by polyacrylamide SDS-8% PAGE. The more oxidized a protein was at the moment of lysis, the slower was the electrophoretic mobility in this assay. PDI was more oxidized in SP than in intact cells (compare lanes 2 and 4), but the change in redox state was reversed by the addition of 10 mM GSH (lane 3).

than in BSO-DEM-treated cells. Therefore, the deprivation of either cytosol or GSH accelerates disulfide formation, eventually leading to the formation of high molecular mass, detergent-insoluble, covalent complexes.

The **Redox State of PDI Differ**s in **SP Cells**—The presence of mixed disulfides between JcM and PDI (14) suggests that formation of disulfide bonds in JcM chains upon DTT removal is catalyzed by PDI. In turn, Ero1α and β are involved in facilitating the oxidation of PDI (14). Therefore, the faster oxidation of JcM chains in SP HeLa cells could be due to an altered redox state of either PDI or Ero1α. To investigate changes in the PDI redox state, we made use of both native gels and alkylation of free thiols with mPEG-maleimide. Electrophoresis in native gels has been shown to discriminate the different redox isoforms of PDI (25), supposedly because of conformational changes. In non-denaturing gels, PDI migrated as a doublet in intact cells (Fig. 5A, lane 3). Treatment with the reducing agent DTT caused a shift toward the faster species, whereas the reverse was obtained exposing cells to diamide (Fig. 5, lanes 4 and 1, respectively). This result suggests that the two bands consisted of different redox isoforms of PDI. Only the slower molecular species was detected in SP cells, suggesting that oxidized PDI is the predominant isoform in SP cells (Fig. 5, lane 2). Fig. 5, lane 5 shows PDI reduced after cell lysis with 10 mM DTT to illustrate the electrophoretic mobility of a PDI molecule that is likely reduced completely.

Similar results are obtained by SDS-PAGE of maleimide-modified samples. As shown in Fig. 5B, alkylation of samples with mPEG-maleimide induces changes in PDI electrophoretic mobility according to the redox state of the molecule. A mobility shift consistent with a more oxidized state can be observed in SP cells (lane 2), whereas the addition of 10 mM GSH could restore the higher mobility of a more reduced PDI (lane 3).

The above findings suggest that cytosolic GSH influences the redox state of PDI, thus modulating its ability to form and isomerize disulfide bonds in cargo proteins.

**GSH Modulates the Redox Status of Ero1α**—Different Ero1α isoforms are detectable in non-reducing gels, including mixed disulfides with PDI and ERP44 and two distinct bands corresponding to monomeric Ero1α, Ox1 and Ox2 (20, 26). The latter two species are clearly redox-sensitive, as treatment with DTT favors the accumulation of Ox1, whereas the oxidant diamide induces Ox2 (26). Although the functional significance of Ox1 and Ox2 remains to be established, their relative abundance provides an assay to monitor changes in the Ero1α redox state.

Therefore, to determine also whether Ero1α is sensitive to GSH, we monitored the rate of conversion of Ox1 into Ox2 after exposure to DTT in intact and SP cells transfected with myc-tagged Ero1α (Fig. 6). In intact cells, the Ox1/Ox2 ratio is only slightly changed during 8 min at 24 °C of incubation without DTT. In contrast, Ox1 disappears very rapidly in SP cells, and already after 4 min, only the Ox2 form is detectable.

The addition of GSH delays the formation of the Ox2 form in digitonin-treated cells, and this inhibitory effect is dose-dependent (Fig. 6, third and fourth panels). In fact, inhibition of
Ox1-Ox2 conversion begins to be detectable at 1 mM GSH, whereas the process was completely restored only at a concentration of GSH (10 mM), which is more similar to that normally present in HeLa cells. These results suggest that GSH can also affect the Ero1α redox state.

**Ero1α Antagonizes the Effects of GSH**—We have shown in Fig. 5 that PDI is more oxidized in SP cells, suggesting a role for cytosolic GSH in modulating the PDI redox state. On the other hand, it has been demonstrated that Ero1α overexpression is able both to accelerate disulfide formation and to modify the PDI redox state (14). Thus, we wondered whether we could see an effect of Ero1α overexpression also in SP cells. To address this issue, we co-transfected HeLa cells with both Ero1α and JcM and then followed the oxidation of the latter after digitonin treatment in the presence of different GSH concentrations.

Confirming previous data (14), Ero1α overexpression accelerates the rate of JcM oxidation in intact cells (Fig. 7, upper panels). Interestingly, however, no significant differences could be seen between Ero1α- and mock-transfected SP cells (Fig. 7, second row of panels from top), suggesting that, in the absence of cytosol, a plateau in the oxidation rate was reached. This result is consistent with the fact that PDI is mainly oxidized in SP cells, whereas in SP cells, it becomes almost completely oxidized (Fig. 5). However, SP cells overexpressing Ero1α needed more GSH than mock-transfected controls to slow down disulfide formation (Fig. 5, third and bottom lines). Thus, in cytosol-deprived cells, Ero1α overexpression was ineffective in accelerating JcM oxidation unless GSH was added, further indicating that in intact cells, Ero1α activity may be counterbalanced by cytosolic GSH.

**Overexpression of Ero1α Induces the Accumulation of Intracellular GSH**—The above results indicate that GSH buffers the oxidative power of Ero1α. This is probably important in living cells, as reduced PDI is needed to isomerize and reduce disulfide bonds during ER quality control (reviewed in Refs. 27 and 28). Indeed, preliminary results from our laboratory indicate that the overexpression of Ero1α inhibits the degradation of several ERAD substrates. In view of the importance of these processes, we decided to determine whether cells are able to respond to excessive ER oxidation by modulating the GSH levels. We found a strong and consistent increase in intracellular GSH in Ero1α-transfected cells, when compared with mock-transfected ones (Fig. 8). The average increase expressed as a percentage of the GSH concentration relative to mock-transfected cells was about 30%. Neither an inactive Ero1α

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**FIG. 6. GSH affected the redox state of Ero1α.** In HeLa cells transfected with myc-tagged Ero1α, after 5 min of incubation with 5 mM DTT, Ox1 was slightly more abundant than Ox2 in all the conditions tested (lane 0, all panels). The conversion to the Ox2 form was slower in intact cells than in SP cells, where almost all Ero1α was present as Ox2 after 4 min (second panel down, lane 4). The addition of GSH decreased the rate of Ox2 formation in a dose-dependent way (lower panels).

**FIG. 7. Ero1α accelerated JcM oxidation only in the presence of GSH.** HeLa cells expressing JcM were co-transfected with either Ero1α or an empty vector (mock). The presence of Ero1α in intact cells accelerated disulfide formation (first row of panels), whereas in SP cells, no significant difference could be discerned between mock and Ero1α-transfected cells (second row of panels). However, the addition of GSH to SP cells revealed a clear role of Ero1α as a GSH antagonist (third and fourth rows of panels).

**FIG. 8. Ero1α overexpression increased intracellular GSH.** A, the cellular content of GSH was measured by high pressure liquid chromatography in HeLa cells transiently transfected with either empty vector (white bar), Ero1α (black bar), the Ero1α mutant C394A (cross hatched bar), or a mutated ribophorin (dotted bar). Only Ero1α gave rise to a significant increase in the intracellular GSH pool. The results are shown as a percentage of mock-transfected cells and represent the average of 3–8 experiments in triplicate, *p < 0.01 (by Student’s t test), significant difference from mock. B, the levels of transgene expression for a representative experiment. Anti-myc or anti-ribophorin antibodies were used to detect wild-type/mutant Ero1α and ribophorin, respectively. The ribophorin doublet reflects differential utilization of an N-glycosylation site. As determined by immunofluorescence, transfection efficiency ranged from 20 to 40%, being generally similar in single experiments, particularly when wild-type and mutant Ero1α vectors were used.

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Formation of Disulfide Bonds Does Not Require Soluble Cytosolic Proteins—The fact that disulfide bonds are efficiently formed in SP cells implies that oxidative equivalents can be generated within the ER, where all JcM chains are localized, in the absence of soluble cytosolic proteins. By oxidizing PDI, members of the Ero1 family play a pivotal role in this pathway, which in turn transfers disulfide bonds to nascent proteins via mixed disulfide intermediates (14, 30). In yeast, oxygen accepts electrons from Ero1p in a reaction requiring FAD (31). The observation that human Ero1 molecules complement the Ero1-deficient yeast strain (32) suggests that an analogous mechanism could be active also in mammalian cells.

Cytosolic GSH Limits Disulfide Bond Formation—The faster rate of disulfide formation in SP cells also implies that cytosolic factor(s) limit oxidative folding. Our experiments indicate that this task is largely fulfilled by GSH. Addition of purified GSH slows down disulfide bond formation in a dose-dependent manner; at 10 mM, a GSH concentration within the physiological range for eukaryotic cells (24), JcM oxidation proceeded at the rate observed in intact cells. GSSG was not active, demonstrating the redox dependence of the phenomena. Cysteine accessibility to alkylating agents and native gels were used to monitor the PDI redox state, whereas for Ero1, we compared the proportions between the redox isofoms Ox1 and Ox2 (20).

Although it is not easy to determine precisely the redox state of PDI, our assays reveal significant differences between intact or SP cells, the latter accumulating PDI species that can be increased by diamide in intact cells. With both techniques, we were able to show the co-existence of different redox isofoms at steady state. This might support the idea that PDI, as the key ER oxidoreductase, is concurrently involved in conflicting redox reactions.

It is noteworthy that the rate of Ero1α oxidation following DTT-induced reduction was much faster in the absence of GSH, suggesting that Ero1α is also a target of GSH. Therefore, the two main elements in the process of disulfide bond formation, Ero1α and PDI, are both influenced by cytosolic GSH. Although we cannot exclude the concurrent involvement of other cytosolic reducing systems, namely thioredoxin and NADPH, our results strongly suggest a role for cytosolic GSH in regulating oxidative folding. Lowering the GSH levels in living cells by exposure to BSO and DEM also accelerated JcM oxidation, albeit less than in SP cells. This difference is intriguing, as it may reflect either the existence of additional ways to buffer the ER redox or the utilization of compensatory mechanisms, such as, for instance, the selective delivery of reduced GSH to the ER lumen by enzymatic or transport mechanisms.

Limiting ER Oxidation—The physiological implications of these observations are clear, considering that not only are disulfide bonds formed in the ER, but they must be extensively isomerized during folding (15) and reduced before the dislocation of ER-associated degradation (ERAD) substrates (16). As both reactions are catalyzed by reduced PDI (33, 34), the activity of Ero1α and Ero1β must be limited. Indeed, the overexpression of Ero1α inhibits the degradation of a wide spectrum of ERAD substrates.² Our results indicate that cytosolic GSH can reduce PDI, and it cooperates with Ero1α, though working in opposite directions, to establish the proper PDI redox state. In agreement, more GSH is needed to balance oxidative folding in SP cells that overexpress Ero1α, whereas the effects of Ero1α overexpression on the rate of oxidative folding are hardly detectable in the absence of GSH.

In addition to GSH, other factors can generate reduced PDI in living cells. For instance, secretory proteins entering the ER with reduced cysteines likely contribute to consume oxidized PDI, and this may explain why cycloheximide, which blocks protein synthesis, slows down degradation of some ERAD substrates. It is also possible that specific PDI reductases exist, perhaps with a non-uniform distribution within the ER lumen or during development.

Is the ER Environment Really Oxidizing?—The notion that GSH has access to the ER lumen (35, 36) suggests that the ER environment could be much less oxidative than normally assumed. It is not clear how GSH negotiates transport across the ER membrane. Its high concentration in the cytosol provides a strong gradient, but likely specific transporters or channels exist for this charged tripeptide (35, 36). Whatever the mechanism of entry, it is likely that GSH rapidly diffuses within the ER lumen, thus providing a reducing milieu also within this organelle. The function of Ero1 would then be to oxidize PDI by means of specific protein-protein interactions. In principle, this mechanism would generate a default toward reduced PDI. In this redox form, PDI can isomerize and reduce disulfides and, in addition, act as a chaperone endowed with unfoldase activity (33). This may reflect the requirements for stringent quality control systems in higher eukaryotes, where development and intercellular communication depend upon the fidelity of protein secretion.

Regulation of the ER Redox—In most cells, disulfide bond formation depends upon the levels of Ero1α. When these become insufficient to cope with entering polypeptides, the unfolded protein response (UPR)-dependent Ero1β expression could furnish the required oxidative power. In many respects, the periplasmic space of prokaryotes is similar to that of the ER. A fundamental difference, though, is that the oxidation and isomerization/reduction pathways are separated in the periplasmic space, being controlled by the DsbB-DsbA and DsbC-DsbD pathways, respectively. In eukaryotes, instead, PDI is thought to mediate both oxidation and reduction. At first sight, this yin-yang mechanism seems to cause unnecessary complications. However, the redox-dependent conformational changes proposed to regulate unfoldase activity of PDI (37) can offer a unique possibility of coupling precise quality control to the elaboration of disulfide bonds. The import of GSH would set a reducing environment also in the ER, counteracted by a series of protein-protein interactions that allow the targeted delivery of oxidative equivalents. Folding would then make the correct disulfides inaccessible and lock the oxidized PDI in a more inert, misfolded state, which would be easily reduced and, after multiple attempts, targeted for degradation. Insufficient ER redox buffering seems to favor formation of high molecular mass, detergent insoluble, covalent aggregates. These molecular species are likely unproductive and could be dangerous for living cells, causing ER storage diseases (38).

Intercompartment Redox Homeostasis—The cytoplasmic connection via GSH might provide a defense against excessive oxidation produced by the oxidative activity of the ER. The observation that the overexpression of Ero1α induces a significant increase in the reduced glutathione content underscores the relevance of intercompartmental redox control. At present, it is unclear whether the increase in GSH depends upon accelerated synthesis or reduced catabolism-release. Pharmacological induction of a robust UPR with tunicamycin or thapsigargin also increased GSH,³ confirming the existence of tight links between the ER and the cytosol. The transcription factor ATF4 has recently been shown to couple ER stress to a general cellular response that increases the production of GSH, thus offering protection toward oxidative stress (39). However, the expression of inactive Ero1α mutants or ribophorin 332, a short-lived ER protein devoid of cysteines (29), did not alter the

³ S. Nerini Molteni, A. Fassio, and R. Sitia, unpublished results.
GSH levels (Fig. 8), thus excluding a major role for cargo-dependent UPR pathways and suggesting that an Ero1-dependent alteration of the ER redox was capable of inducing a cytosolic response. This implies the existence of redox sensors and transducers that transmit information across the ER membrane to modulate GSH metabolism, thus inducing a compensatory response. One attracting possibility is that Ero1 activity generates peroxide (39), thereby eliciting downstream responses to restore the cellular redox homeostasis.

In conclusion, our findings demonstrate that cytosolic GSH counteracts the oxidative power of Ero1 in the ER, likely allowing optimal redox conditions for disulfide isomerization and reduction. The levels of cytoplasmic GSH are influenced by the ER oxidation, implying the existence of a tight ER-cytosolic connection. Redox-dependent, intercompartmental signaling pathways likely play an important role in cell physiology, allowing the exchange of information across the ER membrane. In this way, cells can rapidly adapt to changing synthetic needs, thus reducing the risks of oxidative stress.

Acknowledgements—We thank all of the members of the laboratory, Adam Benham, Ineke Braakman, Anna Teresa Palamara, and Lars Ellgaard, for helpful criticism, suggestions, and discussions, Tania Mastrandrea for secretarial assistance, and Erwin Ivessa and Adam Benham for providing reagents.

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