Selective induction of mitochondrial chaperones in response to loss of the mitochondrial genome

Ryan D. MARTINUS', Graeme P. GARTH', Tracie L. WEBSTER', Peter CARTWRIGHT', Dean J. NAYLOR', Peter B. HOJ' and Nicholas J. HOOGENRAAD

1 School of Biochemistry, La Trobe University, Victoria, Australia
2 Department of Horticulture, Viticulture and Oenology, University of Adelaide, South Australia, Australia

(Received 29 January/28 May 1996) — EJB 96 0117/1

Molecular chaperones are known to play key roles in the synthesis, transport and folding of nuclear-encoded mitochondrial proteins and of proteins encoded by mitochondrial DNA. Although the regulation of heat-shock genes has been the subject of considerable investigation, regulation of the genes encoding mitochondrial chaperones is not well defined. We have found that stress applied specifically to the mitochondria of mammalian cells is capable of eliciting organelle-specific, molecular chaperone response. Using the loss of mitochondrial DNA as a means of producing a specific mitochondrial stress, we show by Western-blot analysis that mtDNA-less (g') rat hepatoma cells show an increase in the steady-state levels of chaperonin 60 (cpn 60) and chaperonin 10 (cpn 10). Nuclear transcription assays show that the upregulation of these chaperones is due to transcriptional activation. There was no effect on the inducible cytosolic Hsp 70, Hsp 72, nor on mtHsp 70 in g' cells, leading us to conclude that stress applied selectively to mitochondria elicits a specific molecular chaperone response. Heat stress was able to provide an additional induction of cpn 60 and cpn 10 above that obtained for the g' state alone, indicating that these genes have separate regulatory elements for the specific mitochondrial and general stress responses. Since the mitochondrial-specific chaperones are encoded by nuclear DNA, there must be a mechanism for molecular communication between the mitochondrion and nucleus and this system can address how stress is communicated between these organelles.

Keywords: molecular chaperone; mitochondria; stress response; heat shock; gene activation.

Molecular chaperones are found in all of the major sub-cellular compartments and have been shown to play essential functions in the folding and subcellular targeting of proteins. In addition, the ability of cells to survive stresses such as elevated temperatures, exposure to heavy metals and amino acid analogues is dependent on the induction of molecular chaperones (reviewed in [11]). Many of the insights into their roles in protein targeting have come from studies on the biogenesis of fungal mitochondrial proteins (reviewed in [2-4]). To date, four mammalian mitochondrial chaperones have been identified. We have isolated cDNAs encoding rat mitochondrial Hsp 70 (mtHsp 70) [5], rat chaperonins 60 and 10 (cpn 60 and cpn 10) [6, 7] and a rat protein homologue of GrpE [8]. In yeast cells, mtHsp70 and GrpE have been shown to be associated with the matrix face of the inner mitochondrial membrane, where they are involved in the import of nuclear-encoded proteins [9, 10]. mtHsp 70 has also been implicated in the stabilisation of mitochondrially encoded proteins as well as in the degradation of mitochondrial proteins [11], whilst cpn 60 and cpn 10 are thought to play important roles in the folding and stabilisation of proteins within the mitochondrion [12-14]. Their importance has been highlighted by gene disruption experiments in Saccharomyces cerevisiae, which have shown that all of the above mitochondrial chaperones are essential for cell viability [10, 15-17]. The upregulation of mitochondrial chaperones in response to metabolic insults such as heat shock, glucose deprivation, amino acid analogues and agents that impair energy metabolism [18-19], suggest that they also play an important role in maintaining homeostasis in mitochondria when a general stress is applied to the cell. However, it is not known if mitochondrial chaperones are induced specifically in response to the application of a selective stress to mitochondria.

Chaperone synthesis can be regulated in part by modulation of the message stability and the frequency of translation initiation, but is regulated mainly through specific transcriptional factors termed heat-shock factors (reviewed in [20]). The binding of heat-shock factors to DNA regulatory sequences, called heat-shock elements, promotes the expression of heat-shock genes, and is thought to be modulated by a number of processes such as oligomerisation, phosphorylation and interaction with heat-shock proteins [20].

Whilst cells have a generalised mechanism for responding to various stresses, at a more specific level, can the cell also respond to a stress which exists in a particular subcellular compartment? It has been shown that inhibition of glycosylation and, therefore, protein folding within the endoplasmic reticulum, results in a specific induction of the endoplasmic reticulum isoform of Hsp 70, Bip [21]. Using complementation cloning in Saccharomyces cerevisiae, Mori et al. [22] identified a putativecdc2+/CDC28-related transmembrane kinase (ERN1) which somehow is able to sense the accumulation of unfolded proteins within the endoplasmic reticulum and communicate with the nu-
nucleus through the activation of an ‘unfolded protein response factor’ which subsequently binds to an ‘unfolded protein response element’ in the upstream region of the KAR2 (Bip) gene. This raises the question whether cells can respond specifically to stress which affects other subcellular compartments. To answer this question, we have devised an experimental system to induce a specific mitochondrial stress, and see whether this results in the selective induction of genes encoding mitochondrial chaperones.

Some cells are able to be totally depleted of their mtDNA by exposure of actively growing cells to ethidium bromide for periods of up to 10–14 generations, producing the g0 state [23]. Once obtained, such cells can be successfully propagated in the presence of pyruvate and uridine in the growth medium [24]. Since the polypeptides normally encoded by mtDNA are essential components for oxidative phosphorylation at the inner mitochondrial membrane [25], cells which lack mtDNA are reliant on glycolysis for their energy demands. In such cells, pyruvate is considered to act as a redox sink (via a pyruvate/lactate couple) which enables the re-oxidation of reducing equivalents (NADH) generated during glycolysis [26]. Uridine is required to bypass a blockage in the de novo pyrimidine biosynthesis pathway at the level of dihydroorotate dehydrogenase [27].

In this paper, the ability to successfully propagate mammalian cells lacking mitochondrial oxidative phosphorylation functions, in growth medium supplemented with pyruvate and uridine, has been exploited to study the mitochondrial molecular chaperone response. We report here that a selective stress aimed at the mitochondrion, such as that produced by the g0 state, is able to elicit a specific molecular chaperone response from the nucleus. We show that both cpm 60 and cpm 10 are induced in these cells, whereas cytosolic Hsp 72 and mtHsp 70 are unaffected by this stress. This implies that not only is there a specific stress-signalling mechanism between the mitochondrion and nucleus but there is also a mechanism whereby only a subset of mitochondrial chaperones can be induced.

**EXPERIMENTAL PROCEDURES**

**Cell line and growth conditions.** A clonal rat hepatoma cell line (H4) was routinely cultured in DMEM medium (Multi Cell, Trace Biosciences, Pty. Ltd) supplemented with 10% (by vol.) fetal calf serum (Multi Ser, Trace Biosciences, Pty. Ltd) in a 5% CO2 atmosphere at 37°C. Viable cells were counted in a hemocytometer (Neubauer improved-type) by trypan blue dye exclusion using a 0.05% (mass/vol.) trypan blue solution (Sigma).

**Ethidium bromide treatment of cultured cells.** A cell line completely devoid of mtDNA (g0) was obtained by growing the parent cells in the presence of ethidium bromide (EtBr, 50 ng/ml) as described previously [23]. During this treatment and for subsequent propagation of the g0 cells, the growth medium was supplemented with pyruvate (1 mM) and uridine (50 μg/ml) [24]. The total ablation of mtDNA was achieved after growing the cells in the presence of EtBr for a period of 10 generations. The presence of mtDNA was monitored by a PCR-based procedure [28]. The sequences of the primers RL7567 and RL8381 used were complementary to the rat mitochondrial genome positions 7567–7589 (light strand) and 8381–8361 (heavy strand), respectively [29]. PCR products were separated by electrophoresis in 1% agarose gels containing EtBr (1 μg/ml). Once the g0 state had been obtained, residual EtBr was removed by extensive washing and the cells cultured in normal growth medium supplemented with pyruvate and uridine without EtBr. Under these conditions, the EtBr has no effect on the integrity of nuclear DNA [30] and the g0 cells have been cultured continuously for more than 12 months.

**Heat-shock treatment of cell cultures.** Parent (g+) and mtDNA-less (g0) hepatoma cells in the logarithmic growth phase were heated by replacement of the growth medium with fresh medium heated to a temperature of 45°C followed by emersion in a waterbath for 20 min. Following heat treatment, cells were centrifuged at 2500 rpm in a bench top centrifuge (MSE) for 5 min and re-suspended in fresh growth medium and incubated at 37°C. For Northern-blot and Western-blot analyses, cells were harvested 2 h and 6 h post-heat shock, respectively.

**SDS/PAGE and Western-blot analysis.** Total cellular protein was solubilised from cell pellets by incubation in 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 (TENT buffer) with 0.4 mM phenylmethanesulfonyl fluoride (PhMeSO,F) for 30 min at 4°C. After vortexing, cell debris and nuclei were removed by centrifugation in an Eppendorf centrifuge (15000 rpm) for 5 min at room temperature. Protein concentrations were determined by the biocinchoninic acid protein assay (Pierce). Equivalent amounts of protein (10 μg) from each sample was separated by SDS/PAGE in 10% gels and transferred to nitrocellulose (0.45 micron, Micron Separations Inc.) by a semi-dry blotting procedure [31]. After visualising the transferred proteins by Ponceau S staining, membranes were sequentially probed with the following antibodies: rabbit polyclonal antibodies against cpm 60 and cpm 10 (kindly donated by Dr A. Giraud, Melbourne, Australia), a mouse monoclonal antibody (N6-F3-5) which recognises both constitutively expressed Hsp 73 and stress-inducible Hsp 72 (kindly donated by Dr W. Welch, San Francisco, USA), a chicken polyclonal antibody prepared against mitochondrial Hsp 70 and a rabbit polyclonal antibody against pyruvate dehydrogenase (E1 α subunit; kindly donated by Dr D. Thorburn, Melbourne, Australia). Antibodies binding to the filters were detected with an appropriate secondary antibody labeled with horseradish peroxidase (Silenus) followed by incubation with enhanced chemiluminescence detection reagent (Amersham) and subsequent exposure on X-Omat film (Kodak).

**Immunofluorescence microscopy.** Cells growing in slide chambers were washed with 137 mM NaCl, 2.7 mM KCl, 8.7 mM NaH2PO4, 1.5 mM NaH2PO4, pH 7.4 (NaCl/P) prior to fixation and permeabilisation in a solution of 50% ethanol/50% acetone (by vol.) for 30 min at room temperature. After several rinses with NaCl/P, containing 0.2% Tween (by vol.) the primary antibody (anti-cpm 60) was added at a dilution of 1:1000 and incubated in a humidified chamber for 4 h at room temperature. After several washes with NaCl/P, containing 0.2% Tween, the cells were incubated with fluorescein-conjugated goat anti-rabbit IgG diluted 1:200 in NaCl/P. Following further washes with NaCl/P, containing 0.2% Tween, the slides were mounted and examined by phase and fluorescence microscopy on an Olympus BH-2 microscope. Direct cytofluorescence of mitochondria was also measured on unfixed cultures of rat hepatoma cells using the mitochondrial-specific stain, rhodamine 123 (Kodak). Cells were incubated for 30 min at 37°C in culture medium containing 10 μg/ml rhodamine 123, washed extensively in NaCl/P, then directly viewed by fluorescence microscopy.

**Nuclear transcription assay.** The nuclear transcription of cpm 60, cpm 10, mtHsp 70 and cytosolic Hsp 72 were measured by the nuclear runoff assay as described by Celano et al. [32]. Nuclear fractions from both g+ and g0 cells were isolated by the following procedure: cells (1–5×106), harvested by centrifugation at 2500 rpm in a bench top centrifuge (MSE), were lysed in sucrose buffer I (0.32 M sucrose, 3 mM CaCl2, 2 mM magne-
Fig. 1. Progressive depletion of mtDNA in rat hepatoma cells (H4) exposed to EtBr (50 ng/ml). After extracting total cellular DNA from cells treated with EtBr for 0, 1, 2 and 4 weeks, samples (20 ng) were subjected to PCR amplification using the primer pair RL7567 and RL3831, which amplifies an 815-bp segment of mtDNA. The PCR products were separated on a 1% agarose gel as follows: untreated cells (lane 1), cells treated for 1 week (lane 2), 2 weeks (lane 3) and 4 weeks (lane 4). The arrow on the right-hand side of the gel indicates the position of the predicted 815-bp PCR product. The DNA size marker is λ DNA digested with both EcoRI and HindIII.

RESULTS

Establishment of rat hepatoma ρ<sup>−</sup> cells. Rat hepatoma cells (H4), when subjected to heat shock, are capable of eliciting a good mitochondrial heat-shock response [7, 19] and were, therefore, used as the parent cell line for the preparation of ρ<sup>−</sup> cells. A cell line devoid of mtDNA was produced by growing the parent cells in the presence of EtBr (50 ng/ml) for a period of 10 generations. The progressive decline in mtDNA levels was monitored by PCR amplification using mtDNA-specific primers. The primers used resulted in the amplification of a 815-bp segment of mtDNA from untreated ρ<sup>−</sup> cells. Indeed, after 4 weeks, no mtDNA was detected (Fig. 1). These cells had an increased population doublings time of 30 h compared to 22 h for the respiratory-competent parent cells, and were unable to be propagated in the absence of the pyruvate and uridine supplementation of the growth medium (Fig. 2). Similar growth characteristics have been documented for human ρ<sup>−</sup> cells [26]. The growth of the parent cell line was not affected by the pyruvate/uridine supplementation of the growth medium (Fig. 2), showing that the stress response in ρ<sup>−</sup> cells is not due to the addition of pyruvate or uridine. Cells have been propagated in the absence of EtBr for more than 12 months and the stability of the cell line was indicated by the absence of detectable mtDNA after this period of time and their continued dependence on pyruvate and uridine for growth.

Molecular chaperone levels in rat hepatoma ρ<sup>−</sup> cells. The level of selected molecular chaperones in the ρ<sup>−</sup> cells was measured immunocytochemically by Western blotting. The steady-state level of cpn 60 and cpn 10 was increased 2.5-fold in ρ<sup>−</sup> cells compared with the parent cell line (Fig. 3). Significantly, the levels of these chaperones could be further increased by heat shock, suggesting that the mitochondrial stress was separately regulated from the classical heat-shock response. The specificity of the mitochondrial stress response was measured by probing the Western blots with a monoclonal antibody which recognises both constitutively expressed cytosolic Hsp 73 and stress-inducible cytosolic Hsp 72. Both parent and ρ<sup>−</sup> cells responded in the normal manner to heat shock, by the induction of cytosolic Hsp 72 (Fig. 3). In contrast, cell extracts from untreated ρ<sup>−</sup> cells showed...
no evidence for an induction of the cytosolic Hsp 72. This result suggests that the elevated levels of cpn 60 and cpn 10 found in q<sup>0</sup> cells is not due to a general stress response in these cells, but represents a specific response to the q<sup>0</sup> state. The upregulation of cpn 60 and cpn 10 in q<sup>0</sup> cells is also not due to a general upregulation in the synthesis of nuclear-encoded proteins imported into the stressed mitochondria. Thus, similar steady-state levels of pyruvate dehydrogenase (E1a subunit) were found in both parent and q<sup>0</sup> cells (Fig. 3). Further, the response to the mitochondrial stress does not affect all mitochondrial chaperones as mtHsp 70 levels were unaltered in q<sup>0</sup> cells. As is the case with the constitutive cytosolic Hsp 70 isoform, Hsp 73, this chaperone was also unaffected by heat shock.

A comparison of the immunofluorescence-staining pattern obtained with cpn 60 antisera with that of rhodamine-123-stained mitochondria demonstrated that cpn 60 is localised within the mitochondria of both parent and q<sup>0</sup> cells (Fig. 4). The mitochondrial localisation of cpn 60 is shown by the punctate staining pattern which is similar to that obtained with rhodamine 123. These immunofluorescence studies of q<sup>0</sup> cells using the anti-(cpn 60) serum revealed that the intensity of the emitted green light was also significantly increased, thereby supporting the immunoblot experiments (Fig. 3). The mitochondrial localisation of cpn 60 was further demonstrated by the immunoblot experiments. Thus, the size of the cpn 60 before and after induction found in q<sup>+</sup> and q<sup>0</sup> cells is that of the mature cpn 60 (57.9 kDa), which is produced by proteolytic processing of the 60.9-kDa precursor within the mitochondrial matrix [35].

In order to determine if the increase in expression of cpn 60 and cpn 10 at the protein level in q<sup>0</sup> cells was due to gene activation, we measured transcription in nuclei isolated from both parent and q<sup>0</sup> cells. There was a sixfold and eightfold induction in cpn 60 and cpn 10 genes in nuclei from q<sup>+</sup> and q<sup>0</sup> cells as described in Experimental Procedures. The data are the mean ± SEM of four independent experiments.

<table>
<thead>
<tr>
<th>Chaperonin</th>
<th>Transcriptional activation of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>q&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>cpn 60</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>cpn 10</td>
<td>2.7 ± 0.4</td>
</tr>
</tbody>
</table>

DISCUSSION

We have shown that rat hepatoma cells can respond to a specific stress applied to the mitochondria by the selective induction of the nuclear-encoded, mitochondrial chaperones, cpn 60 and cpn 10. It has long been known that depletion of ATP
by various mitochondrial respiratory chain inhibitors results in a general heat-shock gene activation (reviewed in [36]). However, as shown in this paper, despite their inability to produce ATP via oxidative phosphorylation, \( q^0 \) cells do not show a general stress response and have apparently become adapted to the loss of mitochondrial ATP production as the inducible cytosolic Hsp 70 (Hsp 72) is not produced in these cells (Fig. 3). A recent report showed that the expression of Hsp 70 was unaltered in a human \( q^0 \) cell line [37]. We also found that the level of mtHsp 70 was not increased in \( q^0 \) cells. In contrast, the elimination of mtDNA and, therefore, the absence of the respiratory proteins encoded by this genome, nevertheless provides a selective stress since there is an induction of mitochondrial chaperones cpn 60 and cpn 10 (Fig. 3). Thus, the 2–3-fold increases in the levels of cpn 60 and cpn 10 were shown to be primarily due to activation of transcription (Fig. 5 and Table 1). The possibility exists that, in addition to the gene activation that we observed, the upregulation of cpn 60 and cpn 10 in \( q^0 \) cells could be further enhanced by post-transcriptional mechanisms, such as stabilisation of mRNA levels; such is the case after inhibition of mitochondrial protein synthesis with thioumiocal, where there is both an increase in the transcription of selected nuclear genes encoding electron transport chain proteins and an increase in their stability [38].

Although cpn 60 and cpn 10 were upregulated in \( q^0 \) cells, not all mitochondrial chaperones were upregulated together, as the level of mtHsp 70 was essentially unaltered. It has been demonstrated that mtHsp 70 is primarily involved in the mechanism of translocation of proteins across the mitochondrial membranes [9]. Its failure to respond to the mitochondrial stress raises questions about its involvement in protein folding within the mitochondria. As mitochondria are the site for protein import into mitochondria is an electrochemical gradient in the absence of oxidative phosphorylation capacity in such cells remains to be elucidated.

The pattern of induction shown for cpn 60 and cpn 10 in \( q^0 \) cells suggest that there are two separate regulatory elements in the promoters of the cpn 60 and cpn 10 genes. Thus, these genes were induced by the \( q^0 \) state but could be further induced by heat shock. In contrast, the Hsp 72 gene is only responsive to heat stress but not the specific mitochondrial stress, and the mtHsp 70 gene was not responsive to either form of stress (comparable to the cytosolic Hsp 73). A precedent for this dual effect is found in yeast where the promoter region of the yeast KAR2 (BiP) gene contains a heat-shock element and a regulatory domain that responds to the presence of unfolded protein in the endoplasmic reticulum [39]. We are currently analysing regulatory sequences in cpn 60 and cpn 10 genes for elements that can respond to the observed general and specific stress signals. The results presented in this paper suggests that there is a specific mechanism whereby a mitochondrial stress can be signalled to the nucleus resulting in the induction of selected mitochondrial chaperones, independent of heat-shock factor activation. Organelle-specific stress responses in yeast have been documented with respect to the endoplasmic reticulum [21] and, recently, with respect to the cytosol, where depletion of a subunit of the signal recognition particle (SRP) has been shown to selectively induce cytosolic Hsp 72 levels [42]. The ability of the nucleus to respond to a stress within the mitochondrial compart- ment suggests that there is a molecular communication mechanism between the mitochondria and the nucleus. Although the endoplasmic-reticulum-specific stress response provides a precedent for a molecular communication pathway, the mechanism for mitochondrial communication is likely to be different, if for no other reason than that signalling has to occur across two membranes in mitochondria compared with one in the endoplasmic reticulum. Based on the findings on endoplasmic reticulum/nuclear communication, the pathway is likely to include a receptor in the mitochondria, a signal-transduction pathway and a specific transcription factor binding to a unique promoter element. The nature of organelle-nuclear communication is likely to be even more complex than this simple model suggests, as evidenced by the discovery of the RTG1 and RTG2 genes controlling interorganelle communication between mitochondria, peroxisomes and nuclei in S. cerevisiae [43, 44].

Thus, an understanding of the mechanism by which mitochondrial stress can result in the activation of genes encoding mitochondrial chaperones will depend on the identification of the appropriate promoter element, transcription factors that bind to this element and the signal-transduction components and receptor that lead to the activation of the transcription factors. We have recently isolated the cpn 60 and cpn 10 genes from rat and found that they are co-regulated by a common, bidirectional promoter (Ryan, M., unpublished results), providing an explanation for the co-ordinate regulation of these genes in the \( q^0 \) cells. The experimental system described here is the first step forwards the identification of the components mediating the organelle-specific induction of mitochondrial stress proteins.

We gratefully acknowledge the provision of a cpn 10 cDNA clone by Michael Ryan (Adelaide, Australia), the helpful suggestions made by Trevor Lithgow (Melbourne, Australia) after critically reading the manuscript, and to Nicola Daly (Melbourne, Australia) for statistical analysis. This work was supported by grants to NJH and PBH from the Australian Research council and the National Health and Medical Research Council of Australia. GPG, TLW and DJN are recipients of Australian postgraduate Awards.

REFERENCES


