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# Comprehensive Invited Review

# The Endoplasmic Reticulum: Folding, Calcium Homeostasis, Signaling, and Redox Control

AGNES GÖRLACH,[1] PETER KLAPPA,[2] and THOMAS KIETZMANN[3]

*Reviewing Editors: Adam Benham and Gabor Banhegyi*



## ABSTRACT

**The endoplasmic reticulum (ER) plays a major role in regulating synthesis, folding, and orderly transport of proteins. It is also essentially involved in various cellular signaling processes, primarily by its function as a dynamic $Ca^{2+}$ store. Compared to the cytosol, oxidizing conditions are found in the ER that allow oxidation of cysteine residues in nascent polypeptide chains to form intramolecular disulfide bonds. However, compounds**

[1]Experimental Pediatric Cardiology, Department of Pediatric Cardiology and Congenital Heart Disease, German Heart Center Munich at the Technical University Munich, Munich, Germany.
[2]Department of Biosciences, University of Kent, Canterbury, United Kingdom.
[3]Faculty of Chemistry, Department of Biochemistry, University of Kaiserlautern, Kaiserlautern, Germany.

**and enzymes such as PDI that catalyze disulfide bonds become reduced and have to be reoxidized for further catalytic cycles. A number of enzymes, among them products of the *ERO1* gene, appear to provide oxidizing equivalents, and oxygen appears to be the final oxidant in aerobic living organisms. Thus, protein oxidation in the ER is connected with generation of reactive oxygen species (ROS). Changes in the redox state and the presence of ROS also affect the $Ca^{2+}$ homeostasis by modulating the functionality of ER-based channels and buffering chaperones. In addition, a close relationship exists between oxidative stress and ER stress, which both may activate signaling events leading to a rebalance of folding capacity and folding demand or to cell death. Thus, redox homeostasis appears to be a prerequisite for proper functioning of the ER.** *Antioxid. Redox Signal.* 8, 1391–1418.

## I. INTRODUCTION: BASIC FUNCTIONS OF THE ENDOPLASMIC RETICULUM

The endoplasmic reticulum (ER) has two major functions. It is responsible for synthesizing and packaging proteins, and also plays a central role in metabolism and in many signaling processes. These reactions also contribute to redox changes; thus they can influence the two functions of biosynthesis and signaling. Furthermore, disturbances in either process can profoundly influence the other. For example, an accumulation of misfolded proteins can alter ER $Ca^{2+}$ homeostasis, whereas a change in the luminal content of $Ca^{2+}$ can affect protein synthesis.

With regard to its function in signaling processes, the ER can receive and transmit signals. The input signals include intracellular messengers such as $Ca^{2+}$, inositol 1,4,5-trisphosphate (InsP3), sphingosine-1-phosphate (S-1-P), reactive oxygen species (ROS), and sterols. In response to these signals, the ER generates output signals such as $Ca^{2+}$, activators of store-operated channels (SOCs), stress signals, arachidonic acid, and various transcription factors (NF-κB, CHOP, ATF6, and SREBPs) (14).

Further, the ER represents one of the largest membrane networks within a cell. A so-called smooth and rough ER can be distinguished. The smooth ER is highly abundant in hepatocytes of the liver, in steroid hormone-producing cells, and in muscle where it is also known as sarcoplasmic reticulum (SR), whereas all other cells contain less smooth ER.

Enzymes in the smooth ER are mainly involved in synthesis of fatty acids, cholesterol, and phospholipids. In hepatocytes these enzymes are also important for xenobiotic metabolism where drugs, chemicals, and carcinogens are converted to more water-soluble compounds. High concentrations of theses compounds (*e.g.*, ethanol) induce a large proliferation of the smooth ER in hepatocytes. By contrast, in muscle the SR has a major function for calcium sequestration.

All eukaryotic cells contain a certain amount of rough ER, which is formed by attachment of ribosomes at the cytosolic surface. It coordinates the synthesis of membrane and organelle proteins and of virtually all proteins to be secreted. Thus, high amounts of rough ER can be found in specialized cells like antibody producing plasma cells where nearly the entire cytosol is filled with ER. Due to its function in protein synthesis and transport, the rough ER represents the first compartment of the so-called secretory pathway. Quality control within this pathway is achieved at the early steps (*i.e.*, within the ER). The enzymes involved in these control steps modify certain proteins in a way that they can be delivered to their proper target sites within the cell or the extracellular environment. These modifications include folding of the nascent polypeptides and posttranslational modifications such as glycosylations or disulfide bond formation. Thus, the ER is not just a passive compartment involved in protein transport but rather an active site responsible for quality control of the cargo. This is underscored by the findings that only properly folded proteins and assembled subunits are transported from the rough ER to the Golgi complex. Failure in protein folding has been shown to be associated with the initiation of signaling pathways from and to the ER that act either to increase the folding capacity or to initiate cell death.

With the appearance of oxygen and the development of aerobic life on earth, reactive oxygen species (ROS) became important factors influencing a number of processes within a cell. Since high amounts of ROS can cause damage to proteins, DNA, and lipids, cells use an array of nonenzymatic and enzymatic detoxification mechanisms. However, the last decade has shown that ROS can also act as signaling molecules mediating changes in $O_2$ tension as well as the response to hormones, growth factors, and mechanical or chemical stress. It is thus easy to envision that ROS and redox changes could affect many aspects of ER function, which we try to review within this article.

## II. REDOX ENVIRONMENT AND ROS GENERATION

In mammalian cells, ROS can be formed in response to toxic reagents or as by-products of $O_2$-utilizing enzymes such as those in the mitochondrial respiratory chain, the arachidonic acid pathway, the cytochrome P450 family, glucose oxidase, amino acid oxidases, xanthine oxidase, NADH/NADPH oxidases, or NO synthases (27, 65). Thus, ROS can be produced by multiple mechanisms in all cellular compartments. The first reactive oxygen species produced in the reduction pathway of oxygen to water is the superoxide anion radical ($O_2^{-}\bullet$), which participates in the generation of other toxic metabolites, most importantly hydrogen peroxide ($H_2O_2$), hydroxyl radicals ($OH\bullet$), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$) (Fig. 1).

Under physiological conditions, excess formation of ROS is prevented by the endogenous antioxidant defense systems.

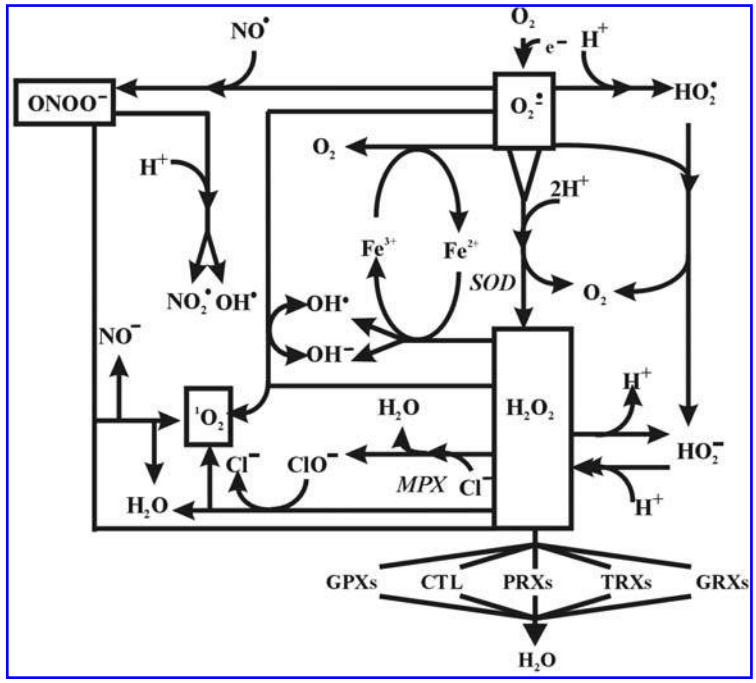


**FIG. 1. Pathways of ROS generation.** When $O_2$ accepts an electron, superoxide anion radicals ($O_2^{-}$•) are formed that can then be dismutated spontaneously or by superoxide dismutases (SOD) to hydrogen peroxide ($H_2O_2$). The $O_2^{-}$• can also be protonated to hydroperoxyl radicals ($HO_2$•) or together with $HO_2$• disproportionate to the basic hydrogen peroxide ($HO_2^{-}$). At neutral pH, $HO_2^{-}$ exists in its protonated form, $H_2O_2$. $H_2O_2$ can be converted in the presence of iron ($Fe^{2+}$) (Fenton reaction) forming hydroxyl radicals (OH•) and hydroxyl anions ($HO^{-}$), which can be reduced by $O_2^{-}$•. $O_2^{-}$• also reacts with $H_2O_2$ or OH• to produce singlet oxygen ($^1O_2$). In addition, $^1O_2$ can be generated from $H_2O_2$ and hypochlorite ($ClO^{-}$) formed by a myeloperoxidase (MPX). Further, $O_2^{-}$• and nitric oxide (NO•) form peroxynitrite anions ($ONOO^{-}$), which break into nitrogen dioxide ($NO_2$•) and OH•. The $ONOO^{-}$ can react with $H_2O_2$ to form $^1O_2$, nitroxyl anion ($NO^{-}$) and water. Since $H_2O_2$ gives rise to the formation of the highly reactive OH•, $ClO^{-}$ and $^1O_2$ it is usually detoxified in cells by the action of glutathione peroxidase (GPX) or catalase (CTL), peroxiredoxins (PRX), thioredoxins (TRX), and glutaredoxins (GRX).

These include superoxide dismutases (SOD), glutathione peroxidases (GPX), catalase, thioredoxin peroxidases (peroxiredoxins), glutaredoxins, the thioredoxin–thioredoxin reductase system and exogenously taken up micronutrients and vitamins (20, 75). In addition, several redox systems like the $NAD^+$/NADH, $NADP^+$/NADPH and oxidized glutathione/reduced glutathione (GSSG/2GSH) contribute to redox homeostasis.

Remarkably, proper folding and disulfide bond formation of proteins appears to be dependent on the redox status within the ER. Although reducing conditions prevail in the cytosol, the lumen of the ER represents a more oxidizing environment, with a higher ratio of oxidized to reduced glutathione [GSSG/GSH] (112, 245). Especially, reduced glutathione (GSH) or protein thiols react with ROS and thus contribute to cellular redox homeostasis (197). In particular, GSH is considered as the major thiol-disulfide redox buffer of cells and often the ratio between GSSG and GSH is used to determine the redox state. In the cytoplasm, the ratio of reduced glutathione to oxidized glutathione is >50:1 (64). By contrast, this ratio is 1:1 to 3:1 in the ER. This oxidizing environment is especially important for the formation of disulfide bonds characteristic for most proteins synthesized in the ER. Reciprocally, disulfides can also be reduced in the ER, and hence redox homeostasis must be maintained.

## III. THE ER AND CALCIUM HOMEOSTASIS

The ER plays a major role in fast physiological signaling due to its ability to act as a dynamic $Ca^{2+}$ store. The ER $Ca^{2+}$ store participates in the generation of rapid intracellular $Ca^{2+}$ signals following chemical activation of plasmalemmal receptors or electrical excitation of the plasma membrane (14, 188). The ER also serves as an important intracellular $Ca^{2+}$ buffer, which is required to remove excess $Ca^{2+}$ accumulated during cellular stimulation (246). The concentration of $Ca^{2+}$ inside the ER lumen ([$Ca^{2+}$]ER) controls ER homeostasis by modulating the activation of ER-located $Ca^{2+}$ release channels, the endomembrane $Ca^{2+}$ uptake mechanism, and numerous enzymatic cascades (Fig. 2). $Ca^{2+}$ levels thousands of times greater than the $Ca^{2+}$ concentration in the cytosol have been determined (25). In living cells, [$Ca^{2+}$]ER varies from around 100 μ*M* up to 800 μ*M* (47, 156, 219, 220, 246, 247). This very high intra-ER $Ca^{2+}$ concentration acts to drive $Ca^{2+}$ movement from the ER lumen to the cytosol and controls the amplitude and velocity of $Ca^{2+}$ release (25).

It has also been suggested that a leak pathway exists where $Ca^{2+}$ is released through an aqueous pore in the translocon that directs newly synthesized proteins into the ER. Normally, it is occluded by the ribosome from the cytoplasmic side and by the chaperone GRP78 (also known as BiP) from the luminal side. Inhibition of protein synthesis has been shown to induce a large increase in the leak of $Ca^{2+}$ (146). In addition, the majority of the ER resident chaperones and foldases are low-affinity, high-capacity $Ca^{2+}$-binding proteins (253). Their involvement in $Ca^{2+}$ storage is supported by the finding that chaperone functions are disturbed upon perturbation of ER-luminal $Ca^{2+}$ (38, 170, 226). In addition, intraluminal $Ca^{2+}$ levels appear to allosterically regulate the availability of intracellular $Ca^{2+}$ release channels and control the velocity of $Ca^{2+}$ uptake (25, 246).

### *A. Regulation of [$Ca^{2+}$]ER by $Ca^{2+}$ release channels*

There are two types of $Ca^{2+}$ release channels, the ryanodine receptors (RYRs) and the InsP3 receptors (InsP3Rs),

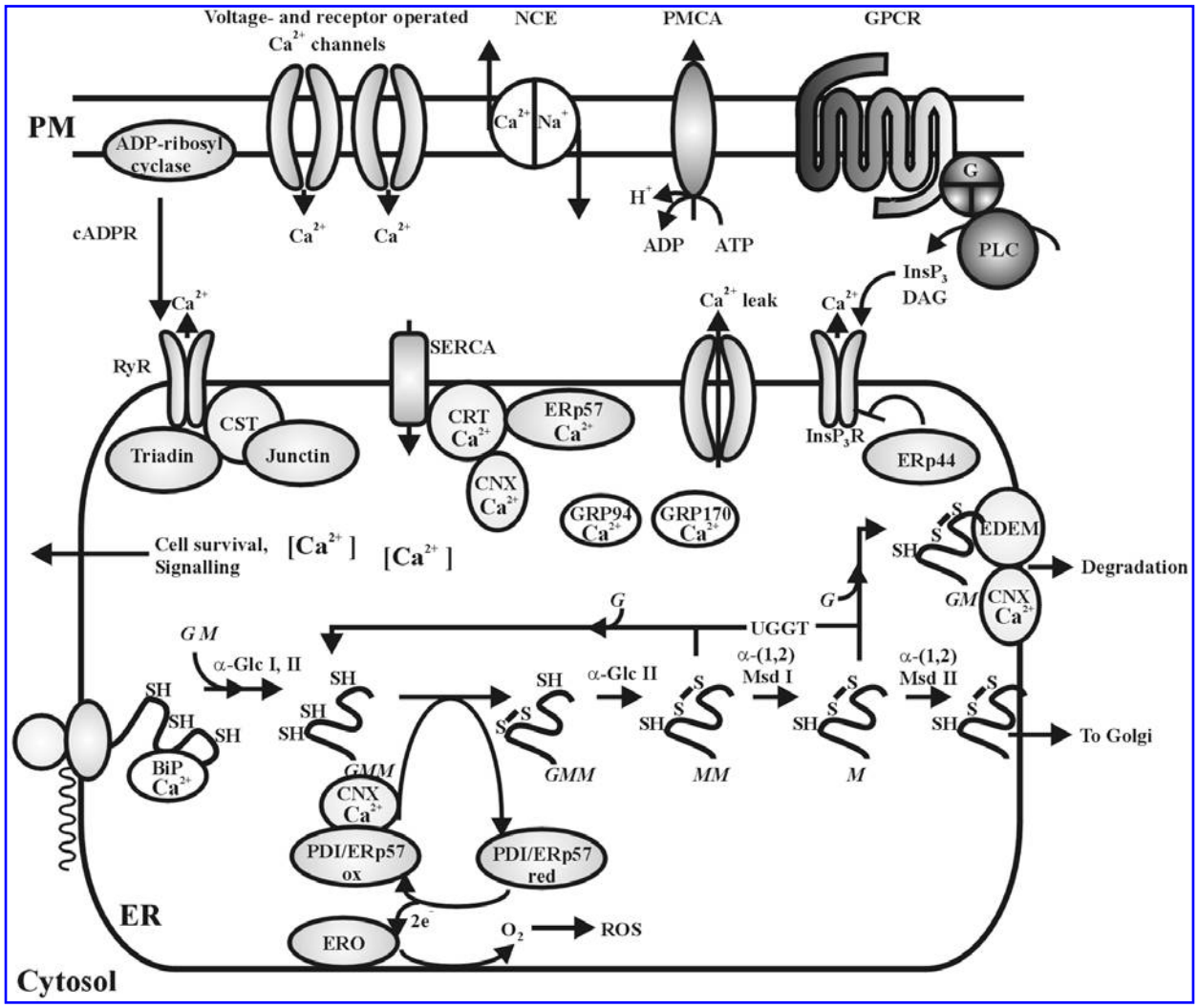


**FIG. 2. Calcium homeostasis and protein folding in the ER.** The ER plays a role as a dynamic $Ca^{2+}$ store. The concentration of $Ca^{2+}$ in the ER is regulated by the activity of SERCA, which takes up the $Ca^{2+}$ that had entered the cytosol via specific channels. $Ca^{2+}$ also required for intracelllular signaling is released by specific release channels, RyR and InsP3R, which are regulated by $Ca^{2+}$-activated second messengers such as cADPR and InsP3, respectively. In addition, a number of chaperones such as calreticulin and calnexin bind $Ca^{2+}$ thus acting as a $Ca^{2+}$ buffer and facilitating the interaction with other chaperones, specific substrates, and other ER proteins, including SERCA, RyR, and InsP3R. $Ca^{2+}$ binding chaperones such as calreticulin (CRT) and calnexin (CNX) provide the link to the other function of the ER in regulating protein synthesis. These chaperones play critical roles in folding of proteins and glycoproteins. This goes hand in hand with disulfide bond formation where oxygen represents the final electron acceptor since CRT and CNX form complexes with PDI or PDI-like catalysts such as ERp57 and bind monoglucosylated, newly synthesized glycoproteins. An α-glucosidase II and UDP-glucose:glycoprotein transferase (UGGT), which recognizes and glucosylates partially unfolded glycoproteins, contribute to de- and reglucosylation of the proteins. In conjunction with the action of α-(1,2)-mannosidase I, properly folded proteins exit this cycle to the Golgi complex. Unproperly folded and UGGT-reglucosylated proteins are subject to proteasomal degradation facilitated by interaction with CNX and the lectin EDEM. See Abbreviations for definitions.

which are sensitive to a number of input signals. One of the most important regulators is $Ca^{2+}$ itself, which can promote the release of $Ca^{2+}$, thus activating either the RYRs or the InsP3Rs, a process known as $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) (14). ClCR can link voltage-operated channels (VOCs) or receptor-operated channels (ROCs) to the release channels on the ER, particularly in cardiac muscle and neurons. Furthermore, CICR can connect the release channels on the ER to set up the intracellular $Ca^{2+}$ waves that spread signals throughout the cell, a process that requires that the release channels on the ER are in a sufficiently excitable state to respond to the $Ca^{2+}$ signals sent from their neighbors (188). The excitability depends on the level of $Ca^{2+}$-mobilizing second messengers such as InsP3 and cyclic ADP-ribose (cADPR), which can enhance the $Ca^{2+}$-sensitivity of the InsP3Rs and RYRs, respectively (14, 246). Another important determinant of excitability is the luminal level of $Ca^{2+}$ (25). Upon increases of $[Ca^{2+}]$ER to a point where the buffering

system becomes saturated, the luminal level of $Ca^{2+}$ will begin to rise. A variety of studies provided evidence that changes in [$Ca^{2+}$]ER directly affect the open probability of RyRs and modulate their sensitivity to activation by both cytosolic $Ca^{2+}$ and caffeine (9, 49, 88). This was further confirmed by direct measurements of the open probability of single RyR channels, where it was demonstrated that an increase of $Ca^{2+}$ on the luminal side of the receptor greatly (4–6 times) increases the channel open probability (33, 86, 87, 216, 217). By inducing conformational changes in the release channels elevated $Ca^{2+}$ levels can further enhance the channels' sensitivity to $Ca^{2+}$ (14, 246). Thus, an increase in sarcoplasmic reticulum's $Ca^{2+}$ load significantly (several fold) elevated the frequency of $Ca^{2+}$ sparks in muscle cells (32, 147, 199).

The permissive effects of [$Ca^{2+}$]ER to RyR gating is regulated by a complicated intraluminal $Ca^{2+}$-sensing machinery associated with RyRs. It seems to consist of a complex of three proteins, triadin-1, junctin, and calsequestrin, which, working in concert, act as a [$Ca^{2+}$]ER sensor and regulate the gating of RyRs according to fluctuations of [$Ca^{2+}$]ER (33, 86, 87).

Thus, these ER release channels function as integrators of information coming in from both the cytoplasm and from within the lumen of the ER. The fact that the excitability of the ER can vary depending on its lumen level of $Ca^{2+}$ is particularly important in neurons.

In nonexcitable cells, inositol 1,4,5-trisphosphate (InsP3) is critical to the generation of intracellular calcium ($Ca^{2+}i$) oscillations (34). Activation of membrane receptors can stimulate phospholipase C to hydrolyze phosphatidylinositol 4,5-bisphosphate and to generate diacylglycerol and InsP3, which leads to the release of $Ca^{2+}$ from the ER (181). The $Ca^{2+}i$ oscillations seem to depend on InsP3 receptors releasing $Ca^{2+}$ in "hotspots" in the ER (138), which subsequently diffuses to adjacent sites in the ER, increasing the local sensitivity of the InsP3R and inducing further $Ca^{2+}$ release. Changes in the sensitivity of the ER to InsP3 are likely to be important in the generation of repetitive $Ca^{2+}I$spikes. Although the [Ca2+]ER regulation of InsP3-induced $Ca^{2+}$ release has been shown in various studies (155, 167, 178) whether InsP3Rs are directly regulated by [$Ca^{2+}$]ER is still under debate (99, 213). However, it has been observed that there is a direct correlation between [$Ca^{2+}$]ER and InsP3-induced $Ca^{2+}$ release, further suggesting a modulatory effect of the filling state of the store on $Ca^{2+}$ release through InsP3Rs (25, 30).

### *B. Regulation of [$Ca^{2+}$]ER by $Ca^{2+}$-binding proteins*

The luminal [$Ca^{2+}$]ER interacts with a large number of $Ca^{2+}$-binding proteins, which can be roughly divided into chaperones and buffers, although there exists considerable overlap between these two functions. The buffers (*e.g.*, calsequestrin, calreticulin) have a very large capacity for $Ca^{2+}$-binding and are responsible for maintenance of the [$Ca^{2+}$]ER in the physiological range. This allows the ER to generate a large number of constant amplitude $Ca^{2+}$ signals without having to depend upon the slower pumping mechanism.

$Ca^{2+}$-sensitive chaperones such as GRP78 (BiP), GRP94 (endoplasmin), calnexin, GRP170/ORP150 or ERp57 contain multiple $Ca^{2+}$-binding sites. $Ca^{2+}$-binding to these chaperones regulates their activity. These chaperones play an important role in protein quality control, and act as a system of defence against any alterations in protein synthesis. (25). Accumulation of unfolded proteins within the ER lumen creates conditions of ER stress, either in forms of unfolded protein response or ER overload response, which leads to the upregulation of ER chaperones (67, 149, 175, 179, 194, 202).

The lectin-like chaperones calreticulin and calnexin (which form the so-called calreticulin/calnexin cycle) contribute to the posttranslational folding of many glycosylated, secreted, or integral membrane proteins synthesized within the ER. The chaperone function of both proteins is $Ca^{2+}$-dependent since their binding to glycoproteins is sensitive to [$Ca^{2+}$]ER (37, 76, 154).In addition, the interactions of calreticulin with two other chaperones, the protein disulfide isomerase (PDI) and ERp57, are also regulated by [$Ca^{2+}$]ER (37). Thus, maintenance of a constant luminal level of $Ca^{2+}$ is essential for the posttranslational processing, folding, and export of proteins (22, 25, 246). Indeed, decreases in the [$Ca^{2+}$]ER below approximately 50 $\mu M$ may completely inhibit chaperone activity. Furthermore, the disruption of ER $Ca^{2+}$ homeostasis by itself triggers ER stress, which may eventually affect cell survival (37).

### *C. Regulation of [$Ca^{2+}$]ER by SERCA*

Although the $Ca^{2+}$ used for signaling is released from the ER stores, its signaling functions depend also on replenishment mechanisms to ensure that the store remains sufficiently filled. $Ca^{2+}$ accumulation into the lumen of the ER is mediated by specific endomembrane $Ca^{2+}$ pumps, Sarco(Endo)plasmic Reticulum $Ca^{2+}$ ATPases (SERCAs) (15, 25, 246). Several subtypes (SERCAs 1–3) are variously expressed in eukaryotic cells.

Considerable evidence indicates an important regulatory role of [$Ca^{2+}$]ER in controlling the SERCA-dependent $Ca^{2+}$ uptake into the ER lumen. In a variety of studies, it was found that a high intravesicular $Ca^{2+}$ level effectively inhibits $Ca^{2+}$ uptake (25, 60, 114). Furthermore, a decrease in [$Ca^{2+}$]ER markedly increased the velocity of SERCA-dependent $Ca^{2+}$ uptake (156, 220). In both cases, agonist-induced $Ca^{2+}$ depletion of the ER led to a remarkable (5–8 times) increase in the velocity of $Ca^{2+}$ uptake, which decreased in parallel with the replenishment of the store. Subsequent studies confirmed that SERCA-mediated $Ca^{2+}$ reuptake into the ER can be regulated exclusively from the luminal side (156, 220, 246).

However, since SERCA is a $Ca^{2+}$-activated enzyme, local rises in cytosolic [$Ca^{2+}$] also have activating effects on the ER $Ca^{2+}$ uptake by SERCA (261). Such a mechanism may be responsible for an increase in [$Ca^{2+}$]ER following stimulation-induced cytosolic $Ca^{2+}$ loads.

Several studies showed that the $Ca^{2+}$ activity of SERCA is also regulated by the ER-resident chaperones calnexin, calreticulin, or ERp57, which inhibit ER $Ca^{2+}$ uptake (118, 189). It was shown that ERp57 promotes disulfide bond formation in the L4 portion of SERCA 2b, which diminishes the activity of the $Ca^{2+}$ pump. Furthermore, the complex of calreticulin-ERp57 binds to SERCA2b in a $Ca^{2+}$-dependent manner. This leads to a more effective inhibition of $Ca^{2+}$ pumps at higher [$Ca^{2+}$]ER. In contrast, decreasing [$Ca^{2+}$]ER resulted in the

dissociation of the calreticulin-ERp57 complex from the SERCA2b, and thus to enhanced activity of the $Ca^{2+}$ pump (25, 29, 118, 143).

Since some losses of [$Ca^{2+}$]ER are always occurring that result in a decline in the signaling capacity of ER stress signaling pathways, the cell employs a store-operated entry mechanism to keep the [$Ca^{2+}$]ER constant (185). This entry mechanism has also been referred to as capacitative $Ca^{2+}$ entry (CCE). When the store is full, entry of $Ca^{2+}$ through the store-operated channels (SOCs) is low, but as soon as the store content of $Ca^{2+}$ starts to decline, entry begins, thus maintaining the internal levels of $Ca^{2+}$. This mechanism can also contribute to $Ca^{2+}$ signaling in particular under conditions of prolonged $Ca^{2+}$ signaling as occurs during stimulation of cell proliferation (13)

There is now substantial evidence that a small specialized region of the ER closely associated with the plasma membrane regulates the entry of $Ca^{2+}$. Activation of receptors in the plasma membrane may stimulate phospholipase C to produce localized microdomains where InsP3 functions to deplete $Ca^{2+}$ in the subsurface ER cisternae. The latter then send a message to the SOCs in the plasma membrane to promote $Ca^{2+}$ entry (14). However, the exact nature of the signal coming from the ER is not completely clear (185). Some of the proposed mechanisms include the generation of a calcium influx factor, exocytosis of vesicles containing SOCs or a conformational-coupling mechanism whereby the release channels in the ER interact directly with the SOC channels in the plasma membrane (14). The latter mechanism resembles that found in skeletal muscle where the L-type $Ca^{2+}$ channels in the plasma membrane directly interact with the type 1 RYRs in the underlying SR membrane. Depolarization in the plasma membrane results in a change in conformation of the L-type $Ca^{2+}$ channels that then lead to opening of the RYR1 channels through a direct protein–protein interaction (201).

### *D. $Ca^{2+}$ and protein synthesis*

$Ca^{2+}$ homeostasis in the ER has also important implications for protein synthesis. $Ca^{2+}$ is required for the maintenance of protein synthesis in almost all mammalian cell types with the exception of reticulocytes. $Ca^{2+}$ sequestered in the ER supported early protein processing, a process that is coupled to the phosphorylation of initiation factor 2 (eIF2) and regulation of the rate of translational initiation (22). When $Ca^{2+}$ is depleted from the ER, glycoproteins are retained in the "high mannose" configuration (35, 138). This seems to allow the addition of a "glucose tag", provided by an UDP-glucose glucosyl transferase (UGGT). Subsequently, the glycoproteins form a complex with calnexin, calreticulin, and PDI-like catalysts, which results in ER retention (96, 177). Upon $Ca^{2+}$ repletion of the ER, the glucose tag is removed and the complexes dissociate, thus allowing the properly folded and transport-competent proteins to exit the ER.

In contrast, relatively little is known regarding potential actions of ER sequestered $Ca^{2+}$ upon the folding of nonglycosylated proteins, the assembly of protein oligomers, or the cotranslational translocation of nascent chains into the lumen. It has been suggested that $Ca^{2+}$ may assist folding of the growing polypeptide chain by neutralizing or temporarily crosslinking carboxyl groups associated with glutamate- or aspartate-rich regions (22). It could also promote subunit assembly or specific proteolytic processing activities. Alternatively, the high $Ca^{2+}$ levels may be required for the activity of the ER folding machinery. High luminal $Ca^{2+}$ concentrations may also be essential for the associations that form between chaperone(s) and peptide chains during co-translational translocation and subsequent folding events (22).

$Ca^{2+}$ sequestered in the ER stores is also required for maintaining optimal rates of translational initiation (21). Depletion of ER $Ca^{2+}$ stores can occur in response to hormones generating InsP3, to EGTA and other chelating agents, to thapsigargin, a sesquiterpene lactone that inhibits SERCA, and to different agents modulating the passage of $Ca^{2+}$ across the ER membrane to the cytoplasm, such as the $Ca^{2+}$ ionophores ionomycin and A23187, unsaturated fatty acids such as arachidonic acid, and various peptide metalloendoprotease antagonists such as carbobenzoxy-Gly-Phe-amide (22). The depletion of [$Ca^{2+}$]ER can result in the inhibition of amino acid incorporation, disappearance of polysomes with accumulation of monosomes and ribosomal subunits, a reduction in the cellular content of 43S preinitiation complex, and the phosphorylation of eIF2α and inhibition of eIF2B (22). However, with the exception of thapsigargin, which is an irreversible inhibitor of [$Ca^{2+}$]ER accumulation, the effects of $Ca^{2+}$-depleting agents can be reversed within several minutes by the addition of supraphysiologic $Ca^{2+}$ concentrations to the extracellular medium under defined conditions.

### *E. $Ca^{2+}$ as messenger in the ER–mitochondria couple*

The function of the ER is closely linked to that of the mitochondria. These two organelles build a dynamic network where they cooperate in the generation of $Ca^{2+}$ signals. Mitochondria modulate the $Ca^{2+}$ signal released from the ER and assist during the recovery phase by rapidly sequestering $Ca^{2+}$ and then later returning it to the ER. Under normal conditions, most of the $Ca^{2+}$ is present in the lumen of the ER except during $Ca^{2+}$ signaling when small amounts are periodically released to the cytoplasm and then resequestered with a part passing through the mitochondria. During normal signaling, therefore, there is a continuous up and down of $Ca^{2+}$ between these two organelles (14). When the normal equilibrium is disturbed, $Ca^{2+}$ moves from the ER lumen into the mitochondria, thereby involving a number of proteins regulating apoptosis. Interestingly, the proapoptotic Bcl-2 homology 3 (BH3) domain-only proteins (Bak, Bax, Bid, Spike) and the antiapoptotic Bcl-3 protein family member Bcl-2 and Bax inhibitor protein 1 localize to the ER membrane. Apoptotic stimulation of Bid induces a conformational change in Bak and Bax, promoting their oligomerization and formation of an ion pore at the ER membrane, resulting in $Ca^{2+}$ efflux. Similarly, Bak and Bax oligomerization in the outer mitochondrial membrane results in $Ca^{2+}$ influx. The ER calcium release appears also to involve an ER transmembrane and stress-induced effector protein called BiP-associated protein (BAP), which is cleaved by caspase-8. Thus, movement of $Ca^{2+}$ to the mitochondria leads to the activation of a number of stress signals that can induce the onset of apoptosis by ac-

tivating formation of the permeability transition pore (PTP). The PTP contributes to the initiation of cell death pathways, either by causing ATP depletion and energetic collapse (186) or by promoting the release of cytochrome c and/or apoptosis-inducing factor (AIF) (for reviews, see Refs. 12, 39, 131).

### *F. ROS and $Ca^{2+}$ in the ER*

Cells have evolved a sophisticated mechanism of intracellular signaling based on localized changes in the oxidation state of specific proteins (198). Since the internal environment of cells is normally highly reducing, an increase in the oxidative state can act as an important intracellular trigger (14).

Oxidative stress from different sources due to enhanced ROS levels causes $Ca^{2+}$ influx into the cytoplasm from the extracellular environment and from the ER or SR through the cell membrane and the ER/SR channels, respectively. Rising $Ca^{2+}$ concentrations in the cytoplasm promote $Ca^{2+}$ influx into nuclei and mitochondria (see above). In the nucleus $Ca^{2+}$ modulates gene transcription and nucleases that control cell apoptosis. Both in the nucleus and cytoplasm, $Ca^{2+}$ can regulate phosphorylation/dephosphorylation of proteins, thus modulating signal transduction pathways (56).

Mitochondrial $Ca^{2+}$ loading can stimulate mitochondrial metabolism with subsequent increased generation of ROS. High ROS generation within mitochondria initiates a sequence of events in which the oxidative stress increases the probability of $Ca^{2+}$ release from the ER. The very close proximity of ER and mitochondria leads to the accumulation of $Ca^{2+}$ by nearby mitochondria, further sensitizing the mitochondria to the ROS, which finally can cause the PTP to open (115). Furthermore, these ROS also can feedback to sensitize the $Ca^{2+}$ release channels on the ER (14).

An important mechanism how ROS can affect ER $Ca^{2+}$ signaling relates to the selective oxidative modification of unique sites of calcium regulatory proteins (248, 249). The relative oxidizing environment of the ER compared to the cytosol was suggested to contribute to the preferred oxidation of ER resident proteins (245).

The SERCA can be inhibited both by oxidation of its sulfhydryl groups (200) and nitration of specific tyrosines on the ATP-binding site (249). Furthermore, hydroxyl radicals can directly affect the ATP-binding site (259), resulting in decreases in ATP-dependent calcium fluxes across membranes and hence decreased ATP consumption. The depletion of calcium in the ER inhibits protein synthesis (223) and processing, leading to the accumulation of partially folded proteins. This is followed by the activation of the transcription of ER chaperone genes, such as GRP78/BiP, GRP94, and calreticulin, to increase the capacity of intracellular calcium stores and to prevent cellular calcium toxicity (144). These findings suggest that oxidative modifications contribute to a decline in the function of the SERCA (184) and an increase in intracellular calcium levels (222). On the other hand, a decreased mitochondrial ATP production, as a result of lower ATP consumption by the ER, might result in decreased ROS production. Thus, reducing equivalents generated in the mitochondrial matrix due to the decreased ATP synthesis can contribute to cytosolic antioxidant defense mechanisms and cellular repair processes that minimize the accumulation of oxidized biomolecules (221). Recently, ROS have also been shown to lead to prolonged complex formation between unfolded SERCA2a and calreticulin, which may be a prerequisite for the inactivation and degradation of SERCA2a (113).

As stated above, SERCA2b activity is modulated by ERp57, an ER luminal oxidoreductase, in a [$Ca^{2+}$]ER- and redox state-dependent manner (143). Since the pump activity of SERCA2b is higher when thiol groups in its luminal loop are reduced, $Ca^{2+}$ uptake by SERCA2b should be enhanced by ERp57 when the ER luminal environment shifts to the reducing condition, thus leading to enhanced [$Ca^{2+}$]ER.

Recently it has been shown that ERp44, a thioredoxin family protein, specifically binds to reduced cysteine residues in the L3V domain of the InsP3R1, which appears to directly inhibit $Ca^{2+}$ release via InsP3R1 (98). Thus, SERCA2b and InsP3R1 seem to work together to increase [$Ca^{2+}$]ER under reducing conditions. This is reasonable since a reduced ER luminal environment is unfavorable for protein folding and increasing [$Ca^{2+}$]ER benefits the function of the many chaperones and oxidoreductases that require a relatively high [$Ca^{2+}$]ER. Indeed, ERp44 was identified as a protein that forms mixed disulfide bonds with Ero1$\alpha$, an ER oxidoreductase (4), and ERp44 was shown to be involved in ER retention of Ero1$\alpha$ (3). Since Ero1 family proteins play pivotal roles in oxidative protein folding (238), ERp44 may play dual roles in protein folding: inhibiting $Ca^{2+}$ release (which reinforces $Ca^{2+}$-dependent chaperones) by inactivating InsP3R1 and supporting disulfide bond formation by reinforcing the Ero1$\alpha$/oxidoreductase system. The fact that ERp44 is induced during the unfolded protein responses (3) fits well with this model (98).

Consistently, there is ample evidence that ROS also affect the ER $Ca^{2+}$ release mechanisms. Oxidized glutathione (97) and ROS derived from xanthine/xanthine oxidase (252) induce $Ca^{2+}$ release from InsP3-mediated stores in endothelial cells. Additionally, superoxide may stimulate InsP3-mediated $Ca^{2+}$ release in vascular smooth muscle cells (227).

As the opening of the PTP is also regulated by thiols (128), it seems that the redox sensitivity of the InsP3R and the PTP leads to enhanced sensitivity of this whole system to oxidative damage by initiating a positive feedback cycle, whereby increased mitochondrial ROS production enhances the local probability of ER $Ca^{2+}$ release and mitochondrial $Ca^{2+}$ loading. This may further enhance ROS generation (52) and sensitize the mitochondria to ROS, culminating in PTP opening (115).

ROS may also modulate the gating of ryanodine-sensitive $Ca^{2+}$ channels in the SR (18, 101) probably through modification of critical thiol groups (127). Furthermore, evidence has been provided that the RyR is sensitive to the local redox potential (61, 256). Redox reactions may play a critical role in controlling the kinetics of the $Ca^{2+}$ release mechanism. ROS may act as redox active signaling molecules to activate $Ca^{2+}$ transport (228). Under mild oxidative stress, small changes to the cellular redox potential resulted in significant stimulation of the RyR and sensitized the $Ca^{2+}$ release mechanism to activation.

Furthermore, ROS have been suggested to act on $Ca^{2+}$ release from the cardiac sarcoplasmic reticulum (SR) by affect-

ing cADPR, which is involved in $Ca^{2+}$ mobilization. ROS appear to have dual effects that are concentration-dependent. At low concentrations (nearly nanomolar levels), ROS enhanced $Ca^{2+}$ release by stimulating the synthesis of cADPR, which in concert with calmodulin sensitizes RyRs. At higher concentrations (micromolar levels), ROS inhibit the function of calmodulin thus inhibiting RyRs (166).

In addition to mitochondria, also other ROS-generating enzymes provide important sources of ROS. The NADPH oxidase, a complex enzyme system consisting of regulatory cytosolic subunits and membrane-bound catalytic subunits, is known to generate large amounts of superoxide in phagocytic cells (6). Several isoforms of NADPH oxidases characterized by the differential expression of homologues primarily of the catalytic subunit gp91phox (NOX2) have been identified in nonphagocytic cells and seem to provide ROS for cellular signaling processes (81, 82, 136). Whereas NOX2 has been identified in the plasma membrane in leukocytes, NOX homologues have been observed intracellularly in some nonphagocytic cells (140). Evidence has been provided that some of the NOX homologues can be also present in the ER and colocalize with calnexin (243) or calreticulin (Fig. 3). Although the functional importance of these observations is not clear to date, recent findings showing that NOX1 and NOX4 interact with PDI suggest an important regulatory function of the ER on cellular ROS production in addition to the interaction with mitochondria (116). Consistently, it has been shown that activation of an endothelial NADPH oxidase increases the sensitivity of endoplasmic reticulum $Ca^{2+}$ stores to InsP3 (109). Possibly, NADPH oxidase-derived ROS may lead to the sensitization of the InsP3R and subsequent $Ca^{2+}i$ oscillations. Furthermore, 7-ketocholesterol induced ROS production by activation of a NOX4-containing NADPH oxidase, which triggered an endoplasmic reticulum stress response with intracellular $Ca^{2+}$ oscillations, and the induction of the expression of the cell death effector CAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) and of the GRP78/BiP chaperone via the activation of IRE-1 as hallmarks of the unfolded protein response (UPR) (181). In line with these observations, it has been shown that superoxide generated by an endogenous NADH oxidase present in the SR increases RyR activity (257). Interestingly, NADH oxidase activity, ryanodine binding, and activation of ryanodine-binding activity induced by NADH copurified in sucrose gradient fractions, suggesting that the RyR1 contains NADH oxidase activity. Although it is not clear to date whether this oxidase is similar to the NADPH oxidases, such an enzyme generating superoxide anions may support a low level of oxidative stress, which increases the open probability of the $Ca^{2+}$ release channel. Interestingly, preconditioning tachycardia induces translocation of cytosolic NADPH oxidase subunits to a SR-enriched fraction isolated from dog ventricular muscle and to concomitantly increase *S*-glutathionylation of RyR2 and enhance $Ca^{2+}$ release (196).

## IV. THE ER AND OXIDATIVE PROTEIN FOLDING

A common feature of any protein is its requirement for a specific three-dimensional structure to fulfil its biological function. In many proteins this native conformation is stabilized by intra- or intermolecular disulfide bonds. For most proteins containing more than one disulfide bond, the correct spatial arrangement of these disulfide bonds is absolutely essential. If the disulfide bonds are not formed or positioned in the correct spatial arrangement, the protein will not attain its functional conformation, resulting in misfolding and malfunction of the protein.

When Anfinsen and colleagues recognized that the conditions required for successful *in vitro* refolding of reduced ribonuclease (high dilution, high pH, etc.) were not physiological, and that the time-scales involved (hours to days) were much longer than those for protein folding in the cell, they proposed specific cellular catalysts (55). Subsequently, they and other groups showed that the refolding of proteins could be catalyzed by microsomal preparations from vertebrate secretory tissues such as liver and pancreas (79). These microsomes predominantly contained enzymes derived from the ER. It is now commonly accepted that the luminal environment of the ER of uni- and multicellular eukaryotes favors the maturation of secreted and membrane-associated proteins by providing the required catalysts and an oxidizing environment suitable for native disulfide bond formation (Fig. 4). It also provides a quality control system through which those proteins are eliminated that are not correctly folded (58). In the case of a protein with an ensemble of incorrect disulfide bonds, which cannot be converted into the native ones, the protein usually is reduced and retrotranslocated into the cytosol where it will be subjected to degradation (58).

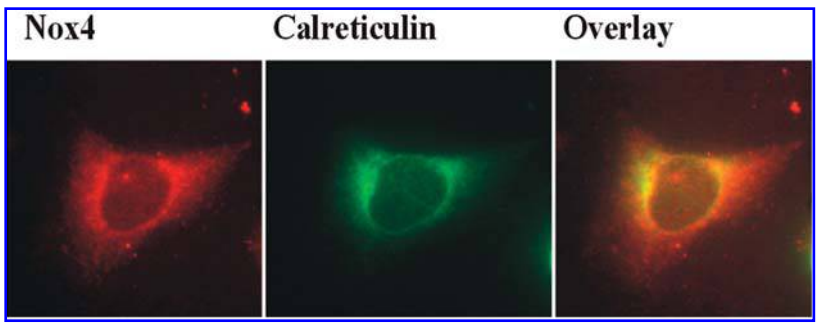


**FIG. 3. NOX4 co-localizes with calreticulin in EaHy926 cells.** NADPH oxidases are important sources of ROS in many cell types. However, their cellular localization in nonphagocytic cells is unclear. In this experimental set-up, co-immunofluorescence was performed on endothelial EaHy926 cells using antibodies against one of the NADPH oxidase subunits, NOX4 (*red*) as well as against calreticulin (*green*). Co-localization of both proteins is indicated in *yellow.* This experiment suggests that the NADPH oxidase subunit NOX4 is localized in the ER in endothelial EaHy926 cells (181a).

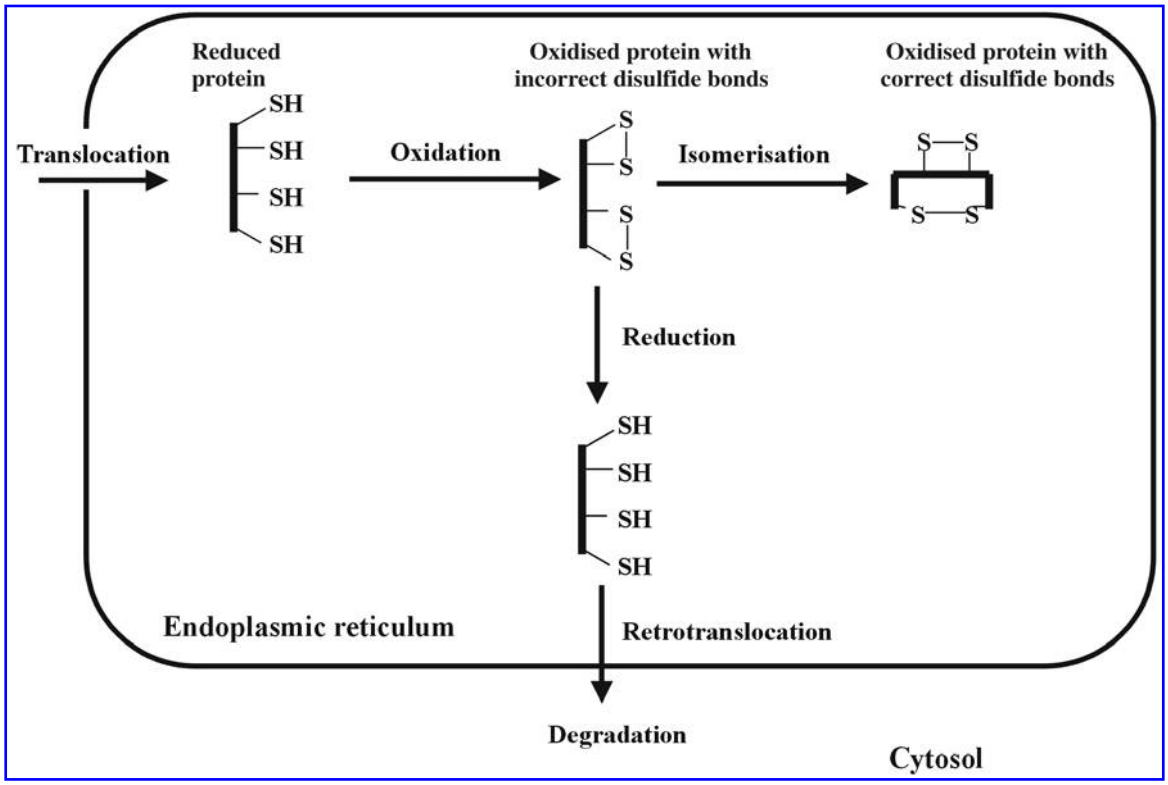


**FIG. 4. General scheme of thiol:dithiol exchange mechanism in the ER.**

Native disulfide bond formation can occur via multiple parallel pathways, and there is growing evidence that a large number of different gene families and redox carriers may play a role in the supply of redox equivalents for protein disulfide bond formation.

## *A. Mechanisms of oxidation, reduction, and isomerization of disulfide bonds*

*a. Oxidation.* Undoubtedly, one of the fundamental functions of the ER is the oxidation of cysteine residues in nascent polypeptide chains to form intramolecular disulfide bonds.

The oxidation process starts with a nucleophilic attack of a thiolate in the substrate on the disulfide bond in the catalyst (step 1), generating a mixed disulfide bond (Fig. 5A). Subsequently this bond is attacked by a second thiolate in the substrate (step 2), thus forming an intramolecular disulfide bond with concomitant release of the reduced catalyst. In addition to the dependence on the $pK_a$ of the thiols in the substrate, the oxidation is also dependent on the stability of the disulfide bond in the catalyst. To act as an oxidant, the reduced form of the enzyme must be stabilized relative to the disulfide state. Furthermore, a futile reaction would occur if the second thiolate in the catalyst attacked the mixed disulfide bond (step 4, Fig. 5A). To act as an efficient thiol–disulfide oxidant, factors that decrease the nucleophilicity of the second thiolate increase the oxidase activity of the catalyst (Fig. 5B). In analogy to the formation of an intramolecular disulfide bond, the nucleophilicity of the second thiolate in the catalyst affects the oxidase activity.

*b. Reduction.* If a protein cannot attain its native ensemble of disulfide bonds, then reduction of the disulfide bonds is required for retrotranslocation and subsequent proteasome-dependent degradation in the cytosol (58). Furthermore, reduction might also be mechanistically linked to isomerisation of disulfide bonds (see below).

Reduction starts with a nucleophilic attack of a disulfide bond in the substrate by a thiolate of the catalyst (step 3), which generates a mixed disulfide bond (Fig. 5A). This bond is then attacked by a second thiolate from the catalyst (step 4), thus generating a disulfide bond in the catalyst and two reduced thiolates in the substrate. Although this process is the reverse reaction of the oxidation, it is important to note that in this case the reaction is dependent on the pKa values of the thiols in the enzyme and the stability of the disulfide bond in the substrate and the enzyme. In contrast to the oxidation process, however, factors that increase the nucleophilicity of the second thiolate in the catalyst also increase its reductase activity. An analogous mechanism can be formulated for the reduction of intermolecular disulfide bonds.

*c. Isomerization.* Proteins with an incorrect ensemble of disulfide bonds very often misfold into nonnative structures. The rate-limiting step for native disulfide bond formation in proteins that contain multiple disulfides is late-stage isomer-

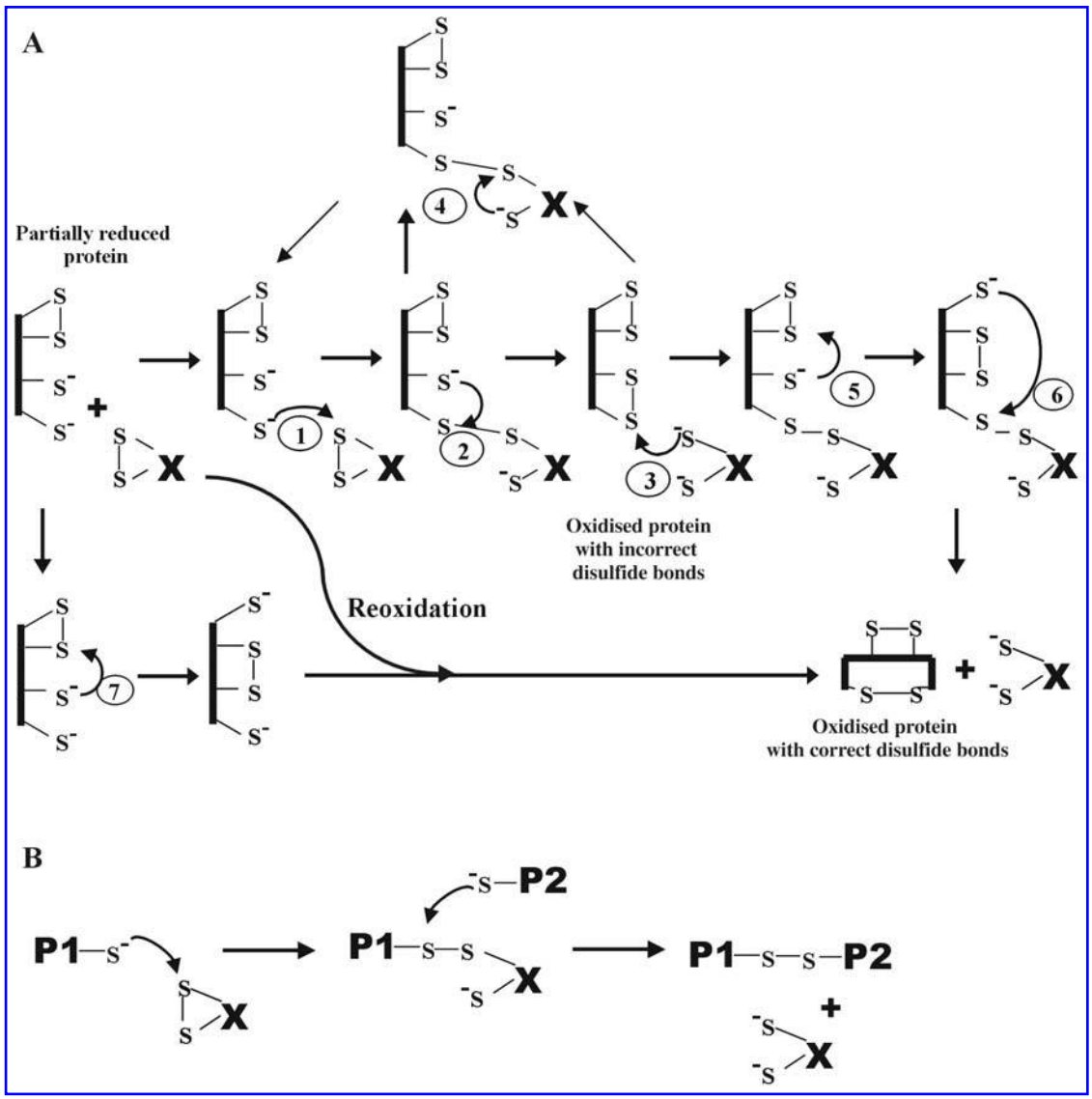


**FIG. 5. Oxidation of thiols, reduction, and isomerization of disulfide bonds.** (**A**) Oxidation and reduction is only shown for one disulfide bond. Isomerization of disulfide bonds can occur directly or via a reduction/reoxidation cycle. The individual steps (1–7) are explained in the text. X symbolizes the catalyst. (**B**) Intermolecular disulfide bond formation. P1 and P2 denote different proteins.

ization reactions, where disulfide bond formation is linked to conformational changes in protein substrates with substantial regular secondary structure. This implies that the conversion of the incorrect disulfide bonds into the native ensemble requires a partial unfolding of the already formed structures.

Isomerization of incorrect disulfide bonds begins with the nucleophilic attack of a disulfide bond in the target protein by a thiolate of the catalyst (step 3), which generates a mixed disulfide bond (Fig. 5A). This process produces a nucleophilic thiolate in the substrate, able to attack another disulfide bond (step 5). Eventually a thiolate will attack the mixed disulfide bond between the substrate and the catalyst (step 6), leading to the formation of a new disulfide bond and the release of the catalyst. It is noteworthy to mention that in this mechanism only one thiolate of the catalyst is involved in the process. In other words, the reaction is insensitive to factors that affect the nucleophilicity of the second thiolate in the catalyst.

In addition to this mechanism, an alternative one that proceeds via a reduction/reoxidation cycle can be formulated (Fig. 5A). This process comprises two separate steps, the first of which is the reduction of the substrate disulfide bond with a nucleophilic attack of a thiolate on an intramolecular disulfide bond (step 7). In a second step, free thiolates in the substrate are reoxidized by the catalyst as described earlier. An important difference to the isomerization mechanism described above is that in this process the second thiolate of the catalyst is essential for the reduction process. In fact, it was shown that isomerization, catalyzed by protein disulfide isomerase, occurs through rapid cycles of oxidation and reduc-

tion (205). Factors that increase the nucleophilicity of the second thiolate therefore increase the reductase activity of the catalyst in the first step, but reduce the oxidase activity in the second step.

It is important to emphasize that oxidation and reduction of disulfide bonds is linked to the transfer of two electrons, whereas the rearrangement of disulfide bonds (isomerization) is electroneutral.

## *B. Factors affecting the oxidase/reductase activity of the catalyst*

Whether a catalyst has oxidase or reductase activity depends strongly on the redox-equilibrium between the dithiol and disulfide form. To act as an oxidase, the dithiol state of the enzyme must be stabilized relative to the disulfide state. The equilibrium constant $K_{ox}$ is usually expressed for the reaction between a dithiol and glutathione:

$$\text{Dithiol} + \text{GSSG} \rightarrow \text{disulfide} + 2\ \text{GSH}$$

$$K_{ox} = \frac{[disulfide]\text{x}[GSH]^2}{[dithiol]\text{x}[GSSG]} \qquad (1)$$

The redox potential can be calculated using the Nernst equation:

$$E_0 = E'_{0(GSH/GSSG)} - \frac{RxT}{nxF}\ \text{x}\ \log K_{ox} \qquad (2)$$

with $R$ = gas constant, $T$ = temperature, $n$ = number of electrons, $F$ = Faraday constant, $E'_{0(GSH/GSSG)} = -0.240$ V at pH 7.0 and 25°C (254).

The ability to act as an oxidase or reductase also depends on the nucleophilicity of the attacking thiolate, which is generated through abstraction of a proton from the thiol:

$$\text{R-SH} \rightarrow \text{R-S}^- + \text{H}^+$$

The $pK_a$ of the thiol is affected by the microenvironment of the thiol and adjacent amino acids. Very often thiol:disulfide oxidoreductases contain a catalytic domain with the active site motif Cys–X–X–Cys at the N terminus of an alpha helix (see below). Using DsbA from *Escherichia coli* as a model system, Huber–Wunderlich and Glockshuber investigated the effect the X–X dipeptide has on the redox properties. They found that replacing the X–X dipeptide of DsbA (Cys30–Pro31–His32–Cys33) with the dipeptides of eukaryotic protein disulfide isomerase (PDI; Gly–His), glutaredoxin (Pro–Tyr), and thioredoxin (Gly–Pro) from *E. coli* resulted in less oxidizing DsbA variants. Furthermore, they also found that the replacement led to a strong increase in the $pK_a$ of the nucleophilic cysteine (Cys30) (110). Lappi and colleagues showed that a conserved arginine residue in several members of the human protein disulfide isomerase family has a very marked effect on the $pK_a$ value of the active site cysteine residues and is critical for the catalytic function of these thiol:disulfide oxidoreductases (137).

## *C. Components involved in oxidative folding in the ER*

*a. Protein disulfide isomerase.* Protein disulfide isomerase (PDI), an enzyme found in the endoplasmic reticulum of eukaryotic cells, was the first catalyst of oxidative protein folding to be identified. Within the last 30 years, the role of PDI in cellular protein folding has been established beyond doubt by the classical methods of biochemistry, cell biology, and molecular genetics. In whole cells, and in cell-free systems for *in vitro* translation and translocation, PDI can be cross-linked to nascent and newly translocated secretory proteins (121, 191). Removal of luminal resident proteins in microsome preparations leads to defective folding of translocated proteins, which is repaired by reconstitution with purified PDI (24). The gene encoding PDI in *Saccharomyces cerevisiae* has been cloned and has been shown to be required for the proper folding, targeting, and secretion of disulfide-bonded proteins (51, 135). However, the precise role of PDI in the cell is still not entirely clear. Whereas Laboissiere ascribed the essential function of yeast Pdi1p to the unscrambling of nonnative disulfide bonds, thus acting as an isomerase (134), other reports demonstrated the vital role of this protein as an oxidase (218, 258).

Experiments using PDI from various sources established that the effectiveness of PDI as a catalyst of native disulfide bond formation in folding polypeptides depends on the ability to catalyze disulfide–dithiol exchange, to bind nonnative proteins, and to trigger conformational changes in the bound substrate, thus allowing access to buried cysteine residues (72, 74). The catalysis of disulfide bond isomerization is most prominent over the noncatalyzed rate in a glutathione buffer that mimics the redox environment of the ER. Under these conditions, the greatest enhancement of rate is for late-stage isomerization process [*i.e.*, the rate-limiting steps for native disulfide bond formation (157)].

PDI seems able to catalyze all of the steps in native disulfide bond formation for all substrate proteins reported. It is still unclear how PDI recognizes all of these different folding states, from essentially unfolded through to substrates with almost native conformations but lacking specific disulfide bonds. Yet PDI does not appear to interact with correctly folded and disulfide-bonded substrate proteins (122).

## *D. Structure–function relationship of PDI*

The cloning and sequencing of rat PDI cDNA (53) led to the recognition of internal sequence homologies within the protein and the suggestion of a structural organization based on duplicated sequence modules. PDI was recognized as a member of the thioredoxin superfamily of proteins, containing four thioredoxin-like domains in the order **a–b–b′–a′** and a C-terminal extension, including a KDEL ER-retention motif. The two homologous catalytic domains, **a** and **a′** with the active site motif -CGHC- are separated by two homologous noncatalytic domains **b** and **b′** (63, 71–73).

Recently a small angle X-ray scattering study of intact human PDI in solution was presented. The restored model reveals that PDI is a short and roughly elliptical cylinder with a molecular mass of 69 kDa and dimensions of 105 x 65 x 40 A. The four thioredoxin-fold domains are arranged in an an-

nular fashion, which accounts for the cooperative properties in both the isomerase and chaperone functions of PDI (142). The crystal structure of yeast Pdi1p reveals that all four domains have a characteristic (βα)βαβαββα thioredoxin fold. They are arranged in the shape of a twisted "U" with the active sites facing each other across the long sides of the "U." The inside surface of the "U" contains the principal peptide binding domain **b′** (123) and is enriched in hydrophobic residues, thereby facilitating interactions with misfolded proteins (230). The most noteworthy difference between the **a** and **a′** domains is the redox state of their active site cysteine residues. Under equilibrium conditions, the two cysteines in the **a** domain are primarily in the oxidized state, forming a disulfide bridge, while their counterparts in the **a′** domain are in the reduced state (230). The equilibrium constant $K_{ox}$ for the oxidation of the cysteine pair in each active site by oxidized glutathione is 17 m*M* for the **a** domain and 1 m*M* for the **a′** domain. The value for the yeast Pdi1p **a′** domain is similar to the ones for human PDI with $K_{ox}$ for both domains being in the range of 1–2 m*M* (44–46, 148). The redox potentials for the different domains are given in Fig. 6.

For yeast Pdi1p, these values predict that the **a** domain is more stable in the oxidized form, while the **a′** domain prefers to be in the reduced form, in good agreement with the structure. One can therefore assume that in yeast Pdi1p the function of the **a′** domain is the oxidation of a substrate protein. This supposition has been confirmed by Tian and colleagues, who showed that mutation of both active site cysteines in the first active site of Pdi1p has less effect on the oxidative folding of denatured ribonuclease than mutation of the cysteines in the second active site (230). Furthermore, in yeast only the **a′** domain, expressed at wild-type levels, could complement a *PDI1* deletion. To show a similar effect, expression levels of the **a** domain had to be significantly higher than those of the **a′** domain. Isomerization reactions, however, seemed to be catalyzed by the **a** domain, since the isomerization-dependent rate of folding of carboxypeptidase Y *in vivo* was reduced by inactivation of the **a** domain but not the **a′** domain (102). Taken together, these results indicate that in yeast Pdi1p the two catalytically active domains fulfill different functions: whereas the **a** domain seems to be involved in isomerization of disulfide bonds, the **a′** domain appears to be required for the oxidation of sulfhydryls (230).

For mammalian PDI, however, the almost identical $K_{ox}$ values and redox potentials indicate that both active sites are more stable in the reduced form, and hence the active site disulfides are supposed to be oxidizing (*i.e.*, weak, unstable). It is therefore tempting to speculate that in human PDI both catalytically active domains fulfill a similar function, probably related to oxidation of thiols.

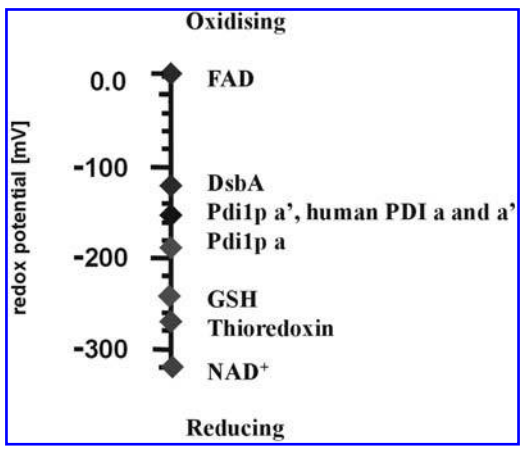


**FIG. 6. Redox potentials of PDI domains.** Redox potentials were calculated from Kox constants for thioredoxin from (148), for DsbA from (266), and for PDI domains from (231).

## *E. Other members of the PDI family*

In addition to previously known members of the PDI-like family members PDI, PDIp, ERp57, ERp72, P5, PDIr, ERp28 (62) and TMX1 (150), several new human PDI-family members have been described recently including ERp18 (2, 124), ERp44 (3, 4), ERp46 (124), ERdj5 (42), TMX2 (152), and PDILT (244), as reviewed previously (54). In total there are now 14 PDI-family members in the human ER, with a wide range of domain architectures and active site chemistries. A similar picture has been established for plants (108). The biological functions of these proteins have not yet been identified in most cases. However, there seems to be significant overlap between the catalytic functions, as demonstrated by experiments in *S. cerevisiae*. Lethal knockouts of yeast Pdi1p can be rescued by overexpression of some specific mammalian PDI-like proteins (85, 130). These findings highlight that different members of the PDI-family can complement each other, at least to a certain degree, and therefore might fulfill similar biological functions. However, it was reported that the ability of the overexpressed Pdi1p homologues to restore viability to a *PDI1*-deleted strain was dependent on the presence of low endogenous levels of one or more of the other homologues. Only Mpd1p was capable of carrying out all the essential functions of Pdi1p, which indicates that the homologues are not functionally interchangeable (164, 165).

Apart from the archetypal mammalian PDI, ERp57 is probably the best-studied member of the PDI-family in multicellular eukaryotes. ERp57 has been shown to act *in vitro* and *in vivo* on glycosylated substrates through interaction with the ER-resident lectins calnexin and calreticulin (168, 265). The **b′** domain of ERp57 is adapted for lectin association and cannot be replaced by that of archetypal PDI (183, 193, 215, 240); however, other domains also contribute to the interaction of ERp57 with the P-loop of calreticulin (215). From these results it has been proposed that the region of ERp57 equivalent to the primary substrate binding site of archetypal PDI is occupied by calreticulin, thus acting as an adaptor molecule that defines the substrate specificity of ERp57 (193). Although *in vitro* ERp57 efficiently catalyzes disulfide reduction, disulfide isomerization, and dithiol oxidation in substrate proteins, *in vivo* its activity is dispensable for co-translational oxidation of cysteines in nascent model glycoproteins. Recent *in vivo* studies indicated that ERp57 is required for the late-stage isomerization of disulfide bonds leading to acquisition of the native conformation (59,117).

### *F. Reoxidation of the catalyst*

Compounds that catalyze the formation of disulfide bonds in proteins become reduced and hence have to be reoxidized to fulfill further catalytic cycles. The final oxidant in eukaryotic systems is most likely molecular oxygen, but other oxidants might be utilized under anaerobic conditions (83). *In vitro* the rate-limiting step for PDI-catalyzed disulfide bond formation is the reoxidation of PDI, which is commonly achieved through the addition of a glutathione redox-buffer, containing reduced (GSH) and oxidized (GSSG) glutathione. It is not clear what the function of glutathione in the context of disulfide bond formation is *in vivo*, but GSSG and GSH occur in the ER at concentrations favorable for the formation of disulfide bonds. The ratio of reduced (GSH) to oxidized (GSSG) glutathione in the secretory pathway is between 1:1 and 3:1. This is considerably more oxidizing than the cytosolic ratio of 30:1 to 100:1 (111) and similar to the optimum for *in vitro* folding of disulfide bond containing proteins. It therefore has been assumed for many years that GSSG is the immediate source of oxidizing equivalents *in vivo*. To achieve this ratio, it was proposed that GSSG is preferentially transported from the cytosol into the ER lumen (112). However, recently it was shown that GSH is transported into mammalian liver microsomal vesicles and that GSSG accumulates in the lumen of the ER (7). How GSH is oxidized to GSSG is not entirely clear, but Cuozzo and Kaiser (43) demonstrated that in yeast the product of the *ERO1* gene could provide oxidizing equivalents to protein thiols and glutathione in the secretory pathway (see below). It also has been suggested that GSSG can be generated in the ER through the action of a flavin-containing monooxygenase (224, 225).

GSSG can either directly form mixed disulfides with the protein substrate, hence acting as the direct oxidant, or reoxidize the oxidative catalyst (*e.g.*, PDI). Directly supporting the involvement of GSSG as an oxidant is the finding that mutant forms of lysozyme are secreted from *S. cerevisiae* with unpaired cysteine residues in mixed disulfides with glutathione (92).

The high levels of glutathione-protein mixed disulfides within the ER lumen suggest that such conversion of an exposed free thiol to protein-SSG is not exceptional (8). One can speculate that as a protein is translocated into the ER, free cysteine residues rapidly form mixed disulfides with GSSG, producing a protein–S–SG. These proteins, containing both glutathione mixed disulfides and free thiols, are excellent substrates for PDI (192). In a subsequent step PDI then catalyzes isomerization reactions to generate an intramolecular protein disulfide bond (Fig. 7). This process is then repeated with further isomerization of intramolecular disulfide bonds by PDI to finally produce a native ensemble of disulfide bonds.

Contradicting a vital role of glutathione in disulfide bond formation is the finding that a yeast strain in which a key enzyme of glutathione synthesis has been eliminated, is still viable (68). This indicates that there are other components present in the ER that provide oxidizing equivalents for disulfide bond formation. However, there is no reason to exclude the possibility that GSSG is part of the normal oxidative pathway for secretory proteins, since in the absence of GSSG a normally minor direct oxidative pathway may become the major pathway (8).

The idea that GSSG is the main oxidant of thiols has been further challenged by the discovery of endoplasmatic reticulum oxidoreductin 1p (Ero1p). Ero1p is a novel FAD-binding

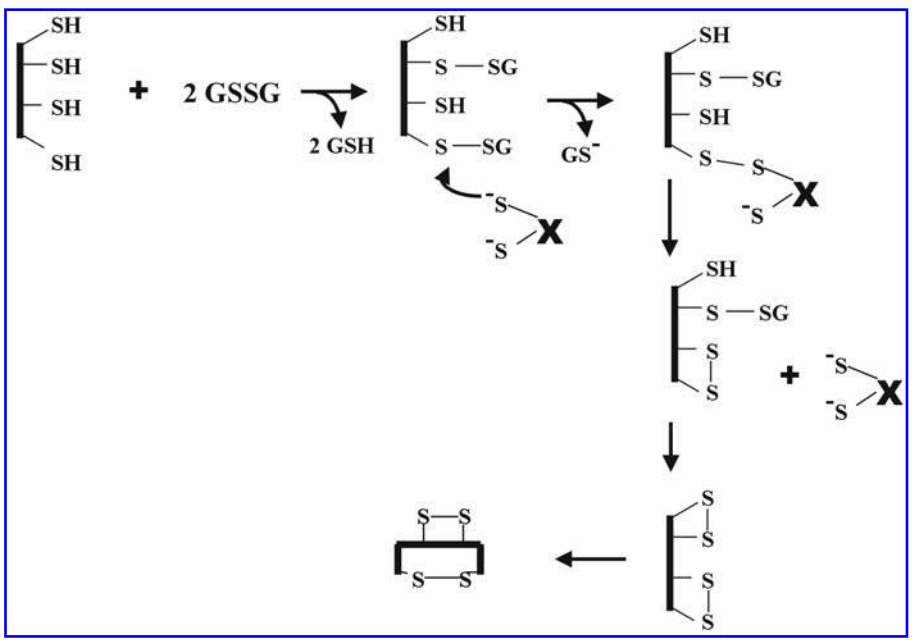


**FIG. 7. Isomerization of disulfide bonds in the presence of glutathione.** GSSG, oxidized glutathione; GSH, reduced glutathione. Final steps of the isomerization process are analogous to the ones described in Fig. 7. X symbolizes the catalyst.

protein essential in *S. cerevisiae* (70, 182), which interacts with yeast Pdi1p and the PDI-like protein Mpd2 (69). In humans, there are two ERO1 isoforms, hERO1-La and hERO1-Lb (26, 173, 174), both of which lack a COOH-terminal tail of ~127 amino acids required by the yeast protein for membrane association (174). *In vivo*, membrane association of Ero1p may allow the protein to be retained in the ER and facilitate cotranslational disulfide bond formation. Ero1p possesses seven conserved cysteine residues that are likely involved in catalyzing electron transfer (70, 182). Consistent with a function in folding in the ER, yeast Ero1p and hERO1-Lb are induced by the unfolded protein response (68, 174, 182), whereas the expression of hERO1-La is stimulated by hypoxia (78).

It is not known whether hEro1-La/b have preferential substrates or differential activities; however, both proteins interact with PDI (26), ERp44 (4) and PDILT (244). Although no evidence for a disulfide-dependent linkage has been shown for PDILT, these interactions appear to form disulfide-linked complexes. It therefore has been speculated that Ero1 is the main oxidant of reduced PDI.

Mutations in the *ERO1* gene in *S. cerevisiae* lead to hypersensitivity to reducing conditions and an accumulation of reduced proteins in the ER, including yeast Pdi1p (68;69). Human and yeast Eros can provide oxidizing equivalents through a set of conserved cysteine residues in a characteristic CXXCXXC motif to PDI (11). Two essential cysteine triads (Cys85–Cys94–Cys99 and Cys391–Cys394–Cys397) cooperate in electron transfer, with Cys94 likely forming mixed disulfides with PDI. Under aerobic conditions the final electron acceptor is most likely molecular oxygen (236, 237), whereas under anaerobic conditions a variety of small and macromolecular electron acceptors including soluble FAD have been reported (83).

Recently, yeast Ero1p has been shown to selectively oxidize the active site cysteine residues in the **a′** domain of yeast Pdi1p (235). Further analysis demonstrated that Ero1p oxidizes the isolated **a** and **a′** domains of Pdi1p at the same rate. However, in full-length Pdi1p, there is an asymmetry in the rate of oxidation of the two active sites (132). This asymmetry is reflected in the redox potential of the individual active site domains, thus resulting in different biological functions: while the **a** domain is predominantly involved in isomerization processes and hence maintained in the reduced state, the **a′** domain is oxidized by Ero1p. Since the two active sites of mammalian PDI do not show this difference in redox potential (see above) it is tempting to speculate that both domains fulfill a similar function (*i.e.*, oxidation of thiols). It is therefore not unreasonable to assume that both active site domains of human PDI are substrates for Eros.

Interestingly, Eros from yeast and mammalian systems do no appear to form covalent complexes with newly synthesized cysteine-rich proteins, which makes it likely that Eros are not involved in direct oxidation of thiols in reduced proteins other than PDI (11). In addition, Ero1p from yeast, and most likely human Eros as well, do not directly oxidize GSH to GSSG. In fact, deletion of a key enzyme of glutathione synthesis suppresses the temperature-sensitive phenotype of an *ERO1* mutation in yeast (68, 69). This result can be explained by assuming that Ero1p and GSH have antagonistic functions: while Ero1p keeps PDI in the oxidized form, thus promoting oxidation of thiols, GSH is required for the reduction of PDI, essential for isomerisation of disulfide bonds.

This supposition has been further strengthened by findings showing that overexpression of Ero1 led to the acceleration of disulfide bond formation and correct protein folding. However, lowering the levels of glutathione within the cell resulted in acceleration of disulfide bond formation but did not lead to correct protein folding (31).

Only human and yeast PDI, the human ER protein ERp44 (4) and the yeast PDI-like protein Mpd2p (69) are substrates for the respective Eros, which suggests that only these proteins are involved in the oxidation of thiols. Since none of the other mammalian members of the PDI family, such as ERp72, ERp57 or P5 have been found to interact with Eros (153), it is tempting to speculate that the function of these enzymes is the isomerization of disulfide bonds, which does not require the catalyst to be in the oxidized state. This is supported by the findings of Jessop and Bulleid, who showed that ERp57 is indeed directly reduced by GSH at physiological concentrations and that ERp57 and glutathione form mixed disulfide bonds in microsomes (117).

The temperature-sensitive phenotype of an *ERO1* mutant in yeast can be suppressed by overexpression of the protein Erv2p (77, 207), a member of the ERV/ALR family of sulfhydryl oxidases. Purified recombinant Erv2p is a FAD-binding protein that catalyzes $O_2$-dependent formation of disulfide bonds. Like Ero1p, Erv2p transfers oxidizing equivalents to Pdi1p by a dithiol–disulfide exchange reaction. For the activity of Erv2p, two pairs of cysteine residues are required which may transfer electrons between substrate protein dithiols and the FAD (84).

Other candidates for the reoxidation of PDI and PDI-like proteins could be the recently discovered quiescin-like sulfhydryl oxidases (QSOXs) (36, 104, 106, 229) (Fig. 8). They contain a thioredoxin domain and a small FAD-binding domain homologous to yeast Erv2p. Although nothing is known about the role these enzymes play *in vivo*, it has been speculated that they could provide a different pathway for disulfide bond formation by directly oxidizing cysteine residues in the substrate protein with the reduction of oxygen to hydrogen peroxide (104, 105). Chicken egg white sulfhydryl oxidase utilizes an internal redox-active cystine bridge and a FAD moiety in the oxidation of a range of small molecular weight thiols such as glutathione, cysteine, and dithiothreitol (104, 105). Although the $K_M$ of this QSOX for the oxidation of reduced glutathione is rather high (20 m*M*) (104), it is not impossible that oxidized glutathione is generated through this pathway. Furthermore, *in vitro* experiments showed that QSOXs are efficient oxidases, but are almost entirely devoid of isomerase activity. It is therefore tempting to speculate that QSOXs might catalyze the oxidation of cysteine residues while PDI and other PDI-like proteins are involved in subsequent isomerization steps. This speculation is in line with recent results that QSOXs show high synergism with mammalian PDI in the activation of reduced RNase (104). Whether the QSOXs directly oxidize the substrate, with PDI catalyzing the isomerization, or whether QSOXs can act as an oxidant for PDI is not clear. However, the complete pathway for protein disulfide bond formation in poxviruses was re-

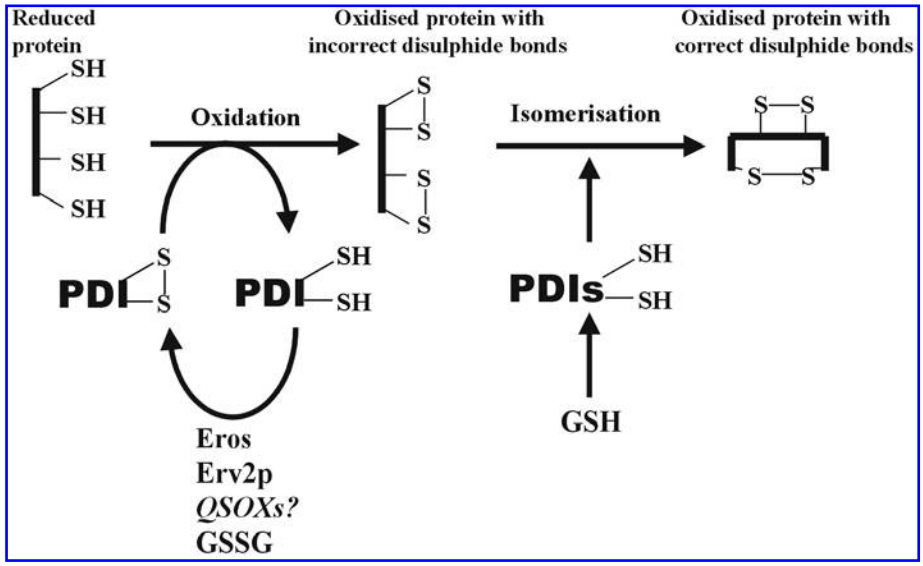


**FIG. 8. Components involved in pathways of disulfide bond formation.** Components specific for multicellular systems are *italicized*. PDIs indicate members of the PDI family. *Question marks* indicate that no clear evidence exists yet, whether QSOX acts as PDI oxidant.

cently established and it was shown that a sulfhydryl oxidase, a small alpha-helical protein with a CxxxC motif and a thioredoxin-like thiol oxidase are sufficient for the formation of disulfide bonds (206).

Although the ALR protein is related to Erv2p, and there may have been a fusion event, no QSOXs have been reported in yeast (10). Thus, it may be speculated that different pathways of native disulfide bond formation might exist in yeast and mammalian systems.

## V. FROM ER TO NUCLEUS: THE UNFOLDED PROTEIN RESPONSE AND MODULATION OF TRANSCRIPTION FACTOR FUNCTIONS

After protein synthesis, posttranslational modifications and folding within the ER contribute to maturation of the protein. However, some proteins never reach maturity and those misfolded proteins are commonly recognized as such in the ER lumen with the help of the lectin EDEM (Fig. 2) (95, 157). Folding failures are then retrotranslocated to the cytosol, where they are submitted to ubiquitination and proteasomal degradation, a process referred to as ER-associated degradation (ERAD).

Further, if ER function is perturbed by various pathological conditions, the entry of newly synthesized proteins may exceed the folding and modification capacity, resulting in accumulation of unfolded proteins and ER stress. This accumulation leads to an activation of signaling events known as the unfolded protein response (UPR) which should rebalance folding capacity and folding demand within the ER (Fig. 9). To that aim, the folding capacity of the ER is increased, whereas the folding load is decreased. The latter is achieved by downregulating transcription and translation of genes encoding secretory proteins (89) as well as increasing the ERAD of slowly folding or misfolded proteins (234). By contrast, the folding capacity is enhanced after enlargement of the ER (50) and the synthesis of ER resident molecular chaperones and foldases (129).

In response to ER stress, three distinct signaling components involving double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK), also called pancreatic eukaryotic initiation factor 2α (eIF2α) kinase (PEK), the inositol requiring kinase 1 (IRE1), and activating transcription factor 6 (ATF6) are triggered (Fig. 9). In addition, $Ca^{2+}$ is released from the ER to activate apoptotic signaling pathways.

Under nonpathological conditions, all three components associate with the abundant luminal chaperon BiP (also known as glucose-regulated protein 78, GRP78; or Kar2p in yeast) and this interaction keeps these signaling molecules in an inactive state. Once unfolded proteins accumulate in the ER, BiP preferentially associates with the unfolded proteins instead of PERK, ATF6, and IRE1, resulting in activation of their downstream signaling molecules (for further excellent reviews, see Refs. 203 and 242).

### *A. PERK signaling*

PERK is an ER-resident serine/threonine protein kinase with an ER-luminal domain associated with BiP. Accordingly, PERK may be activated through dissociation from BiP (16) leading to dimerization and trans-autophosphorylation of the cytosolic kinase domain that promotes phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) (119) (Fig. 9). Although, phosphorylation of eIF2α subsequently inhibits translation–initiation, and thus protein synthesis, to prevent further influx of nascent proteins into an already saturated ER lumen (89, 190), its phosphorylation

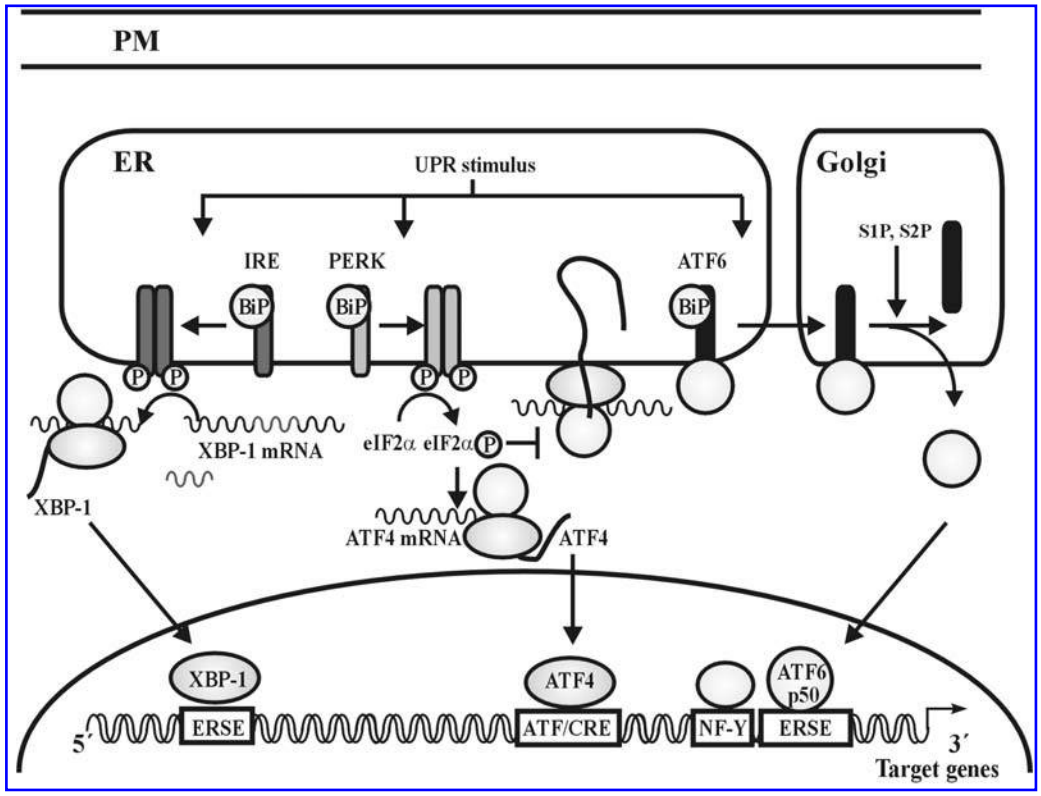


**FIG. 9. The unfolded protein response.** Stimulation of the unfolded protein response (UPR) results in dissociation of BiP from either IRE, PERK, or ATF6. Subsequent dimerization and activation of IRE lead to XBP1 mRNA splicing, translation of the spliced RNA, and activation of XBP1-regulated genes mainly involved in ER-associated degradation (ERAD). PERK dimerization and activation leads to phosphorylation of eIF2α, which activates ATF4 translation and inhibits translation in general. BiP dissociation from ATF6 allows its transport to the Golgi where cleavage by S1P and S2P facilitate release of ATF6p50, which induces genes involved in augmentation of folding capacity. See Abbreviations for definitions.

induces translation of activating transcription factor ATF4 and subsequent expression of the ATF4 target genes, growth arrest, and DNA damage-34 (GADD34) and CAAT/Enhancer binding protein (C/EBP) homologous protein (CHOP) (5).

In addition, another direct PERK target appears to be the bZIP Cap'n'Collar transcription factor nuclear factor erythroid-2 (NF-E2)-related factor-2 (Nrf2), which appears to integrate a variety of responses to oxidative stress. Under basal (reducing) conditions, Nrf2 is found in an inactive cytoplasmic complex with the cytoskeletal anchor Keap1, which targets Nrf2 for ubiquitination and proteasomal degradation (41). Interestingly, a recent study has revealed an intimate correlation between accumulation of ROS and ER stress (90). Whereas tunicamycin, an inhibitor of protein N-linked glycosylation, only weakly induced accumulation of ROS in wild-type cells, the same treatment induced marked accumulation of ROS in cells lacking PERK. In addition, Nrf2-/- cells are very sensitive to ER stress (41). Thus, when there is ER stress, PERK phosphorylates Nrf2, resulting in dissociation of the Nrf2–Keap1 complex, nuclear localization of Nrf2, which, after heterodimerically partnering with other transcription factors, activates transcription through the antioxidant response element (ARE) (162). The ARE controls expression of genes involved in xenobiotic metabolism such as glutathione *S*-transferase A1 and A2, γ-glutamylcysteine synthetase, heme oxygenase 1, NAD(P)H:quinone oxidoreductase, and UDP-glucuronosyl transferase (162).

Furthermore, ER stress may also act on the level of Keap1. Keap1's ability to bind to Nrf2 is regulated by critical cysteine residues (267, 268), which could be targeted by ROS. Indeed, it was shown that the C151, C273, and C288 residues are critical in Keap1-dependent ubiquitination and proteasome-mediated degradation under basal conditions (267, 268).

Together, two mechanisms may account for Nrf2 activation: direct phosphorylation of Nrf2 by PERK, PKC, MAPK, or PI3K (1, 40, 161, 176), or modification of Keap1 at C273 and C288, which prevents capturing of Nrf2 and proteasomal degradation (48, 250).

Further, it was shown that the transcription factor NF-κB can be activated in response to ER stress (175). Since this response was inhibited by the use of antioxidants, these findings underline the link between ER stress and redox homeostasis. However, the redox-sensitivity of NF-κB activation appears to be cell-type dependent (reviewed in Refs. 19 and 141) and the action of the antioxidants such as *N*-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) may also occur independently from their antioxidant properties (91). Thus, NF-κB activation does not seem to be an universal response to oxidative stress.

### *B. ATF-6 signaling*

The second signaling pathway is mediated by the basic leucine zipper-type transcription factor, ATF6, of which two homologous proteins, ATF6α and ATF6β/cAMP-response-element-binding protein (CREB)-related protein (CREB-RP)/G13 have been identified. ATF6α is synthesized as a type II transmembrane precursor protein with a molecular mass of 90 kDa (p90 ATF6), and anchored to the ER membrane where it is retained by BiP. In response to ER stress, BiP dissociation (209) and underglycosylation (103) lead to the transport of ATF6 into the Golgi complex (Fig. 9). Here ATF6 is sequentially cleaved by two proteases. Site-1 protease (S1P), a serine protease, cleaves ATF6 in the luminal domain and the N-terminal part is cleaved by the metalloprotease site-2 protease (S2P) (262)). The processed form of ATF6 (p50) translocates to the nucleus and binds to the ATF/cAMP response element (CRE) and to the ER stress responsive element (ERSE) together with NF-Y, and subsequently activates target genes. Many genes encoding ER chaperones, including BiP, glucose-regulated protein 94, and calreticulin contain an ERSE in their promoter regions and are induced by ATF6α (264).

Further, ATF6β (93) may provide negative regulation of ATF6α, whereas another transcription factor with similarity to ATF6 named OASIS acts as activator via ATF/CRE promoter elements instead of ERSEs (125).

Interestingly, the proteases S1P and S2P are also involved in the processing of the transcription factor sterol response element binding protein (SREBP), which is activated when cholesterol levels decrease (80) SREBPs (SREBP1a, SREBP1c, SREBP2) belong to the basic helix–loop–helix (bHLH) transcription factors and are also found in the ER membrane together with the tryptophan (W)–aspartic acid (D) repeat (WD repeat) domain of SREBP cleavage-activating protein (SCAP) and the insulin-induced gene 1 protein (INSIG-1) (107). Thus, a decrease in cholesterol levels and SREBP activation may be linked to the UPR since cholesterol also functions as an important membrane component. Indeed, it was found that SREBP2 can form a complex with ATF6α and the histone deacetylase complex 1 (HDAC1) at the low-density lipoprotein receptor (LDLR) promoter, thus suppressing its expression (266). Thus, this modulatory action may adjust membrane fluidity during ER stress.

Recently, a liver-specific transcription factor called CREBH forming complexes with ATF6α has been characterized, which plays a role in ER stress and inflammation (269). CREBH, like ATF6 and SREBP, is activated by regulated intramembrane proteolysis (RIP) involving S1P and S2P. Transit of activated CREBH to the nucleus activates expression of serum amyloid P-component and C-reactive protein. Further, proinflammatory cytokines induced cleavage of CREBH in the liver *in vivo* (269). Thus, CREBH represents a link how ER stress may initiate a tissue-specific reaction like activation of an acute inflammatory response in liver. It is tempting to speculate that similar regulatory loops may exist, which couple ER stress to tissue-specific reactions.

### *C. IRE1 signaling*

The third signaling pathway is composed of IRE1 and the bZIP transcription factor of the ATF/CREB family X-box binding protein-1 (XBP-1) (Fig. 9). In mammals, IRE1α (231) and IRE1β (251) consist of an unfolded protein sensor domain in the ER lumen and a cytosolic serine threonine kinase and ribonuclease domain. Under normal conditions BiP associates with the luminal domain of IRE1 (16, 120). When proteins accumulate in the ER lumen, dissociation of BiP leads to dimerization of IRE1, its autophosphorylation and activation of the RNase activity (208, 214, 231). Once activated, IRE1 cuts 26 nucleotides out of the XBP-1 mRNA. The remaining ends are then relegated by an RNA ligase, which is known as Rlg1p in yeast, but still to be identified in mammals. However, IRE1-mediated splicing of XBP-1 mRNA results in encoding of transcriptionally active XBP-1 protein (28, 210, 264). In the nucleus, XBP-1 can transactivate targets with ERSE or mammalian UPRE upstream activating sequences in conjunction with the general transcription factor NF-Y (264).

## VI. REDOX HOMEOSTASIS AND ER FUNCTIONS

Accumulating evidence has indicated a crosstalk between generation of ROS and the ER stress response (Fig. 10). Although a redox imbalance can be caused by affecting PERK and ATF-4 functions because they induce genes that are involved in elimination of ROS (40, 90), most ROS in the ER are generated via Ero1. Indeed, disulfide-bond formation involving Ero1 contributes significantly to the total production of ROS in the cell (90, 238). In addition, overexpression of both Ero1 proteins shifts the redox state of PDI towards the oxidized form, which thus also influences oxidation of PDI substrates (153, 158). During disulfide bond formation, electrons pass several thiol–disulfide exchange reactions, the thiols of the substrate, PDI and Ero1 from where they finally reach molecular oxygen (238). The incomplete reduction of oxygen results in formation of superoxide anion radicals, which can then be dismutated to $H_2O_2$ or converted to other ROS (see above).

That superoxide formation and its dismutation by superoxide dismutase are important for ER function is underlined by findings from transgenic mice expressing mutant CuZn-superoxide dismutases. These mice show aggregate formation leading to extensive dilation of the ER and degeneration of motor neurons within the spinal cord, similar as in patients with amyotrophic lateral sclerosis (159, 211, 232, 239). Whether the ER dilation seen in these mice is the direct effect of ROS on protein folding or the more indirect result via activation of signaling events leading to accumulation of unfolded proteins in the ER is not clear now. However, the CuZn-superoxide dismutase-induced degeneration of neurons can be blocked by overexpression of the cytosolic chaperone heat-shock-protein 70 (HSP70) (23, 255), suggesting that cytosolic ROS generation and protein folding in the ER are connected in a more indirect manner. This is further supported by the finding that mutant CuZn-superoxide dismutase induces BiP (GRP78) levels (232).

The connection between oxygen, ROS, energy metabolism, and ER resident proteins is also underlined by the fact that some of the glucose regulated proteins (GRPs) (212)

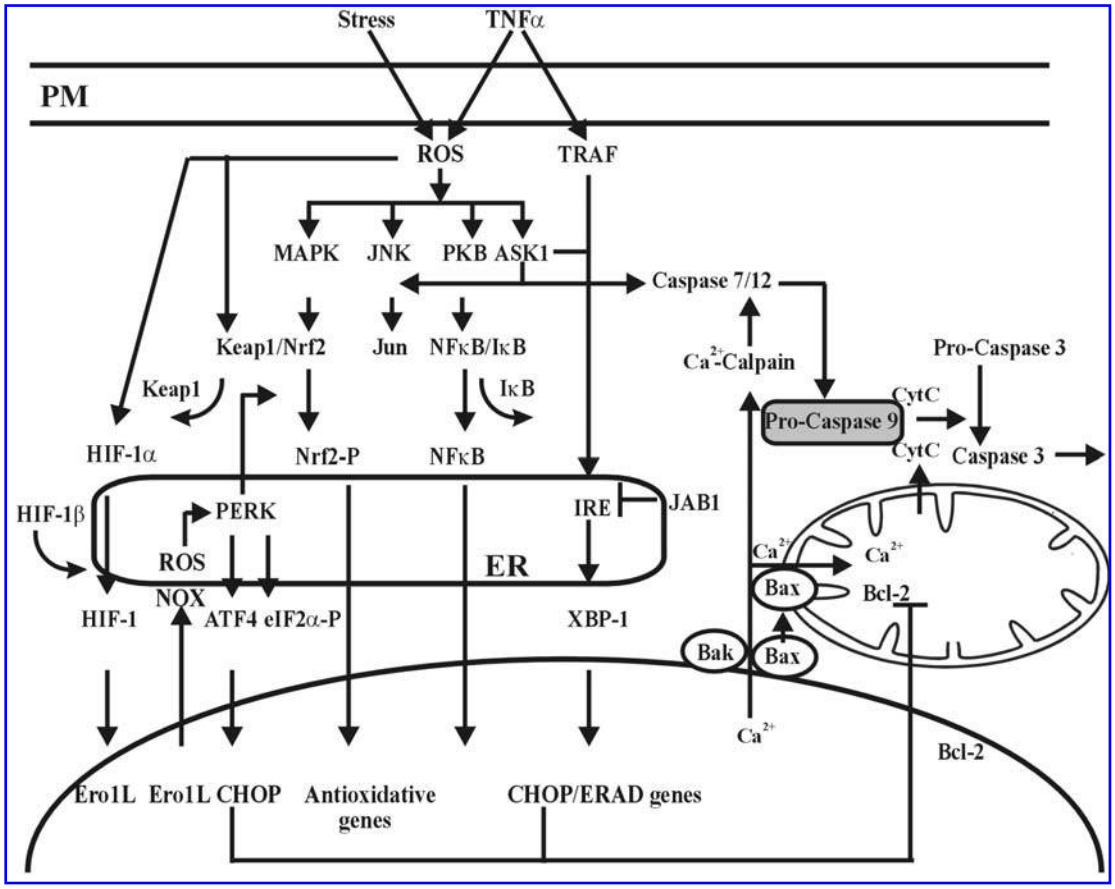


**FIG. 10. Reactive oxygen species-mediated signaling between cellular compartments.** Stress situations or cytokines like TNFα change intracellular levels of ROS. Once ROS levels are changed, they modulate the activity of a number of kinases such as MAP kinases, PKB, and ASK1, as well as the activity of the transcription factors HIF-1α, Keap1, and NFkB. In addition, TNFα via ROS and ASK1 can activate IRE and thus XBP-1-dependent genes. Activation of HIF can induce Ero1L expression and thus ROS production in the ER, which can also be achieved by NOX proteins. Further ROS formed in the ER activates PERK and subsequently Nrf2, eIF2α, and ATF-4. Redox-regulation of ASK1 is achieved by thioredoxin, an interacting partner of ASK1 that forms also a complex with IRE1α and TRAF2. Interestingly, JAB-1 may be a negative feedback regulator since it interacts with |IRE1α. Apoptotic changes in ROS levels can initiate ASK1 and IRE1α activation and subsequently caspase-7 and caspase-12, as well as JNK activation, contribute to cell death. Apoptotic stimulation of proapoptotic Bak and Bax at the ER membrane promotes formation of an ion pore, resulting in $Ca^{2+}$ efflux. Released $Ca^{2+}$ activates the protease calpain, which again contributes to caspase-7 and -12 activation. Similarly, Bak and Bax oligomerization in the outer mitochondrial membrane results in $Ca^{2+}$ influx, which can induce the onset of apoptosis by promoting the release of cytochrome c. See Abbreviations for definitions.

are also known as oxygen regulated proteins (ORPs) (94, 133, 171, 172). In line with this, severe hypoxia or anoxia could also activate ATF4 (17, 57). Hypoxia-dependent expression of GRP170/ORP150 appears to have an important cytoprotective role and act as inhibitor of apoptosis (172). In addition, GRP170/ORP150 expression promoted VEGF expression and angiogenesis in wounds of diabetic mice (171). Interestingly, the hypoxia-induced expression of GRP78 was mediated by protein kinase C, thus linking expression of ER proteins to oxygen sensing signaling pathways (126). However, aside from ATF4, hypoxia primarily acts via a family of transcription factors known as hypoxia-inducible factors (HIF). These proteins consist of an oxygen-sensitive α-subunit and a constitutive β-subunit (100). From the three factors known to date, HIF-1 is the best characterized and has its own targets distinct from those of ATF4 (205). Although ER stress and anoxia induce ATF4, the latter was shown to be independent of HIF-1. By contrast, the $O_2$-sensitive HIF-1α subunit localizes to the perinuclear ER membrane under normoxia, where ROS such as hydroxyl radicals are generated in an iron-dependent Fenton reaction (145), thus linking ER function to the HIF-1 pathway. Inhibition of the Fenton reaction under hypoxia results in stabilization of HIF-1α and its migration to the nucleus, where it dimerizes with HIF-1β and induces transcription of its target genes (145). Ero1 is the most likely candidate for the production of these ROS (238). Interestingly, Ero1Lα but not Ero1Lβ appears to be under transcriptional control from HIF-1 (78). Thus, ROS-dependent HIF-1α regulation may be part of a feedback cycle contributing to thiol-oxidation in the ER via adaptation of Ero1Lα transcription to the overall cellular redox status.

However, prolonged ER stress and activation of the UPR may also contribute to apoptosis via accumulation of ROS. This is illustrated by the finding that decreased expression of the PERK homolog in *C. elegans* reduced its life span. This is in line with data showing that ER stress induces accumulation of ROS in PERK or ATF-4 knockout cells to higher levels than in wild-type cells (90). The role of ROS within regulation of cell death and life span is then further supported by the finding that repression of Ero-1 restored normal life span in *C. elegans* with repressed PERK expression (90).

Further, TNFα induced the UPR and cell death in a ROS-dependent fashion in murine fibrosarcoma L929 cells (260). However, UPR induction by pretreatment with tunicamycin substantially inhibited TNFα-induced ROS accumulation and cell death. Within that scenario, the tumor necrosis factor receptor-associated factor 2 (TRAF2) may be one of the components linking the UPR and cell death. TRAF2 interacts with the cytosolic domain of IREα (263). TRAF2 also binds to apoptosis signal-regulating kinase 1 (ASK1). TRAF2 has also been shown to promote caspase-7 and caspase-12 activation (160, 187, 263), although the redox connection appears to be with ASK1. Within this scenario formation of a trimeric complex consisting of IREα, TRAF2, and ASK1 activates ASK1 (151, 163, 241). ASK1, which activates c-Jun N-terminal kinase (JNK) and also p38 MAP kinase (p38) pathways (66, 195) can also be activated via ROS. This redox-regulation is achieved by thioredoxin, another interacting partner of ASK1. Oxidative stress disrupts the ASK1-thioredoxin complex by oxidization of thioredoxin and thereby activating ASK1 (233) and subsequently JNK, p38, and cell death. Interestingly, the Jun activation domain-binding protein (JAB-1, also known as COP9 signalosome subunit 5) may be a negative feedback regulator since it was shown that it interacts with IRE1α (159) and that constitutively active JAB-1 mutants inhibited XBP-1 splicing and GRP78 transcription (169). Thus, ER stress and oxidative stress may induce cell death by using the same molecular complex consisting of IREα/TRAF2/ASK1/thioredoxin with JAB1 as negative regulator.

## VII. CONCLUSION

Although it is clear that the ER has a central role in the oxidative folding of proteins, the precise mechanisms that lead to the native structure of a disulfide bond-containing protein, as well as the cellular components required are not yet understood in their entirety. The reason for this uncertainty is that in addition to well-known key enzymes, like PDI, other catalysts seem to contribute to this process in parallel and potentially overlapping pathways.

From the study of uni- and multicellular organisms, it is clear that similar components and mechanisms exist in both systems. To understand the organization of disulfide bond formation at a molecular level *in vivo,* the key question is therefore how the different cellular components contribute to this fundamental cell biological process. This is important for the understanding of protein folding and misfolding *in vivo*, and how these processes promote cell survival, or cell death associated with a number of diseases including diabetes mellitus, atherosclerosis, Alzheimer's, Huntington's and Parkinson's diseases (see Ref. 203 for a comprehensive list). From this perspective it is apparent that eukaryotic cells expressing immense quantities of correctly folded secretory proteins containing disulfide bonds would also be at risk for ROS accumulation from prolonged ER stress. These cells are presumably exposed to levels of ER-derived oxidative stress proportional to the number of nascent disulfide bonds being formed. If cells expressing high amounts of proteins contain mutations either within the proteins to be secreted or the proteins responsible for folding, it would result in an enormous amount of ER-retained and misfolded proteins that are over the threshold of ERAD capacity. In addition, the continuously acting folding/refolding processes and the UPR would deplete the cells of GSH and lead to ROS accumulation, which can aggravate deleterious processes by disruption of ER $Ca^{2+}$ homeostasis. The consequence of ER retention, $[Ca^{2+}]$ER depletion and ROS accumulation is cell death. Thus, the detailed understanding of these mechanisms may contribute to the development of new therapeutic target for diseases associated with enhanced ROS levels and ER pathologies.

## VIII. ABBREVIATIONS

α-GlcI,II, α-glucosidase I and II; α-(1,2) Msd I,II, α-1,2 mannosidase I and II; AIF, apoptosis-inducing factor; ASK1, apoptosis signal-regulating kinase 1; BAP, BiP-associated protein; Bax, Bcl-associated factor, Bcl, B-cell lymphoma; bHLH, basic helix–loop–helix; BiP/GRP78, immunoglobulin heavy chain binding protein/glucose regulated protein 78; cADPR, cyclic ADP-ribose; CCE, capacitative $Ca^{2+}$ entry; CHOP, CAAT/Enhancer binding protein (C/EBP) homologuos protein; CICR, $Ca^{2+}$-induced $Ca^{2+}$ release; CRE, ATF/cAMP responsive element; CRT, calreticulin; CST, calsequestrin; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; ERAD, ER associated degradation; EDEM, ER degradation-enhancing α-mannosidase-like protein; ERSE, ER stress response element; ERO, endoplasmatic reticulum oxidoreductase; G, glucose; GADD growth arrest and DNA damage; GPCR, G-protein coupled receptor; GPX, glutathione peroxidases; GRP, glucose-regulated protein; GSSG/GSH, oxidized to reduced glutathione; HDAC1, histone deacetylase complex 1; HIF, hypoxia-inducible factors; HSP, heat-shock-protein; InsP3, inositol 1,4,5-trisphosphate; InsP3R, inositol-1,4,5-trisphosphate receptor; INSIG-1, insulin-induced gene 1 protein; IRE, inositol requiring kinase; JAB-1, Jun activation domain-binding protein; JNK, c-Jun N-terminal kinase; Keap, Kelch-like erythroid-cell-derived protein with CNC homology; LDLR, low-density lipoprotein receptor; M, mannose; MAPK, mitogen activated protein kinase; NAC, *N*-acetylcysteine; NCE, Na+, $Ca^{2+}$- exchanger; NOX, NADPH oxidase; Nrf2, nuclear factor erythroid-2 (NF-E2)-related factor-2; ORP, oxygen regulated protein; PDI, protein disulfide isomerase; PDTC, pyrrolidine dithiocarbamate; PEK, pancreatic eukaryotic initiation factor 2α kinase; PERK, RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase; PMCA, plasma membrane $Ca^{2+}$-ATPase; PKB, protein kinase B/Akt, PTP, permeability transition pore; QSOXs, quiescin-like sulphydryl oxidases; ROCs,

receptor-operated channels; ROS, reactive oxygen species; RyR, ryanodin receptor; S-1-P, sphingosine-1-phosphate; SCAP, SREBP cleavage-activating protein; SOCs, store-operated channels; SOD, superoxide dismutases; SP, site protease; SR, sarcoplasmic reticulum; SREBP, sterol response element binding protein; TRAF2, tumor necrosis factor receptor-associated factor 2, UPR, unfolded protein response; UGGT, UDP-glucose glycoprotein glycosyl transferase; VOCs, voltage-operated channels; XBP, X-box binding protein.

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Address reprint requests to:
*Dr. T. Kietzmann*
*Fachbereich Chemie, Abteilung Biochemie*
*Universität Kaiserslautern*
*Erwin Schrödinger Str. Geb 54*
*D-67663 Kaiserslautern, Germany*

*E-mail:* tkietzm@gwdg.de

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