Fusion of mitochondria in mammalian cells is dependent on the mitochondrial inner membrane potential and independent of microtubules or actin

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Abstract Mitochondrial fusion is a poorly characterized process which has mainly been studied in yeast and *Drosophila* but is thought to occur in all eukaryotes. Until now, there was only indirect evidence to support such a process in mammalian cells. In this study, using a cell fusion system, we found that mitochondrial fusion occurs rapidly in mammalian cells and is completed in less than 24 h. We report that the fusion of mitochondria requires an intact mitochondrial inner membrane potential but is independent of a functional cytoskeleton.
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Key words: Mitochondrial fusion; Membrane potential; Actin; Tubulin

1. Introduction

Mitochondria are complex semi-autonomous organelles surrounded by a double membrane and have their own genome and protein synthesis machinery. They perform a variety of important functions including reactions of the tricarboxylic acid cycle, oxidative phosphorylation and ATP production. Moreover, they are key players in the cascade that leads to apoptosis [1–4].

In recent years, the view of mitochondrial morphology has changed from that of individual organelles anchored on the cell's cytoskeleton to a dynamic and organized network of tubule-like mitochondria. This network maintains its shape by movement, fission and fusion. Mitochondrial dynamics has been extensively studied in yeast [5]. Among the proteins that control the fission in mammals are dynamin-related protein 1 (Drp1, first identified as Dnm1p in yeast) and Fis1p [6]. Fission of mitochondria also appears to be involved in different cellular events including apoptosis [2,7]. The machinery of mitochondrial fusion is less well characterized although a few proteins have now been shown to play a role in this event [8]. During *Drosophila melanogaster* spermatogenesis, mitochondria in spermatids aggregate, fuse, and elongate beside the growing flagella. Investigation of this process permitted the identification of the *fuzzy onions* (*fzo*) gene that encodes a transmembrane GTPase necessary for mitochondrial fusion. *Fzo* mutant males are defective in this developmentally regulated mitochondrial fusion and are sterile [9]. *Fzo* has homologues in mammals, nematodes, and yeast. The yeast homologue was characterized [10] and shown to be required for mitochondrial fusion [11]. Inhibition of mitochondrial fusion in yeast results in loss of mitochondrial DNA, and a fragmented mitochondrial morphology. This could be suppressed by a compensatory mutation in the fission machinery protein Dnm1p [12,13]. A genetic screen based on this finding was used to identify a new component required for mitochondrial fusion, Ugo1 [12]. In mammals, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) were found to be homologous to Fzo1p [14] but in contrast to *Drosophila* Fzo, they are ubiquitously expressed [15]. However, their ability to drive mitochondrial fusion is unclear [14,15].

Up to now, evidence for mitochondrial fusion in mammals has been provided by light and electron microscopy studies (for a extended review see [16]), complementation studies both in cells [17–21] and in vivo in mice [22]. The results from those studies are in part contradictory, some studies showing complementation between fused mitochondria using respiration-competent and respiration-deficient mitochondria [17,18,21] whilst others demonstrated a lack of complementation [19] or very rare events [20]. So could mammalian mitochondria merge? Here, using a cell fusion assay, we show that mammalian mitochondria do fuse and that this process occurs within less than 2 h. Mitochondrial fusion requires an intact mitochondrial inner membrane potential but does not rely on the cytoskeleton. Co-transfection with human hFis1 protein or a dominant negative mutant of human Drp1 also suggests that mitochondrial fusion is independent of its complementary process, fission.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium high glucose (DMEM high glucose), fetal calf serum, glutamine, penicillin, streptomycin, tetracycline, nocodazole, cytochalasin D, cycloheximide, TRITC-phalloidin and carbonyl cyanide *m*-chlorophenyl hydrazone (cccp) were purchased from Sigma (St. Louis, MO, USA). G418 was purchased from Promega (Catalys, Switzerland) and hygromycin from Calbiochem (Juro, Switzerland). Fugene was purchased from Roche (Roche,

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Abbreviations: cccp, carbonyl cyanide *m*-chlorophenyl hydrazone; $\Delta\psi_m$, mitochondrial inner membrane potential; mtCFP, cyan fluorescent protein targeted to the mitochondrial matrix; mtYFP, yellow fluorescent protein targeted to the mitochondrial matrix; PEG, polyethylene glycol

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Switzerland), tetramethyl rhodamine (TMRE) and taxol (paclitaxel) from Molecular Probes (Eugene, OR, USA) and the caspase inhibitor zVAD was from Enzyme Systems Products (Livermore, CA, USA). All other chemicals were purchased from either Sigma or Fluka (Buchs, Switzerland).

2.2. Cloning and plasmids

pEYFP-Mito and pECFP-Mito were purchased from Clontech (Palo Alto, CA, USA). To generate pTRE-ECFP-Mito, the eCFP-Mito coding sequence was subcloned by polymerase chain reaction (PCR) and inserted into the *Eco*RI site of pTRE (Clontech). pCI HA-Drp1(K38A) was constructed by PCR subcloning of the Drp1(K38A) coding sequence from pcDNA3-HA-Drp1(K38A) (gift from A. Van der Bliek) into the *Eco*RI/*Xba*I site of pCI (Promega, Madison, WI, USA). During the PCR subcloning, we added an alternative HA tag (MQDLPGNDNSTAGL) to the N-terminal end of Drp1(K38A). pCI-HA-FisFL was produced by amplifying the cDNA of hFis1 from a human liver cDNA library (Serono, Switzerland) and subcloning the PCR product into the *Eco*RI/*Xba*I site of pCI. During the PCR subcloning, we added an HA tag (as above) to the N-terminal end of hFis1 (for further information see James et al., submitted). All PCRs were performed with Pwo or Tgo polymerase (Roche, Rotkreuz, Switzerland), the clones were verified by DNA sequencing.

2.3. Cell culture and transfections

HeLa and Madin–Darby canine kidney (MDCK) Tet-Off cells (Clontech) were cultured at 37°C in a humidified incubator in the presence of 5% CO_2/95% air, in DMEM high glucose supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. G418 was added to MDCK Tet-Off cells according to the manufacturer's instructions. Cells were transfected with either calcium phosphate according to Jordan et al. [23] or Fugene according to the manufacturer's instructions (Roche). MDCK Tet-Off cells with eCFP targeted to mitochondria (mtCFP) stably transfected (MMCS) were obtained by co-transfection of pTRE-eCFP-mito and pTK-Hyg (10:1) and selected with hygromycin (400 μg/ml) in the presence of tetracycline (1 μg/ml) to keep off mtCFP expression. HeLa cells stably transfected with mtYFP tet-inducible (HMYS) were obtained by co-transfection of pTRE-eYFP-mito and pTet-Off. The cells were then selected with G418 (800 μg/ml) in the presence of tetracycline (1 μg/ml) and individual fluorescent clones were selected by fluorescence-activated cells sorting.

2.4. Cell fusion

For cell fusion experiments, MMCS and HMYS cells were cultivated for 4 days before experiment without tetracycline to induce mtCFP or mtYFP expression respectively. Twenty-four hours before fusion MMCS were plated to 70% confluence in individual glass bottom dishes (3 cm dishes, MaTek, MA, USA). Four hours before cell fusion HMYS or transiently transfected HeLa cells were plated (70% confluence) in the same dishes. They were allowed 3 h to attach then cycloheximide was added to a final concentration of 50 μg/ml; the cells were then left for another hour. To fuse cells, we treated them with a mix of prewarmed 50% w/v polyethylene glycol (PEG) 6000/DMEM for 2–3 min at 37°C (this is a modification of a protocol described in [24]). The cells were then washed gently four times with phosphate-buffered saline and placed back in culture medium with cycloheximide. The cells were observed 1–24 h post fusion. To assess cell fusion in certain experiments, we transfected HMYS cells with pDsRed1 (Clontech), which drives expression of a red fluorescent cytosolic protein. The presence of blue mitochondria in a cell with red cytosolic fluorescence was indicative of a fusion between MMCS and HMYS cells. Cells observed 24 h after cell fusion were treated with the pan-caspase inhibitor zVAD (100 μM) to prevent cell death due to cycloheximide treatment.

2.5. Drug treatments and activity assays

To disrupt the tubulin network we treated cells with nocodazole (5 μg/ml). To cause forced polymerization of the tubulin network we treated cells with taxol (1 μM). To cause impairment of the actin network, we treated cells with 10 μM cytochalasin D. To disrupt mitochondrial inner membrane potential, we treated cells with 75 μM or 10 μM cccp.

To inhibit protein synthesis during our experiments we added cycloheximide (50 μg/ml) to cell culture media 1 h prior to fusion. In order to ensure arrest of protein synthesis, we performed a fusion experiment adding radiolabeling medium after fusion (DMEM Cys and Met were replaced by ^{35}S-labeled Cys and Met, Amersham). Cells were collected at different times after fusion by resuspending them in 1× sample buffer (10% glycerol, 2.0% SDS, 10 mM EDTA, 60 mM Tris, 100 mM dithiothreitol, 0.05% bromophenol blue). Samples were centrifuged through glass wool to break genomic DNA, heated and loaded on a 12% SDS–PAGE gel. The gel was stained with Coomassie blue to ensure equal loading of samples and dried and autoradiographed.

If not specified otherwise, all drugs were added 1 h before fusion and kept in cell culture medium after fusion. Concentrated stocks of drug were made in dimethylsulfoxide, ethanol or methanol, they were freshly diluted in medium before each experiment, final solvent concentration never exceeded 0.1%.

2.6. Quantitative analysis of the fusion process

To analyze the fusion process quantitatively, we estimated in each polykaryon the percentage of mtYFP-labeled mitochondria that contained mtCFP, those were considered fused. Since precise determination of percentage is difficult, we divided the polykarya into the five following categories: 0–20%, 21–40%, 41–60%, 61–80%, 91–100%. We determined the percentage of fused mitochondria in cells fixed at a given time after fusion.

2.7. Fluorescence and live microscopy

We used an inverted microscope (Axiovert 135TV, 100× objective, Zeiss) equipped with a 50 W Hg lamp which allowed us to visualize living cells on glass bottom dishes. To achieve complete separation of eCFP and eYFP fluorescence, we used the following filter set: CFP set: excitation filter D414/30×, beam splitter 455 DCLP, emission filter HQ 487/15×; YFP set: excitation filter HQ500/14×, beam splitter 51017bs (Chroma, USA), emission filter 51017 m (Chroma).

For confocal microscopy, we used a Zeiss inverted 200M microscope with a scanhead QLC100 Nipkow disk (Yokogawa) equipped with a three line argon laser 457/488/514 (Laser Physics) with AOTF control of laser lines (Visitech). We took pictures with a Coolsnap HQ Camera (Roper Scientific). Image acquisition and analysis was performed with Metamorph/Metafluor 4.1.2 software (Universal Imaging).

For video microscopy, we used a Zeiss Axiovert S1000TV fluorescence microscope, a 50 W Hg lamp attenuated by transmission neutral-density filters (Omega Optical, Brattleboro, VT, USA), a CCD camera C4742-95-12NRB (Hamamatsu-City, Japan) and Open Lab Software (Improvision, Coventry, UK). The temperature (37°C) and atmosphere (5% CO_2) control was done with the CTI-3700/37-2-Digital system (PeCon, Erbach-Bach, Germany). Exposure time: 200–300 ms; delay between pictures: 30 s; recording time: 15 min; frames/s: 2.

For some experiments we replaced standard DMEM by DMEM without phenol red. Pictures were processed in Adobe Photoshop 6.0 or converted into QuickTime movies using Open Lab.

3. Results

3.1. Mitochondria fuse and mix their matrix contents

To investigate mitochondrial fusion, we decided to fuse two cell lines, MDCK and HeLa, each displaying mitochondria labeled either with eCFP (MDCK) or with eYFP (HeLa). Both cell lines expressed the eCFP or eYFP genes under the control of a Tet-responsive promoter. We chose an inducible promoter to avoid continuous expression of these proteins, which appeared to be deleterious for the cells. The fluorescent proteins were targeted to the matrix space of the mitochondria. Upon fusion of the two cell types, we analyzed mitochondria for the presence of one or both fluorescent proteins. Mitochondria that displayed both eYFP and eCFP were considered to result from a fusion between eCFP- and eYFP-labeled mitochondria. We fused the cells with the addition of PEG-saturated medium for 2–3 min. In some experiments, cell fusion was detected by the diffusion of a cytosolic marker, the protein DsRed, that was transiently expressed in HeLa

cells only (Fig. 1A, DsRed1). Moreover, formation of polykarya was easily observed by phase contrast microscopy and was indicative of cell fusions. The first mitochondria that appeared to display both fluorescences (eYFP+eCFP) were detected as soon as 90 min after PEG addition and were clearly observed at 2–4 h post cell fusion (Fig. 1A,B). At 6 h post cell fusion, a large part of the polykarya had all of their mitochondria labeled with both fluorescent proteins; however, the distribution of the proteins was still different. A complete mitochondrial fusion, which means that both proteins are homogeneously distributed throughout the polykaryon mitochondria, was observed 24 h after cell fusion (Fig. 1C).

To exclude the possibility that bilabeled mitochondria resulted from the superposition of two differently colored mitochondria, confocal microscopy was used. We performed the recording on a single focal plan that allowed us to have a z resolution of 1 μm, which is almost the width of a standard mitochondrion (Fig. 1D). This result confirmed that eYFP and eCFP were localized to only one mitochondrion, suggesting that fusion of mitochondria was responsible for this co-localization. However, this co-localization could have resulted from the import of de novo synthesized eYFP or eCFP to mitochondria already containing either of these proteins. However, this hypothesis is unlikely since the cells were

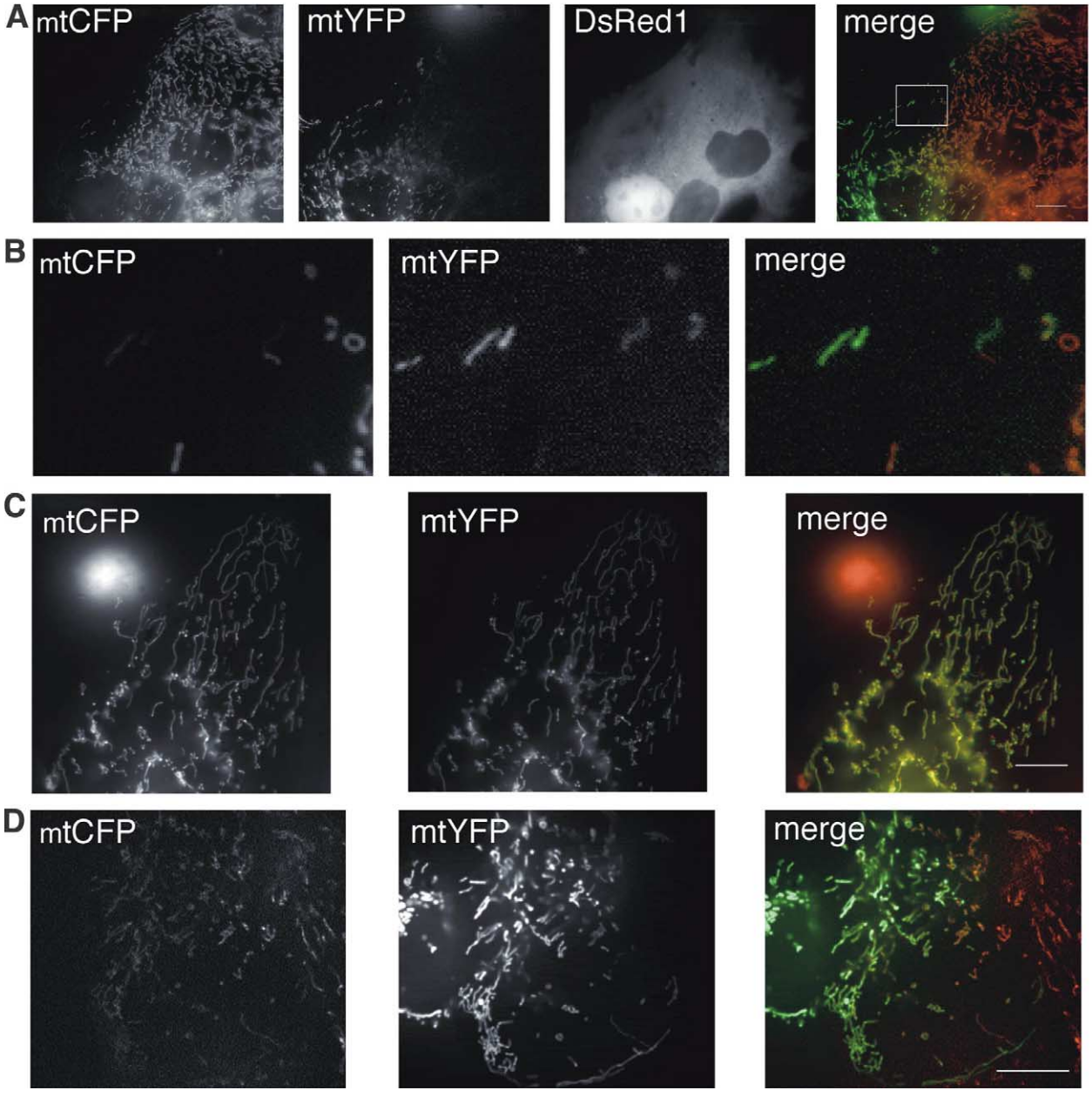
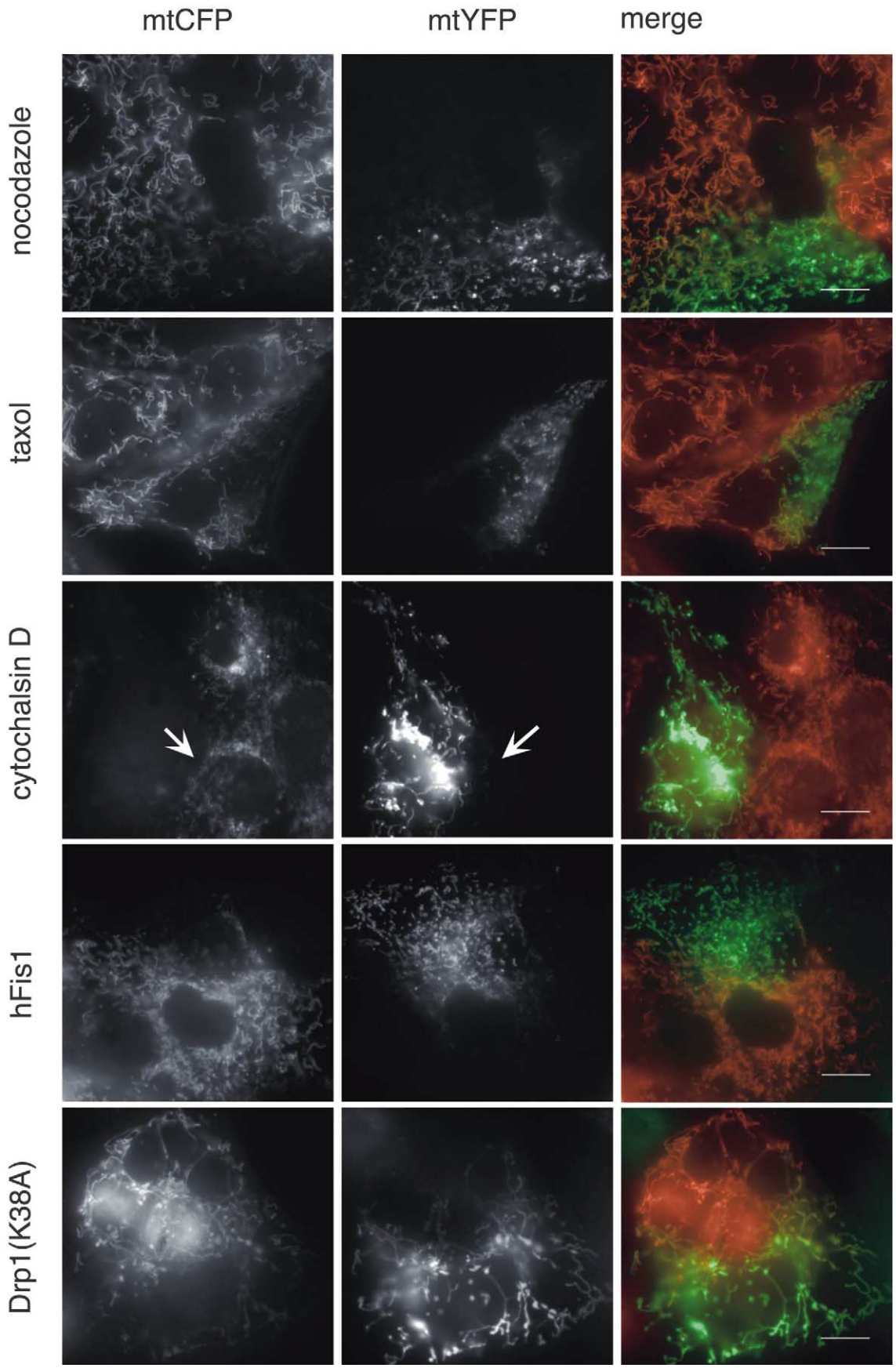


Fig. 1. Mitochondria fuse and mix their matrix content. A: MMCS cells fused with HMYS cells pictured 4 h after fusion. MtCFP picture: mitochondria from the MMCS cell; mtYFP picture: mitochondria from the HMYS cell; DsRed picture: the red fluorescent protein, transiently expressed in HMYS cell, has diffused in the MMCS cells; merge of mtCFP (red) and mtYFP (green) images: fused mitochondria appear in yellow. B: Higher magnification of the boxed area in A merge. C: Twenty-four hours after fusion in polykarya, all mitochondria have fused. D: Confocal image taken 3 h after fusion of a MMCS cell (right part of the picture) with a HeLa cell transiently transfected with mtYFP (left part of the picture); this represents a single plane image with a z resolution of 1 μm; overlay with artificial color, as described above. Scale bar 10 μm.


mtCFP
mtYFP
merge
nocodazole
taxol
cytochalsin D
hFis1
Drp1(K38A)

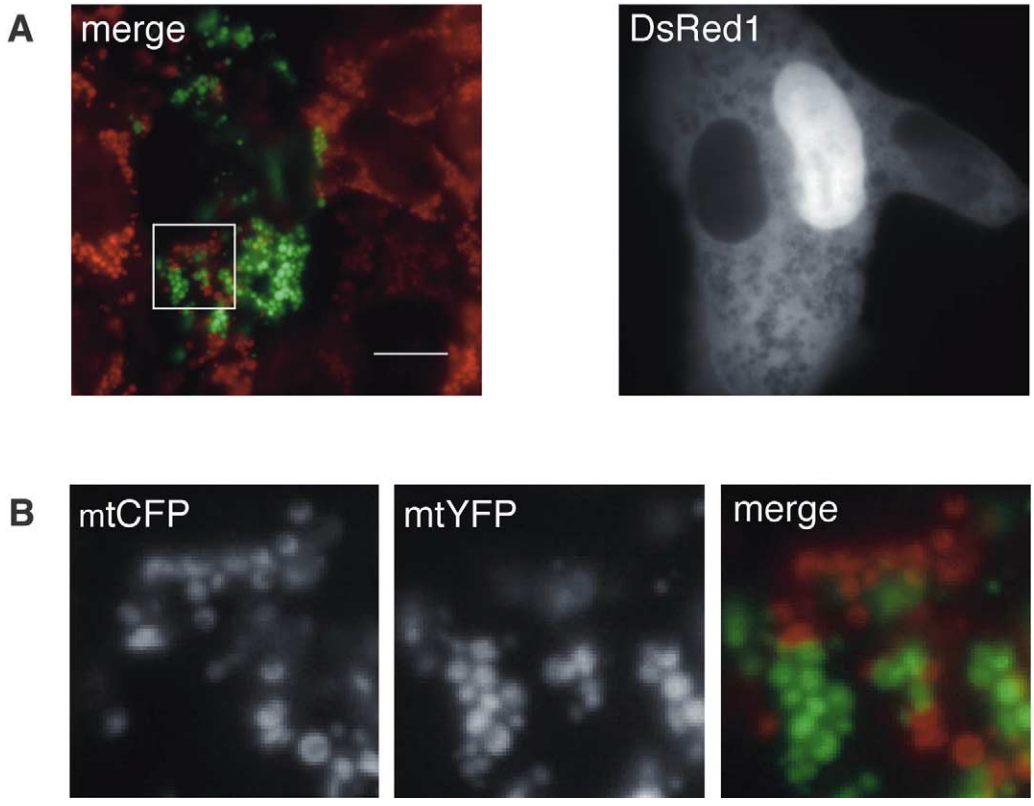


Fig. 3. Effect of $\Delta\psi_m$ disruption on mitochondrial fusion. A: Cells treated with 75 μM cccp. Note the absence of mitochondrial fusion in a polykaryon resulting from the fusion of two MMCS cells (left and right side) and one HMYS cell (in the middle). The fusion of the three cells is confirmed by the diffusion in the MMCS cells of DsRed1 protein transiently expressed in the HMYS cell. B: Higher magnification of the boxed area in A. Cells were pictured 2 h after cell fusion. Scale bar 10 μm.

treated with 50 μg/ml cycloheximide 1 h before fusion to inhibit protein synthesis. This treatment was shown to inhibit efficiently protein synthesis (data not shown).

To further confirm that mitochondrial fusion was occurring, we performed video microscopy recording of fused cells 1 h following PEG treatment. We observed mitochondria moving in the cell and eventually fusing, events that resulted in the mixing of eCFP and eYFP. This observation unambiguously proved mitochondrial fusion (see supplemental data).

3.2. *Mitochondrial fusion occurs in cells displaying an abnormal actin or tubulin network*

Since mitochondria have been reported to move on either actin or microtubule networks depending on cell type and/or cell compartments, we investigated if we could impair mitochondrial fusion by altering these networks. Treatment of cells with 5 μM nocodazole 1 h before fusion completely disrupted the microtubule network (assayed with an antibody against β-tubulin, data not shown) and modified the mitochondrial morphology (Fig. 2, nocodazole). However, disruption of the microtubule network did not prevent mitochondrial fusion (Fig. 2, nocodazole). Forcing microtubule polymerization with taxol treatment (1 μM) 1 h before fusion affected the microtubule network and cell morphology but did not prevent mitochondrial fusion (Fig. 2, taxol).

We also investigated if disruption of the actin network could inhibit mitochondrial fusion. We used cytochalasin D (10 μM), which has three major primary effects in vitro: capping of the barbed end of the actin filament, disrupting the supramolecular organization of actin filaments and promoting depolymerization of filaments undergoing rapid length change (confirmed by actin staining with TRITC-phalloidin, data not shown, see also [25]). Firstly, we observed that treating the cells with this drug 1 h before fusion had a considerable effect on both the cellular and mitochondrial morphology (data not shown). The mitochondrial fusion took place but the process seemed to be slowed down. Most of the fused mitochondria were found near the site at which the cell membranes fused (Fig. 2, cytochalasin D). Quantitative experiments confirmed that mitochondrial fusion was significantly slowed down. Results from three independent fusion experiments show that 3 h after fusion, in cytochalasin D-treated cultures, 63% of the polykarya ($n = 30$) have less than 20% of their yellow mitochondria fused whereas in control cells 89% of the polykarya

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Fig. 2. Effect of cytoskeleton alteration, Drp1K38A or hFis1p overexpression on mitochondrial fusion. Cells treated with: nocodazole (5 μM), note the change in mitochondrial morphology; taxol (5 μM) and cytochalasin D (10 μM), note the reduced number of fused mitochondria (arrow indicates two fused mitochondria). Nocodazole- and taxol-treated cells were pictured 4 h after cell fusion, cytochalasin D 3 h after cell fusion. Cells overexpressing: hFis1, note the fragmentation of mitochondria induced by expression of the protein; Drp1K38A, note interconnected mitochondria induced by expression of the protein. Cells were pictured 4 h after cell fusion. Scale bar 10 μm.

($n = 28$) had more than 20% of their yellow mitochondria fused.

3.3. Fragmented or elongated mitochondria are still able to fuse

It is well accepted that a balance between fusion and fission events is necessary to maintain mitochondrial morphology. However, it is not clear if there is a cross-talk between these two processes. We tested whether fusion of mitochondria was stimulated when one of the cell types used contained fragmented mitochondria. In yeast, mitochondrial fission is mediated through Fis1p, a protein essential for mitochondrial fission [26]. In this study, we obtained mitochondrial fission by overexpressing hFis1, the mammalian homologue of Fis1p (James et al., submitted). Following fusion of cells transfected with hFis1 with cells displaying mitochondria with a normal morphology, we found that mitochondrial fusion was not accelerated compared to the kinetics of mitochondrial fusion occurring in cells displaying normal mitochondria (Fig. 2, hFis1).

We also tested whether mitochondrial fusion was reduced when one of the cell types displayed interconnected mitochondria. Drp1(K38A), a dominant negative mutant of Drp1, a protein known to play a role in mitochondrial fission, was overexpressed in order to obtain an interconnected mitochondrial network. Mitochondrial fusion was not reduced by overexpression of the Drp1 mutant (Fig. 2, Drp1(K38A)).

3.4. Mitochondrial fusion requires an intact mitochondrial membrane potential

Treatment of cells with cccp, a protonophore that disrupts the mitochondrial membrane potential ($\Delta\psi_m$) and uncouples oxidative phosphorylation, leads to mitochondrial fragmentation ([26] and our unpublished data). We wondered if this effect resulted from impaired fusion of mitochondria rather than fission. Cells were treated with 75 μM cccp, a concentration that completely disrupts the $\Delta\psi_m$, for 1 h. The $\Delta\psi_m$ disruption was confirmed using TMRE (40 nM), a mitochondrial staining dye whose accumulation in the matrix is dependent on $\Delta\psi_m$ (data not shown). In fused cells 2 h after cytoplasmic fusion, mitochondria were fragmented and no mitochondrial fusion was observed (Fig. 3A,B). At later time points (12 h), fused cells displayed mixed unicolor-labeled mitochondria but no mitochondria displayed both colors indicating that no mitochondrial fusion occurred in cells treated with cccp (data not shown).

4. Discussion

Since their discovery, mitochondria have been described as organelles able to change their shape through fusion and fission events [16]. Nevertheless, if the dynamic nature of the mitochondria and their ability to fuse and divide has been clearly established in different eukaryotes [6,8], the fusion of mammalian mitochondria still remains a matter of debate [27]. One of the problems encountered when mitochondria are observed by light microscopy or fluorescence microscopy using a single fluorescent mitochondrial marker is to distinguish between fusion events and transient contacts between mitochondria. In our study, we developed an assay based on the mixing of two fluorescent proteins in the mitochondrial matrix. This procedure allowed us to clearly show that mitochondrial merging does occur in mammalian cells and is completed in less than 24 h. So it could be said that this process is fast compared to the time needed for functional complementation; restoration of normal morphology and respiratory activities in hybrids of two respiration-deficient cell lines following fusion required more than 10 days [21]. However, mitochondrial merging is clearly slower than the diffusion of the cytosolic protein DsRed1. Our data are consistent with recent data published by Legros et al. [28] and with the observation that mitochondrial DNA from wild type cytoplasts spreads throughout mitochondria from ρ^0 cells within 6 h after fusing the cells [29].

Our results also shed some light on the mechanism of the fusion process. Studies on a number of cell types showed that mitochondria co-localize with microtubules, actin microfilaments and intermediate filaments [5]. Depending on the cell type, they can be transported across the cytoplasm using both microtubule-based [30–33] and actin-based motors [31,34–37]. Yet our results clearly show that the mitochondrial fusion process in itself is independent of both actin and microtubule cytoskeleton. However, we consistently observed that following actin disruption, the kinetics of mitochondrial fusion was significantly slowed down. At 3 h post cell fusion, the percentage of fused mitochondria rarely exceeded 20% when cytochalasin D was added to the cultures, whereas in its absence, more than 50% of mitochondria had fused. Because cytochalasin D triggers cell detachment, we have not been able to monitor mitochondrial fusion later than 5 h after actin depolymerization. In cells treated with cytochalasin D, the mobility of mitochondria was clearly altered and we think that this could be responsible for a decrease in the probability that two mitochondria meet and fuse. In contrast to cytochalasin D, agents that destabilize microtubules did not modify the kinetics of mitochondrial fusion, suggesting that actin, more than microtubules, is required for the mobility of mitochondria in those mammalian cells.

Importantly, we found that an intact $\Delta\psi_m$ is required for mitochondrial fusion to occur, since decreasing $\Delta\psi_m$ with cccp resulted in a rapid fission of mitochondria and absence of mitochondrial fusion. This result is in agreement with the results recently published by Legros et al. [28]. The mechanism by which the $\Delta\psi_m$ would permit mitochondrial fusion is unclear and is currently being investigated.

Studies in yeast suggested that mitochondrial fusion is of particular importance in the inheritance and maintenance of the mitochondrial genome [38]. Another role of this process would be to allow complementation of mitochondrial gene products, as recently shown in mammals [21,22]. Mitochondria have also been proposed to be interconnected and thereby form an electrically united system that would facilitate energy distribution throughout the cell [39]. Mitochondrial fusion and fission might also play a role in other physiological process such as apoptosis ([2,7] and James et al., submitted). Our results on the rapid kinetics of mitochondrial fusion support the hypothesis where mitochondria are considered as a single functional unit [27,37].

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