The mitochondrial UPR – protecting organelle protein homeostasis

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Summary
Mitochondria are required for numerous essential metabolic processes including the regulation of apoptosis; therefore, proper maintenance of the mitochondrial proteome is crucial. The protein-folding environment in mitochondria is challenged by organelle architecture, the presence of reactive oxygen species and the difficulties associated with assembly of the electron transport chain, which consists of components encoded by both the mitochondrial and the nuclear genomes. Mitochondria have dedicated molecular chaperones and proteases that promote proper protein folding, complex assembly and quality control. Work in cultured mammalian cells and Caenorhabditis elegans has yielded clues to the mechanisms linking perturbations in the protein-folding environment in the mitochondrial matrix to the expression of nuclear genes encoding mitochondrial proteins. Here, we review the current knowledge of this mitochondrial unfolded protein response (UPRmt), compare it with the better understood UPR of the endoplasmic reticulum and highlight its potential impact on development and disease.

Key words: Mitochondria, Molecular chaperones, Proteases, Protein homeostasis

Introduction
Maintenance of protein-folding homeostasis is essential for all organisms and requires molecular chaperones, which promote a functional protein-folding environment by preventing the aggregation of newly synthesized, newly imported or stress-denatured proteins, as well as by facilitating efficient folding and complex assembly of nascent polypeptides. (Bukau et al., 2006; Young et al., 2004). Studies of chaperone expression in response to unfolded protein accumulation have revealed a conserved signaling paradigm, where signaling is repressed by free chaperones that are not engaged by client proteins. For example, in Escherichia coli, the transcription factor σ32 is repressed by the Hsp70 chaperone DnaK (Guisbert et al., 2004). Upon accumulation of unfolded proteins, DnaK preferentially interacts with clients and releases σ32 to activate transcription of the heat-shock operon. The subsequent increase in chaperones re-establishes protein-folding homeostasis and the excess of free DnaK is able to interact with σ32 to repress the response (Straus et al., 1990; Tilly et al., 1983).

In compartmentalized eukaryotic cells, several pathways have evolved independently to ensure the integrity of the protein-folding environments in the cytosol, the endoplasmic reticulum (ER) and the mitochondria. All three compartments encounter nascent, unfolded polypeptides and each has a repertoire of compartment-specific chaperones to promote efficient folding. Unfolded protein stress is sensed in a compartment-specific manner and signaled to the nucleus for induction of the expression of compartment-specific chaperone genes. The cytosolic heat-shock response, which maintains protein-folding homeostasis in the cytosol, is mediated predominantly by the heat-shock factor (HSF) family of transcription factors (Fig. 1). Similarly to the bacterial response described above, Hsp70 binds to the transactivating domain of HSF1, thus repressing its transcriptional activity. Following either heat shock or any other condition that perturbs protein folding within the cytosol, Hsp70 preferentially interacts with the accumulating unfolded proteins, thus releasing HSF1 and allowing it to transcriptionally activate genes with promoters containing HSF1 binding sites, such as Hsp70, Hsp90 and proteasome subunits (Perisic et al., 1989; Shi et al., 1998).

Signaling within the UPRER is significantly complicated by the need to transduce the signal across the ER membrane to the nucleus (Fig. 1). The UPRER is a tripartite signaling pathway that is regulated by three ER-localized transmembrane proteins. Inositol-requiring 1 (IRE1), protein-kinase-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) all monitor the protein-folding status in the ER lumen through direct interactions with the ER chaperone-binding immunoglobulin protein (BiP) (Ma and Hendershot, 2001; Ron and Walter, 2007). In the most conserved branch of the UPRER, IRE1 is activated by its dissociation from BiP; this results in IRE1 oligomerization and activation of its cytosolic domain, which contains a sequencespecific RNase activity that splices the X-box binding protein 1 (XBP1) mRNA. The spliced XBP1 is then translated into a basic leucine zipper (bZip) transcription factor that traffics to the nucleus, where it upregulates genes encoding ER chaperones and the ER-associated degradation machinery (Calfon et al., 2002; Yoshida et al., 2001).

Here, we review current results from both mammalian cell culture and Caenorhabditis elegans on a conceptually similar pathway referred to as the mitochondrial unfolded protein response (UPRmt). The UPRmt is a stress response that activates transcription of nuclear-encoded mitochondrial chaperone genes to promote protein homeostasis within the organelle. We focus on recently identified components required for signaling the response and on the underlying biological conditions where it is active.
Unique challenges to mitochondrial protein-folding homeostasis

Mitochondria have dedicated machinery that functions to promote accurate protein folding and complex assembly within the organelle. Matrix-localized chaperones are required for protein import and facilitate protein folding, whereas proteases localized in the matrix and inner membrane degrade proteins that fail to fold or assemble correctly (Tatsuta and Langer, 2008). Mitochondria-localized chaperones include an Hsp70 family member, mtHsp70, as well as the orthologues to GroEL and GroES (Hsp60 and Hsp10), which are required for protein folding and complex assembly within the organelle. The UPRmt expands the folding capacity of the organelle during times of stress to maintain proteins in a folding or assembly-competent state, preventing deleterious protein aggregation.

UPR mt signaling in mammalian cells

Pioneering experiments from the Hoogenraad laboratory showed that disturbing the stoichiometry of mitochondrial- and nuclear-encoded proteins by selectively lowered expression from the mitochondrial genome or overexpression of a nuclear-encoded aggregation-prone protein targeted to the mitochondrial matrix results in increased expression of HSP60 and the mitochondrial protease ClpP in cultured mammalian cells (Martinus et al., 1996; Zhao et al., 2002). Importantly, the response was shown to be organelle specific because ER-specific chaperones are not induced. Furthermore, this transcriptional program appeared to have a role in maintaining protein homeostasis, because the upregulation of HSP60 and ClpP reduces mitochondrial protein aggregation (Zhao et al., 2002). Therefore, these studies provided the first clue to the existence of a mitochondrial UPR.

The first component identified to be required for HSP60 upregulation was the transcription factor CHOP (C/EBP homology protein) because a CHOP binding site was found in the HSP60 promoter, and subsequently, its dimerization partner C/EBPβ was identified (Fig. 2). Further support for a role of CHOP was illustrated by an increase in HSP60 transcription when CHOP is overexpressed and, conversely, expression of a dominant-negative CHOP repressed the response (Zhao et al., 2002). CHOP and C/EBPβ are not only required for induction of the stress response,
transcription of both is increased in response to mitochondrial stress and has been suggested to be an early event in the pathway (Horibe and Hoogenraad, 2007; Zhao et al., 2002). Analysis of the CHOP and C/EBPβ promoters revealed the presence of an activator protein-1 (AP-1) site in both, suggesting the involvement of the Jun transcription factor, which is known to bind AP-1 sites. Horibe and Hoogenraad demonstrated the involvement of JNK2, a kinase upstream of Jun, because it becomes phosphorylated during mitochondrial stress, and a JNK2 inhibitor attenuated the response. CHOP, C/EBPβ, and Jun are unlikely to be the only transcription factors involved in the UPRmt because promoters with CHOP binding sites that are activated in response to mitochondrial stress have two additional conserved sequences, the so-called mitochondrial UPR elements (MUREs) (Aldridge et al., 2007). This reveals the importance of stress-responsive transcription factors and kinases in the UPRmt and suggests the potential for a crosstalk between UPRmt signaling and other stress responses. However, such studies leave open the question of the identity of the sensing mechanisms that recognize the perturbation in the mitochondrial protein-folding environment.

**UPRmt signaling in C. elegans**

A genetic approach was taken in *C. elegans* where we generated reporter animals in which the expression of GFP is under the control of a mitochondrial chaperone gene promoter. Under normal conditions, GFP expression from the hsp-60 promoter is at a basal level, but increases significantly when mitochondrial stress is induced (Haynes et al., 2007; Yoneda et al., 2004). The UPRmt in *C. elegans* is activated by RNAi-mediated knockdown of factors required for mtDNA expression, mirroring the findings made in mammalian cells (Martinus et al., 1996). The worm UPRmt is also activated by knockdown of mitochondrial chaperones and proteases (Yoneda et al., 2004). A genome-wide RNAi approach was taken to identify components required to signal the UPRmt. Cellular components localized in the mitochondrial matrix, inner membrane, cytosol and nucleus that compose a plausible signal transduction pathway were identified (Fig. 3). Consistent with a role for these components in protection of the mitochondrial protein-folding environment, knockdown or deletion of all components sensitizes organisms to mitochondrial stress, resulting in altered mitochondrial morphology, slowed development, shortened lifespan and reduced mitochondrial function (Benedetti et al., 2006; Haynes et al., 2007; Haynes et al., 2010).

**ClpP and ClpX**

Detection of unfolded proteins within the mitochondrial matrix is probably the initial signaling event that triggers the UPRmt. The ATP-dependent protease ClpXP was shown to be required for induction of mitochondrial chaperone genes in response to stress (Haynes et al., 2007). We showed that it is localized within the matrix and potentially serves as the sensor by recognizing and degrading unfolded proteins that have accumulated beyond the mitochondrial chaperone protein-folding capacity (Haynes et al., 2007; Haynes et al., 2010). Eukaryotic ClpXP is involved in protein quality control because bacterial homologues perform a similar function to peptides in the degradation of substrates (Flynn et al., 2004; Yu and Houri, 2007). Furthermore, in the absence of ClpXP, proteolysis of unfolded mitochondrial proteins is impeded (Haynes et al., 2010). The requirement for a quality control protease in signaling the UPRmt is intriguing, but a plausible mechanism by which proteolysis could contribute to signal transduction was not revealed until the discovery of the involvement of a mitochondrial peptide transporter.

**HAF-1**

HAF-1 is a mitochondrial inner-membrane-localized ABC transporter shown to have a positive role in mitochondrial chaperone induction in response to stress (Fig. 3). HAF-1 is homologous to the yeast protein Mdl1, which is also located in the mitochondrial inner membrane and to the TAP transporter located in the mammalian ER membrane, where both act as peptide transporters (Shepherd et al., 1993; Young et al., 2001). The orientation of HAF-1 in the inner mitochondrial membrane suggested that it fulfills a similar function in pumping peptides from the matrix to the inner membrane space. Using purified mitochondria, we showed that HAF-1 is required for mitochondrial peptide efflux (Haynes et al., 2010) similarly to Mdl1 (Young et al., 2001). The HAF-1-dependent recovery of peptides in mitochondrial supernatants is consistent with the observation that once they cross the inner membrane, small peptides (<20 amino acids) are able to diffuse freely through the mitochondrial outer membrane into the cytoplasm (Young et al., 2001). HAF-1 is required for development and normal lifespan in the presence of mitochondrial stress, supporting the functional importance of its role in mitochondrial protein homeostasis (Haynes et al., 2010). These observations suggest a model whereby ClpXP-mediated mitochondrial unfolded protein degradation generates peptides that are pumped out of the organelle and contribute
somehow to downstream signaling in the UPR\textsuperscript{mt} and promote the return of the organelle to homeostasis.

The requirement for the quality control protease ClpXP suggests the UPR\textsuperscript{mt} in \textit{C. elegans} relies on principles common to other UPRs, in which organelle-specific chaperone occupancy regulates signaling (Prahlad and Morimoto, 2009). However, in the case of the worm UPR\textsuperscript{mt}, our findings suggest that free chaperones do not directly repress the downstream transcription factor because they are in separate compartments (see below), but, more likely, mitochondrial chaperones bind to unfolded proteins, thus preventing their degradation by ClpXP into peptides that contribute to the stress response. It is interesting to note that this proposed sensing mechanism of the UPR\textsuperscript{mt} uses components found in bacteria because according to the endosymbiotic theory, both mitochondria and bacteria originate from a common precursor. However, the downstream signal transduction mechanisms require specific proteins that most likely arose as a consequence of the compartmentalized nature of the eukaryotic cell.

**Downstream components of the UPR\textsuperscript{mt} pathway: ZC376.7, DVE-1 and UBL-5**

Three nuclear components of the UPR\textsuperscript{mt} pathway have been identified that regulate downstream steps. ZC376.7 is a basic leucine zipper protein that resides in the cytoplasm of unstressed cells (Haynes et al., 2010). Upon induction of mitochondrial unfolded protein stress, ZC376.7 accumulates in the nucleus through a step that depends on ClpP and the mitochondrial peptide transporter HAF-1. How HAF-1-mediated peptide efflux affects ZC376.7 is presently unknown, but its regulation is required for worm development during stress (Haynes et al., 2010). Worms lack a conspicuous homolog of CHOP; given that both ZC376.7 and CHOP are small basic leucine zipper proteins, it is tempting to speculate on the parallels in their function in the UPR\textsuperscript{mt}.

DVE-1 is a conserved DNA binding protein with a homeobox domain. The homologous mammalian proteins SATB1 and SATB2 are global chromatin organizers implicated in transcriptional control (Galande et al., 2007). In response to perturbations in the mitochondrial protein-folding environment, DVE-1 redistributes within nuclei and binds to the \textit{hsp-60} promoter potentially remodeling chromatin structure to promote ZC376.7 binding and transcriptional activation. Transcriptional upregulation of mitochondrial chaperone genes leads to their subsequent import into mitochondria, thus relieving stress and re-establishing homeostasis.

**Sources of mitochondrial unfolded protein stress**

In wild-type \textit{C. elegans}, UPR\textsuperscript{mt} reporter gene activity is observed during the L3–L4 stage of larval development, which is approximately 30–40 hours after hatching and when the germline begins to proliferate. During the L3–L4 stage, a burst of mitochondrial biogenesis is known to take place (Tsang and Lemire, 2002). If protein folding is not perturbed by mutation or pharmacological agents, the UPR\textsuperscript{mt}, which is promoted by the physiological increase in unfolded protein load is relatively short-
lived and attenuates when mitochondrial biogenesis subsides (Yoneda et al., 2004). However, when the folding environment is perturbed, for example by RNAi-mediated knockdown of mitochondrial chaperones, a dramatic increase in UPR\textsuperscript{mt} signaling is observed at the L3–L4 stage of development and beyond (Yoneda et al., 2004). Although it is clear that the knockdown of molecular chaperones causes unfolded protein stress, this rather broad perturbation does not provide any information on the identity of the unfolded proteins triggering the response.

An interesting clue comes from earlier studies that examined conditions that activate the UPR\textsuperscript{mt} in mammalian cell culture (Martinus et al., 1996). Cells cultured in the presence of ethidium bromide, which inhibits mitochondrial genome replication and transcription, experience strong activation of their UPR\textsuperscript{mt} most likely as a result of the accumulation of orphaned subunits from nuclear-encoded proteins of the electron transport chain. This point was supported in follow-up experiments in \textit{C. elegans}, where knockdown of individual components of hetero-oligomeric complexes was shown to activate the UPR\textsuperscript{mt}, presumably by promoting accumulation of orphaned subunits (Yoneda et al., 2004). Moreover, previous studies have shown that paraquat, an insecticide known to result in high levels of ROS, can also activate the UPR\textsuperscript{mt} (Cocheme and Murphy, 2008; Yoneda et al., 2004). Finally, direct evidence that accumulation of misfolded proteins activates the UPR\textsuperscript{mt} was shown in cell culture by targeting a mutant, aggregation-prone protein to the mitochondrial matrix (Zhao et al., 2002).

**Unfolded protein stress in diseases associated with mitochondrial dysfunction**

Numerous diseases are associated with mitochondrial dysfunction and failure of protein folding or assembly might contribute to their pathogenesis. These include spastic paraplegia, Parkinson’s disease, Friedreich’s ataxia and cancer, as well as disorders caused by mutations in the mitochondrial genome, such as Leigh Syndrome and Leber’s hereditary optic neuropathy (Wallace, 2005). In addition to certain specific characteristics (some of which are discussed further below), these conditions have important features in common, such as increased ROS generation, mitochondrial DNA mutation accumulation and a reduction in metabolic outputs, including ATP production, all of which secondarily affect protein-folding homeostasis in the mitochondria.

**Spastic paraplegia**

Spastic paraplegia is a neurodegenerative disease often associated with mitochondrial dysfunction. Among the known 13 mutations that cause spastic paraplegia, two impair the mitochondrial chaperonin Hsp60 and the protease Sgp7 (Casari et al., 1998; Hansen et al., 2002). Sgp7 is an AAA+ protease localized within the inner mitochondrial membrane and is required for the processing and the quality control of electron transport chain components as well as for mitochondrial ribosome biogenesis (Nolden et al., 2005). The SPG13 mutation in the \textit{HSP60} gene encodes a protein with compromised chaperone activity (Bross et al., 2008). Perhaps not surprisingly, knockdown of either HSP-60 or SGP-7 activates the UPR\textsuperscript{mt} in \textit{C. elegans} (Yoneda et al., 2004). Another intriguing study has shown that in patients with \textit{HSP60} mutations, ClpP expression is reduced as the disease progresses (Hansen et al., 2008). Assuming that the role of ClpP in the UPR\textsuperscript{mt} is conserved in humans, this feature would contribute to further deterioration in mitochondrial protein-folding capacity.

**Friedreich’s ataxia**

Friedreich’s ataxia (FA) is a neurodegenerative disease caused by a trinucleotide expansion within the first intron of the gene encoding frataxin, resulting in reduced expression (Rouault and Tong, 2005). Frataxin is a highly conserved mitochondrial-localized protein required for iron–sulfur (Fe–S) cluster biogenesis and intracellular iron homeostasis (Babcock et al., 1997). Reduced frataxin expression affects the assembly and function of proteins that require Fe–S clusters as a cofactor, including aconitase and several proteins within the electron transport chain (Rotig et al., 1997). Additionally, several cytosolic and nuclear proteins involved in DNA repair also require Fe–S clusters (Veatch et al., 2009). Interestingly, in a \textit{C. elegans} model of FA, both the cytosolic heat-shock response and the UPR\textsuperscript{mt} are activated, indicating the accumulation of unfolded proteins within mitochondria and the cytosol (Ventura et al., 2009), which might be due to an accumulation of folding intermediates lacking Fe–S cofactors. Furthermore, in a mouse model of FA, ClpP expression was shown to increase over time (Guillon et al., 2009), similarly to what was shown earlier in cell culture following overexpression of a model misfolded protein (Zhao et al., 2002). These data provide support for a role of the UPR\textsuperscript{mt} in protecting the mitochondrial protein-folding environment when subcellular levels of frataxin and Fe–S clusters are reduced.

**Cancer**

Many tumors and cancer cell lines have increased levels of mitochondrial chaperones consistent with an activated UPR\textsuperscript{mt} and an accumulation of unfolded proteins (Ghosh et al., 2008; Kang et al., 2007). In most cases, the exact underlying stress is unclear, but alterations that affect mitochondrial biology found in cancer cells are numerous, ranging from the increase in protein-altering ROS to genomic instability. Commonly observed genomic alterations include increased point mutations in the mitochondrial (Chatterjee et al., 2006) and nuclear genomes, as well as aneuploidy, all of which can disrupt protein assembly by altering the stability and stoichiometry of protein complexes (Luo et al., 2009).

Protein-destabilizing mutations in three of the four components of the succinate dehydrogenase complex cause head and neck tumors, as well as renal cell carcinomas (Baysal et al., 2000; Gogvadze et al., 2008). Additionally, numerous missense mutations that directly affect the folding and stability of mitochondrial protein complexes are found in cancer cells and are thought to enhance tumor growth (Petros et al., 2005) and metastasis (Ishikawa et al., 2008). Several mechanisms have been proposed to explain how mitochondrial dysfunction confers an advantage to tumor cells, including increased ROS signaling, suppression of apoptosis and reduced aerobic respiration, leading to increased glycolysis—the so-called Warburg effect (Gogvadze et al., 2008). Regardless of whether mitochondrial dysfunction itself is pathogenic or not, it appears that it is prevalent in cancers, suggesting that cancer cells might depend on cellular pathways that protect mitochondrial function, such as the UPR\textsuperscript{mt}.

**Conclusions and Perspectives**

The UPR\textsuperscript{mt} is a stress-response pathway that adapts the protein-folding capacity of the mitochondrial matrix to the load of unfolded proteins in normal physiology and disease states. The protein-folding load of the mitochondrial matrix is monitored, and accumulation of unfolded proteins is signaled to the nucleus with subsequent upregulation of the respective chaperone-encoding genes. The UPR\textsuperscript{mt} is organized such that changes in chaperone
expression are intimately associated with the degradation of a percentage of accumulating unfolded proteins, thereby ensuring protein-folding homeostasis at the level of both protein folding and removal.

Important questions regarding regulation of the UPRmt as well as the precise signaling mechanisms within the pathway remain to be resolved. Of particular interest is the mechanism by which ClpP-dependent proteolysis and HAF-1 activity or peptide efflux impact nuclear activities of the downstream transcription factors. For example, the existence of a cytosolic signaling molecule that might interact with effluxed peptides to propagate the signal has been hypothesized, although none has been reported to date (Haynes et al., 2010; Young et al., 2001; Zhao et al., 2002).

A more comprehensive determination of UPRmt transcriptional outputs dependent on each required transcription factor will potentially shed light on the global cellular response to mitochondrial stress and dysfunction (Aldridge et al., 2007). Further details of the conditions that activate the response and how unfolded proteins are recognized will provide a better understanding of upstream events in the UPRmt, as well as the underlying causes of mitochondrial dysfunction. Finally, all of the signaling components identified in *C. elegans* have mammalian orthologues and they should be examined in the cell culture system generated by Hoogenraad and colleagues to determine the extent of conservation in UPRmt pathways of different organisms.

What can studies of the UPRmt and its signaling components in higher eukaryotes reveal about mitochondrial biology and disease states involving mitochondrial dysfunction? If ClpXP acts as the stress sensor, ClpXP substrates are likely to represent some of the primary protein-folding defects within that particular organelle or specific cell type. One prediction is that different forms of stress will perturb the folding of different substrates, all of which are able to activate the signaling pathway. Identification of ClpXP substrates might thus provide important insights into the underlying causes of mitochondrial alterations or rearrangements found in a variety of biological scenarios.

The UPRmt is only one of the stress responses cells use to protect mitochondrial function. It will be interesting to learn how UPRmt signaling integrates with the retrograde response to respond to lower mitochondrial metabolic output (Liu and Butow, 2006). Furthermore, recent work has shown that terminally defective mitochondria can be degraded through the autophagy pathway that requires the proteins PINK1 and Parkin (Geisler et al., 2010; Narendra et al., 2010). It is possible that the UPRmt is the initial response to protect mitochondrial function and re-establish homeostasis, but organelles that are irreparably damaged are targeted by mitophagy. Furthermore, as damage accumulates and the majority of organelles become irreparable, programmed cell death pathways might be used. A possible mechanism was proposed whereby accumulating unfolded proteins within mitochondrial contribute to mitochondrial permeability transition, an early event in apoptosis (He and Lemasters, 2002). However, the relationship between the UPRmt, mitophagy and apoptosis remains to be examined.

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