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INVITED REVIEW

Redox Regulatory Mechanisms in Cellular Stress Responses

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• *Background* Reactive oxygen species are produced in a highly localized and specific pattern in biological stress responses. The present review examines the redox regulatory aspects of a number of molecular stress response mechanisms in both prokaryotes and eukaryotes.
• *Scope* The present review provides examples representing both the cytoplasmic stress response, often studied as the heat shock response, as well as the stress response of the endoplasmic reticulum, known as the unfolded protein response. The examples have been selected to illustrate the variety of ways that redox signals mediate and affect stress responses.
• *Conclusions* Redox regulatory mechanisms are intricately embedded in both the cytoplasmic and endoplasmic reticulum stress responses at multiple levels. Many different stimuli, both internal and external, activate endogenous production of reactive oxygen species as a necessary part of the intracellular communication system that activates stress responses.

Key words: Reactive oxygen species, regulation of molecular stress response, heat shock response, unfolded protein response.

INTRODUCTION

Aerobic organisms respond to both stress and normal physiological stimuli, such as hormones, by producing reactive oxygen species (ROS), generally in defined subcellular locations. While much of the earlier literature focused on the deleterious effects of ROS, there is growing recognition that ROS, as well as reactive nitrogen and sulfur species, are as fundamental a part of the basic communication chemistry of cells and organisms as calcium (Ghezzi *et al*., 2005). Production of ROS serves highly localized and specific signalling functions both in stress responses and in normal physiological processes, as well as in development (Joo *et al*., 2001; Foreman *et al*., 2003; Inoue *et al*., 2004; Wu *et al*., 2005).

One might ask why and how such seemingly toxic ions and molecules can serve as essential signals. The answer may be that it is the very mechanisms that tightly control their intracellular concentrations that make calcium and ROS useful signalling molecules. It has been suggested that the low solubility of calcium phosphate and the importance of phosphate in cellular metabolism necessitated the early evolution of mechanisms for maintaining extremely low intracellular calcium concentrations (Sanders *et al*., 1999). Similarly, it has been conjectured that the initial evolution of organisms under reducing conditions necessitated the development of mechanisms for maintaining a reducing environment in cells when oxygen began to accumulate in the atmosphere (Sitia and Molteni, 2004). As with calcium, the redox homeostatic mechanisms that maintain the intracellular reducing environment make it possible to use ROS signals with high spatial resolution both within and between cells.

Calcium and ROS share with protein phosphorylation and other enzymatic modifications the capacity to markedly alter protein conformation, the former by its binding properties and the latter by its ability to oxidize sulfur residues in proteins. But unlike enzymatic modifications, both calcium and ROS act on proteins directly. The mechanisms that minimize the calcium concentration and maintain the internal reducing environment are ubiquitous within cells and rapidly quench transient increases in either. In the case of calcium, these include a variety of calcium-binding proteins and mechanisms for externalizing calcium and for sequestering it within internal compartments. For oxidants, these range from maintaining high levels of reducing small molecules, such as glutathione and ascorbate, to the many enzymes, such as superoxide dismutase, catalase and other peroxidases, that further reduce ROS to water. The foregoing properties, together with the existence of multiple transducers, such as calcium-binding proteins and protein disulfide isomerases, make both calcium and ROS ideal local signals for the differential expansion and reorientation of cells. Thus mechanisms that initiate the production of local calcium spikes, bursts and oscillations, as well as localized bursts of intra- and intercellular ROS, are central to understanding not only the stress physiology of cells, but how they change, move, and grow (Joo *et al*., 2001; Foreman *et al*., 2003; Liszkay *et al*., 2004; Mori and Schroeder, 2004; Felty *et al*., 2005).

The best-studied ROS-mediated processes in plants are their responses to pathogens and stress (Mullineaux and Karpinski, 2002; Dietz, 2003; Mahalingam and Fedoroff, 2003; Overmyer *et al*., 2003; Foyer and Noctor, 2005). But the line between normal physiological responses and stress responses is arbitrary, particularly in plants, which continuously adapt to changes in environmental conditions

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such as light, temperature, humidity and salinity. Thus the study of redox mechanisms in stress responses provides insight into the molecular physiology of adaptive responses in general. In the ensuing narrative, I have assembled several reasonably well-understood examples from the prokaryotic and eukaryotic literature of the kinds of molecular mechanisms that connect oxidative signals with cellular responses. Although the illustrative examples are drawn primarily from bacteria and yeast, these are strongly conserved stress response systems whose principle players are present in animals as well as plants. But the details differ from organism to organism, hence specific effectors and the precise topology of their interconnections must be ascertained for each system.

REDOX SENSORS AND EFFECTORS

Aerobic organisms assure a highly reducing cytosolic environment primarily by maintaining a marked excess of the reduced over the oxidized forms of glutathione and ascorbate (May, 1998; Smirnoff, 2000; Stone, 2004; Ghezzi *et al*., 2005). Under these conditions, cytosolic proteins are generally maintained in their reduced forms. By contrast, intracellular compartments such as the endoplasmic reticulum (ER), as well as the extracellular milieu, which is the apoplast in plants, is substantially more oxidizing (Noctor *et al*., 2002; Nardai *et al*., 2003).

Among ROS, H_2O_2 is the longest-lived and freely membrane permeable. As a consequence, H_2O_2 has long been used to elicit oxidative stress responses on the assumption that it penetrates cells. The literature is rife with experiments measuring cellular responses to the external application of H_2O_2. However, because the cytoplasm is highly reducing, a transmembrane gradient is rapidly established, with intracellular concentrations well below the extracellular (Stone, 2004). Hence the immediate targets of oxidizing stimuli are likely to be at the cell surface, as suggested by the observation that heterotrimeric G protein signalling mediates the oxidative burst in *Arabidopsis* (Joo *et al*., 2005). More importantly, cells respond rapidly to external stimuli of many kinds, including oxidants, by activating the endogenous production of ROS from membranes and intracellular compartments. The endogenous production of ROS, first characterized in mammalian neutrophils and termed the 'oxidative burst' (Dahlgren and Karlsson, 1999; Forman and Torres, 2001), is also a characteristic plant response to a variety of noxious stimuli, as well as hormones (Bolwell, 1996; Mahalingam and Fedoroff, 2003).

Because the intracellular ROS response to a particular stimulus, be it hormonal, chemical or physical, can be highly localized, the search for redox 'sensors' has produced very few useful generalizations. Certain kinds of proteins have active site cysteine residues or other active site configurations that facilitate direct interaction with H_2O_2 (Xu *et al*., 2002; Gupta and Luan, 2003; Stone, 2004). Phosphatases contain readily oxidizable (low pK_a) active site cysteine residues (Stone, 2004). Because phosphatases are involved in regulation of kinases, redox regulation of phosphatase activity can, in turn, regulate the activity of its target kinases (Tonks, 2005). But there are also examples of kinases that are directly redox regulated by thioredoxins and peroxiredoxins (Martinsuo *et al*., 2003; Veal *et al*., 2004). As I will try to illustrate through the examples collected in subsequent sections, perhaps the most striking attributes of redox regulation are its variety and its pervasiveness. Redox regulation of transcription, for example, is effected through several different direct and indirect mechanisms, ranging from the oxidation or reduction of a transcription factor, to the redox regulation of a step far upstream in a signalling cascade from the transcription factor itself.

Many different cellular processes are subject to redox regulation both in immediate response to oxidative stress and as a temporally extended response that enhances the organism's ability to withstand subsequent stress. The critical processes of protein biosynthesis and folding in the ER and cytoplasm, as well as the transfer of proteins between organelles and out of the cell as secreted proteins, are performed by a host of folding proteins, termed chaperones, which include protein disulfide isomerases that promote the controlled oxidation of cysteines to form disulfide bonds (Noiva, 1999; Papp *et al*., 2003; Young *et al*., 2004).

Cells monitor protein misfolding closely, triggering rapid responses to a variety of abiotic stresses, including heat and oxidative stress, as well as inhibitors of protein synthesis and modification (Rao and Bredesen, 2004). These responses, termed the heat shock response (HSR) for cytoplasmic chaperones and the unfolded protein response (UPR) for ER proteins, result in the rapid adjustment of chaperone levels and the redox activation or accumulation of stress-specific chaperones that bind to proteins temporarily until the folding machinery recovers (Sitia and Molteni, 2004; Winter and Jakob, 2004; Young *et al*., 2004; Schroder and Kaufman, 2005). Unchecked, the accumulation of misfolded proteins becomes catastrophic, leading to cell death (Schroder and Kaufman, 2005). Indeed, the accumulation of misfolded proteins is a central feature of human neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's diseases (Rao and Bredesen, 2004).

Cellular stress responses are triggered extremely rapidly. Among the most rapid chemical responses are calcium transients and the endogenous activation of ROS production, followed by rapid fluctuations in the abundance of certain transcripts (Papp *et al*., 2003; Rao and Bredesen, 2004; Mahalingam *et al*., 2005). In the present discussion, I will focus on the emerging evidence that redox regulatory processes are central to activating the response processes in general, and the accumulating data suggesting that the production of ROS by internal enzyme systems is a necessary component of such responses. My purpose here is to show both the complexity and the versatility of the redox regulatory mechanisms that are critical to maintaining the intracellular environment. As well, my objective is to show how several aspects of stress responses, such as redox homeostasis, protein

folding and protein synthesis, are interconnected by redox mechanisms.

REDOX REGULATION OF TRANSCRIPTION

OxyR protein

The by now paradigmatic redox-regulated transcription factor is the bacterial OxyR protein (Zheng *et al*., 1998). The OxyR protein is both redox sensor and effector, as illustrated in Fig. 1, activating transcription of genes encoding antioxidant proteins upon oxidation (Storz *et al*., 1990). OxyR is activated by the formation of a disulfide bond between Cys199 and Cys208 and deactivated by enzymatic reduction by glutaredoxin 1 (Zheng *et al*., 1998; Lee *et al*., 2004). Oxidation changes the DNA binding properties of OxyR, converting the protein to a transcriptional activator that participates in the recruitment of RNA polymerase (Toledano *et al*., 1994). Recent studies suggest that OxyR is destabilized by the formation of the intramolecular disulfide bond, which then facilitates the conformational change back to the reduced form (Lee *et al*., 2004). However, there is also evidence that the transcriptional activity of OxyR is controlled solely by the oxidation state of the Cys199 residue and that the different oxidized derivatives of the protein exhibit subtle differences in co-operativity and ability to bind to DNA (Kim *et al*., 2002).

Yap1 protein

The Yap1p protein of yeast is perhaps the most extensively studied eukaryotic redox regulator of transcription. Hydrogen peroxide (H_2O_2) treatment induces the synthesis of many new proteins in yeast and the Yap1 bZIP transcription factor is an important determinant of resistance to oxidative stress (Georgiou, 2002). The phospholipid hydroperoxidase Gpx3 is the primary oxidant sensor (Delaunay *et al*., 2002). H_2O_2 oxidizes Cys36 of Gpx3, forming a sulfenic acid intermediate that reacts with the Cys598 of Yap1 to form a transient disulfide-linked complex (Fig. 2). The transient complex rearranges to regenerate reduced Gpx3 and oxidized Yap1 with two disulfide bridges connecting Cys303 to Cys598 and Cys310 to Cys629 (Delaunay *et al*., 2002; Wood *et al*., 2003). Oxidized Yap1 rapidly accumulates in the nucleus because a nuclear export signal in the C-terminal domain is obscured by the formation of the intramolecular disulfide bonds (Wood *et al*., 2003, 2004). Yap1 stimulates transcription of many genes, including an the essential *TRX2* gene, which encodes a thioredoxin necessary for the reduction of Yap1, whose presence in turn allows it to be exported from the nucleus by the beta-karyopherin-like exportin, Crm1p (Yan *et al*., 1998; Wood *et al*., 2004).

Curiously, there is evidence that Yap1 activates transcription differently in response to the oxidants H_2O_2 and diamide (Coleman *et al*., 1999). This appears to be attributable to the requirement of the internal disulfide bonds for recruitment of the Rox3p transcriptional mediator for transcriptional activation of some promoters, but not others (Gulshan *et al*., 2005). The *YBP1* gene encodes Ybp1p, a Yap1-interacting protein that is required for Yap1 activation in response to H_2O_2, but not diamide (Veal *et al*., 2003). Thus it appears that redox regulation by the Yap1 transcription factor is influenced not only by the structure of the protein itself, but also through the involvement of additional interacting nuclear proteins.

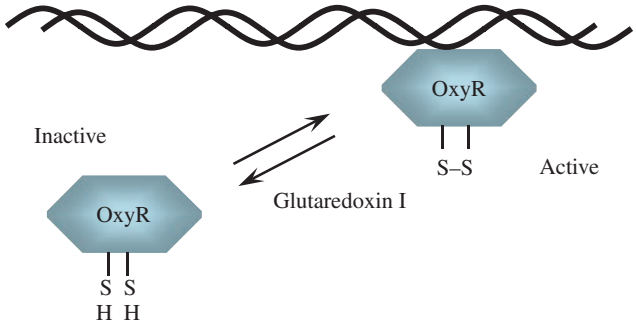

FIG. 1. The prokaryotic OxyR is converted to a transcriptional activator upon oxidation. Reduction by glutaredoxin I returns it to the inactive form.

Transcriptional cofactor NPR1

The nuclear localization of the plant stress-activated transcriptional cofactor NPR1 is redox-controlled by a somewhat different mechanism than Yap1 (Fig. 3). Plants exhibit an increase in pathogen resistance, termed systemic acquired resistance (SAR) upon prior exposure to pathogens, abiotic stress and salicylic acid (SA), as well as certain SA homologues (Pieterse and Van Loon, 2004). The transcriptional regulatory factor NPR1, which is at the heart of SAR, is activated when SA accumulates in cells in response to stress. In unstressed cells, NPR1 is maintained in the cytoplasm in a large complex comprising disulfide-bonded intermolecular oligomers (Mou *et al*., 2003). Upon induction of the SAR, the intermolecular disulfide bonds are reduced, releasing monomeric NPR1, which then moves into the nucleus to interact with TGA transcription factors and activate defence gene expression (Fig. 3). Mutations of either Cys82 or Cys216 render the protein both constitutively monomeric and nuclear and constitutively activate expression of defence genes (Mou *et al*., 2003).

Interaction between the NPR1 protein and certain TGA transcription factors has also been reported to be redox-regulated. Thus an intramolecular disulfide bond between Cys260 and Cys266 in TGA1 prevents interaction with NPR1 (Despres *et al*., 2003). Reduction of the disulfide bond permits TGA1 to interact with NPR1, which, in turn, stimulates its DNA-binding activity. SA promotes the reduction of NPR1 and TGA1 during SAR, although the mechanism is not understood (Despres *et al*., 2003; Fobert and Despres, 2005). Recent evidence suggests that SA functions as a transient respiratory uncoupler and inhibitor of mitochondrial electron transport, which would stimulate mitochondrial production of ROS (Norman *et al*., 2004).

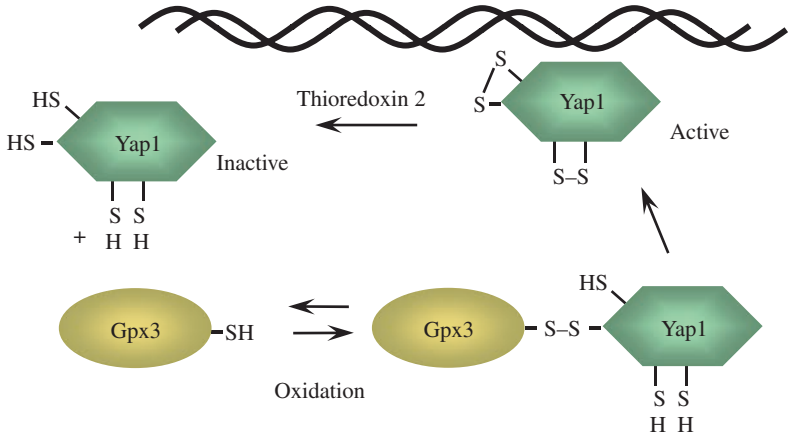

FIG. 2. Upon oxidation, the yeast oxidant sensor Gpx3, a phospholipid hydroperoxidase, forms a transient disulfide-linked complex with the Yap1 transcription factor, an important determinant of resistance to oxidative stress. The complex rearranges to regenerate reduced Gpx3 and oxidized Yap1 with two disulfide bonds. Oxidized Yap1 rapidly accumulates in the nucleus and stimulates transcription of many genes, including the *TRX2* gene, which encodes the thioredoxin necessary for the reduction of Yap1.

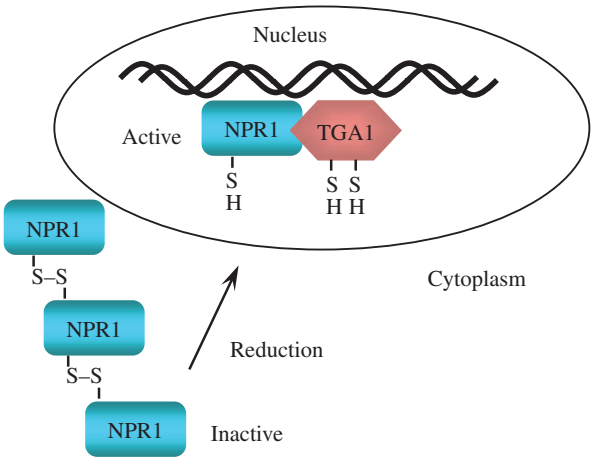

FIG. 3. In unstressed *Arabidopsis* cells, NPR1 is maintained in the cytoplasm as a disulfide-bonded oligomer. Reduction of the disulfide bonds during SAR releases monomeric NPR1, which moves into the nucleus to interact with reduced TGA1 transcription factor to activate defence gene expression.

The apparent paradox that SA activates ROS production, but stimulates NPR1 and TGA1 by a reductive mechanism may be resolved by the observation that quenching of the early stress-induced oxidative burst by ozone occurs concomitantly with a rapid increase in the transcript levels of *Arabidopsis* genes encoding antioxidant proteins, such as peroxidases and glutathione-S-transferases (Joo *et al*., 2005; Mahalingam *et al*., 2005). Thus SA's effect may be indirect because NPR1-induced defence response gene expresssion peaks somewhat later in the stress response, possibly as a consequence of the build-up of antioxidants that enhance the general cellular reducing capacity or expression of genes encoding proteins, such as a thioredoxin or a redox-regulated chaperones, which directly reduces the NPR1 complex (Cumming *et al*., 2004).

The redox sensor for the SAR has not yet been identified. However, a recent report describes an *Arabidopsis* protein, OXS2, with the anticipated properties of such a sensor. OXS2 cDNA confers tolerance to the oxidizing agent diamide on yeast cells expressing it (Branvillain and Ow, pers. commun.). It is a zinc-finger transcription factor that is cytoplasmic under non-stress conditions by virtue of a C-terminal nuclear export sequence. Under stress conditions, the protein remains in the nucleus and activates genes involved in flowering time to promote early reproduction. Although its molecular mechanism is not yet understood, it is possible that OXS2 nuclear localization is directly activated by the ROS produced in response to ABA and both biotic and abiotic stresses.

NFκB

There is substantial evidence for redox regulation of mammalian stress-activated transcription factors, although the relationship between redox sensor and effector can be much less direct than it is in bacteria and lower eukaryotes. Treatment of cultured mammalian cells with the pro-inflammatory cytokine IL-1β or H_2O_2 activates NFκB (Marangolo *et al*., 2001). In unstimulated cells, NFκB is bound to by the inhibitory protein IκBα (Kamata and Hirata, 1999). Phosphorylation of IκBα by the IκB kinase (IKK) complex leads to its dissociation from NFκB and its proteasomal degradation, which allows NFκB to move into the nucleus and activate gene expression. NFκB-inducing kinase (NIK) is a further upstream kinase that phosphorylates IKK, and there is evidence that NIK is the actual target of redox regulation (Zhang *et al*., 2001; Li and Engelhardt, 2006). The signalling cascade initiated by IL-1β involves recruitment and activation of NIK by an adaptor protein, TNF receptor-associated factor 6 (TRAF6), and H_2O_2 increases the strength of this interaction, as does inhibition of phosphatase activity (Qian *et al*., 2001; Li and Engelhardt, 2006). This suggests that H_2O_2 could act indirectly by inhibiting an NIK phosphatase. The small GTPase Rac1 (Abo *et al*., 1991) is also required for IL-1β-stimulated activation of NFκB (Jefferies *et al*., 2001).

Both inhibition of the membrane-bound NADPH oxidase activity by diphenylene iodonium (DPI) and silencing of Rac1 gene expression by a silencing RNA (siRNA) construct inhibited IL-1β-stimulated NIK-TRAF6 complex formation, indicating that the Rac1-dependent NADPH oxidase is the cellular source of the ROS. Thus it appears that IL-1β activates the membrane-bound NADPH oxidase to produce ROS, which, in turn, activate NIK in the pathway to inactivation of IκB, releasing NFκB from inhibition.

It remains unclear whether NIK is the direct target of redox regulation or whether ROS act indirectly by inhibiting an NIK phosphatase, or both (Li and Engelhardt, 2006). This rather complex example shows that the redox-regulated component of a signalling pathway can be relatively far removed from the target protein. As well, it raises interesting questions about the nature of the ROS sensor (Stone, 2004). The affinity of NIK for TRAF6 may be enhanced directly by a disulfide bond-mediated conformational change in the protein, or it may be indirectly stimulated by reducing the activity of a regulatory phosphatase. Protein phosphatases commonly have readily oxidizable cysteine residues in their active sites and can be reversibly inactivated by oxidation (Tonks, 2005). As well, there are examples of kinases whose activity is directly redox-regulated (Martinsuo *et al*., 2003). Both mechanisms provide an interface between redox and phospho-regulation.

p53 tumour suppressor

The intensively studied p53 tumour suppressor is a stress-activated DNA-binding protein that promotes transcription of a large number of target genes whose protein products are involved in DNA repair, the cell cycle and apoptosis (Mills, 2005). In response to stress signals, p53 is activated by post-translational modifications, primarily phosphorylation, that affect its stability, interaction with the negative regulator Mdm2 and other proteins, and its ability to interact with DNA (Hainaut and Hollstein, 2000).

The DNA-binding activity of p53 is substantially enhanced by a non-covalent interaction with a bifunctional protein which has been identified both as an abasic endonuclease excision repair enzyme (APE) and a nuclear redox factor (Ref-1) that activates a number of nuclear proteins (Jayaraman *et al*., 1997; Gaiddon *et al*., 1999; Ueno *et al*., 1999; Tell *et al*., 2005). Ref-1 is most active in its reduced form, which is regenerated by a thioredoxin-thioredoxin reductase mechanism (Ueno *et al*., 1999; Seemann and Hainaut, 2005). There is evidence that association with Ref-1 both stabilizes p53 to degradation and promotes formation of tetramers, enhancing its DNA-binding activity (Hanson *et al*., 2005; Seemann and Hainaut, 2005). Significantly, Ref-1 promotes both the tetramerization of dimers and the resolution of larger p53 aggregates to active tetramers (Hanson *et al*., 2005).

How Ref-1 affects p53 activity is not known, although its ability to activate the DNA-binding activity of the Jun protein has been traced to reduction of Cys252 of the Jun protein, which involves the Cys65 and Cys93 residues of the Ref-1 protein (Walker *et al*., 1993). It has been reported that supplementation of cells with selenomethionine, which is incorporated as selenocysteine into selenoproteins, such as thioredoxin reductase and glutathione peroxidase, enhances p53 reduction and activates its DNA-binding activity and that both are blocked by a dominant negative mutation in Ref-1 (Seo *et al*., 2002). These observations are consistent both with a direct disulfide isomerization mechanism, as well as a chaperone-like interaction between Ref-1 and p53.

Heat shock protein Hsf1

The eukaryotic transcription factor that appears to be most directly redox regulated is heat shock factor (Hsf1), a highly conserved transcription factor which mediates the transcription of a complex of genes in response to heat, oxidative stress and a variety of other stressors. Both heat shock and oxidative stress induce the expression of a variety of heat shock proteins, among the most abundant of which are the molecular chaperones Hsp90 and Hsp70 (Young *et al*., 2004). It has been known for some time that the transcriptional response to heat shock does not require new protein synthesis and can be inhibited by reducing agents, suggesting redox activation of a pre-existing protein (Huang *et al*., 1994). Stress mediates the trimerization, nuclear localization and binding of Hsf1 to the promoters of genes containing heat shock motifs (Zuo *et al*., 1994).

The precise molecular mechanism of heat and oxidant-mediated activation are as yet somewhat unclear. It has been reported that purified Hsf1 multimerizes and binds to DNA upon either heating or oxidation with H_2O_2 (Ahn and Thiele, 2003). The same investigators further showed that the stress-inducible DNA-binding activity and nuclear localization are abrogated by mutations of Cys35 and Cys105, although they provided no direct evidence for intermolecular disulfide bond formation (Ahn and Thiele, 2003). Others have argued that the oxidant-mediated activation is indirect, presenting evidence that the formation of intramolecular disulfide bonds in Hsf1 itself inhibits trimerization and DNA-binding, but can be reversed by either dithiothreitol or thioredoxin (Jacquier-Sarlin and Polla, 1996; Manalo *et al*., 2002). Possibly trimerization is promoted by the oxidation of these cysteine residues, but inhibited by the formation of intramolecular disulfide bonds between them; key experiments remain to be done. However, evidence that endogenous production of ROS is necessary for induction of the heat shock response comes from the observation that a dominant negative allele of Rac1, the small GTPase necessary for the activation of ROS production by membrane-bound NADPH oxidase (Abo *et al*., 1991), inhibits the stress-induced activation of Hsf1 (Ozaki *et al*., 2000).

Drosophila and most vertebrates have one or a few *Hsf* genes; by contrast, plants have at least three families of Hsfs, each with several members. Rice, tomato and *Arabidopsis* each have 18–23 *Hsf* genes, some of which appear to encode co-activators, co-repressors or determinants of

intracellular location (Nover *et al*., 2001; Baniwal *et al*., 2004). Experiments in tomato suggest that one of the proteins, Hsf1A, is a master regulator of the heat shock response, while similar experiments in arabidopsis show that at least two *Hsf* genes must be mutated for a substantial effect on the heat shock response (Mishra *et al*., 2002; Lohmann *et al*., 2004). Little is known, as yet, about the molecular mechanisms regulating either the structure or the localization of plant Hsfs.

REDOX REGULATION IN PROTEIN FOLDING

Heat shock protein Hsp33

Although heat-shock proteins, as their name implies, were first discovered in studies of proteins induced by subjecting organisms to elevated temperatures, they are part of a complex system of molecular chaperones that enables proteins to fold correctly and protects cells from the damaging effects of protein aggregation (Young *et al*., 2004). Recent studies have revealed that several heat shock proteins, including the prokaryotic Hsp33 and the eukaryotic Hsp70, are redox-regulated (Papp *et al*., 2003; Winter and Jakob, 2004). While expression of the prokaryotic Hsp33 protein is induced by heat stress, it is functionally activated by oxidation (Chuang and Blattner, 1993; Graumann *et al*., 2001). Hsp33 is a small (32·8 kDA) cytoplasmic protein that is present in inactive, monomeric form under reducing conditions (Fig. 4). It contains four conserved cysteine residues in its C-terminal domain that bind a zinc atom with high affinity (Jakob *et al*., 2000). Binding of zinc to Hsp33 stabilizes the α-helical C-terminus, masks the substrate-binding site, and blocks dimerization (Winter and Jakob, 2004).

Upon oxidation, Hsp33 is activated by dimerization through the formation of two intramolecular disulfide bonds and the release of zinc (Graumann *et al*., 2001). Curiously, the zinc atom also appears to be necessary for optimal dimerization, since zinc-free Hsp33 dimerizes slowly and incompletely (Jakob *et al*., 2000). During oxidation, the cysteine residues first form two intramolecular disulfide bonds, which then rearrange to intermolecular disulfide bonds, dimerizing and activating Hsp33 to bind to a variety of unstructured protein substrates (Fig. 4). Both heat and oxidizing conditions promote Hsp33 activation. The physiological function of Hsp33 appears to be that of a 'holdase', a chaperone that binds to proteins under conditions in which the normal 'foldases,' which are the DnaK/DnaJ/GrpE proteins in *Escherichia coli*, are inactivated (Winter *et al*., 2005). Substrate-bound oxidized Hsp33 is reduced by the glutaredoxin and thioredoxin systems, but releases its substrate only in the presence of the DnaK/DnaJ/GrpE foldase system, which facilitates protein refolding (Hoffmann *et al*., 2004).

Heat shock protein Hsp70

While the redox regulation of eukaryotic chaperones is not nearly as well-understood as that of the prokaryotic

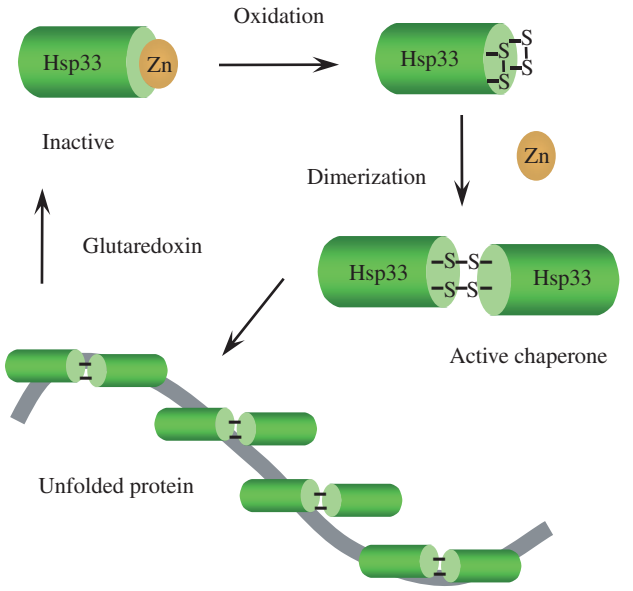

FIG. 4. The prokaryotic heat shock protein Hsp33 is an inactive Zn-containing monomer under reducing conditions. Upon oxidation, Hsp33 dimerizes through formation of two intramolecular disulfide bonds and the release of zinc, activating its chaperone function. Oxidized Hsp33 is reduced by glutaredoxin.

Hsp33 proteins, it was recently reported that mammalian Hsp70 is activated by *S*-glutathionylation, which oxidizes an internal thiol to an intramolecular disulfide with the tripeptide glutathione (Hoppe *et al*., 2004; Shelton *et al*., 2005). The glutathionylated Hsp70 was more effective in preventing protein aggregation than the reduced form (Hoppe *et al*., 2004), providing a post-translational mechanism for regulating chaperone activity. While the precise molecular connections are not yet understood, both the inducible Hsp70 and its constitutive homologue Hsc70 are integral to the function of the immune system and are activated under oxidative stress conditions (Callahan *et al*., 2002; Menoret *et al*., 2002; Li *et al*., 2005). Designated immunochaperones, certain Hsps, including Hsp70, are also enhancers of anti-tumour immunity (Facciponte *et al*., 2006).

Hsp70 activity is modified by a number of proteins, including the co-chaperone Hsp70-interacting protein (HIP) (Young *et al*., 2004). An *Arabidopsis* HIP, designated AtTDX, has recently been characterized and shown to be a bifunctional protein with a HIP and a thioredoxin domain (Vignols *et al*., 2003). AtTDX interacts with the ATPase domain of the yeast Hsp70 chaperone protein Ssb2. H_2O_2 treatment disrupted the AtTDX-Ssb2 interaction, but only if the thioredoxin domain of AtTDX was intact (Vignols *et al*., 2003). Mutation of either active site cysteines in the thioredoxin domain of AtTDX or of the single highly conserved cysteine residue in the ATPase of the Ssb2 protein also prevented H_2O_2-mediated dissociation of the complex (Vignols *et al*., 2003). The foregoing observations provide examples of both direct and indirect redox regulation of eukaryotic molecular chaperones.

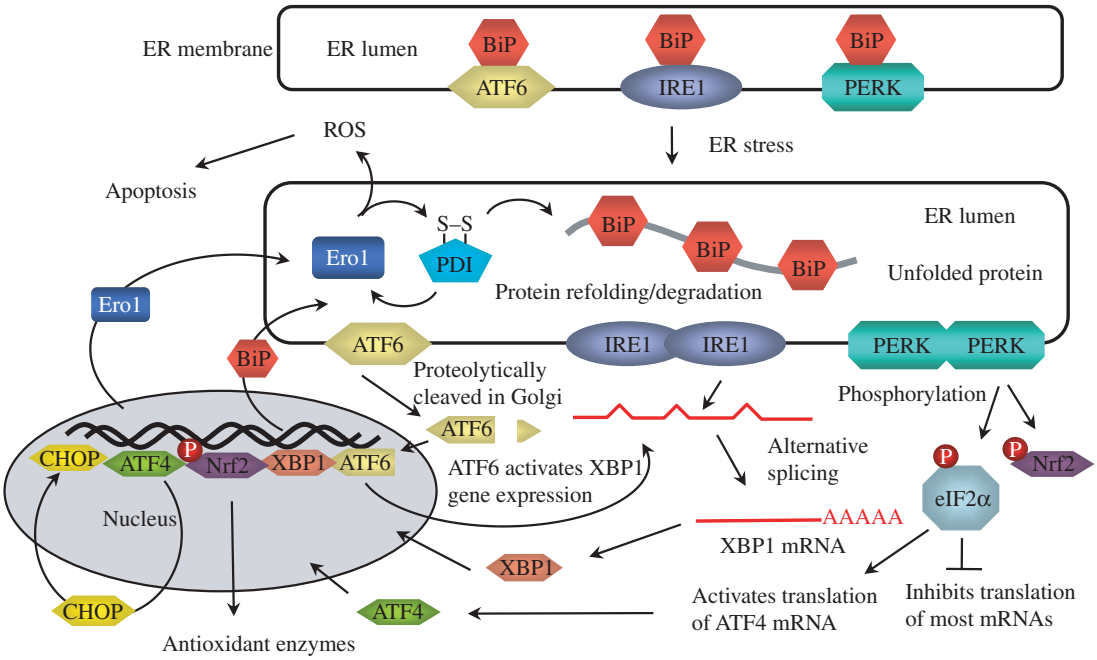

FIG. 5. BiP is a resident ER chaperone and regulator in the UPR. The UPR is triggered by a signalling system that is held in check by the binding of BiP to the lumenal domains of two kinases (PERK and IRE1) and the ATF6 transcription factor. When unfolded proteins accumulate, BiP dissociates from the regulators, promoting the oligomerization and activation of the kinases and the translocation of ATF6 to the Golgi complex, where it is activated by proteolytic cleavage. ATF6 translocates to the nucleus and activates ER stress response genes, including BiP. PERK interconnects the UPR, inhibition of protein synthesis and degradation, and the oxidative stress response. PERK phosphorylates eIF2α, inhibiting translation of most mRNAs, but stimulating translation of the mRNA encoding the ATF4 transcription factor, which in turn up-regulates expression of the pro-apoptotic transcription factor CHOP. CHOP activates expression of Ero1, which together with PDI and BiP, promote protein folding and degradation. PERK also phosphorylates the transcription factor Nrf2, which activates expression of genes encoding antioxidant proteins and enzymes. IRE1, a kinase and ribonuclease, promotes the alternative splicing of the mRNA encoding XBP1, induced by ATF6, to produce the highly efficient UPR transcriptional activator XBP1, which activates BiP gene expression. Prolonged activation of the UPR leads to futile cycling of Ero1-PDI, accumulation of ROS and apoptosis.

Protein disulfide isomerase (PDI)

PDI is a protein thiol-disulfide oxidoreductase with unique properties (Noiva, 1999). Depending on its cellular location, its substrate, and its partners, it can reduce, oxidize, or isomerize disulfide bonds in proteins. Its primary structure contains four domains with thioredoxin-like structure, two of which contain the vicinal cysteine pairs characteristic of protein-thiol oxidoreductases (Noiva, 1999). PDI interacts with a large variety of proteins, showing little sequence specificity for peptide binding. In the oxidizing environment of the ER, PDI oxidizes and isomerizes disulfide bonds, but it acts as a reductant in the cytoplasm, endosomes and the plasma membrane. In addition, there is evidence that PDI serves as a chaperone, accelerating the folding of proteins that lack disulfide bridges by a mechanism that is independent of its redox activity (Quan *et al*., 1995).

Misfolded proteins trigger the ER stress response, termed the unfolded protein response (UPR; Fig. 5). Proteins which are activated or whose expression is induced during the UPR promote the refolding or, alternatively, the degradation of misfolded proteins by ER-associated protein degradation (ERAD) (Travers *et al*., 2000). Under conditions of substrate excess, PDI acts as an anti-chaperone, promoting the formation of large aggregates and promoting either ERAD or refolding (Puig and Gilbert, 1994; Puig *et al*., 1994; Zhao *et al*., 2005). The prolonged accumulation of misfolded proteins in the lumen of the ER and sustained activation of the UPR triggers cell death (Haynes *et al*., 2002, 2004).

The UPR-induced chaperone immunoglobulin heavy chain-binding protein (BiP) and PDI can interact to facilitate protein refolding (Puig and Gilbert, 1994), an observation that has been put to practical use to facilitate higher recombinant protein expression levels without triggering cell death consequent on the excessive accumulation of protein aggregates (Xu *et al*., 2005). Moreover, there is evidence that PDI participates in the toxicology of cholera toxin by acting as a redox-driven chaperone (Tsai *et al*., 2001). Reduced PDI binds to and unfolds the cleaved cholera toxin A chain, releasing it upon oxidation.

Luminal ER glycoprotein Ero1p

Correct disulfide bond formation during oxidative protein folding in the ER requires both PDI and the luminal ER glycoprotein Ero1p (Pollard *et al*., 1998). Ero1p acts catalytically to initiate disulfide bond formation by oxidizing the paired cysteine residues of the active sites in PDI, which then transfers the oxidizing equivalents

to folding proteins (Tu *et al*., 2000; Tu and Weissman, 2004). Ero1p functions as a flavin adenine dinucleotide (FAD)-dependent oxidase, using molecular oxygen as an electron sink (Tu *et al*., 2000). Oxidation of PDI occurs through the formation of an intermolecular disulfide bond between Ero1p and PDI, allowing Ero1p and PDI to catalyse protein folding even in a reducing environment (Tu *et al*., 2000).

There is evidence that prolonged activation of the UPR leads to the accumulation of ROS from protein oxidation, as well as from mitochondria, contributing to ER stress-induced cell death (Haynes *et al*., 2004). A factor in the accumulation of toxic levels of ROS during the UPR appears to be the depletion of glutathione by the futile cycling of the Ero1p–PDI protein oxidation cycle (Haynes *et al*., 2004).

Immunoglobulin heavy chain-binding protein (BiP)

BiP, also identified as a glucose-regulated protein (GRP78), is a central molecular chaperone and regulator in the UPR (Haas and Wabl, 1983; Schroder and Kaufman, 2005). The UPR, illustrated in Fig. 5, is a complex cellular response to the accumulation of misfolded proteins that includes transient inhibition of protein synthesis and the up-regulation of genes coding for antioxidant proteins, chaperones and proteins that promote degradation of misfolded proteins (Harding *et al*., 2003; Schroder and Kaufman, 2005). The UPR is triggered by a signalling system that is held in check by the binding of BiP, an abundant ER resident chaperone, to two kinases (PERK and IRE1) and a transcription factor ATF6 (Bertolotti *et al*., 2000; Urano *et al*., 2000; Shen *et al*., 2002). Eukaryotic initiation factor 2α (eIF2α) kinase or RNA-activated protein kinase (PKR)-like ER kinase (PERK), IRE1, a kinase and ribonuclease, and the bZIP transcription factor ATF6 are ER resident transmembrane proteins (Cox *et al*., 1993; Bertolotti *et al*., 2000; Iwawaki *et al*., 2001; Shen *et al*., 2002, 2005).

In unstressed cells, BiP binds to the lumenal domains of PERK and IRE1 and the accumulation of misfolded proteins prompts its dissociation, promoting the oligomerization and activation of the kinases (Bertolotti *et al*., 2000). Mammalian cells contain two IRE1 proteins, IRE1α and IRE1β (Tirasophon *et al*., 2000). IRE1β represses protein synthesis by cleaving 28S rRNA (Iwawaki *et al*., 2001). IRE1α activates BiP gene expression by promoting the alternative splicing of the mRNA encoding the X-box binding protein 1 (XBP1), induced by ATF6, to produce the highly efficient UPR transcriptional activator XBP1 (Tirasophon *et al*., 1998; Yoshida *et al*., 1998, 2001). The ATF6 transcription factor is also actively released from its association with BiP in response to misfolded proteins (Shen *et al*., 2005). It is then translocated to the Golgi complex, where it is activated by proteolytic cleavage (Shen *et al*., 2002). The activated ATF6 then translocates to the nucleus and activates ER stress response genes, including BiP (Yoshida *et al*., 2000). BiP functions as a molecular chaperone, stimulating protein refolding and degradation; it has also been reported to function as a lumenal calcium storage protein (Lievremont *et al*., 1997).

Kinase PERK

PERK is a central regulatory hub that interconnects the UPR, inhibition of protein synthesis and degradation, and the oxidative stress response (Harding *et al*., 2003; Cullinan and Diehl, 2005). PERK phosphorylates eIF2α, inhibiting translation of most mRNAs, but stimulating translation of the mRNA encoding the ATF4 transcription factor, which in turn up-regulates expression of the pro-apoptotic protein C/EBP homologous protein (CHOP) (Harding *et al*., 2000). CHOP activates expression of the gene encoding the Ero1 protein, increasing ROS production and oxidative stress, and promotes reactivation of PERK (Marciniak *et al*., 2004).

However, inhibition of ROS production during the UPR induced by tumour necrosis factor α (TNFα) inhibits CHOP induction, as well as alternative splicing of XBP1 mRNA (Xue *et al*., 2005). Silencing of the Nox4 NADPH oxidase gene has also been reported to prevent oxysterol induction of the UPR in human muscle cells (Pedruzzi *et al*., 2004). The mechanism by which the UPR protective effect is exerted is through PERK phosphorylation and activation of the NF-E2-related factors 1 and 2 (Nrf1 and Nrf2), which are critical transcription factors in protection from oxidative stress (Leung *et al*., 2003; Cullinan and Diehl, 2004). Nrf2 promotes expression of genes encoding antioxidant enzymes, including genes in the glutathione biosynthetic pathway (Hayes and McMahon, 2001; Leung *et al*., 2003). Nrf1, which activates expression of mitochondrial transcription factor A (Tfam), required for mitochondrial DNA transcription and replication, is activated by redox-regulated phosphorylation by Akt kinase (also known as protein kinase B) (Piantadosi and Suliman, 2006). Redox-sensitive components in this signalling pathway include the Akt kinase and the PtdIns(3,4,5)P3 phosphatase PTEN (Leslie *et al*., 2003; Murata *et al*., 2003; Kwon *et al*., 2004; Meuillet *et al*., 2004; Lee *et al*., 2005). Hence ROS production appears to be necessary for the induction of the UPR by some agents and serves, in part, to protect cells from excessive ROS build-up associated with oxidative protein folding by up-regulating expression of antioxidant proteins.

SUMMARY AND CONCLUSIONS

Redox regulatory mechanisms, just a few of which have been reviewed here, are intricately embedded in both the cytoplasmic and ER stress responses at multiple levels. Moreover, it is becoming increasingly evident that many different stimuli activate endogenous production of ROS and that these are a necessary part of the intracellular communication system that activates the stress response. While the ER and cytoplasmic stress responses have not yet been studied as extensively in plants as they have been in yeast and mammalian cells, both stress responses are strongly conserved among eukaryotes and the broad

outlines are similar in yeast, plants and animals (Jelitto-Van Dooren *et al*., 1999; Baniwal *et al*., 2004; Zuppini *et al*., 2004; Iwata and Koizumi, 2005; Kamauchi *et al*., 2005; Kirst *et al*., 2005). It has been reported that the cell death inhibitor BAX inhibitor-1 (BI-1) can function to suppress the ER stress-induced apoptotic response in both plants and animals (Chae *et al*., 2004; Huckelhoven, 2004). Finally, it has recently been reported that many genes coding for ER stress proteins, including BiP2 and several PDIs, are primary targets of NPR1 transcriptional regulation in the arabidopsis SAR (Wang *et al*., 2005). Moreover, the observation that *bip2* knock-out mutant plants are hypersensitive to treatment with SA analogues suggests that SA-induced expression of secreted pathogenesis-related proteins requires the BiP chaperone (Wang *et al*., 2005). Thus there appears to be a clear linkage between the ER and cytoplasmic stress responses in plants.

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