Tom20-mediated mitochondrial protein import in muscle cells during differentiation

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Grey, Janice Y., Michael K. Connor, Joseph W. Gordon, Masato Yano, Masataka Mori, and David A. Hood. Tom20-mediated mitochondrial protein import in muscle cells during differentiation. Am J Physiol Cell Physiol 279: C1393–C1400, 2000.—Mitochondrial biogenesis is accompanied by an increased expression of components of the protein import machinery, as well as increased import of proteins destined for the matrix. We evaluated the role of the outer membrane receptor Tom20 by varying its expression and measuring changes in the import of malate dehydrogenase (MDH) in differentiating C2C12 muscle cells. Cells transfected with Tom20 had levels that were twofold higher than in control cells. Labeling of cells followed by immunoprecipitation of MDH revealed equivalent increases in MDH import. This parallelism between import rate and Tom20 levels was also evident as a result of thyroid hormone treatment. Using antisense oligodeoxynucleotides, we inhibited Tom20 expression by 40%, resulting in 40–60% reductions in MDH import. In vitro assays also revealed that import into the matrix was more sensitive to Tom20 inhibition than import into the outer membrane. These data indicate a close relationship between induced changes in Tom20 and the import of a matrix protein, suggesting that Tom20 is involved in determining the kinetics of import. However, this relationship was dissociated during normal differentiation, since the expression of Tom20 remained relatively constant, whereas imported MDH increased 12-fold. Thus Tom20 is important in determining import during organelle biogenesis, but other mechanisms (e.g., intramitochondrial protein degradation or nuclear transcription) likely also play a role in establishing the final mitochondrial phenotype during normal muscle differentiation.

mitochondrial biogenesis; antisense oligodeoxynucleotides; thyroid hormone; translocases of the outer membrane

THE MITOCHONDRION POSSESSES a 16-kb genome that encodes a small portion of its constituent proteins. The remaining proteins are encoded by nuclear genes and synthesized on free ribosomes in the cytosol (2). These nuclear-encoded preproteins are translocated into the mitochondria by an import machinery that is well characterized in Neurospora crassa and Saccharomyces cerevisiae (20), the components of which are beginning to be described in mammals (1, 10, 11, 17, 26, 31, 37, 42). The major complexes comprising the import machinery include translocases of the outer membrane (Toms) and translocases of the inner membrane (Tims) (28, 32, 35). Tom20, Tom22, Tom37, and Tom70 are referred to as import receptors, and they are involved in the initial recognition of preproteins at the surface of the organelle (28, 32, 35). These form dynamic protein oligomers. The Tom20-Tom22 and Tom37-Tom70 heterodimers bind preproteins with their cytosolic domains and initiate import into the mitochondria (21). Certain preproteins are targeted primarily to the Tom37-Tom70 heterodimer through the action of an ATP-requiring cytosolic chaperone termed the mitochondrial import stimulation factor (MSF) (11), whereas others are directed to Tom20-Tom22 via a heat shock protein 70 (HSP70)-preprotein complex in a process that does not require ATP (23). The Tom37-Tom70 subunits mainly recognize preproteins with hydrophobic internal presequences (5), which are then transferred to the Tom20-Tom22 subunits and translocated into the mitochondria. The Tom20-Tom22 heterodimer has a highly acidic cytosolic domain (3) that preferentially binds preproteins possessing basic, cleavable amino-terminal presequences (5), but also those containing a variety of other targeting signals (36), thus implicating this heterodimer in the import of most mitochondrial proteins (28).

Previous studies have shown that when striated muscles such as heart and skeletal muscle undergo mitochondrial biogenesis, there is an increased expression of Tom20 as well as other components of the import machinery, coincident with an increased import of malate dehydrogenase (MDH), a protein destined for the matrix space (6, 39). However, these studies could not conclude that the induction of Tom20 was responsible for the accelerated import observed. To address this, we used muscle cell differentiation-induced mitochondrial biogenesis (27, 38). In this model, increases in nuclear-encoded mitochondrial enzymes have been reported to be four- to sixfold, whereas ultrastructural

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indexes of mitochondrial inner membrane density increase about twofold (27). Whether this increase in mitochondrial protein is due to transcription or posttranscriptional mechanisms remains unknown. To begin an evaluation of the role of protein import in determining mitochondrial phenotype during differentiation, we manipulated Tom20 levels using expression constructs and antisense oligodeoxynucleotides (ODNs) to assess whether specific changes in Tom20 expression could be associated with changes in the import of a typical matrix protein. Although many studies have examined protein import in vitro (see Refs. 20 and 28 for reviews) relatively few (12, 15, 25, 34, 41, 44) have documented rates of import in intact mammalian cells. In view of the role of Tom20 at the intersection of the MSF-HSP70 chaperone-mediated import pathway, we hypothesized that Tom20 could function as a point of regulation in the import process. This may be particularly relevant to investigate in muscle cells, in view of reported mitochondrial myopathies that are associated with an import malfunction (14, 16). Relevant to this is the recent finding that deletions of the outer membrane import receptor, Tom70, led to mitochondrial DNA deletions and rearrangements, potentially leading to a respiratory chain deficiency (18).

**METHODS**

**Materials.** The pCAGGs vector was used for human Tom20 (hTom20) overexpression was originally described by Niwa et al. (30). Primary antibodies against metaxin and MDH were supplied by Dr. P. Bornstein (University of Washington, Seattle, WA) and Dr. K. Freeman (McMaster University, Hamilton, Ontario, Canada), respectively. Secondary antibodies were purchased from Sigma (St. Louis, MO). The mitochondrial transcription factor A (TFAM) construct was provided by Dr. Rudolf Wiesner (University of Cologne, Germany). The Bel-2 antibody was obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Lipofectamine and 3,3,5-triiodothyronine (T$_3$) were obtained from GIBCO BRL (Burlington, Canada) and Sigma, respectively.

**ODN synthesis.** Two different regions of the rat Tom20 cDNA (GenBank accession no. Q62760) were used in the synthesis of phosphorothioated antisense ODNs (Dalton Chemical Laboratories, Toronto, Canada). These regions either overlapped the start codon from position -4 to +12 (5’-CCGGCCACCATCTTC-3’; designated ODN 12) or were located further downstream from +62 to +77 (5’-CGGTCAAAAGTAGATGC-3’, designated ODN 77). A sense ODN corresponding to positions -4 to +12 (5’-GAAGATGTTGGGCCCGG-3’, designated 12S) was synthesized as a control.

**Cell culture and preparation of cell extracts.** C2C12 myoblasts were cultured in 100-mm dishes in 10 ml of growth medium (DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin) at 37°C under an atmosphere of 5% CO$_2$, 95% air. At a confluence of 60–70%, the cells were washed twice with Dulbecco’s phosphate-buffered saline (DPBS), switched to a low serum medium (DMEM containing 5% heat-inactivated horse serum and 1% penicillin-streptomycin), and scraped from the plates to prepare cell extracts. The protein concentrations were determined using the Bradford assay (4).

**Transfection.** For the transfection of Tom20, C2C12 cells were cultured as described above, then were transiently transfected with 20 μg of control plasmid or Tom20 expression construct (pCAGGS-hTom20) using a poly-l-ornithine-DMSO shock method (33). Cells were then switched to low serum conditions and allowed to differentiate for the indicated times. For transfection of the ODNs, C2C12 cells were cultured in 24-well plates as described above. After 24 h, the cells were rinsed with 1 ml of DMEM, and each well was incubated with 0.3 ml DMEM containing Lipofectamine at a final concentration of 10 μg/ml, and ODNs at a concentration of 200 nM for 5 h at 37°C. The medium containing Lipofectamine was then replaced with growth medium lacking bacterial antibiotics and, after 24 h, the cells were switched to low serum conditions and allowed to differentiate for the indicated times. The cells were then scraped and the resulting extracts were subjected to 8% SDS-PAGE and analyzed by immunoblotting.

**Metabolic labeling of cells and immunoprecipitation.** Cultivation and transfection of C2C12 cells were performed as described above, except that 150-mm dishes and 40 μg of plasmid DNA were used for transfection. After 6 days, C2C12 myotubes were washed twice with DPBS and then incubated at 37°C for 1.5 h in 6 ml of cysteine-methionine-free medium. The myotubes were then radiolabeled with 100 μCi of [35S]methionine-[35S]cysteine (ICN, Montreal, Canada) for either 0.5 or 1 h. The myotubes were washed with cold DPBS containing 100 μg/ml cycloheximide and permeabilized with TENT buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100). The resulting cell lysate was then centrifuged at 10,000 g for 10 min at 4°C. MDH was immunoprecipitated with 7 μl of MDH antiserum and 100 μl of a 10% suspension of protein A-Sepharose (Amersham-Pharmacia Biotech, Baie D’Urfé, Canada). The protein samples were subjected to 10% SDS-PAGE and fluorography, dried, and quantified by electronic autoradiography.

**Immunoblotting.** Cell extracts were subjected to 8% (for MDH and metaxin), 10% (for Bel-2), or 12% (for Tom20) SDS-PAGE as described above and then electrophoretically transferred to nitrocellulose membranes (Hybond-C, Amersham). The membranes were blocked with 5% skim milk-PBS and incubated separately with anti-Tom20 (1:1,000), anti-metaxin (1:1,000), anti-MDH (1:1,000), or anti-Bel-2 (1:100) antibodies. The Tom20 signal was detected using anti-rabbit IgG coupled to alkaline phosphatase (1:1,000). Antibodies. The Tom20 signal was detected using anti-rabbit, peroxidase-coupled IgG (1:1,000) using the ECL chemiluminescence detection system (Amersham-Pharmacia Biotech). The blots were then quantified by laser densitometry (39).

**Protein import in vitro.** To evaluate the functional role of Tom20 in mediating the import of matrix and outer membrane proteins into mitochondria in vitro, import reactions were performed with isolated rat muscle intermyofibrillar mitochondria. The isolation of mitochondria and the import of MDH and Bel-2 were performed as described previously (39, 40). For the import of TFAM, the construct was linearized with Xho I, transcribed with T7 RNA polymerase, and the mRNA was translated in a rabbit reticulocyte lysate. Import was performed for 15 min (30°C). In all cases, the import reactions were performed in the presence or absence of Tom20 antibody (200 ng/ml).
Cytochrome-c oxidase enzyme activity. C2C12 myotubes were scraped from 100-mm plates at 0, 2, 4, 6, and 8 days of differentiation in ice-cold DPBS, pelleted, and resuspended in 200 μl of extraction buffer (100 mM NaKPO₄, 2 mM EDTA, pH 7.2). Cells were sonicated (4 × 10 s) and subjected to one freeze-thaw cycle. After a clearing spin (5 min, 4°C), the supernate was used in the enzyme assay. Approximately 350–400 mg of cellular protein were incubated with a reduced cytochrome c solution (1 mg/ml), and cytochrome-c oxidase enzyme activity (nmol·min⁻¹·mg protein⁻¹) was determined spectrophotometrically (λ = 550 nm), as done previously (13).

Statistics. Statistical analysis was done using Student's paired t-test. The data are expressed as means ± SE, and values of *P* < 0.05 were used to indicate statistical significance.

RESULTS

Protein expression in C2C12 cells during cellular differentiation. When C2C12 myoblasts were switched to low serum conditions, they fused and formed myotubes that contracted asynchronously. During this differentiation period (0–8 days), total cellular protein increased progressively from 467 ± 75 to 1,913 ± 151 μg/plate (*n* = 6). As an indicator of mitochondrial biogenesis, cytochrome-c oxidase enzyme activity was used, since it contains multiple protein subunits derived from both the nuclear and the mitochondrial genomes (19). Enzyme activity increased approximately threefold from 78.2 ± 8.1 to 237.9 ± 14.1 nmol·min⁻¹·mg protein⁻¹ (*n* = 6–7 samples/day) between 0 and 6 days and remained constant at 8 days of differentiation. In contrast, Tom20 was expressed at a constant level per microgram of protein at all indicated time points during differentiation (Fig. 1A). Levels of the 35-kDa precursor MDH (pMDH) increased as a function of differentiation time, as did levels of mature, imported MDH (14-fold) and metaxin (7-fold; Fig. 1, B and C). Bcl-2 protein levels declined to ~40% of that found at day 0 by days 6–8 (Fig. 1D). These data reveal that the expression of Tom20, metaxin, mature MDH, and Bcl-2 is differentially regulated during mitochondrial biogenesis induced during muscle cell differentiation. Further, they indicate that dramatic increases in the level of the mature form of MDH can occur during normal differentiation even in the absence of changes in Tom20 concentration.

Effect of T₃. We have previously shown that T₃ treatment results in an upregulation of both the import rate as well as the expression of components of the import machinery in cardiac mitochondria when administered in vivo (6). Here we used T₃ treatment to investigate the inducibility of the protein import system as a whole in skeletal muscle cells. T₃ treatment resulted in a significant increase in Tom20 and mature MDH levels compared with vehicle-treated cells at days 4 and 8 of differentiation (Fig. 2, A and C). In contrast, metaxin (Fig. 2B), pMDH (Fig. 2C), and Bcl-2 (Fig. 2D) protein levels were not significantly altered. These results indicate that T₃ treatment of cultured skeletal muscle cells can bring about specific alterations in mitochondrial protein import.
drial composition, in particular related to the mitochondrial import pathway.

**Tom20 expression and MDH import.** To evaluate whether changes in only one component (Tom20) of the import machinery could influence the rate of mitochondrial protein import in intact cells, we forced the overexpression of Tom20, and we inhibited Tom20 expression in normal differentiating cells using an antisense ODN approach. Evidence for the overexpression of Tom20 in C2C12 cells is shown in Fig. 3A. Increases (*P*, 0.05) in Tom20 protein levels of 2.4 ± 0.1- and 1.8 ± 0.4-fold compared with control are evident at 4 and 8 days of differentiation, respectively (Fig. 3A). The amount of newly synthesized MDH translocated into the mitochondria of the C2C12 myotubes overexpressing Tom20 was also assessed under the same conditions. The results show that increases in MDH import of 1.9 ± 0.3- and 1.6 ± 0.3-fold (*P* < 0.05) above control cells at 30 and 60 min of metabolic labeling, respectively, were observed in cells in which Tom20 was overexpressed (Fig. 3B). No consistent effect of Tom20 overexpression on pMDH levels was detected. These data also appear to indicate that overexpression of human Tom20 adequately complemented the mouse mitochondrial import machinery.

To investigate the effect of a selective reduction of Tom20 expression on MDH import into mitochondria, antisense ODNs were designed. Preliminary experiments indicated that there was no difference in the effect of 100 and 200 nM ODN concentrations on Tom20 levels in C2C12 cells. A 40% reduction (*P* < 0.05) in Tom20 levels was observed with the transcription of both antisense ODNs 12 and 77 relative to the sense ODN (12S) and nontransfected (NT) cells (Fig. 4). No difference in Tom20 levels was observed between 12S and NT cells, indicating no nonspecific inhibition due to the presence of the ODN in culture (Fig. 4, NT and 12S). We then assessed the effect of the reduction in Tom20 levels on the expression of mature MDH in the same cell samples in which Tom20 was selectively reduced. Mature MDH levels in cells treated with either ODN 12 or 77 were reduced to 40–45% of the control (12S) value (Fig. 4), whereas no significant effect on pMDH was noted in the same experiments. Thus a reduction in the mature MDH level paralleled the change observed in Tom20 expression.

Taken together, when the experimentally induced increases (via T3 and forced overexpression) and decreases (via ODN-mediated inhibition) in Tom20 levels were compared with MDH import, a high correlation was found (*r* = 0.82; graph not shown), suggesting the existence of a direct relationship between Tom20 expression and protein import into the matrix.

**Effect of Tom20 inhibition on import into the matrix and outer membrane.** To evaluate the importance of Tom20 in the import of proteins into the matrix and outer membrane, the import of the matrix proteins MDH and TFAM (Fig. 5, A and C) was compared with that of the outer membrane protein Bcl-2 (Fig. 5B) in response to inhibition of function with Tom20 anti-

![Fig. 2](http://ajpcell.physiology.org/)

Fig. 2. Effects of thyroid hormone treatment on the expression of Tom20, metaxin, MDH, and Bcl-2 in C2C12 cells. C2C12 cells were treated with 100 nM 3,3',5'-triiodothyronine (T3) or NaOH (vehicle) and allowed to differentiate for 4 and 8 days. Cell extracts (20 μg) from both T3-treated (solid bars) and vehicle (open bars) cells were subjected to SDS-PAGE and immunoblotting. Each graph represents the summary of 3–4 separate immunoblots for Tom20 (A), metaxin (B), MDH (C), and Bcl-2 (D). Insets: typical immunoblots showing vehicle (V) vs. T3-treated cells on day 8 of cellular differentiation. *P* < 0.05; T3 vs. vehicle.
body. TFAM import was most dramatically reduced, to only 35 ± 9% of control (P < 0.05), whereas MDH import was reduced to 59 ± 3% of control (P < 0.05). In contrast, Bcl-2 import into the outer membrane was unaffected (102 ± 6% of control) at this concentration of Tom20 antibody (Fig. 5D).

DISCUSSION

Tom20 is part of a hetero-oligomeric protein import receptor that mediates the import of cytosolic preproteins into the mitochondria. In yeast, this import receptor functions as a Tom20-Tom22 heterodimer, and it appears to be involved in the import of all preproteins known to use the general import machinery (22, 28). A lack of Tom20 causes cell growth arrest as well as a reduction in the mitochondrial capacity for oxidative phosphorylation and protein synthesis (28). In contrast to Tom20 deficiency, Tom20 overexpression can be achieved in skeletal muscle by a program of increased contractile activity (39). This treatment induces the expression of other members of the mitochondrial protein import machinery as well (e.g., MSF, mitochondrial HSP70), and the result is a coincident increase in the capacity for precursor protein import, as measured in vitro in isolated mitochondria. This is likely an important contributing process to the overall increase in mitochondrial biogenesis observed (39). A similar parallel relationship between Tom20 levels and in vitro measured protein import capacity has been shown in cardiac mitochondria as a result of T3 administration in vivo (6). These results suggest that changes in the expression of mitochondrial import machinery components are important in accelerating the translocation of precursor proteins into the mitochondria. The extension of those in vitro import assays was to evaluate the functional role of Tom20 in an intact cellular environment. In most in vitro studies the preprotein is synthesized in a reticulocyte lysate and imported into isolated mitochondria. However, this situation does not reflect changes that may occur in the cytosol of an intact cell during the differentiation process or in response to physiological perturbations. Relatively few studies have examined mitochondrial protein import in vivo (12, 15, 25, 29, 34, 41, 44), and none have done so under the conditions of enhanced mitochondrial biogenesis. Given that a rapid increase in mitochondrial biogenesis occurs in intact muscle cells during differentiation (27, 38), we used this model to study protein import. Under these conditions, we wanted to determine whether specific changes in the expression of Tom20 alone could alter the import of a matrix-des-}

Fig. 3. Tom20 overexpression and mitochondrial MDH import. C2C12 cells were transfected with pGem4Blue (C) or pCAGGS-hTom20 (T20) and allowed to differentiate. A: cell extracts (100 μg) from 100-mm dishes were electrophoresed using SDS-PAGE and subjected to immunoblot analyses using anti-Tom20 antibody (n = 8). Inset: typical immunoblot of Tom20 expression. B: cells cultured in 150-mm dishes were allowed to differentiate for 6 days and then labeled with [35S]cysteine-[35S]methionine for 30 or 60 min. The radiolabeled proteins were immunoprecipitated with MDH antiserum, electrophoresed using SDS-PAGE, and then analyzed by fluorography (n = 10). Inset: typical autoradiogram following 60 min of labeling. *P < 0.05, Tom20 vs. control; d, day.

Fig. 4. Antisense oligodeoxynucleotide (ODN)-mediated inhibition of Tom20 expression and mitochondrial MDH import. C2C12 cells cultured in 24-well plates were transfected with either 12, 77, or 12S ODNs as described in METHODS. After 4 days of differentiation, cell extracts were made and subjected to SDS-PAGE and immunoblotting. Top: immunoblots of Tom20 and MDH expression in nontransfected (NT) C2C12 cells or in cells transfected with ODNs. Bottom: summary of mature MDH (solid bars) and Tom20 expression (open bars) in ODN-treated cells (n = 4–5 experiments). *P < 0.05, ODN 12 or ODN 77 vs. ODN 12S.
MDH paralleled that of Tom20 during both forced overexpression, as well as during conditions of reduced expression, of the import receptor. In addition, this proportionality was also evident when a mature MDH level was measured under conditions of mitochondrial biogenesis induced by T3 treatment. These effects on MDH import were independent of changes in precursor MDH (pMDH) levels, and they indicate that the rate of protein import can be directly modified by changes in the expression of the single mitochondrial outer membrane import receptor Tom20. This not only confirms its role in the import process, but suggests its involvement in determining the kinetics of the process.

However, we also present some evidence for a dissociation between the expression of Tom20 and the level of imported MDH. This result was apparent at days 4, 6, and 8 of normal differentiation, in which the level of imported MDH far exceeded the change in Tom20 level during the same period. In this case, levels of pMDH were also increased along with the imported form of the protein, suggesting that events upstream of import (e.g., transcription) may be involved in regulating the import process, independent of Tom20 levels. In addition, the large accumulation of MDH in the mitochondrial matrix during normal differentiation could be a result, in part, of a reduced rate of intramitochondrial protein degradation (i.e., increased protein stability) as differentiation proceeds. These interesting possibilities remain to be evaluated.

It is established that Tom20 is involved in mediating the import of a variety of proteins destined for different mitochondrial compartments (28). Antibody inhibition studies have shown that the import of matrix and outer membrane proteins can be reduced by applying high concentrations of Tom20 antibody (24). However, using low amounts of antibody, our data suggest that the sensitivity of the import process directed toward the matrix in response to Tom20 inhibition is greater than that of import into the outer membrane, at least with respect to the proteins used in the present study. These data indicate that Tom20 does mediate MDH import into mitochondria, and they support the contention that the interaction of Tom20 with preproteins differs (5, 41). It is of interest to relate these results with those of Dekker et al. (7), in which it was reported that only one in four Tom complexes formed a stable supercomplex, with membrane-spanning preproteins linking all the Tim core complexes. If these overabundant Tom20 complexes that are not involved in interacting with Tim proteins are uniquely involved in the import of precursor proteins into the outer membrane, then this suggests that import into the outer membrane may follow different import kinetics and regulatory mechanisms than import into the matrix, even though Tom20 is involved in both processes. Our data certainly suggest that Tom20 plays a regulatory role in the import of MDH into the matrix under most conditions.

We also investigated the expression pattern of two mitochondrial outer membrane proteins, metaxin and Bcl-2, during muscle cell differentiation. The gradual decline in Bcl-2 concentration is similar to the pattern observed in differentiating ML-1 cells (43) and is consistent with the idea that Bcl-2 is involved at an early myogenic stage in promoting muscle cell proliferation (8). In contrast to the decline in the levels of Bcl-2, the expression of metaxin, an import receptor subunit that is a homologue of Tom37, was increased during differentiation. At this time, the role of metaxin and its relationship to Tom20 and the import process itself is not defined. Nonetheless, the expression pattern of mitochondrial proteins observed during muscle differentiation indicates that proteins destined for the same mitochondrial compartment (e.g., the outer membrane) are subjected to different regulatory influences.

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References


