Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity

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Summary

The mammalian homologues of yeast and *Drosophila* Fzo, mitofusin (Mfn) 1 and 2, are both essential for mitochondrial fusion and maintenance of mitochondrial morphology. Though the GTPase domain is required for Mfn protein function, the molecular mechanisms of the GTPase-dependent reaction as well as the functional division of the two Mfn proteins are unknown. To examine the function of Mfn proteins, tethering of mitochondrial membranes was measured in vitro by fluorescence microscopy using green fluorescence protein- or red fluorescent protein-tagged and Mfn1-expressing mitochondria, or by immunoprecipitation using mitochondria harboring HA- or FLAG-tagged Mfn proteins. These experiments revealed that Mfn1-harboring mitochondria were efficiently tethered in a GTP-dependent manner, whereas Mfn2-harboring mitochondria were tethered with only low efficiency. Sucrose density gradient centrifugation followed by co-immunoprecipitation revealed that Mfn1 produced oligomerized ~250 kDa and ~450 kDa complexes in a GTP-dependent manner. The ~450 kDa complex contained oligomerized Mfn1 from distinct apposing membranes (docking complex), whereas the ~250 kDa complex was composed of Mfn1 present on the same membrane or in the membrane-solubilized state (cis complex). These results were also confirmed using blue-native PAGE. Mfn1 exhibited higher activity for this reaction than Mfn2. Purified recombinant Mfn1 exhibited ~eightfold higher GTPase activity than Mfn2. These findings indicate that the two Mfn proteins have distinct activities, and suggest that Mfn1 is mainly responsible for GTP-dependent membrane tethering.

Key words: Mitochondria, Membrane fusion, GTPase, Mitofusins, Fzo1

Introduction

Mitochondria are very dynamic and change shape depending on cell environment, physiology, developmental stage and pathologic condition (Yaffe, 1999; Jensen et al., 2000; Griparic and van der Bliek, 2001; Shaw and Nunnari, 2002; Karbowski and Youle, 2003; Scott et al., 2003; Westermann, 2003; Chen and Chan, 2004). In living cells, mitochondrial movement in the cytoplasm depends on the cytoskeletal structures, and mitochondria frequently divide and fuse with each other (Nunnari et al., 1997; Yaffe, 1999).

Mitochondrial morphology is maintained under a dynamic balance between division and fusion; inhibition of mitochondrial fusion results in mitochondrial fragmentation by progression of mitochondrial division whereas an arrest of mitochondrial division causes the formation of highly connected and elongated mitochondria (Bleazard et al., 1999; Sesaki and Jensen, 1999). In mammalian cells, the induction of apoptosis activates mitochondrial division to form fragmented mitochondria, which is apparently related to the progression of apoptosis (Frank et al., 2001). Furthermore, mitochondrial morphology is controlled by the mitochondrial membrane potential ($\Delta\Psi$) across the inner membrane; dissipation of $\Delta\Psi$ inhibits mitochondrial fusion to induce mitochondrial fragmentation, and re-establishment of $\Delta\Psi$ recovers the filamentous network structures (Legros et al., 2002; Ishihara et al., 2003).

Mitochondrial GTPase protein fuzzy onion (Fzo) in *Drosophila* or yeast Fzo1, and the mammalian homologues Mitofusin 1 and 2 (Mfn1 and Mfn2), function in the mitochondrial fusion reaction (Hales and Fuller, 1997; Hermann et al., 1998; Rapaport et al., 1998; Santel and Fuller, 2001; Rojo et al., 2002; Eura et al., 2003; Mozdy and Shaw, 2003; Santel et al., 2003). They are anchored to the mitochondrial outer membrane through the C-terminal membrane-binding domain, extruding the N-terminal GTPase domain to the cytoplasm (Fritz et al., 2001; Rojo et al., 2002). In *Drosophila*, Fzo protein is specifically expressed in the testis, and Fzo mutations inhibit spermatogenesis-specific mitochondrial fusion to cause male infertility (Hales and Fuller, 1997). In the *fzo1* mutant strain of *Saccharomyces cerevisiae*, mitochondrial fusion is impaired; thus, mitochondria are fragmented, resulting in the loss of mitochondrial DNA, which indicates that mitochondrial fusion is important for the maintenance of functional mitochondria (Hermann et al., 1998; Rapaport et al., 1998). An active GTPase domain is essential for the function of yeast and *Drosophila* Fzo proteins (Hales and Fuller, 1997; Hermann et al., 1998).

In mammals, two Fzo homologues, Mfn1 and Mfn2, are widely expressed in many tissues (Rojo et al., 2002; Eura et al., 2003; Santel et al., 2003), although their expression levels in each tissue clearly differ: in the brain, Mfn2 is

predominantly expressed over Mfn1, and the converse is true in the heart and testis (Eura et al., 2003). Gene disruption experiments and RNA interference (RNAi) experiments in cultured cells revealed that both Mfn proteins and their active GTPase domains are essential for the mitochondrial fusion reaction (Chen et al., 2003; Eura et al., 2003). A recent report demonstrated that mutations mainly within or close to the GTPase domain of Mfn2 cause Charcot-Marie-Tooth neuropathy type 2A (Züchner et al., 2004). These results, together with the finding that a significant fraction of the two exogenously expressed Mfn proteins forms complexes, suggest that both Mfn proteins function cooperatively (Chen et al., 2003; Eura et al., 2003). A heptad-repeat region at the C-terminal portion of Mfn1 was recently shown to form a dimeric antiparallel coiled-coil structure, which mediates mitochondrial tethering before the fusion reaction (Koshiba et al., 2004). The functional differences between the two Mfn proteins and their mechanisms of action in mitochondrial membrane fusion, however, are unknown. Here, we demonstrate that Mfn1 tethers mitochondrial membranes, depending on GTP hydrolysis, with a higher efficiency than Mfn2, which corresponds to changes in the oligomeric states of the Mfn-containing complex. These properties reflect differences in the GTPase activity of the Mfn proteins; purified Mfn1 exhibited ~eightfold higher activity than Mfn2. We demonstrated a functional difference in the membrane tethering step using an in vitro system.

Materials and Methods

Materials and cell culture

Rabbit polyclonal and monoclonal (16B12) antibodies against hemagglutinin (HA) were obtained from BabCO and monoclonal antibodies against FLAG (M2) were obtained from Sigma. All nucleotides were purchased from Roche Molecular Biochemicals. The mammalian expression plasmids of rat Mfn1 and Mfn2 with HA or FLAG-tags at their C-termini were previously described (Eura et al., 2003). The expression plasmids of mitochondrial green fluorescent protein (pSu9-GFP) and mitochondrial red fluorescent protein (pSu9-RFP) were as described earlier (Ishihara et al., 2003). HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2. Transfection was performed using Lipofectamine (Lifetech) and the cells were incubated for 24 hours after transfection.

Assay for GTP-dependent mitochondrial tethering and Mfn complex formation

Immunoprecipitation

The membrane fractions were suspended in immunoprecipitation (IP) buffer: 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl and protease inhibitors [0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml α2-macroglobulin, protease inhibitor cocktail complete EDTA-free (Roche Molecular Biochemicals)]. They were solubilized with 1% digitonin. The lysates were cleared twice by centrifugation at 14,000 ***g*** for 5 minutes in a microfuge, and mixed with polyclonal anti-HA antibodies and Protein A-Sepharose (Amersham Pharmacia) at 4°C for 2 hours. The resin was washed three times with IP buffer containing 0.5% digitonin. Proteins were eluted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to SDS-PAGE and subsequent immunoblotting using monoclonal anti-HA or anti-FLAG. The bands on the filter were analyzed and quantified by LAS1000 and the Image Gauge program (Fuji Film Co.).

Preparation of mitochondrial fractions

The expression plasmids of HA-tagged or FLAG-tagged Mfn proteins were separately transfected into HeLa cells, which were cultured in 10 cm dishes. The cells were suspended in 800 μl homogenization buffer [10 mM HEPES-KOH buffer (pH 7.4) containing 0.22 M mannitol, 0.07 M sucrose and protease inhibitors], and homogenized with ten strokes using a syringe with a 27-gauge needle. The post-nuclear supernatant (PNS) was recovered by centrifugation at 200 ***g*** for 5 minutes. The mitochondria-enriched pellet was recovered by centrifugation of the PNS at 4000 ***g*** for 5 minutes, and washed once by the homogenization buffer. For salt treatment, mitochondria were washed twice with homogenization buffer containing 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl (wash-mit). These fractions were resuspended in the reaction buffer [homogenization buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MgOAc_2$, 1 mM dithiothreitol and protease inhibitors].

Complex formation in detergent-solubilized lysates

The PNS or mitochondrial fractions in the reaction buffer were solubilized by 1% digitonin on ice for 30 minutes. The solubilized lysates (50 μl each) from the cells expressing HA-tagged and FLAG-tagged Mfn proteins were mixed, and incubated with or without 0.4 mM GTP at 30°C for the indicated times. After incubation, 400 μl IP buffer containing 1% digitonin were added, the lysate was cleared twice by centrifugation at 14,000 ***g*** for 5 minutes, and subjected to immunoprecipitation using polyclonal anti-HA. The immunoprecipitates were analyzed by SDS-PAGE and subsequent immunoblotting using monoclonal anti-FLAG as above.

Complex formation on intact mitochondria

PNS or mitochondrial fractions (50 μl each) from the cells expressing HA- or FLAG-tagged Mfn proteins were mixed, and incubated with or without 0.4 mM GTP at 30°C. After incubation, the mitochondria-enriched pellet was recovered by centrifugation at 4000 ***g*** for 5 minutes, suspended in 500 μl IP buffer, and solubilized with 1% digitonin on ice for 30 minutes. The lysate was cleared twice by centrifugation at 14,000 ***g*** for 5 minutes and subjected to immunoprecipitation.

Immunoisolation of membrane vesicles containing Mfn proteins

PNS fractions from the cells expressing either HA-tagged or FLAG-tagged Mfn proteins were sonicated three times for 10 seconds, and the supernatants containing vesiculated membranes were recovered by centrifugation at 4000 ***g*** for 5 minutes twice to remove unbroken organelles. They were mixed (50 μl each), and incubated with or without 0.4 mM GTP at 30°C for 1 hour. After incubation, 400 μl IP buffer without detergent was added and samples were centrifuged at 1500 ***g*** for 5 minutes twice to remove membrane aggregates. The supernatant was mixed with polyclonal anti-HA antibodies and Protein A-Sepharose. The resin was recovered by centrifugation at 200 ***g*** for 1 minute, washed three times with IP buffer without detergent and analyzed by immunoblotting using monoclonal anti-FLAG antibodies.

Microscopic analysis of mitochondrial binding

Mitochondrial fractions from the cells coexpressing mit-GFP and FLAG-tagged Mfn1, or mit-RFP and FLAG-tagged Mfn1, were mixed and incubated with or without 1 mM GTP at 30°C for 30 minutes. These samples were analyzed by confocal microscopy (BioRad). The number of RFP-mitochondria that were connected with GFP-mitochondria out of the total RFP-mitochondria were counted and expressed as the percentage binding efficiency.

Sucrose density gradient centrifugation

Mitochondria from HeLa cells expressing HA- or FLAG-tagged Mfn proteins were prepared and incubated with GTP as described above, and solubilized with 1% digitonin in 500 μl IP buffer. The cleared lysate was layered on 11 ml of 5-25% (w/v) linear sucrose gradient in 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, protease inhibitors and 1% digitonin, and centrifuged at 174,000 ***g*** for 12 hours at 4°C (RPS40T, Hitachi). The centrifuged solution was separated into 28 fractions and subjected to immunoblotting.

Blue-native PAGE

Blue-native PAGE (BN-PAGE) was performed as previously described (Suzuki et al., 2002). Mitochondria from HeLa cells expressing HA-tagged Mfn1 were incubated with GTP as described above. The digitonin-solubilized mitochondria (80 μl) was mixed with 10 μl sample buffer [5% Coomassie Brilliant blue G-250, 100 mM Bis-Tris (pH 7.0) and 500 mM 6-aminocaproic acid] and 10 μl glycerol, and then electrophoresed through 5-16% polyacrylamide gradient gels. The gels were subjected to immunoblotting using anti-FLAG or anti-HA antibodies.

Purification of recombinant Mfn proteins

The BL21(DE3)-harboring expression plasmid of N-terminal His_6-tagged Mfn1 or Mfn2 (Eura et al., 2003) was cultured in 2×YT containing 20 μg/ml kanamycin. Isopropyl thiogalactoside (0.3 mM) was added and the cells were cultured for another 2 hours at 37°C. The bacterial cells were recovered and disrupted by freeze-thawing and sonication in lysis buffer [20 mM HEPES-KOH buffer (pH 7.4) containing 250 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor mix]. The lysate was centrifuged at 100,000 ***g*** for 1 hour (RP42, Hitachi), and the precipitate was solubilized by the lysis buffer containing 1% Triton X-100. After removal of insoluble materials, the supernatant was incubated with TALON resin (Clontech) in the presence of 10 mM imidazole. The resin was washed with 20 mM imidazole and His-Mfn protein was eluted with imidazole (30-200 mM).

Assay for GTP binding and GTPase activities

GTP binding

The PNS (300 μl) from HeLa cells expressing FLAG-tagged Mfn proteins were solubilized in reaction buffer containing 1% digitonin and the cleared lysate was incubated with 10 μl GTP-agarose beads (Sigma) at 30°C. The resin was washed three times with IP buffer containing 1% digitonin. The proteins were resolved by SDS-PAGE and analyzed by immunoblotting using anti-FLAG antibodies.

GTP hydrolysis

GTP hydrolysis activity was analyzed as described previously (Warnock et al., 1996). Purified His_6-tagged Mfn proteins (0.1 μg) were incubated in 20 μl of 20 mM HEPES-KOH buffer (pH 7.4) containing 2 mM $MgCl_2$, 1 mM dithiothreitol and 1% Triton X-100 with 1 μCi of [α-^{32}P]GTP at 37°C. At the indicated time, 1 μl of the reaction mixture was removed and spotted on the polyethyleneimine-cellulose filter (Merck). The reaction products were resolved by thin-layer chromatography in 1 M $LiCl_2$ and 2 M formic acid. The products were analyzed and quantified using a Bioimage Analyzer BAS2000 (Fuji Film Co.).

Results

Mfn1-stimulated tethering of mitochondria depends on GTP hydrolysis

Previous studies indicated that externally expressed Mfn proteins concentrate at the contact faces of connected mitochondrial outer membranes, suggesting that Mfn proteins are involved in mitochondrial binding (Rojo et al., 2002; Eura et al., 2003; Santel et al., 2003). We examined this in more detail using isolated mitochondria. Green fluorescent protein (GFP) or DsRed (red fluorescent protein; RFP) fused with a mitochondrial-targeting signal (mit-GFP or mit-RFP, respectively) was separately coexpressed with FLAG-tagged rat Mfn1 in HeLa cells. The isolated mitochondria were mixed, incubated with or without GTP, and examined by confocal microscopy (Fig. 1A).

In contrast to the filamentous structures in HeLa cells, the isolated GFP- or RFP-labeled mitochondria had spherical structures, possibly because of fragmentation occurring during the isolation procedures (Fig. 1Ba), as reported previously (Fuchs et al., 2002). After incubation with GTP at 30°C, approximately 30% of the RFP-labeled mitochondria bound to the GFP-labeled mitochondria (Fig. 1Bb-e and 1C). By contrast, the binding efficiency between GFP-labeled and RFP-labeled mitochondria was significantly lower when incubated without GTP (Fig. 1Ba), with GTPγS, or at 0°C (Fig. 1C). GTP weakly, but reproducibly, stimulated tethering of mitochondria prepared from mock-transfected cells, indicating that the results obtained with exogenously expressed Mfn proteins reflected the character of endogenous Mfn proteins (Fig. 1C and Table 1). Exogenous expression of Mfn1-FLAG increased the GTP-dependent mitochondrial tethering reaction two- to eightfold.

We further confirmed this membrane-binding reaction by immunoprecipitation of the fragmented mitochondrial membrane vesicles. PNS fractions were prepared from HeLa cells expressing Mfn1-HA or Mfn1-FLAG. They were vesiculated by sonication, mixed and incubated in the presence or absence of GTP. The Mfn1-HA harboring vesicles were precipitated by anti-HA antibodies and the Mfn1-FLAG harboring vesicles recovered in the immunoprecipitates were identified by western blotting using anti-FLAG antibodies (Fig. 1D,E). The Mfn1-FLAG harboring vesicles were coprecipitated with the Mfn1-HA harboring vesicles to a significant extent only in the presence of GTP. The recovery was low when the mitochondrial vesicles that do not carry Mfn1-HA or those

Table 1. GTP- and Mfn1-dependent mitochondrial binding activity

	Mock			Mfn1-FLAG		
Experiment number	0 minutes	w/o GTP	GTP	0 minutes	w/o GTP	GTP
1	16.1±1.0	22.6±2.0	29.2±5.5	19.8±2.3	29.1±0.5	45.4±6.4
2	9.1±2.3	16.3±3.9	18.1±3.2	5.27±1.32	15.3±2.5	29.9±2.8
3	12.3±2.0	15.1±0.8	16.5±2.1	11.3±2.6	20.4±4.6	29.7±3.7

Mitochondrial binding activity in % (see Materials and Methods); w/o, without.

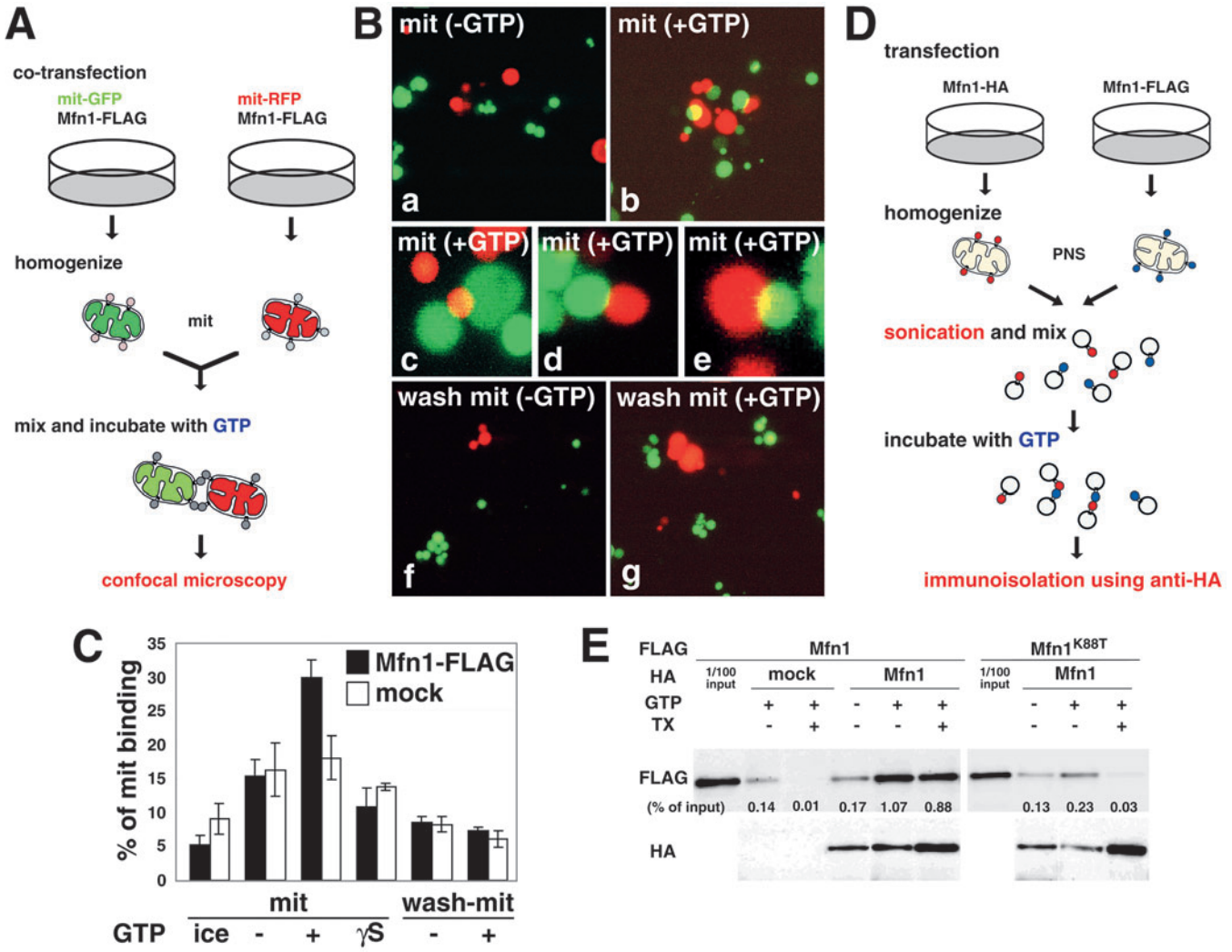

Fig. 1. GTP and Mfn1-dependent mitochondrial tethering. (A) Schematic drawing of the mitochondrial tethering reaction using isolated mitochondria. (B) Mitochondria (mit, a-e) or salt-washed mitochondria (wash mit, f and g) were prepared from HeLa cells coexpressing mit-GFP and Mfn1-FLAG, or mit-RFP and Mfn1-FLAG. Mitochondria labeled with GFP or RFP were mixed, and incubated with (b-e and g) or without (a and f) GTP at 30°C. The reaction mixtures were analyzed by confocal microscopy. Magnified images of bound mitochondria are shown (c-e). (C) GFP-labeled mitochondria and RFP-labeled mitochondria prepared from the cells expressing Mfn1-FLAG (filled bars), or without Mfn1-FLAG (open bars) were incubated as in B, and the number of bound mitochondria were counted as described in Materials and Methods. (D) Schematic drawing of pull-down assay for the binding reaction between sonicated mitochondrial vesicles. (E) The PNS fractions from HeLa cells expressing Mfn1-HA or Mfn1-FLAG were sonicated, mixed and incubated in the presence or absence of GTP. The reaction mixtures were then treated with or without Triton X-100 and subjected to immunoprecipitation using anti-HA antibody. The immunoprecipitates were analyzed by immunoblotting using anti-FLAG antibody. In a separate experiment, Mfn1^{K88T}-FLAG-harboring mitochondria were used in lieu of Mfn1-FLAG-harboring mitochondria.

carrying the GTPase domain-mutated Mfn1-FLAG(Mfn1^{K88T}) were used as the binding partner (Fig. 1E).

These results indicated that Mfn1-FLAG activation of the mitochondrial tethering reaction was dependent on GTP hydrolysis. Furthermore, pre-treatment of the mitochondria with 150 mM NaCl clearly impaired the GTP-dependent tethering reaction (Fig. 1Bg and 1C), although the expressed Mfn1-FLAG protein remained firmly bound to the mitochondrial membrane after the salt wash, suggesting that some peripheral membrane proteins are required for the mitochondrial tethering reaction (see 'wash mit' in Fig. 2B). On the other hand, stimulation of mitochondrial docking was rarely observed for the mitochondria harboring Mfn2-FLAG (data not shown). These results indicate that Mfn1 stimulates mitochondrial membrane tethering in a GTP-dependent manner.

GTP-dependent interaction in trans Mfn proteins expressed on distinct mitochondria

We examined whether Mfn1 proteins localizing in distinct mitochondria interact in trans in the GTP-dependent mitochondrial tethering process. For this purpose, HA- or FLAG-tagged Mfn1 was separately expressed in HeLa cells and the post nuclear supernatant or mitochondrial fractions prepared from them were incubated with or without GTP. The mitochondria were then solubilized with 1% digitonin and

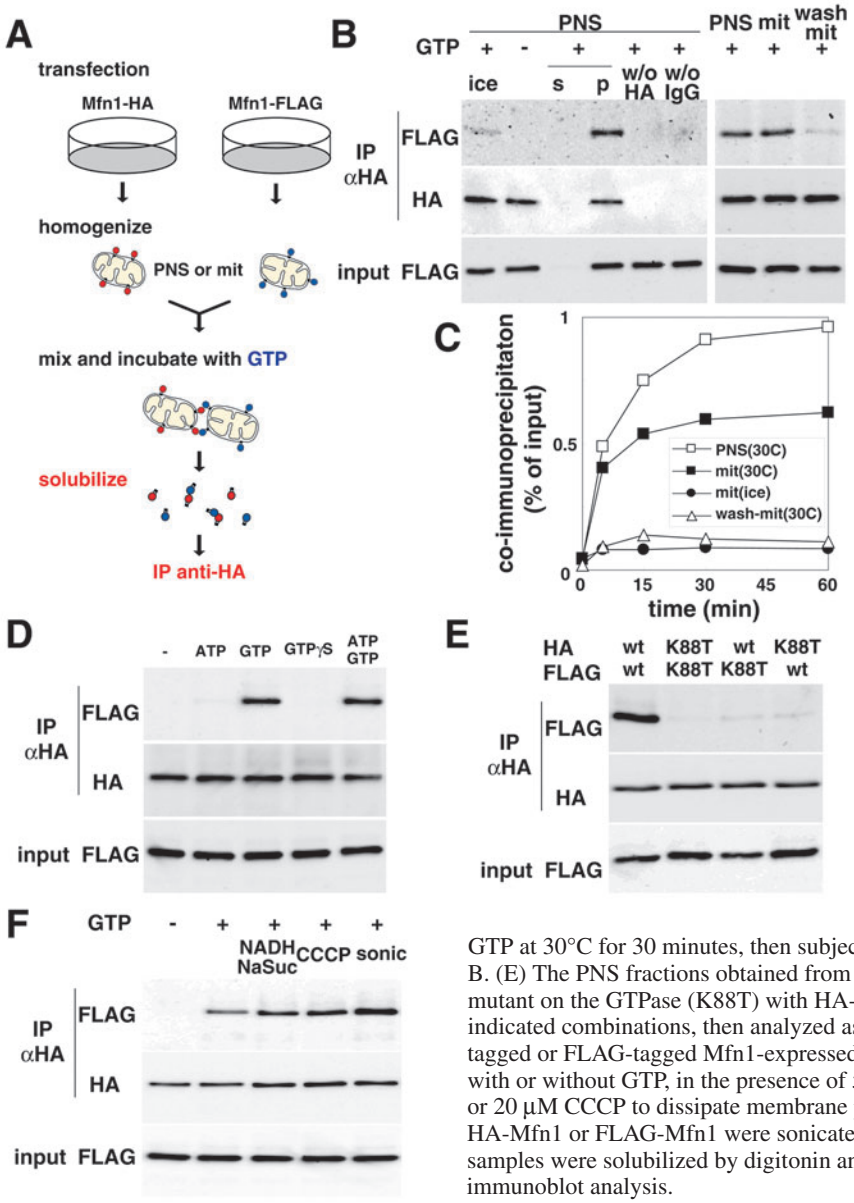

Fig. 2. GTP-dependent formation of trans-mitochondrial complex with Mfn1 protein. (A) Assay for the formation of Mfn1-mediated trans-mitochondrial complex. (B) Post nuclear supernatant (PNS), mitochondria (mit) or salt-washed mitochondria (wash mit) fractions were prepared from HeLa cells expressing HA-tagged or FLAG-tagged Mfn1. Fractions with HA-tagged and FLAG-tagged Mfn1 were mixed, and incubated with or without GTP at 0°C or 30°C for 30 minutes. In a reaction with PNS, mitochondria (p) and the supernatant (s) were separated by centrifugation after the reaction. All these fractions were solubilized with 1% digitonin and subjected to immunoprecipitation using anti-HA antibodies. The immunoprecipitates were subjected to SDS-PAGE and subsequent immunoblotting using anti-FLAG and anti-HA antibodies. As controls, the reaction mixtures using mock-transfected cells instead of Mfn1-HA (w/o HA), or immunoprecipitation without antibodies (w/o IgG) were analyzed. The reaction mixture prior to immunoprecipitation was also analyzed by immunoblotting by anti-FLAG (input). (C) Time course of co-immunoprecipitation. PNS or mitochondria as prepared in B were incubated for the indicated time intervals at 0°C or 30°C. The recovery of Mfn1-FLAG from the reaction mixture is shown. (D) The PNS fractions with HA-tagged or FLAG-tagged Mfn1 were mixed and incubated with 1 mM ATP, GTP, GTPγS or 1 mM ATP plus 1 mM GTP at 30°C for 30 minutes, then subjected to immunoprecipitation and immunoblotting as in B. (E) The PNS fractions obtained from HeLa cells expressing wild-type Mfn1 (wt) or its mutant on the GTPase (K88T) with HA- or FLAG-tag were incubated with GTP in the indicated combinations, then analyzed as in B. (F) The PNS fractions obtained from HA-tagged or FLAG-tagged Mfn1-expressed cells were mixed and incubated at 30°C for 1 hour with or without GTP, in the presence of 5 mM NADH plus 20 mM sodium succinate (NaSuc), or 20 μM CCCP to dissipate membrane potential. In a separate experiment, PNS fractions with HA-Mfn1 or FLAG-Mfn1 were sonicated, then mixed and incubated as above (sonic). These samples were solubilized by digitonin and subjected to immunoprecipitation and subsequent immunoblot analysis.

subjected to immunoprecipitation using anti-HA antibodies and subsequent immunoblotting with antibodies against HA or FLAG (Fig. 2A). Mfn1-FLAG coprecipitated with Mfn1-HA and this interaction was time-dependent, temperature-dependent (Fig. 2B,C), GTP-dependent (Fig. 2B,D), and required the active GTPase domain of Mfn1 (Fig. 2E). GTPγS or ATP failed to support this binding reaction (Fig. 2D). These results indicated that Mfn1 proteins on different mitochondrial membranes interact in trans depending on the GTP hydrolysis activity (the 'docking complex'). On the other hand, a homotypic Mfn2-Mfn2 interaction proceeded, but with extremely low efficiency, and a heterotypic Mfn1-Mfn2 interaction proceeded with moderate efficiency (see Fig. 5).

Interestingly, and consistent with the results shown in Fig. 1, when the mitochondria were treated with 150 mM NaCl, co-immunoprecipitation efficiency was significantly decreased ('wash mit' in Fig. 2B,C), suggesting that the membrane tethering reaction of Mfn1 required additional factors that were peripherally associated with the mitochondria.

The mitochondrial fusion reaction in cultured cells is strongly inhibited by a protonophore, carbonyl cyanid *m*-chlorophenylhydrazone (CCCP), indicating that the reaction depends on the ΔΨ across the inner membrane (Legros et al., 2002; Ishihara et al., 2003). The GTP-dependent tethering reaction of the Mfn1-harboring mitochondrial membranes, however, was not affected by CCCP or by the respiratory substrate NADH with sodium succinate (Fig. 2F). Formation of the complex was also observed with the fragmented mitochondrial vesicles (Fig. 2F 'sonic'). These results suggest that the GTP-dependent membrane tethering reaction, but not the subsequent ΔΨ-dependent membrane-fusion reaction, occurred in the present in vitro assay system.

GTPase-dependent interaction of Mfn1 protein in a membrane-free system

As described above, Mfn1 mediated tethering of mitochondrial membranes in a GTP-dependent manner. We then examined

whether the formation of the Mfn1 complex also proceeded in a detergent-solubilized, membrane-free system. HA- and FLAG-tagged Mfn1 proteins were expressed separately in HeLa cells, their post nuclear supernatant or mitochondria were solubilized with digitonin, and they were then mixed and incubated with or without GTP (Fig. 3A). Immunoprecipitation using anti-HA antibodies revealed that Mfn1-FLAG coprecipitated with Mfn1-HA in a temperature- and time-dependent manner (Fig. 3B,C). ATP and GTPγS failed to activate the interaction (Fig. 3D). The binding reaction did not proceed when either or both mitochondria carried the GTPase-mutant form of Mfn1 (Fig. 3E). Taken together, these findings indicated that homotypic interaction of Mfn1 in the membrane-free system also depended on GTP hydrolysis. Of note, the coprecipitation efficiency was higher compared with the binding assay on the membrane level (Fig. 3F 'mit'), which suggests that Mfn proteins that were somehow masked on the membranes were revealed by detergent to become binding-competent. Even in this case, however, the apparent binding efficiency in the presence of detergent was as low as ~10%. This is mainly because heterotypic complexes containing both Mfn1-HA and Mfn1-FLAG proteins (i.e. the Mfn1-HA/Mfn1-FLAG complex) were immuno-selected among the complexes composed of various combinations of HA- and FLAG-tagged

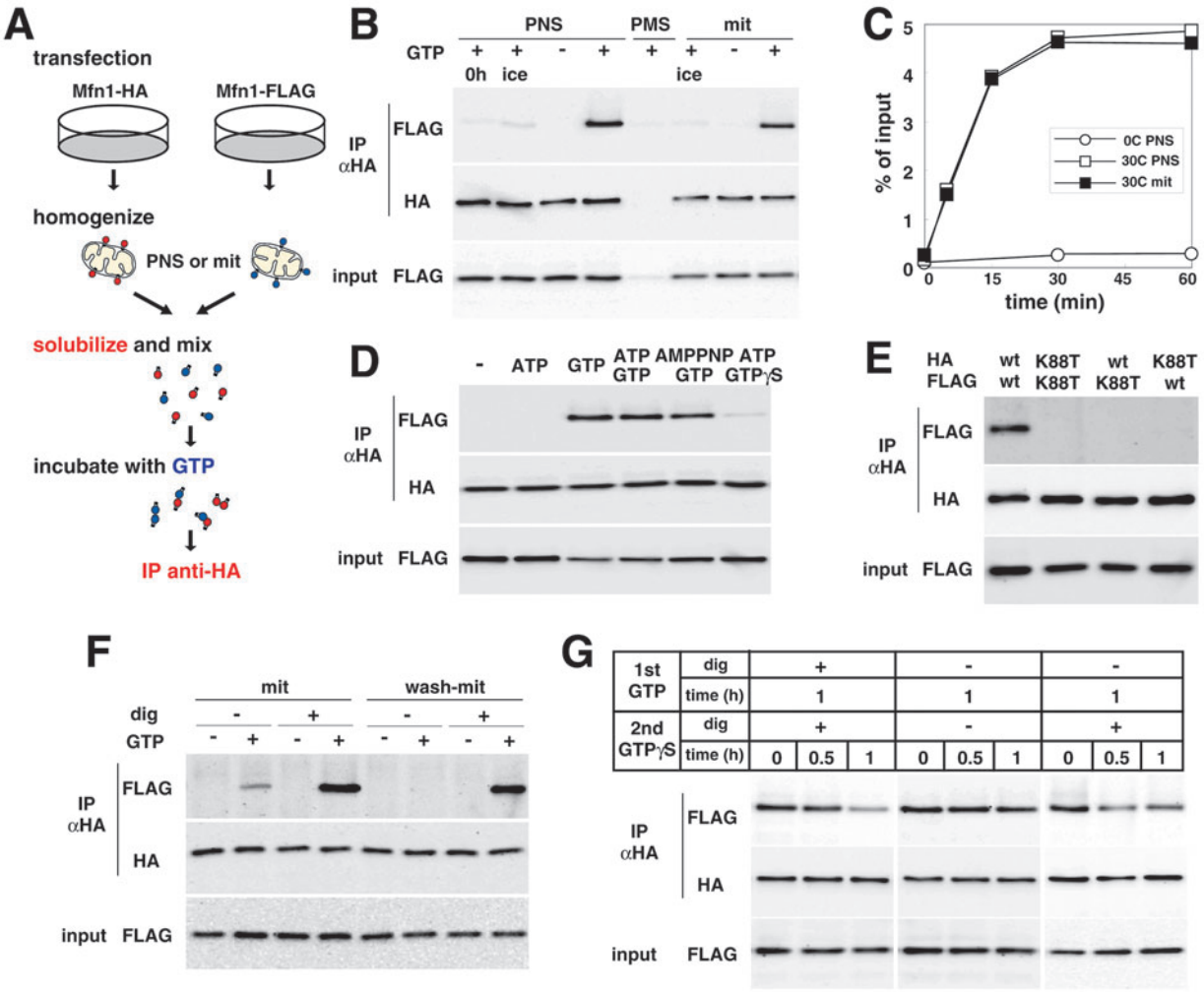

Fig. 3. Complex formation of Mfn1 protein in solubilized conditions. (A) Assay for GTP-dependent homotypic Mfn1 complex formation. (B) PNS, post mitochondrial supernatant (PMS) and mitochondria fractions (mit) were prepared from HeLa cells expressing HA-tagged or FLAG-tagged Mfn1, and solubilized by digitonin. The lysates with HA-tagged or FLAG-tagged Mfn1 were mixed, and incubated with or without GTP, at 0°C or 30°C for 1 hour. The reaction mixtures were subjected to immunoprecipitation using anti-HA antibodies, and analyzed by immunoblotting using anti-FLAG and anti-HA antibodies. The reaction mixtures were also analyzed by immunoblotting using anti-FLAG antibodies (input). (C) Time course of co-immunoprecipitation. The solubilized PNS or mitochondrial fractions with HA-tagged or FLAG-tagged Mfn1 were mixed and incubated with GTP on ice or at 30°C. Co-immunoprecipitated Mfn1-FLAG by anti-HA antibodies was quantified. (D) Solubilized mitochondrial fractions with HA-tagged or FLAG-tagged Mfn1 were mixed and incubated with 1 mM nucleotides as indicated. The reaction mixtures were analyzed as in B. (E) Wild-type Mfn1 (wt) or the mutant K88T with HA or FLAG tags were mixed in the indicated combinations and incubated with GTP. The reaction mixtures were analyzed as in B. (F) The mitochondria (mit), or salt washed mitochondria (wash mit) were solubilized by digitonin. The solubilized (dig+) or intact (dig–) mitochondria were incubated with or without GTP, and analyzed as in B. (G) The mitochondria harboring Mfn1-HA or Mfn1-FLAG were incubated in the digitonin-solubilized (dig+) or unsolubilized (dig–) conditions with 0.4 mM GTP at 30°C for 1 hour (1st GTP). After incubation, 2 mM GTPγS or digitonin was added and further incubated at 30°C for the indicated time intervals (2nd GTPγS), then subjected to immunoprecipitation and immunoblotting.

Mfn1 proteins (see Discussion). Interestingly, 150 mM NaCl treatment, which completely inhibited the membrane tethering reaction, was not inhibitory in the membrane-solubilized condition, and inhibition of membrane tethering by 150 mM NaCl pretreatment was relieved by membrane solubilization (Fig. 3F).

Recovery of the GTP-induced complex in the detergent-solubilized, membrane-free system was decreased when the complex was further incubated in the presence of GTPγS, indicating that the complex was disassembled by incubation with GTPγS (Fig. 3G, left panel). On the other hand, the docking complex formed on the membrane was not dissociable (Fig. 3G, middle panel). However, when incubated with GTPγS followed by membrane solubilization (Fig. 3G, right panel), disassembly of the complex occurred, suggesting that GTPγS-induced dissociation was inhibited by the membrane structure.

GTP-dependent change in oligomerization states of the Mfn1-containing complex

In gel-filtration chromatography, yeast Fzo1 is fractionated as a ~500 kDa complex in the presence of Triton X-100 (Rapaport et al., 1998) and mammalian Mfn1 is fractionated as a ~350 kDa complex in the presence of digitonin (Santel et al., 2003), suggesting that Fzo proteins form oligomeric complexes. We therefore probed GTP-induced changes of the Mfn1-containing complex using sucrose density gradient centrifugation. Mitochondria isolated from the cells expressing Mfn1-FLAG or Mfn1-HA were solubilized by digitonin, mixed together, and incubated with or without GTP as shown in Fig. 3, then subjected to sucrose density gradient centrifugation in digitonin.

In the absence of GTP, Mfn1-FLAG and Mfn1-HA were both recovered mainly in fractions 8-10 (peak 1; Fig. 4A 'w/o'). After incubation with GTP, Mfn1-FLAG and Mfn1-HA co-sedimented in fractions 11-13 (peak 2; Fig. 4A, 'GTP'). The same results were also obtained for mitochondria carrying only the Mfn1-FLAG protein (Fig. 4B). The shift from peak 1 (~180 kDa) to peak 2 (~250 kDa) depended on the GTP incubation time (Fig. 4B). By contrast, GTPγS incubation did not affect the distribution of Mfn1 in the sucrose density gradient (Fig. 4B, GTPγS 1h). When a GTPase mutant, $Mfn1^{K88T}$-FLAG, was examined as above, it remained in peak 1, even after incubation with GTP (Fig. 4B). Consistent with the immunoprecipitation results shown in Fig. 3G, Mfn1-FLAG in the ~250 kDa complex gradually disassembled to form the ~180 kDa complex when further incubated with GTPγS (Fig. 4B). Thus, the GTP-dependent change from the ~180 kDa complex to the ~250 kDa complex was reversible. These results indicated that Mfn1 in the detergent-solubilized state formed an oligomeric complex of ~250 kDa that was dependent on GTP hydrolysis and corresponded to the complex detected by co-immunoprecipitation in Fig. 3.

We then examined the oligomeric state of the Mfn-containing complex that tethers the mitochondrial membranes in trans (docking complex) in a GTPase-dependent manner. The mitochondria harboring Mfn1-FLAG were incubated with or without GTP, solubilized with digitonin and subjected to sucrose density gradient centrifugation (Fig. 4C). When incubated with GTP, Mfn1-FLAG sedimented towards the bottom at around fractions 11-13 (peak 2; ~250 kDa) and fractions 14-16 (peak 3; ~450 kDa) in an incubation time-dependent manner (Fig. 4C). As described above, washing of the mitochondria with 150 mM NaCl inactivated the membrane tethering process. When the salt-washed and Mfn1-FLAG-harboring mitochondria were incubated with GTP prior to the membrane solubilization, however, Mfn1-FLAG also sedimented closer to peaks 2 and 3 (Fig. 4D, 'wash mit'). These results indicated that the formation of the oligomerized species on the individual membranes was GTP dependent, which also sedimented towards the bottom fractions.

To characterize the complexes in peaks 1-3, the mitochondria harboring HA- or FLAG-tagged Mfn1 were mixed in the presence or absence of digitonin, incubated with GTP, and subjected to sucrose density gradient centrifugation. The fractions corresponding to peaks 1, 2, and 3 were collected and subjected to immunoprecipitation with anti-HA antibodies. The precipitates were analyzed by immunoblotting with anti-FLAG antibodies (Fig. 4E). When detergent-solubilized mitochondria were incubated with GTP, the Mfn1-HA/Mfn1-FLAG complex was recovered mainly in peak 2; immunoprecipitation efficiency was increased from ~10% to ~30% after centrifugation (Fig. 4E, left panel, '+dig, mit'). By contrast, the Mfn1-HA/Mfn1-FLAG complex was recovered mainly in peak 2 and peak 3 when mitochondria were incubated in the presence of GTP prior to solubilization with digitonin (Fig. 4E, middle panel, '–dig mit'). Centrifugation increased the efficiency of co-immunoprecipitation for peak 3 from ~1% to ~5% and for peak 2 to 1.4%. These results indicated that peak 3 partly represented the Mfn1 oligomeric complex tethering distinct apposing membranes (the docking complex) and peak 2 is mainly composed of the Mfn1 oligomeric complex that was formed in cis on individual mitochondria (the cis complex). As expected from the above experiments (see Fig. 2B and Fig. 3F), the Mfn1-FLAG/Mfn1-HA complex was not detected in any peaks in the density gradient when 150 mM NaCl-washed docking-compromised mitochondria were used for the reaction (Fig. 4E, right panel, '–dig, washed'), indicating that the cis complex is also contained in the ~450 kDa complex.

We further examined the complex states of Mfn1 using blue-native PAGE (BN-PAGE), which allows separation of the protein complexes in the membrane under native conditions (Fig. 4F). In the absence of GTP incubation, Mfn1-HA migrated as an ~180 kDa complex. Markedly, when the mitochondria incubated with GTP prior to solubilization were analyzed, Mfn1-HA migrated mainly as an ~450 kDa complex. When solubilized prior to GTP incubation, by contrast, Mfn1-HA migrated as an ~250 kDa complex. Thus, these results were consistent with those obtained using sucrose density gradient centrifugation.

Together, these results indicated that mitochondrial membranes are tethered through a ~450 kDa Mfn1 docking complex, whereas the ~250 kDa complex seemed to represent the Mfn1 complex that was formed in cis on individual membranes or in detergent-solubilized membranes. The ~250 kDa cis complex seemed to be reversibly dissociated from the ~180 kDa steady-state complex.

Two Mfn proteins exhibit distinct activity in the GTP-dependent membrane tethering reaction

In mammalian cells, Mfn1 and Mfn2 are both essential for

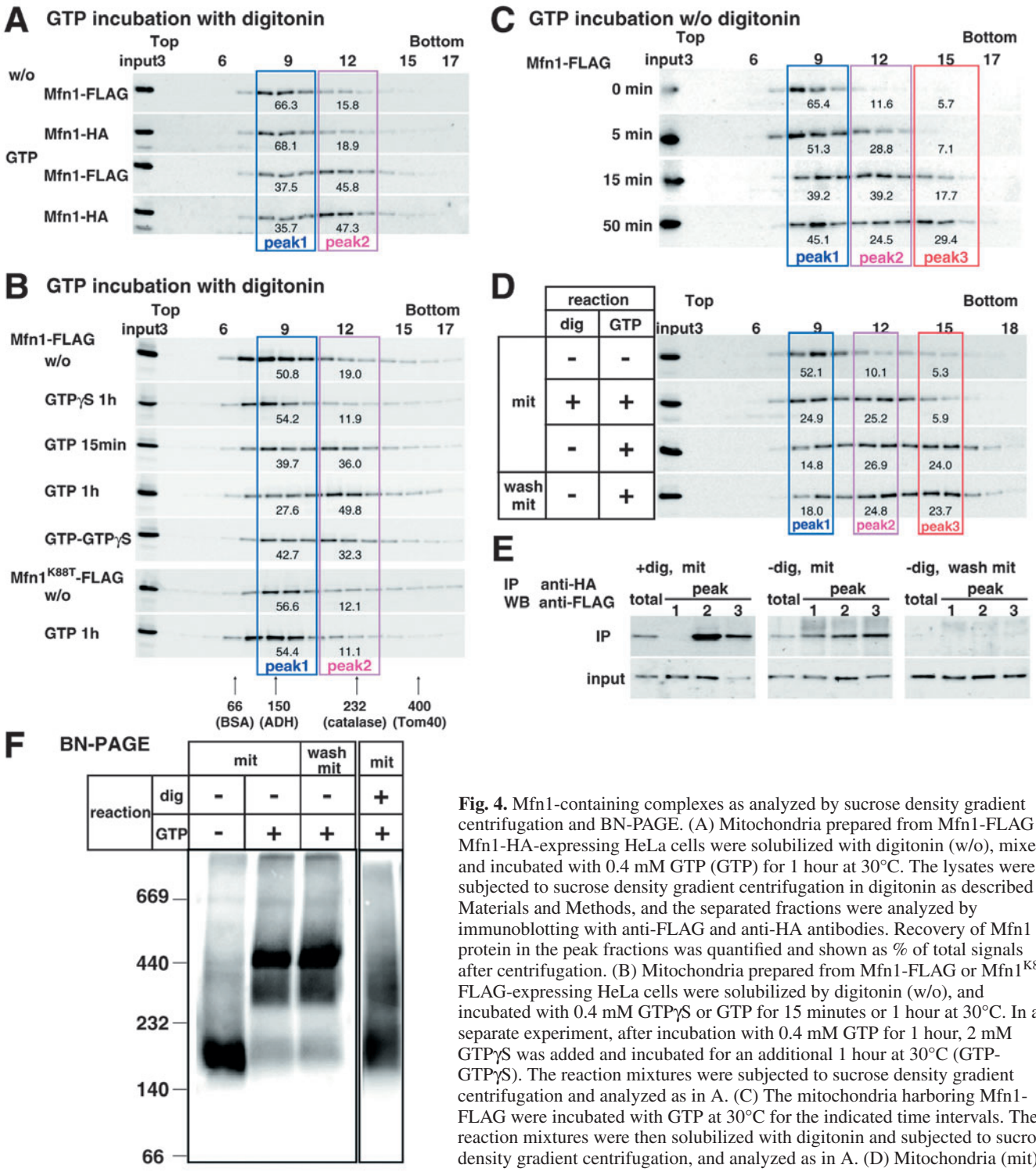

Fig. 4. Mfn1-containing complexes as analyzed by sucrose density gradient centrifugation and BN-PAGE. (A) Mitochondria prepared from Mfn1-FLAG or Mfn1-HA-expressing HeLa cells were solubilized with digitonin (w/o), mixed, and incubated with 0.4 mM GTP (GTP) for 1 hour at 30°C. The lysates were subjected to sucrose density gradient centrifugation in digitonin as described in Materials and Methods, and the separated fractions were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies. Recovery of Mfn1 protein in the peak fractions was quantified and shown as % of total signals after centrifugation. (B) Mitochondria prepared from Mfn1-FLAG or Mfn1^{K88T}-FLAG-expressing HeLa cells were solubilized by digitonin (w/o), and incubated with 0.4 mM GTPγS or GTP for 15 minutes or 1 hour at 30°C. In a separate experiment, after incubation with 0.4 mM GTP for 1 hour, 2 mM GTPγS was added and incubated for an additional 1 hour at 30°C (GTP-GTPγS). The reaction mixtures were subjected to sucrose density gradient centrifugation and analyzed as in A. (C) The mitochondria harboring Mfn1-FLAG were incubated with GTP at 30°C for the indicated time intervals. The reaction mixtures were then solubilized with digitonin and subjected to sucrose density gradient centrifugation, and analyzed as in A. (D) Mitochondria (mit) or salt-washed mitochondria (wash mit) were prepared from Mfn1-FLAG expressing HeLa cells, and incubated in the presence (+dig) or absence (–dig) of digitonin with (+GTP) or without (–GTP) GTP for 30 minutes at 30°C. The incubated mitochondria (for –dig) were solubilized by digitonin and subjected to sucrose density gradient centrifugation as in A. (E) Mitochondria (mit) or salt-washed mitochondria (wash mit) were prepared from HeLa cells expressing Mfn1-HA or Mfn1-FLAG, mixed, and incubated with GTP at 30°C for 1 hour (–dig). The membranes were solubilized by digitonin. Separately, mitochondria solubilized by digitonin (+dig) were also incubated with GTP. The reaction mixtures were subjected to sucrose density gradient centrifugation as above. Fractions 8 and 9 (peak 1), 11 and 12 (peak 2) or 14 and 15 (peak 3) were analyzed by immunoprecipitation using anti-HA antibodies, and subsequent immunoblotting using anti-FLAG antibodies as in Fig. 2. (F) BN-PAGE of mitochondria harboring Mfn1-FLAG or Mfn1-HA. Mfn1-harboring mitochondria were incubated with GTP as in D, and subjected to BN-PAGE. The separated protein complexes were analyzed by immunoblotting using anti-HA antibody. Marker proteins used were serum albumin (66 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), apoferritin (440 kDa) and thyroglobulin (669 kDa).

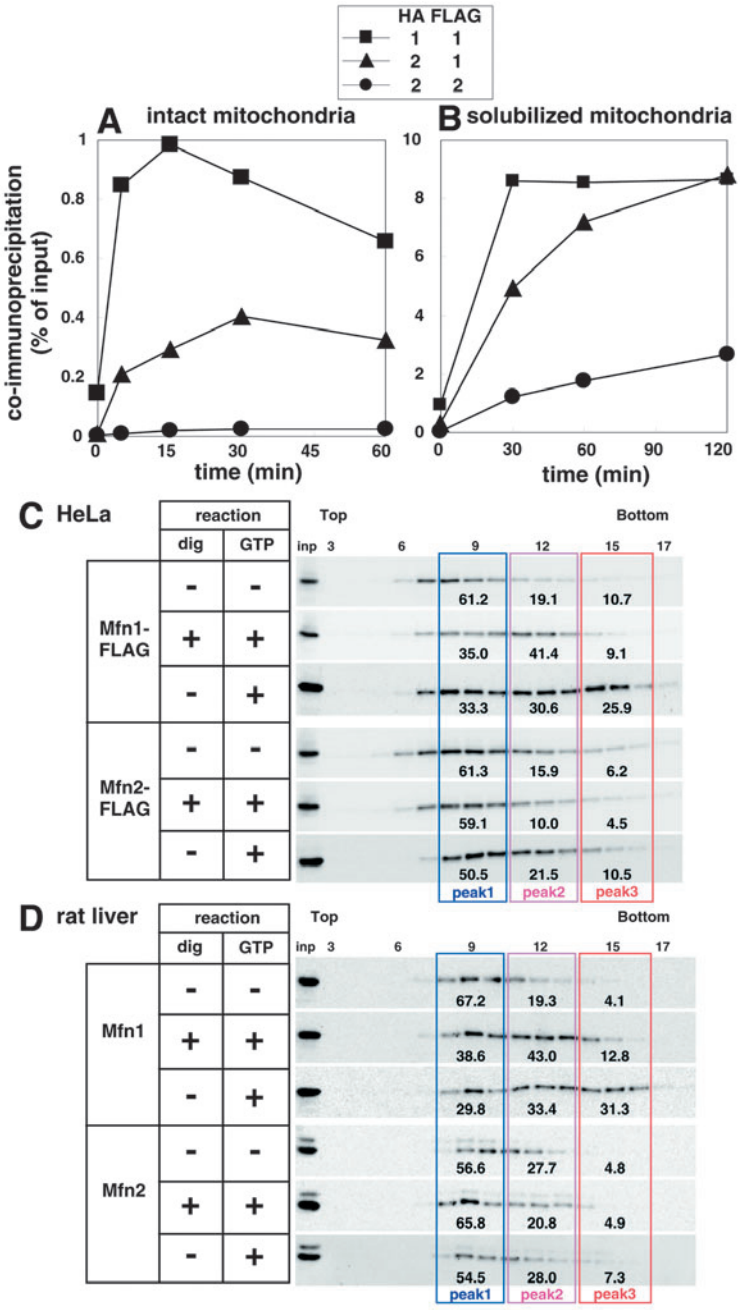

Fig. 5. Comparison of activity of Mfn1 and Mfn2 for GTP-dependent complex formation. (A,B) HA-tagged or FLAG-tagged Mfn1 or Mfn2 was separately expressed in HeLa cells, and the intact mitochondria (A) or digitonin-solubilized mitochondria (B) were mixed in the indicated combinations and incubated with 0.4 mM GTP at 30°C for the indicated times. The complex formation was analyzed by immunoprecipitation using anti-HA antibodies and subsequent immunoblotting by anti-FLAG or anti-HA antibodies as in Fig. 2. The immunoprecipitated bands were quantified. (C) The solubilized (+dig) or intact (–dig) mitochondria from the HeLa cells expressing Mfn1-FLAG or Mfn2-FLAG were mixed, and incubated with or without GTP. The incubation mixtures with intact mitochondria were solubilized by digitonin. All the reaction mixtures were then analyzed by sucrose density gradient centrifugation in the presence of digitonin as in Fig. 4. (D) The solubilized (+dig) or untreated (–dig) rat liver mitochondria were incubated with or without GTP. The reaction mixtures were analyzed by sucrose density gradient centrifugation in the presence of digitonin as in Fig. 4. Recovery (%) of Mfn proteins in the peak fractions is shown.

mitochondrial fusion and are assumed to be involved in distinct reaction steps (Chen et al., 2003; Eura et al., 2003). The difference in their function, however, remains obscure. We therefore examined differences of the two Mfn isoforms in the GTP-dependent complex formation reaction.

Mitochondria harboring HA-tagged Mfn1 or Mfn2 and those harboring FLAG-tagged Mfn1 or Mfn2 were mixed in different combinations, incubated in the presence of GTP, solubilized with detergent, and subjected to immunoprecipitation using anti-HA antibodies followed by immunoblotting with anti-FLAG antibodies (Fig. 5A). Formation of homotypic or heterotypic mitochondrial tethering complexes occurred in a GTP-dependent (not shown) and time-dependent manner, although with distinct differences in efficiency (Fig. 5A). Homotypic interaction of Mfn1-harboring mitochondria occurred with high efficiency compared with Mfn2-harboring mitochondria. A heterotypic interaction between Mfn1- and Mfn2-harboring mitochondria also occurred with moderate efficiency. Thus, Mfn1 and Mfn2 exhibited distinct activity in a GTP-dependent mitochondrial tethering reaction, suggesting that they are differentially involved in the mitochondrial fusion reaction. Similar results were obtained for the interaction of Mfn proteins in a detergent-solubilized system; homotypic interaction of Mfn1 proceeded at an approximately 20-fold higher rate than that of Mfn2 (Fig. 5B).

The mitochondrial tethering complex of Mfn1-FLAG sedimented mainly to fractions 14-16 (peak 3; Figs 4C and 5C), and the complex formed under detergent-solubilized, membrane-free conditions sedimented mainly to fractions 11-13 (peak 2; Fig. 4A,B and Fig. 5C). A significant change in the sedimentation pattern of Mfn2-FLAG, however, was not observed after incubation with GTP (Fig. 5C), consistent with the results of immunoprecipitation in which Mfn2 exhibited much lower complex-forming activity compared to Mfn1.

We then examined whether endogenous Mfn proteins behaved similarly to exogenously expressed proteins. Rat liver mitochondria were solubilized with digitonin, incubated with or without GTP, and subjected to sucrose density gradient centrifugation. The separated fractions were then analyzed by western blotting using antibodies specific for Mfn1 or Mfn2 (Eura et al., 2003). In the absence of GTP, endogenous Mfn1 was recovered mainly in fractions 8-10 (peak 1). Incubation with GTP changed the sedimentation pattern and a species sedimenting to fractions 11-13 (peak 2) was newly formed (Fig. 5D). GTP-induced production of peaks 2 and 3 was not obvious for Mfn2 (Fig. 5D). When rat liver mitochondria were incubated with GTP prior to solubilization, the Mfn1 complex sedimenting to fractions 14-16 (peak 3) was newly formed. These results were consistent with those obtained for the exogenously expressed and tagged Mfn proteins. We thus concluded that the results obtained using tagged Mfn proteins are physiologically relevant in that they reflected the nature of endogenous Mfn proteins.

Mfn1 and Mfn2 exhibit distinct GTPase activity

The results described above indicated that GTP hydrolysis was required for the Mfn1-dependent membrane tethering reaction. Because characterization of GTPase activity in Mfn proteins has not been reported, we compared their GTP-binding and hydrolysis activities.

FLAG-tagged Mfn1 or Mfn2 was expressed in HeLa cells and digitonin-solubilized cell lysates were incubated with GTP-agarose. Mfn2 bound to GTP-agarose depending on the time of incubation (Fig. 6A), whereas introduction of a point mutation in the GTP-binding domain severely impaired the binding ($Mfn2^{K109T}$; Fig. 6B), indicating that Mfn2 was correctly folded and specifically bound to GTP. On the other hand, only a small fraction of Mfn1 was recovered to GTP-agarose (Fig. 6A,B); nevertheless, its binding activity also depended on a functional GTP-binding domain. These results indicated that the two Mfn isoforms have distinct GTP-binding properties.

We then measured the GTP hydrolysis activity of the Mfn isoforms. His_6-tagged Mfn proteins were expressed in *E. coli* and purified from the detergent-solubilized membrane fractions using Ni-beads (Fig. 6C). The isolated proteins were incubated with [α-^{32}P]GTP, and the reaction products were analyzed by thin-layer chromatography using polyethyleneimine-cellulose. Time-dependent GTP-hydrolysis was observed for both Mfn proteins, although Mfn1 exhibited approximately eightfold higher activity than Mfn2 (Fig. 6D,E). Taken together, Mfn1 had higher GTPase activity than Mfn2, whereas Mfn2 exhibited higher affinity for GTP than Mfn1. These properties seemed to reflect the efficiency of the GTP-dependent membrane tethering reaction.

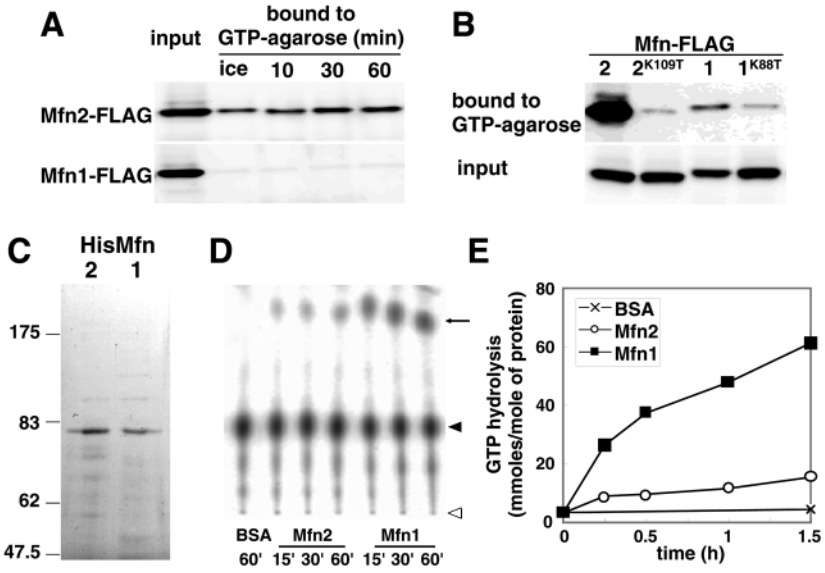

Fig. 6. GTP-binding and hydrolysis activity of two Mfn proteins. (A) FLAG-tagged Mfn2 or Mfn1 was expressed in HeLa cells and their PNS were solubilized by digitonin. The lysates were incubated with GTP-agarose beads on ice for 60 minutes, or at 30°C, for the indicated times. The bound proteins and PNS used (5%) were analyzed by immunoblotting using anti-FLAG antibodies. (B) FLAG-tagged Mfn2, $Mfn2^{K109T}$, Mfn1, or $Mfn1^{K88T}$ was incubated with GTP-agarose at 30°C for 1 hour and bound proteins were analyzed by immunoblotting using anti-FLAG antibodies. (C) His_6-tagged Mfn2 and Mfn1 proteins were expressed in *E. coli* and purified as described. The purified proteins were analyzed by SDS-PAGE followed by Coomassie Brilliant blue staining. (D,E) His-tagged Mfn2 or Mfn1 was incubated with [α-^{32}P]GTP at 37°C for the indicated times, and analyzed by thin layer chromatography followed by digital autoradiography as described, using bovine serum albumin as a control. The positions of GDP, GTP, and the origin are shown by an arrow, filled arrowhead, and open arrowhead, respectively. GTP and GDP radioactivity was quantified using an image analyzer (E).

Discussion

Mfn1 and Mfn2 are both essential for mitochondrial fusion reactions, because mitochondria depleted of either Mfn protein fail to fuse after the cell fusion reaction (Chen et al., 2003; Eura et al., 2003). Their functional division as well as the mechanisms of action in the membrane fusion reaction, however, remains unclear. Although the fusion reaction depends on the functional GTP-binding domain of Mfn proteins (Hales and Fuller, 1997; Hermann et al., 1998; Chen et al., 2003), there is no direct evidence for the functional involvement of GTPase activity in mitochondrial fusion.

In the present study, we demonstrated that purified recombinant Mfn1 exhibited ~eightfold higher GTPase activity than Mfn2, whereas Mfn2 exhibited a higher affinity for GTP. Reflecting these properties, both proteins exhibited distinct activity in the in vitro reconstituted mitochondrial docking reaction. To our knowledge, this is the first demonstration of a functional difference between the two Mfn proteins, and the difference is reflected in the GTP-dependent mitochondrial tethering reaction; binding of mitochondria through homotypic interaction of Mfn1 was more than 100-fold higher than that of Mfn2.

Exogenously expressed Mfn proteins are detected by electron microscopy in the contact region of connected mitochondria (Rojo et al., 2002; Santel et al., 2003; Eura et al., 2003) and the C-terminal cytoplasmic segment of Mfn1 forms a dimeric, antiparallel coiled-coil structure (Koshiba et al., 2004), suggesting that Mfn proteins function in connection with the mitochondrial outer membrane. Fluorescent microscopic observation of GFP- or RFP-labeled isolated mitochondria indicated that their binding in vitro was significantly increased in the presence of GTP when Mfn1 was expressed on both mitochondrial membranes (Fig. 1). Fused mitochondria with merged images of GFP and RFP were not detected in this condition, even when mitochondria harboring both Mfn proteins were used (data not shown). Mfn1- and GTP-dependent membrane binding was also observed using fragmented mitochondrial vesicles (Fig. 1D,E).

When Mfn1-HA-harboring mitochondria and Mfn1-FLAG-harboring mitochondria were incubated prior to membrane solubilization and analyzed by immunoprecipitation after digitonin solubilization, both constructs coprecipitated in a GTP hydrolysis-dependent manner. The immunoprecipitation efficiency decreased significantly when either or both mitochondria were replaced by Mfn2-harboring mitochondria.

These results indicated that mitochondrial membranes were tethered through direct interaction of Mfn proteins depending on GTP hydrolysis, and the homotypic Mfn1-Mfn1 interaction produced the most efficient binding. We concluded that hydrolysis of GTP by Mfn proteins regulates mitochondrial tethering through a trans oligomeric complex formation. Because Mfn proteins contain domains with a potential coiled-coil structure, the coiled-coil structure might be involved in this interaction, or conformational changes of the Mfn-containing complex might contribute to the interaction. The tethering reaction was severely compromised by washing the mitochondria with 150 mM NaCl, suggesting that some unidentified peripheral factors regulate GTP-dependent Mfn functions.

GTP-dependent mitochondrial tethering through Mfn proteins was demonstrated in vitro by co-immunoprecipitation, although the efficiency of the immunoprecipitation was low: ~1% for intact mitochondria and ~10% for digitonin-solubilized mitochondria (see Figs 2 and 3, respectively). Recovery of HA-tagged Mfn1 by anti-HA antibodies from digitonin-solubilized mitochondria was greater than 50%. It should be noted, however, that the in vitro assay mixture contained homotypic Mfn1-HA/Mfn1-HA and Mfn1-FLAG/Mfn1-FLAG complexes and heterotypic Mfn1-HA/Mfn1-FLAG complexes. As such, recovery of the heterotypic complexes by anti-HA antibodies should be decreased. In this context, when the heterotypic complex was concentrated by sucrose density gradient centrifugation, the complex was immunoprecipitated with an efficiency of ~30% (Fig. 4E). Therefore the efficiency of ~10% for the immunoprecipitation obtained in the present study is not unreasonably low. By contrast, recovery of the heterotypic trans complex on the membrane level by immunoprecipitation was much lower. This might be because the interaction of Mfn proteins in the membrane is hindered by the membrane structure, in addition to the reasons described above.

In the sucrose density gradient centrifugation, the GTP-primed mitochondrial tethering complex of Mfn1 was mainly sedimented to the ~450 kDa fractions (peak 3), although without incubation with GTP, Mfn1 sedimented to the ~180 kDa fractions (peak 1), which is similar to the size of endogenous Mfn proteins (Fig. 5D). These complexes were also detected by BN-PAGE. The ~450 kDa mitochondrial tethering complex, however, was not detectable for the Mfn2-harboring mitochondria. These results suggest that Mfn1 is involved in the initial membrane tethering process, whereas Mfn2 is not involved in this process or may negatively regulate this reaction.

There was an efficient interaction of Mfn proteins when they were incubated with GTP after solubilization with digitonin. As is the case for the membrane tethering reaction, a homotypic combination of Mfn1 exhibited higher activity than did Mfn2. This interaction proceeded even when using salt-washed mitochondria, suggesting that the membrane tethering process was bypassed by membrane solubilization and resulted in direct mixing of Mfn proteins. Incubation with GTP in the solubilized condition changed the Mfn1 complex from ~180 kDa to ~250 kDa, and this complex tended to disassemble into the ~180 kDa complex by further incubation with GTPγS. By contrast, the ~450 kDa mitochondrial docking complex was not dissociated in the presence of GTPγS. The ~450 kDa docking complex was not formed, however, when solubilized mitochondrial membranes were incubated with GTP, indicating that the docking complex is formed through interactions between distinct apposing mitochondrial membranes. Taken together, Mfn1 protein exhibited three distinct states; a steady-state complex of ~180 kDa, the ~450 kDa complex and the ~250 kDa complex. The trans-complex tethering apposing mitochondria and the complex formed in cis on the docking-compromised mitochondria were both contained in the ~450 kDa complex. The ~250 kDa complex detected when mitochondria harboring Mfn1-HA and Mfn1-FLAG were incubated with GTP prior to membrane solubilization mainly represented the ‘cis complex’ because Mfn1-FLAG present on the apposing membrane was not concentrated in peak 2 after immunoprecipitation with anti-HA antibodies (see Fig. 4E, middle panel). The ~250 kDa complex was also formed GTP-dependently after membrane mixing by detergent solubilization (see Fig. 4A,B and D). Therefore, we speculate that the ~250 kDa complex is an intermediate formed on the same side (cis side) of the mitochondrial membrane, or formed after mixing two adjacent membranes by the mitochondrial fusion reaction, which is destined to produce the ~180-kDa steady-state Mfn complex.

In the secretory and endocytic pathways, the vesicle and target membranes have different SNARE proteins, and their specific interaction across the two membranes activates the membrane fusion reaction (Chen and Scheller, 2001). By analogy with the SNARE-mediated membrane fusion reaction, the Mfn proteins form a trans-mitochondrial complex in the membrane tethering reaction, and after completion of mitochondrial fusion, the trans-Mfn complex disassembles on the fused membranes to return to the steady-state complex, possibly *via* the cis-intermediate complex. The C-terminal coiled-coil region has been shown to be involved in the mitochondrial tethering (Koshiba et al., 2004). Together with our present results, we are tempted to speculate that GTP hydrolysis should bring about structural changes in Mfn proteins, which facilitates interaction of their C-terminal coiled-coil regions in trans. In striking contrast to the SNARE-mediated fusion reaction, however, efficient mitochondrial tethering occurs through a homotypic Mfn1-Mfn1 interaction, but not a heterotypic Mfn1-Mfn2 interaction. These results are consistent with our previous results obtained with RNAi experiments, in which the Mfn1-deficient mitochondria failed to fuse with Mfn2-deficient mitochondria in cultured cells (Ishihara et al., 2003).

What is the function of Mfn2? Mfn2 exhibits only weak GTPase activity and GTP-dependent oligomerization activity. Nevertheless, it is essential for the mitochondrial fusion reaction in cultured cells (Chen et al., 2003; Eura et al., 2003). Furthermore, expression of the GTPase-domain mutant of Mfn2 ($Mfn2^{K109T}$) induces mitochondrial fragmentation and clustering in the junction between adjacent mitochondria (Eura et al., 2003), suggesting that the mutant arrests the mitochondrial fusion reaction at a certain stage, resulting in the accumulation of docked mitochondrial intermediates. Mitochondrial fusion should occur in multiple processes: mitochondrial movement in the cytoplasm, tethering of mitochondrial membranes, outer membrane fusion, docking and fusion of the inner membrane, and mixing of the matrix contents containing mitochondrial DNA. In this context, it was

demonstrated with yeast mitochondria that outer and inner membrane fusion events are separable and mechanically distinct (Meeusen et al., 2004). Nevertheless, Fzo1 plays essential and fundamental roles in the fusion of both outer and inner membranes. These results may support our conclusion that two Mfn proteins function in different steps in mammalian mitochondrial fusion reaction. One possibility is that Mfn2 affects the mitochondrial binding to couple the reaction with the following process in the fusion reaction. Another possibility is that Mfn1 functions mainly in mitochondrial tethering, whereas Mfn2 functions in the following steps, such as mixing of juxtaposed outer membranes or facilitating docking of the inner membranes. It was reported, in Mfn1-deficient cells, that mitochondria are fragmented and fail to bind to each other (Chen et al., 2003), supporting our conclusion that Mfn1 functions in mitochondrial tethering. By contrast, loss of Mfn2 leads to the formation of larger mitochondrial fragments. Similarly, our experiments with RNAi demonstrated that Mfn2 depletion in HeLa cells induced thick and fragmented mitochondrial tubules (Eura et al., 2003), suggesting that Mfn2 functions in a later process of the fusion reaction. In addition, our previous experiments demonstrated that Mfn2 depletion, but not Mfn1 depletion, compromises intermixing of mitochondria during the cell fusion reaction. These results suggest that, in addition to the fusion reaction, Mfn2 has other distinct functions.

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