Visualization of Mitochondrial Protein Import in Cultured Mammalian Cells with Green Fluorescent Protein and Effects of Overexpression of the Human Import Receptor Tom20*

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The presequence of the ornithine transcarbamylase precursor (pOTC) was fused to green fluorescent protein (GFP), yielding pOTC-GFP and pOTCN-GFP containing the presequence plus 4 and 58 residues of mature ornithine transcarbamylase, respectively. When GFP cDNA was transfected into COS-7 cells, the cytosol and nucleus were fluorescent. On the other hand, pOTC-GFP cDNA gave strong fluorescence of a unique mitochondrial pattern. After fractionation of cells expressing pOTC-GFP with digitonin, fluorescence was recovered mostly in the particulate fraction. Immunoblot analysis showed that processed GFP was present in the particulate fraction, whereas pOTC-GFP was recovered in both the soluble and particulate fractions. pOTC-GFP and pOTCN-GFP synthesized in vitro were imported efficiently into the isolated mitochondria. Single and triple amino acid mutations in the presequence resulted in impaired mitochondrial import and in a loss of mitochondrial fluorescence. Perinuclear aggregation of fluorescent mitochondria was observed when the human mitochondrial import receptor Tom20 (hTom20) was coexpressed with pOTC-GFP. Overexpression of hTom20 (not ΔhTom20, which lacks the anchor sequence) resulted in stimulated mitochondrial import of pOTC-GFP in COS-7 cells. When pOTC-GFP cDNA was microinjected into nuclei of human fibroblast cells, mitochondrial fluorescence was detected as early as 2–3 h after injection. These results show that GFP fusion protein can be used to visualize mitochondrial structures and to monitor mitochondrial protein import in a single cell in real time.

Most mitochondrial proteins are initially synthesized on free ribosomes as larger precursors with NH2-terminal presequences that function as mitochondrial targeting and import signals and are released into a cytosolic pool. The precursors are then imported rapidly into the mitochondria and proteolytically processed to the mature form. The whole process of synthesis of mitochondrial proteins and their translocation, processing, folding, and assembly involves many factors in the cytosol, mitochondrial membrane, and matrix compartments (see Refs. 1–4 for reviews).

In animals, most studies have been performed in an in vitro system in which the precursor proteins synthesized in reticulocyte lysate were imported into isolated mitochondria. Although preprotein import can be separated from preprotein synthesis in vitro, there is no proof that these processes are separated in the intact cell. Therefore, there is a need for procedures that will enable protein import to be investigated in the intact cell. So far, only a limited number of pulse-labeling and pulse-chase studies in cultured cells have been performed (5–8). The green fluorescent protein (GFP)† from the jellyfish Aequoria victoria yields a strongly fluorescent signal in heterologous cell types and has been used as a marker of gene expression and for visualizing subcellular organelles and protein translocation in living cells (see Ref. 9 for a review). Rizzuto et al. (10, 11) constructed a chimeric protein in which the mitochondrial targeting presequence of cytochrome oxidase subunit 8 was fused to GFP and showed that this chimera is targeted to mitochondria and gives the organelle-associated fluorescence.

Here, we constructed two chimeric proteins (pOTC-GFP and pOTCN-GFP) in which the presequence of the human ornithine transcarbamylase precursor (pOTC) plus 4 and 58 mature ornithine transcarbamylase residues, respectively, were fused to GFP and show that they were targeted to and imported into the mitochondria with proteolytic processing, but that only pOTC-GFP became strongly fluorescent in the organelle. Mutant pOTC-GFP fusion proteins with inactive mitochondrial import signals failed to give the mitochondrial fluorescence. Coexpression of the human mitochondrial import receptor Tom20 (hTom20) with pOTC-GFP resulted in perinuclear aggregation of fluorescent mitochondria and in stimulation of mitochondrial import of pOTC-GFP. Microinjection of the fusion cDNA into human fibroblast cells is also described.

EXPERIMENTAL PROCEDURES

Materials—Anti-human ornithine transcarbamylase antibody was raised in a rabbit by injecting Escherichia coli cell-expressed and purified human ornithine transcarbamylase. Anti-hTom20 antiserum was raised in a rabbit by injecting the soluble domain of hTom20 (12). Construction of Plasmids—The Xba/HindIII fragment of the GFP S65T mutant in phGFP-S65T (CLONTECH, Palo Alto, CA) was cloned into the same restriction sites of pGEM-3Zf (+) (Promega, Madison, WI), which contained the presequence of ornithine transcarbamylase.

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When Tom20 alone (endogenous or overexpressed) was immunolocalized, goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Vector Laboratories, Inc.) was used as secondary antibody. Fluorescin isothiocyanate fluorescence was photographed with a NIBA filter.

Mitochondria of living COS-7 cells were stained with 5 μg/ml rhodamine 123 in DMEM. After washing with DMEM, the cells were directly photographed.

Double Staining for Nuclei and GFP—Nuclei of COS-7 cells were stained with 10 μg/ml 4,6-diamino-2-phenylindole dihydrochloride in PBS. 4,6-Diamino-2-phenylindole dihydrochloride fluorescence was photographed with a WU filter (excitation, 330–385 nm; emission, >420 nm), and GFP fluorescence was photographed as described above.

Quantitation of Green Fluorescence in Cultured Cells—COS-7 cells were cultured and transfected in 35-mm dishes under the same conditions as described above. The cells were harvested and washed twice with PBS and then resuspended in ice-cold PBS. Cell fractionation was performed essentially as described previously (8). Briefly, the cell suspension was mixed with an equal volume of ice-cold 0.5% digitonin in PBS, held on ice for 2 min, and centrifuged at 15,000 × g for 2 min. The pellet was dissolved in 0.5% Triton X-100 in PBS, and the insoluble material was removed by centrifugation. Green fluorescence was measured with a Hitachi F3010 fluorescence spectrophotometer (excitation, 488 nm; emission, 511 nm).

Immunoblot Analysis—Culture, transfection, and fractionation of COS-7 cells were performed as described above, except that 10-cm dishes were used and 10 μg of plasmid was used for transfection. The whole cell extracts or fractionated cell extracts (40 μg of protein) were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred onto a nitrocellulose membrane. Antiserum against A. victoria GFP (CLONTECH) or against human ornithine transcarbamylase were used as primary antibodies. Enhanced detection was performed with the ABC-PO kit (Vector Laboratories, Inc.).

In Vitro Import of pOTC-GFP and pOTCN-GFP into Isolated Mitochondria—The EcoRI fragments of pCAGGS-pOTC-GFP and pCAGGS-pOTCN-GFP were cloned into the EcoRI site of pGEM-3Zf(+). The resulting plasmids, pGEM-3Zf(+)-pOTC-GFP and pGEM-3Zf(+)-pOTCN-GFP, were used for in vitro translation. In vitro translation in rabbit reticulocyte lysate and import into the isolated rat liver mitochondria were performed as described previously (16).

Microinjection of cDNA Plasmids—Normal human fibroblasts were grown in DMEM supplemented with 10% fetal bovine serum at 37 °C. Capillary microinjection of plasmid cDNA was carried out by the method described previously (17). After the indicated periods of incubation, cells on coverslips were fixed with 4% formaldehyde in PBS and examined for green fluorescence.

RESULTS

Detection of GFP Fluorescence in COS-7 Cells Transfected with GFP, pOTC-GFP, and pOTCN-GFP cDNAs—cDNAs encoding GFP, pOTC-GFP, and pOTCN-GFP in the potent mammalian expression vector pCAGGS were transfected into COS-7 cells, and GFP fluorescence was observed with a fluorescence microscope. When GFP or pOTC-GFP was expressed, fluorescence was detected in cells 8 h after transfection, and both the number of fluorescent cells and the fluorescence intensity increased with time (Fig. 2A). In the cells expressing GFP, the fluorescence was distributed throughout the whole cell including the nucleus (Fig. 2B). On the other hand, in the cells expressing pOTC-GFP, strong particulate fluorescence characteristic of mitochondria was observed. The results indicate that pOTC-GFP was correctly targeted to the mitochondrion and folded to become fluorescent. When transfected with pOTCN-GFP, the construct containing a substantial piece of mature ornithine transcarbamylase, mitochondrion-specific fluorescence was not seen, and instead, weak, diffuse, and irregular fluorescence was observed in the whole cell.

The cells were fractionated with digitonin into soluble and particulate fractions, and the fluorescence in these fractions was measured (Fig. 2C). In the cells expressing GFP, fluorescence was recovered almost completely in the soluble fraction. The fluorescence increased up to 48 h post-transfection and reached a plateau. On the other hand, in the pOTC-GFP-expressing cells, ~80% of the fluorescence was recovered in the
particulate fraction, and the remainder was in the soluble fraction. The fluorescence reached a near maximum level at 24 h. pOTCN-GFP gave very weak fluorescence that was recovered mostly in the soluble fraction.

**Demonstration of Mitochondrial Localization of Expressed pOTC-GFP**—The COS-7 cells expressing pOTC-GFP were visualized for GFP fluorescence and for endogenous Tom20 using immunocytochemical analysis. Mammalian Tom20 (12, 18, 19) is a homolog of Saccharomyces cerevisiae Tom20/Mas20 and Neurospora crassa Tom20/Mom19, which are import receptors located on the mitochondrial outer membrane (see Ref. 20 for a review). The pattern of GFP fluorescence coincided with that of the Tom20 stain (Fig. 3). These patterns were also similar to that of rhodamine 123-stained mitochondria.

**Biochemical Localization of Expressed GFP Proteins**—The intracellular localization of expressed GFP proteins and their
processing were analyzed by immunoblot analysis (Fig. 4). In the cells expressing GFP, a larger portion of GFP protein was recovered in the soluble fraction than in the particulate fraction. When pOTC-GFP was expressed, unprocessed pOTC-GFP and the processed mature GFP were detected. The processed GFP was recovered almost completely in the particulate fraction, whereas pOTC-GFP was recovered in both fractions. When the standard mitochondrial precursor protein pOTC was expressed, the precursor and mature forms were detected. The mature form was recovered mostly in the particulate fraction, whereas the precursor was recovered mostly in the soluble fraction. This confirms that the mitochondria were partitioned into the particulate fraction, the fraction where the activity for processing pOTC into mature ornithine transcarbamylase is compartmentalized. When pOTCN-GFP was expressed, a larger amount of the unprocessed form and a smaller amount of the processed form were present. Distribution of the two forms was similar to that of the pOTC-GFP products. The processed form of pOTCN-GFP in the particulate fraction was not fluorescent, suggesting that this form is not properly folded.

In Vitro Import of pOTC-GFP and pOTCN-GFP into Isolated Mitochondria—pOTC-GFP and pOTCN-GFP were synthesized in rabbit reticulocyte lysate and subjected to the in vitro import assay. Both precursors were efficiently imported into isolated rat liver mitochondria with concomitant proteolytic processing (Fig. 5). Import of pOTC-GFP was more efficient than that of pOTCN-GFP. However, the processed form of pOTC-GFP was resistant to proteinase K in the presence of Triton X-100, whereas the processed form of pOTCN-GFP was digested by proteinase K. These results, together with those of Figs. 2 and 4, show that pOTC-GFP was imported into the mitochondria, processed, and folded into the fluorescent conformation. On the other hand, pOTCN-GFP was efficiently imported and processed, but was not folded into the fluorescent conformation and appeared to be degraded faster than the processed pOTC-GFP. The NH2-terminal ornithine transcarbamylase sequence of 58 amino acid residues presumably prevented the folding of GFP.

Localization of pOTC-GFP Fusion Proteins with Mutated Presequences—Human ornithine transcarbamylase precursors carrying mutations in the presequence portion (a single amino acid mutant with Arg-23 replaced by Gly and a triple mutant with Arg-15, Arg-23, and Arg-26 replaced by Gly) were shown to be inactive in mitochondrial import in vitro (21) and in cultured cells (6). We constructed pOTC-GFP cDNAs with the same mutations, designated pOTCm1-GFP and pOTCm3-GFP. Both pOTC-GFP mutants synthesized in vitro were not imported into isolated mitochondria (data not shown). When these mutants were expressed in COS-7 cells, mitochondrial-specific fluorescence was not observed (Fig. 6A). Fluorescence in the soluble and particulate fractions of the cells expressing the mutant pOTC-GFP fusion proteins was very low (Fig. 6B). Immunoblot analysis showed that both mutants remained unprocessed and were not much degraded (Fig. 6C). Thus, despite their presence, the unprocessed mutant precursors showed little fluorescence and were recovered both in the soluble and particulate fractions. The unprocessed precursors in the particulate fraction might be associated with particulate components in the cell or might be aggregated.

Perinuclear Aggregation of Mitochondria by Overexpression of hTom20—A dramatic change in mitochondrial fluorescence was observed when hTom20 was coexpressed with pOTC-GFP in COS-7 cells (Fig. 7). The fluorescent mitochondria formed gigantic aggregates adjacent to the nucleus (Fig. 7, panels a and b). These fluorescent structures were seen in most transfected cells. These structures were shown to be mitochondria because a similar structure was stained with anti-hTom20.
antibody, although the cytosol was also stained weakly (Fig. 7B, panel a). Here, only hTom20, and not pOTC-GFP, was expressed. Thus, the aggregation of mitochondria does not depend on expression of pOTC-GFP. This perinuclear aggregation was not observed when DhTom20, which lacks the transmembrane domain of hTom20 (12), was expressed (Fig. 7A, panel c). DhTom20 was stained in the whole cytosol and the nucleus (Fig. 7B, panel b). These results suggest that mitochondria with overexpressed hTom20 associate with each other and form large aggregates.

Stimulation of Mitochondrial Import of pOTC-GFP by Overexpression of hTom20 in COS-7 Cells—The effect of hTom20 overexpression on mitochondrial import of pOTC-GFP in COS-7 cells was studied by immunoblot analysis (Fig. 8). When pOTC-GFP was expressed alone in COS-7 cells, unprocessed pOTC-GFP as well as processed GFP were detected. When increasing amounts of hTom20 were coexpressed, the amount of pOTC-GFP decreased and that of GFP increased in a dose-dependent manner. These results show that overexpression of hTom20 stimulates mitochondrial import of pOTC-GFP. Overexpression of ΔhTom20 had little effect on pOTC-GFP import. Therefore, hTom20-induced mitochondrial aggregation is apparently associated with an enhanced protein import activity rather than mitochondrial dysfunction.

Expression of Microinjected pOTC-GFP cDNA in Human Fibroblasts—pOTC-GFP cDNA was microinjected into nuclei of human fibroblasts. Mitochondrion-specific fluorescence appeared as early as 2–3 h after microinjection and increased with time up to 4 h (Fig. 9). Microinjected cDNA molecules are expected to be transcribed immediately and synchronously, and thus, mitochondrial import of pOTC-GFP can be monitored more rapidly and more accurately.

**DISCUSSION**

GFP yields strong green fluorescence in many cell types and can be directly observed in living cells. Rizzuto et al. (10) first reported that a chimeric GFP harboring a mitochondrial targeting sequence can be targeted to the mitochondria and make the organelle strongly fluorescent. This chimeric GFP was successfully used to show the structural alterations of mitochondria in rat hepatocytes induced by aspirin (22). GFP was also used to visualize nuclear translocation of the glucocorticoid receptor (11, 23, 24) and as a probe to identify and characterize peroxisome assembly mutants in the yeast Pichia pastoris (25).

In this paper, we constructed chimeric GFP proteins with mitochondrial targeting sequences and analyzed their mitochondrial import, processing, and fates by a combination of fluorescence microscopy, cell fractionation, fluorescence quantification, immunoblot analysis, and *in vitro* import. We showed that pOTC-GFP expressed in COS-7 cells was correctly targeted to the mitochondria, proteolytically processed, and folded into a fluorescent form. pOTCN-GFP was also imported...
into the mitochondria and proteolytically processed, but failed to give mitochondrial fluorescence. Mitochondrial import of pOTC-GFP was inhibited by uncoupling of mitochondria (data not shown). Mutant pOTC-GFP fusion proteins with mutated presequences could not be imported into the mitochondria both in vitro and in cultured cells. Therefore, mitochondrial import of pOTC-GFP resembled that of natural precursor proteins (see Refs. 2, 3, and 26 for reviews).

We showed that alteration of mitochondrial morphology can be easily detected in living cells using pOTC-GFP. Thus, the perinuclear aggregation of mitochondria induced by hTom20 overexpression would not have been readily observed without the use of the chimeric GFP protein. Furthermore, this mitochondrial aggregation is not a result of its dysfunction, but is associated with stimulated protein import. This stimulation is noteworthy because overexpression of one component of the import receptor complex may result in import inhibition. This
stimulation is also noteworthy because there has been no method to assess the stimulatory effects of the putative import factors in cultured animal cells to which genetic analysis cannot be easily applied. Further studies on fine structures of the mitochondrial aggregates, the molecular basis of the aggregation, and the functional activities of the aggregated mitochondria remain to be performed.

Finally, we showed that mitochondrial fluorescence can be observed as early as 2–3 h after cDNA microinjection into human fibroblasts. This method may be used to analyze structural changes in mitochondria and protein translocation into the organelle in fibroblasts from patients with various mitochondrial diseases.

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