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Mitochondrial assembly: protein import

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The protein import process of mitochondria is vital for the assembly of the hundreds of nuclear-derived proteins into an expanding organelle reticulum. Most of our knowledge of this complex multisubunit network comes from studies of yeast and fungal systems, with little information known about the protein import process in mammalian cells, particularly skeletal muscle. However, growing evidence indicates that the protein import machinery can respond to changes in the energy status of the cell. In particular, contractile activity, a powerful inducer of mitochondrial biogenesis, has been shown to alter the stoichiometry of the protein import apparatus via changes in several protein import machinery components. These adaptations include the induction of cytosolic molecular chaperones that transport precursors to the matrix, the up-regulation of outer membrane import receptors, and the increase in matrix chaperonins that facilitate the import and proper folding of the protein for subsequent compartmentation in the matrix or inner membrane. The physiological importance of these changes is an increased capacity for import into the organelle at any given precursor concentration. Defects in the protein import machinery components have been associated with mitochondrial disorders. Thus, contractile activity may serve as a possible mechanism for up-regulation of mitochondrial protein import and compensation for mitochondrial phenotype alterations observed in diseased muscle.

Skeletal muscle: Exercise: Protein import: Mitochondrial disease

The assembly of an organelle as intricate as the mitochondrion requires the integration of several vital processes, including the transcription of hundreds of mitochondrial-associated genes, the translocation and targetting of newly synthesized proteins and the assembly of proteins into a functional unit. Since the mitochondrial genome is limited in the number of genes it can encode, the nucleus, in coordination with the mitochondria, is given the crucial task of being the major provider of these essential polypeptides. The important function of trafficking these nuclear-transcribed and cytosolically-synthesized proteins into the organelle is carried out by several translocation processes, each consisting of a distinct network of multisubunit proteins, collectively referred to as the mitochondrial protein import machinery (Truscott *et al*. 2003). Why is the study of mitochondrial assembly and protein import so important? The failure to incorporate an appropriate combination of gene products can lead to reduced synthesis of ATP, or the accelerated production of reactive oxygen species, both of which can lead to cell death, or mitochondrial disease.

Mitochondria exist as different subfractions within striated muscle

The mitochondrion is subdivided into four compartments, including the intermembrane space, the matrix and the outer and inner membranes. In striated muscle mitochondria exist within separate cellular compartments as two functionally and biochemically distinct groups: subsarcolemmal (SS) mitochondria, which accumulate beneath the sarcolemma; intermyofibrillar (IMF) mitochondria, which are found interspersed among the myofibrils (Hoppeler, 1986; Cogswell *et al*. 1993). In general, numerous studies indicate that, while the SS mitochondria are more concentrated and closely opposed to each other around the perimeter of the cell, they occupy a lower percentage of the total mitochondrial volume (approximately 10–20,

Abbreviations: Hsp, heat-shock protein; IMF, intermyofibrillar; SS, subsarcolemmal; Tim, component proteins of the translocases of the inner mitochondrial membrane; Tom, component proteins of TOM; TOM, translocases of the outer mitochondrial membrane.

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depending on tissue and species) than the IMF mitochondria (Hoppeler, 1986). It is also well established that SS mitochondria are more adaptable than IMF mitochondria, in that the concentration of SS mitochondria is readily changed in response to conditions of muscle use and disuse (Hood, 2001). In addition, in certain mitochondrial diseases the histochemical appearance of ragged-red fibres is a result of the abnormal proliferation of SS mitochondria under the sarcolemma (Rifai *et al*. 1995). Thus, in response to the same cellular stimulus SS mitochondrial volume expansion within the cell is differentially affected compared with IMF mitochondria.

Protein targetting to the mitochondria

Initial studies of mitochondrial protein import were performed in the yeast *Saccharomyces cerevisiae* and the fungus *Neurospora crassa*. These investigations continue to contribute immensely to our understanding of the components of the protein import machinery. In mammalian cells work has progressed much more slowly, with research just beginning to identify the essential components of this complex protein trafficking system.

The process begins with the synthesis of precursor proteins, which are transcribed in the nucleus, escorted to the mitochondria and unfolded via a specialized group of molecular chaperones within the cytosol (Komiya *et al*. 1996; Fig. 1). The most important of these chaperones are the cytosolic heat-shock protein (Hsp) 70 and mitochondrial import stimulation factor. These chaperones interact with precursor proteins depending on the presence of specific targetting information within the primary structure of the protein. The targetting signal can be found as cleavable N-terminal extensions termed presequences, or embedded within the mature polypeptide as a non-cleavable sequence (Neupert, 1997). The type of signal contained in the precursor protein not only dictates its localization within the mitochondria, but also the translocation pathway that it will take and its energy requirement. For example, ATP-dependent mitochondrial import stimulation factor-mediated import shows a preference for preproteins with an internal signal, whereas cytosolic Hsp70 translocation is not ATP dependent and binds to preproteins containing a presequence (Komiya *et al*. 1996; Fig. 1).

The translocases of the outer mitochondrial membrane machinery

A multimeric receptor complex termed the translocases of the outer mitochondrial membrane (TOM; comprising component proteins (Tom); Fig. 1) exists in the outer mitochondrial membrane to interact with chaperones and their associated precursor proteins (also termed precursors). This complex is the only entry gate for precursors destined for the mitochondria. Precursors are recognized by the three receptor proteins Tom20, Tom22, and Tom70 present on the outer mitochondrial surface. Tom70 generally binds to precursor proteins with an internal targetting signal (Neupert, 1997; Pfanner & Geissler, 2001). A tetratricopeptide repeat domain present in Tom70 acts as a landing site for cytosolic chaperones, thereby facilitating the translocation of precursor proteins across the outer membrane (Young *et al*. 2003). These protein–protein interaction domains have also been identified in several other TOM components, including Tom20 (Abe *et al*. 2000) and Tom34 (Nuttall *et al*. 1997).

The remaining TOM complex is composed of Tom40 and the accessory proteins Tom5, Tom6 and Tom7 (Dekker *et al*. 1998). Together with Tom22, these proteins form a 400 kDa conducting channel known as the general import pore (Dekker *et al*. 1998; van Wilpe *et al*. 1999). Using blue native gel electrophoresis, Model *et al*. (2001) have shown in yeast that the biogenesis of the 400 kDa general import pore into the outer membrane is preceded by two assembly intermediates of 250 and 100 kDa. This technique should prove useful in future work to establish the steps in the assembly of multisubunit complexes in mammalian cells, and whether these steps can be accelerated under conditions of mitochondrial biogenesis.

Additional components implicated in the mammalian translocation pathway include OM37 and Tom34. OM37 is a 37 kDa protein of the outer membrane that serves as a docking site for mitochondrial import stimulation factor-mediated precursor proteins (Komiya *et al*. 1996). In contrast, Tom34 is characterized as a peripheral membrane protein primarily found within the cytosol (Chewawiwat *et al*. 1999). Although the precise role of this protein remains enigmatic, its association with numerous cytosolic proteins suggests that it may have a chaperone-like role to facilitate precursor import into mitochondria (Yang & Weiner, 2002).

The translocases of the inner mitochondrial membrane machinery

The next portion of the translocation pathway involves the movement of precursors from the TOM complex across the intermembrane space to the inner membrane. This process involves a component protein (Tim) of the translocases of the inner mitochondrial membrane complex, Tim50; a protein with a single transmembrane segment consisting of a large hydrophilic domain located in the intermembrane space (Mokranjac *et al*. 2003). Tim50 appears to interact with translocation intermediates destined for the matrix, and it is involved in transferring precursors between the TOM and translocases of the inner mitochondrial membrane complexes (Geissler *et al*. 2002; Yamamoto *et al*. 2002). The Tim23 complex consists of Tim23, Tim17 and Tim44 subunits, and it serves to accept precursors containing a cleavable N-terminal sequence, and direct their entry into the inner membrane or the matrix (Pfanner & Geissler, 2001; Fig. 1). This process is facilitated by several lower-molecular-weight proteins in the intermembrane space, such as Tim8 and Tim13, that serve as chaperones to pass precursors to the Tim23 complex (Leuenberger *et al*. 1999). In contrast, preproteins such as metabolite carriers with internal targetting signals do not enter the matrix via the Tim23 channel, but transverse the intermembrane space to be directed to the Tim22 complex, and are subsequently incorporated into the inner

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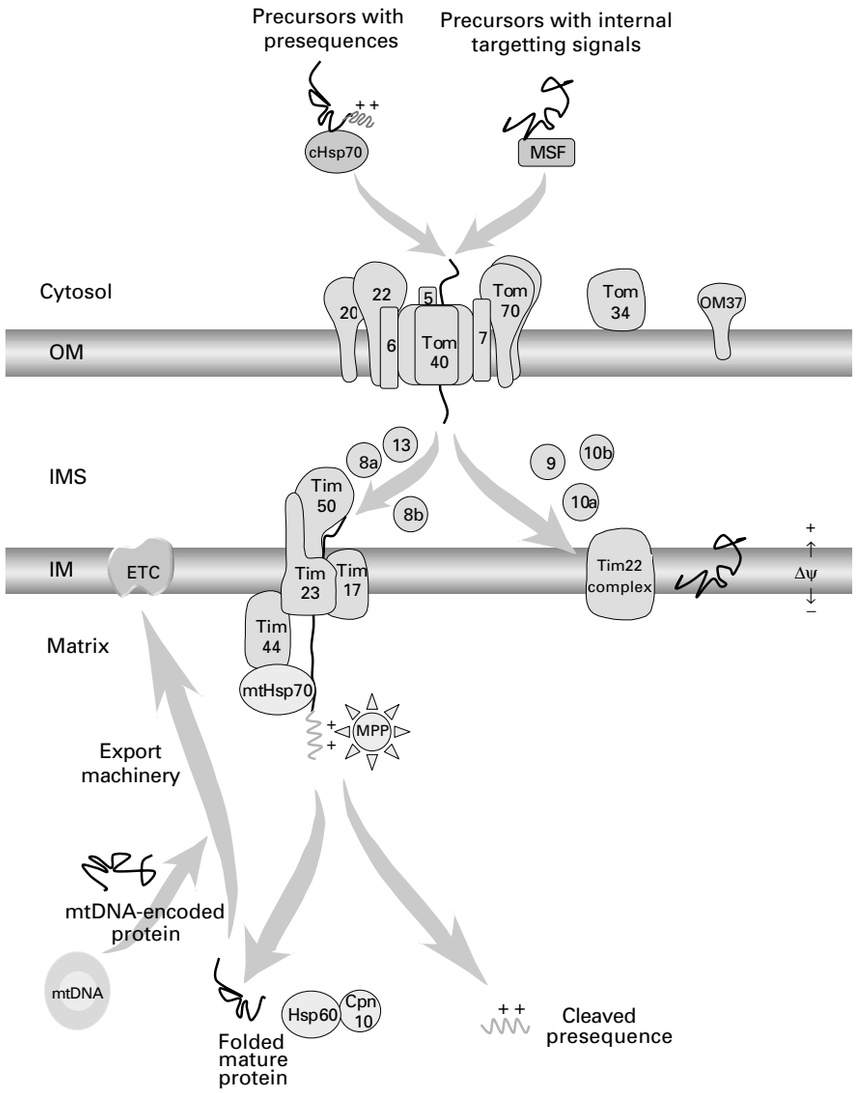

Fig. 1. Protein import into mitochondria. The translocases of the outer mitochondrial membrane (TOM) complex consists of component proteins (Tom) that include three receptor proteins, Tom20, Tom22 and Tom70, as well as Tom40, Tom5, Tom6 and Tom7. Precursors with a cleavable N-terminal presequence are directed from the cytosol to the outer membrane (OM) by cytosolic heat-shock protein (Hsp) 70. Precursors with internal targetting signals are chaperoned to the TOM complex by mitochondrial import stimulation factor (MSF). The precursor associates with Tom20, Tom22 and Tom70 receptors and is transferred to the intermembrane space (IMS) via the Tom40 complex. One of the component proteins (Tim) of the translocases of the inner membrane (IM) complex, Tim50, and the smaller Tim isoforms subsequently direct the precursor either to the Tim22 channel to be inserted into the IM, or to the Tim23 channel to be pulled into the matrix via the ATP-driven action of mitochondrial Hsp70 (mtHsp70) and the membrane potential ($\Delta\psi$). Once inside the matrix, the presequence is cleaved by mitochondrial processing peptidase, and refolded by Hsp60 and its co-chaperonin chaperonin (Cpn) 10 into a mature protein. Subsequently, both nuclear and mitochondrially-encoded proteins are inserted into the IM by the mitochondrial export machinery. ETC, electron transport chain. (Modified from Hoogenraad *et al.* 2002; Truscott *et al.* 2003.)

membrane. Similar to Tim23, this complex consists of several other proteins, including Tim9 and Tim10, which transfer precursors to Tim22 (Koehler *et al.* 1998; Sirrenberg *et al.* 1998).

Translocation, processing and assembly events in the matrix

Once precursors enter the Tim23 channel, they are pulled into the matrix by mitochondrial Hsp70 using the energy

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derived from the hydrolysis of ATP. Tim44 is a closely associated protein that binds mitochondrial Hsp70, thereby increasing the concentration of mitochondrial Hsp70 at the translocation site (Rassow *et al*. 1994; Schneider *et al*. 1994). The mechanism by which mitochondrial Hsp70 drives preproteins into the matrix is still under considerable debate. This translocation across the inner membrane is also dependent on the electrophoretic effect of the membrane potential (inside negative) on the positively-charged presequence (Martin *et al*. 1991). In addition, the phospholipid cardiolipin in the inner mitochondrial membrane is also imperative for protein translocation, since it appears to orient the precursor in the correct position to interact with the Tim44–mitochondrial Hsp70 complex (Leenhouts *et al*. 1996).

Once the preprotein enters the matrix, the N-terminal signal sequence is cleaved by the mitochondrial processing peptidase (Gakh *et al*. 2002). Some precursors require additional processing, and this reaction is catalysed by a second metalloprotease, the mitochondrial intermediate peptidase (Isaya *et al*. 1992; Kalousek *et al*. 1992). The resulting mature protein possesses a lower molecular weight than the precursor protein, and is refolded by Hsp60 and its co-chaperonin chaperonin 10 (Fig. 1). The mature protein can then be assembled into functional components of the electron transport chain. This assembly step is done in combination with protein subunits derived from mitochondrial DNA, and is facilitated by the export machinery of the mitochondria (Fig. 1) that translocates proteins from the matrix to the inner membrane. Several protein export and assembly machinery components have been identified, including oxidase assembly 1, cytochrome c oxidase 18 (Truscott *et al*. 2003) and SURF-1 (Tiranti *et al*. 1998). The absence of SURF-1 in man is the primary cause of Leigh syndrome, a severe neurodegenerative disorder (Tiranti *et al*. 1998). The effects of exercise on the expression of this class of assembly proteins have not yet been investigated.

Mitochondrial protein import in muscle

Studies with mitochondria isolated from skeletal muscle were initially designed to explore the possibility that the different biochemical features of SS and IMF mitochondria are, in fact, attributable to altered rates of protein import. Isolated IMF mitochondria have been found to exhibit rates of protein import that are 2–3-fold greater than those in SS mitochondria (Takahashi & Hood, 1996). These rates closely match differences in mitochondrial respiration and ATP production. When mitochondrial respiration is experimentally reduced using known inhibitors of the electron transport chain, rates of import are correspondingly decreased. In addition, when interaction of the precursor protein with the phospholipid cardiolipin is impaired with adriamycin, protein import is reduced, particularly in the SS mitochondria. This effect corresponds with the higher cardiolipin content in SS, compared with IMF mitochondria (Takahashi & Hood, 1996).

Protein import and contractile activity

Chronic exercise, or experimentally-induced contractile activity, are powerful stimuli for mitochondrial biogenesis in muscle. Part of the reason for this adaptation is now known to be the result of alterations in the expression of protein import machinery components (Ornatsky *et al*. 1995; Takahashi *et al*. 1998; Gordon *et al*. 2001). Among the changes shown to be induced are increases in cytosolic chaperones Hsp70 and mitochondrial import stimulation factor, as well as intramitochondrial chaperones Hsp60, mitochondrial Hsp70 and chaperonin 10 (Ornatsky *et al*. 1995; Neufer *et al*. 1996; Takahashi *et al*. 1998). In addition, cardiolipin, a unique phospholipid found within the inner mitochondrial membrane, is rapidly increased with chronic contractile activity. This change occurs at a rate that is faster than the increase in mitochondrial protein accumulation (Takahashi & Hood, 1993), suggesting that the assembly of the mitochondrial reticulum in response to exercise occurs first by the synthesis of membrane phospholipids, followed subsequently by the insertion of membrane proteins. This pattern is similar to that found in other experimental systems (Hallman & Kankare, 1971; Aithal & Tustanoff, 1975).

In relation to outer membrane import receptors, Takahashi *et al*. (1998) have shown that Tom20 protein levels are increased following 7 d of contractile activity. These changes are functionally relevant, since they correspond with an acceleration of malate dehydrogenase and mitochondrial transcription factor A import rates into the mitochondrial matrix (Takahashi *et al*. 1998; Gordon *et al*. 2001). The increased rate of import of mitochondrial transcription factor A into the matrix is particularly noteworthy, since this protein is an essential mitochondrial DNA transcription factor. Once imported, mitochondrial transcription factor A promotes the transcription and replication of the mitochondrial genome, leading to increased mitochondrial DNA copy number (Williams *et al*. 1986) and increased transcription of cytochrome c oxidase subunits (Gordon *et al*. 2001).

Thus, chronic contractile activity promotes mitochondrial biogenesis in part because of the induction of: (1) cytosolic molecular chaperones that transport precursors to the matrix; (2) outer membrane import receptors such as Tom20; (3) inner membrane phospholipids; (4) matrix chaperonins such as mitochondrial Hsp70, which pulls the precursors into the matrix in 'ratchet-like' fashion; (5) matrix chaperonins that refold the mature protein for subsequent compartmentation in the matrix or inner membrane. The physiological importance of these adaptations is an increased capacity for import (i.e. an increase in the maximum velocity of the transport process). As with any other transport or enzymic step, this increase in capacity represents an improved sensitivity at any given substrate (i.e. precursor protein) concentration (Fig. 2). In this case the precursor protein is provided within the cell by the combined efforts of both transcription and translation. Thus, even if these processes were unaffected by contractile activity, the increased capacity for import would result in a greater translocation of existing cytosolic precursor proteins into the mitochondrial matrix. An alternative interpretation is that the increased capacity for import would permit lower rates of transcription and translation, but would allow the same rate of import into the organelle (Fig. 2).

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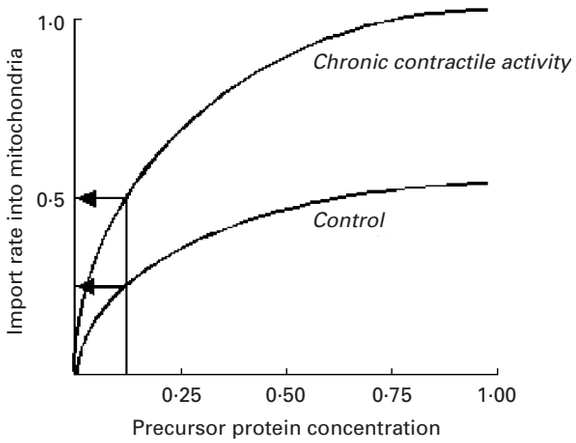

Fig. 2. Hypothetical response of contractile activity-induced changes in protein import. At any given concentration of precursor protein, contractile activity will result in a greater import rate into the mitochondrial matrix. Alternatively, the same rate of protein import can be achieved at a lower concentration of precursor protein provided by upstream transcriptional and translational events.

Interestingly, this adaptation does not hold true for all precursors. The import of Bcl-2 into the mitochondrial outer membrane remains unchanged by identical contractile activity conditions that produce an increased import into the matrix (Takahashi *et al*. 1998). Further work on this compartment-specific adaptation is required in order to examine import rates into the intermembrane space and inner membrane.

It is also noteworthy that most of the effects of contractile activity on mitochondrial protein import have also been shown to occur in cardiac cells in response to thyroid hormone treatment (Craig *et al*. 1998; Schneider & Hood, 2000). This finding reinforces the concept that adaptations in the protein import pathway are a normal component of the adaptive responses of the organelle during mitochondrial biogenesis, even to markedly different stimuli.

Importance of specific protein import machinery components

The role of assigning a specific function to individual components of the protein import machinery has proven difficult in mammalian cells because of the lack of availability of cloned mammalian homologues. The first subunit to be identified was the surface receptor protein Tom20 (Goping *et al*. 1995; Seki *et al*. 1995). In yeast cells human Tom20 has been shown to complement respiratory defects in cells lacking this protein (Goping *et al*. 1995; Seki *et al*. 1995). Overexpression of Tom20 by 2–3-fold in C2C12 muscle cells grown in culture results in a parallel increase in the import of malate dehydrogenase, suggesting that Tom20 alone could accelerate protein import into the matrix (Grey *et al*. 2000). In addition, a forced reduction in Tom20 expression using antisense oligonucleotides leads to equivalent decrements in import, measured in intact cells using immunoprecipitation of the radiolabelled precursor protein (Grey *et al*. 2000). Antibody inhibition studies have also substantiated this finding, since pre-incubation of isolated mitochondria with a Tom20 antibody has been shown to inhibit the import of matrix proteins malate dehydrogenase and mitochondrial transcription factor A, but to have little effect on the import of Bcl-2 (Grey *et al*. 2000). Thus, the import of matrix-destined proteins appears to be largely dependent on the expression of Tom20. Whether other outer membrane protein components share similar importance is not yet established in mammalian cells. However, the critical role of Tom20 for import adds relevance to the study of Tom20 expression. The upstream promoter region of the human Tom20 gene contains potential binding sites for nuclear respiratory factors 1 and 2 (Hernandez *et al*. 1999), which are known to be vital for mitochondrial biogenesis (Scarpulla, 2002). Since these transcription factors are transactivated by PPARγ co-activator-1α, and this protein is rapidly increased by contractile activity (Irrcher *et al*. 2003), this process may be a mechanism for the exercise-induced increase in Tom20 expression, and protein import, that occurs.

Similar overexpression experiments with mitochondrial Hsp70 in H9C2 myocytes have been performed to examine the potential role of this protein in the import pathway. Surprisingly, 2–3-fold increases in mtHsp70 levels using stably transfected cells produce only a very modest 13% increase in import (Colavecchia *et al*. 2003). This finding suggests that mitochondrial Hsp70 is normally found in excess within the organelle, and additional levels of the protein contribute little to the enhancement of the import rate. Further work to establish the threshold level of mitochondrial Hsp70, as well as other import machinery components using RNA interference analysis is needed to identify the critical proteins involved in the adaptation of muscle cells to stimuli that evoke mitochondrial biogenesis.

The importance of protein import in mitochondrial disorders

The aetiology of mitochondrial disorders can be classified into two categories: those arising from genetic mutations in the nuclear genome; those attributed to mutations in mitochondrial DNA (Zeviani *et al*. 1998). Given the importance of protein import into mitochondria, it is quite possible that mutations arising in the nuclear genes encoding the protein import machinery can reduce the energy supply within a cell and lead to mitochondrial disease. In the fungus *Podospora anserina* a mutation in the Tom70 receptor has been shown to result in mitochondrial DNA deletions during senescence (Jamet-Vierny *et al*. 1997). In man a defect in the gene encoding the deafness and dystonia protein 1 has been shown to result in a neurodegenerative disorder termed Mohr-Tranebjaerg syndrome (Koehler *et al*. 1999). Deafness and dystonia protein 1 is homologous to the yeast Tim8p family of proteins that is responsible for escorting proteins from the intermembrane space into the matrix (Koehler *et al*. 1999). Deafness and dystonia protein 1 (Tim8a) interacts with Tim13 to aid in

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the translocation of Tim23 (Rothbauer *et al*. 2001). The mutation in deafness and dystonia protein 1 caused by a cysteine → tryptophan transition (C66W) impairs its proper folding, leading to the inability of deafness and dystonia protein 1 to associate with Tim13, and the insufficient import of Tim23 into the inner membrane (Hofmann *et al*. 2002).

Patients with multiple mitochondrial defects exhibit an altered expression of several Hsp. Analyses of skeletal muscle tissue from patients with mitochondrial myopathies reveal distinct differences in the expression of Hsp60 among the two mitochondrial subfractions of skeletal muscle (Carrier *et al*. 2000). Hsp60 levels are reduced in SS mitochondria, while IMF mitochondria display an overabundance of the protein. Interestingly, Hsp60 is undetectable in ragged-red fibres with a proliferation of SS mitochondria, suggesting that the proteins may be vital for normal organelle biogenesis (Carrier *et al*. 2000). In another study fibroblasts from a patient with multiple mitochondrial disease were examined to determine whether protein import defects could account for the impaired mitochondrial function found in this condition (Rungi *et al*. 2001). In contrast to what was originally believed, protein import rates into mitochondria were found to be normal. These normal rates coincided with unaltered levels of Tom20, but markedly reduced levels of both mitochondrial Hsp70 and Hsp60. Once again, these data point to an important role for Tom20 in maintaining the rate of protein import, even in conditions of mitochondrial dysfunction.

Cultured cells partially, or completely, depleted of mitochondrial DNA (ρ^- or ρ^0 respectively) by ethidium bromide treatment have also been used as a model of mitochondrial disease (Desjardins *et al*. 1985; King & Attardi, 1989). These cells, which exhibit impaired membrane potential and reduced ATP synthesis, display an up-regulation of many nuclear-encoded genes, including components of the protein import machinery (Martinus *et al*. 1996). Interestingly, protein import does not appear to be impaired (Nelson & Schatz, 1979; Herzberg *et al*. 1993) in these respiratory-deficient cells, suggesting the involvement of a compensatory response of the protein import apparatus to maintain protein import and mitochondrial assembly under conditions of energetic deficiency. More work relating the energy state of the cell and protein import in specific human mitochondrial diseases is warranted.

Summary

Mitochondrial biogenesis requires the cooperative interaction of both the nuclear and the mitochondrial genomes. The mitochondrial protein import pathway is responsible for translocating the hundreds of nuclear-derived proteins, and it is a vital step in the expansion of the mitochondrial reticulum. Protein import is a process that is inducible in response to exercise (Takahashi *et al*. 1998; Gordon *et al*. 2001). This adaptation is brought about by the altered expression of several of the protein import machinery components such as Tom20, which appears to be one of the most important proteins within this translocation pathway. The identification of more mammalian homologues, along with their functional characterization in both healthy muscle and muscle from patients with mitochondrial disease, will help in the evaluation of the potential role of exercise in improving any import pathway defects that may exist.

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