The **Chop** Gene Contains an Element for the Positive Regulation of the Mitochondrial Unfolded Protein Response

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We have previously reported on the discovery of a mitochondrial specific unfolded protein response (mtUPR) in mammalian cells, in which the accumulation of unfolded protein within the mitochondrial matrix results in the transcriptional activation of nuclear genes encoding mitochondrial stress proteins such as chaperonin 60, chaperonin 10, mtDnaJ, and ClpP, but not those encoding stress proteins of the endoplasmic reticulum (ER) or the cytosol. Analysis of the chaperonin 60/10 bidirectional promoter showed that the CHOP element was required for the mtUPR and that the transcription of the chop gene is activated by mtUPR. In order to investigate the role of CHOP in the mtUPR, we carried out a deletion analysis of the chop promoter. This revealed that the transcriptional activation of the chop gene by mtUPR is through an AP-1 (activator protein-1) element. This site lies alongside an ERSE element through which chop transcription is activated in response to the ER stress response (erUPR). Thus CHOP can be induced separately in response to 2 different stress response pathways. We also discuss the potential signal pathway between mitochondria and the nucleus for the mtUPR.

**RESULTS**

**Transcriptional activation of chop**

We have previously developed an experimental model for production of a mtUPR and have shown that a mutant of the mtUPR suggesting that the chop promoter contains a mtUPR response element. Similarly, the erUPR also results in the transcriptional activation of the chop gene and it has recently been shown that elevation of CHOP in erUPR culminates in the elevation of the pro-apoptotic factor BIM and apoptosis [12].

In this paper, we describe the identification of an mtUPR response element and components of a signaling pathway that leads to the transcriptional activation of the chop gene in response to the accumulation of unfolded protein in the mitochondrial matrix of mammalian cells. In an accompanying paper [13], we describe features of the promoters of mtUPR responsive genes that are activated by CHOP and C/EBPβ in response to the accumulation of unfolded proteins in mitochondria.
Identification of an mtUPR element in the chop promoter

A deletion analysis of the chop promoter between bases −1015 and +17 (zero being the transcriptional start site) was carried out by assaying promoter activity using the luciferase reporter enzyme. MtUPR activity was measured by comparing the activity obtained from cells transfected with OTCΔ compared with empty vector and erUPR activity was measured by adding tunicamycin to cells transfected with the promoter-LUC construct. Deletions between −1015 and −325 had no effect on chop transcriptional activity (Fig 1C), whereas a further deletion of 103bp essentially ablated mtUPR inducible chop promoter activity. With respect to erUPR inducibility of chop, the critical element appears to lie between −105 and +1 bp (Fig 1C and Fig 2). A sequence comparison of chop promoters from human, bovine, mouse, and rat shows that this region between −278 and −222 contains an AP-1 site (Figure 2A), whereas the previously identified ERSE [14,15] lies between −105 bp and +1 bp (data not shown). The ERSE element consists of two transcription factor binding sites, ATF-6 [15] and NF-Y [16,17]. We deleted the AP-1, ATF-6, and NF-Y sites to determine if any of these sites were required for the regulation of CHOP expression in response to mtUPR. The deletion of the AP-1 site ablated the mtUPR responsiveness (Fig 1C). In contrast, the deletion of either ATF-6 or NF-Y elements, although substantially reducing the erUPR responsiveness (Fig 1C), did not remove the mtUPR responsiveness. Conversely the deletion of the AP-1 site had no effect on the erUPR responsiveness of the chop promoter, although the deletion of the NF-Y site did reduce the overall activity of the chop promoter.

As shown in Figure 2A, the promoter region flanking the AP-1 site is highly conserved in other mammalian chop promoters. These flanking regions may contain additional information for the activation of the chop gene by mtUPR. One of these regions contains a sequence homologous to a putative element, N30, previously identified in a homology search of promoter regions in a range of animal species [18] (Fig 2A, boxed sequence). Deletion of this element had a partial effect on the mtUPR responsiveness of the chop promoter (Fig 1C).

Since we previously showed that CHOP induces transcription of mtUPR responsive genes in combination with C/EBPα [9], it was of interest to note that the promoter of c/ebpb gene also contains an AP-1 site with highly conserved nucleotides (CCCA) in the region flanking the AP-1 site (Fig 2B). This site in the c/ebpb gene is also highly conserved between human, mouse, and rat promoter (Fig 2B) and therefore, we should expect both chop and c/ebpb transcription to be elevated by mtUPR. This was confirmed by Western blot analysis (Fig 2C). It has recently been shown that CHOP combines with C/EBPx or β to activate BIM transcription and apoptosis in response to erUPR [12]. However, the c/ebpb promoter does not contain an AP-1 site (data not shown). This raises the question whether mtUPR also induces apoptosis.

Involvement of JNK2 in mtUPR signaling

Since it is well-known that c-Jun, which is activated by JNK (c-Jun N-terminal kinase), binds to the AP-1 site [19] and it has been reported that the activation of JNK-dependent ATF2 (activated transcription factor 2) is important for the signaling from mitochondria to nucleus during both the genetic and metabolic stresses of mitochondria [3,20], we therefore investigated the effect of mtUPR on the phosphorylation of JNK1 and JNK2 (Fig 3A). The expression of OTCΔ in COS-7 cells had a substantial effect on the phosphorylation of JNK1 and 2 (Fig 3A). To further test the
potential role of JNK1 and JNK2 in mtUPR signaling, we
determined the effect of the MEK inhibitor PD98059 [21,22] on
JNK phosphorylation in response to expression of OTC
A s shown in Figure 3A, the inhibitor completely blocked mtUPR
dependent phosphorylation of JNK2, but had only a small effect
on JNK1 phosphorylation. These experiments were followed up
by measuring the effects of PD98059 on OTC
dependent
activation of the mtUPR responsive promoters
yme1l1 [13]
(Figure 3B) and
mpp [13] (Fig 3C). As shown in Figure 3B and
C, 10 µM MEK inhibitor inhibited the OTC
inducible
activation of the promoter-luciferase reporter constructs in
transfected COS-7 cells. This suggests that mtUPR signaling
utilizes the MEK/JNK2 pathway.

DISCUSSION
The evolution of the eukaryotic cell facilitated the development of
increased metabolic and functional complexity by dividing cells
into distinct, membrane enclosed compartments. However, these
organelles/compartments are extremely crowded, both in terms of
small solutes and macromolecules. Thus, it has been estimated that
the cytosol has a protein concentration of around 350 mg/ml [23]
and the concentration inside the mitochondrial matrix may
approach 500 mg/ml [24]. Not surprisingly then, the cell has
evolved stress response mechanisms which come into play under
conditions where unfolded proteins accumulate, such as the heat
shock response [25]. Equally, the cell has evolved mechanisms to
respond to the accumulation of unfolded proteins in organelle
compartments such as the ER, which has become known as the
UPR [14,15,26,27]. This response, which was initially discovered
in baker’s yeast [28] has been extensively investigated and is
characterized by the transcriptional regulation of a large group of
genes and post transcriptional regulation of proteins involved in
quality control of the secretory pathway [14,15,26,27].

We discovered an equivalent stress response pathway in
mitochondria of mammalian cells [9,10] and originally called it
the Mitochondrial Stress Response. More recently, Ron and
colleagues discovered the response in
C.elegans [11] and more
appropriately called the response the mtUPR, distinguishing it
from the erUPR, as we have done in this paper. Surprisingly, the
mtUPR has not been found in fungi and appears to be an
organelle specific stress response found only in multi-cellular
organisms.

We originally found that in the mammalian mtUPR responsive
gene cpn60/10, the CHOP and C/EBP
transcription factors were
involved in transcription regulation [9]. However, since mtUPR
also led to the transcriptional regulation of chop, this suggested that
the induction of the chop gene is an early event in mtUPR. We
were also intrigued by the finding that although there appears to
be little overlap in the mtUPR and erUPR, both responses led to
the induction of chop transcription. In this paper we describe the
identification of an mtUPR response element in the promoters of
both chop and c/ebp
genes. This element is an AP-1 site,
suggesting that mitochondrial to nuclear signaling of the
accumulation of unfolded proteins in the mitochondrial matrix is
through a JNK pathway. We show, using a specific MEK
inhibitor, that this signaling is through JNK2 and that an
inhibition in the phosphorylation of JNK2 also inhibits mtUPR.
We suggest that the cell can discriminate between organelle

Figure 2. Chop and c/ebp
promoters contain AP-1 sites and are inducible by mtUPR. (A) Nucleotide sequence alignment of the mammalian chop
promoters (~278 to ~222) from human, bovine, mouse and rat. Bold letters show the highly conserved bases of the AP-1 site and the asterisks show
the highly conserved sequence surrounding the AP-1 site in chop promoters. The position of the putative novel element of N 30 [18] is shown in the
box. (B): Nucleotide sequences of mammalian c/ebp
promoter region around AP-1 site (Human, Mouse, and Rat) is compared with the chop
promoter sequence (~278 to ~233). The numbers refer to the distance from transcription initiation site of human chop or human, mouse, and Rat c/
ebp. The asterisks indicate the conserved nucleotides around the AP-1 site. (C): C/EBP
expression in response to mtUPR. Extracts from cells
transfected with vector or OTCΔ were subjected to western blotting and probed with antibodies against C/EBPβ and tubulin as control and show
that C/EBPβ, like CHOP is induced by expression of OTCΔ.
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specific unfolded protein responses through different pathways to activate genes that harbor different stress elements within their promoters. Recently, it has been reported that JNK2 is a positive regulator of the cJun transcription factor [29], and can regulate both mitochondrial and lysosomal death pathways in mouse embryonic fibroblasts [30]. This, taken together with the data presented here, suggests that the JNK2 pathway may play a significant role for the communication from mitochondria to the nucleus in response to mtUPR. Since both mtUPR and erUPR activate transcription of a distinct set of genes, yet both induce CHOP, it is apparent that additional factors besides CHOP and C/EBPβ account for the specificity of the mtUPR. This specificity is provided for the erUPR by the transcription factors ATF6 and NFY [14,15]. The question of the specificity of mtUPR is further explored in the accompanying paper [13].

Recently, Benedetti et al. [31] have carried out a search for genes involved in signaling of mtUPR in *c. elegans* and discovered the involvement of the *ubl-5* gene, encoding the ubiquitin-like protein 5. Whether this pathway exists in mammalian cells, or whether this pathway in *c. elegans* intersects with the pathway we describe here is currently unknown, as is the question whether the CHOP based response described in this paper operates in *c. elegans*.

**MATERIALS AND METHODS**

**Materials**

Tunicamycin was purchased from Sigma Chemical (St Louis, USA). MEK inhibitor, PD98059, anti-C/EBPβ, and anti-pJNK were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). All reagents were of reagent grade quality.

**Plasmid construction, transfection and promoter analysis**

Mammalian expression vectors of wild-type OTC and deletion mutant OTCΔ were constructed as described previously [9]. Transfection efficiencies were between 72 and 85% as determined by transfections with a GFP construct. Based on the human genome sequence information of NCBI, the promoter region of CHOP (from −1015 to +17) was amplified by PCR [32] from human genomic DNA (Promega, Madison, USA) using 5'-CTTTTGGGAGATCTACGGGGCCTAGAAGGAGACCACCC-3' and 5'-GATACGCTCAGAGGTTAGACTTAAGTCTCTGACCTCGG-3' as the upper and lower primers, respectively [mutated nucleotides to introduce *Bgl*II and *Hind* III are underlined], and cloned into *Bgl*II-*Hind* III sites of the
pGL3-Basic vector (Promega, Madison, USA), which contains the firefly luciferase coding sequence but lacks eukaryotic promoter or enhancer elements. For the GFP assay of promoter constructs, luciferase was replaced by GFP cDNA using the firefly luciferase coding sequence but lacks eukaryotic

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