Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria

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Changes in mitochondrial morphology that occur during cell cycle, differentiation, and death are tightly regulated by the balance between fusion and fission processes. Excessive fragmentation can be caused by inhibition of the fusion machinery and is a common consequence of dysfunction of the organelle. Here, we show a role for calcineurin-dependent translocation of the profission dynamin related protein 1 (Drp1) to mitochondria in dysfunction-induced fragmentation. When mitochondrial depolarization is associated with sustained cytosolic Ca^{2+} rise, it activates the cytosolic phosphatase calcineurin that normally interacts with Drp1. Calcineurin-dependent dephosphorylation of Drp1, and in particular of its conserved serine 637, regulates its translocation to mitochondria as substantiated by site directed mutagenesis. Thus, fragmentation of depolarized mitochondria depends on a loop involving sustained Ca^{2+} rise, activation of calcineurin, and dephosphorylation of Drp1 and its translocation to the organelle.

fission | phosphatase | subcellular localization | calcium | cyclosporine A

Mitochondria are complex organelles that can exist in a network, shaped by continuous fusion and fission (1). This equilibrium is tightly regulated to ensure proper mitochondrial content in daughter cells and is impaired during apoptosis, when mitochondria fragment and cluster in the perinuclear region (2). Apoptotic fission is associated with remodeling of the cristae, characterized by opening of their tubular junction. This causes the complete release of proapoptotic factors such as cytochrome *c*, required in the cytosol for the activation of downstream effector caspases (3).

Several proteins, including evolutionarily conserved, dynamin-related GTPases, regulate mitochondrial fusion and fission. Fusion relies on the inner membrane protein optic atrophy 1 (Opa1) and on the outer membrane proteins mitofusin (Mfn) 1 and 2. Fission requires the additional step of translocation of dynamin-related protein 1 (Drp1) from the cytosol, where it associates with microtubules, to mitochondria where it presumably docks on hFis1, its adaptor in the outer membrane. Oligomerization of Drp1 is believed to provide the mechanical force to constrict mitochondrial membranes and to fragment the organelle (1, 4).

Current understanding of the regulation of mitochondrial fusion and fission is limited. Fusion of the inner membrane requires the electrical component of the proton electrochemical gradient, sustained by proton pumping at complexes of the respiratory chain. Dissipation of the electrochemical gradient results in blockage of fusion *in vitro* and *in situ* (1). The small GTPase Rab32 functions as a protein kinase A anchoring protein (AKAP) on mitochondria and coordinates mitochondrial fission, suggesting a role for cAMP and protein phosphorylation (5). The inner membrane rhomboid protease Parl, which participates in the production of the soluble intermembrane space form of Opa1 that regulates apoptosis (6, 7), is specifically phosphorylated on its vertebrate-specific Pβ domain to block its self-cleavage and mitochondrial fragmentation (8). Additionally, cyclin dependent kinase (Cdk) 5 has been reported to promote mitochondrial fragmentation in neurons, albeit the molecular nature of the mediator of its effects on mitochondrial shape is unknown (9). A step forward in our understanding of the integration of mitochondrial shape changes by cellular cues came with the discovery that Drp1-dependent mitochondrial fragmentation is controlled by phosphorylation at two different conserved sites, serine 616 and 637, by Cdk1 and protein kinase A (PKA) (10–12). The two sites seem to have opposing effects on mitochondrial shape, because phosphorylation on S616 by Cdk1 promotes mitochondrial fission during mitosis, while dephosphorylation of S637 by the Ca^{2+}-dependent phosphatase calcineurin promotes mitochondrial fission and is involved in the propagation of apoptosis (13). Interestingly, phosphorylation of these two residues does not affect the GTPase activity of Drp1, or it has marginal effects on it, raising the question of the link between phosphorylation and Drp1-dependent changes in mitochondrial shape. Finally, sumoylation stabilizes the mitochondrial pool of Drp1 and enhances fission, further substantiating the importance of posttranslational modification in regulating morphology of the organelle (14).

Mitochondrial fragmentation is a common feature of several pathological conditions where they are dysfunctional. Inhibition of the fusion machinery has been suggested as the underlying cause of these morphological changes (15, 16). However, it is unclear whether inhibition of mitochondrial fusion is the only mechanism that leads to fragmentation of the dysfunctional organelle or if it is accompanied by a controlled increase in fission. Along this line, the relative role of Drp1 in this process and how it is recruited on dysfunctional mitochondria are completely unknown.

To address these questions, we investigated the relationship between mitochondrial function and shape. We show that inducers of mitochondrial depolarization trigger Ca^{2+}, calcineurin-dependent dephosphorylation of Drp1, driving its translocation to mitochondria and fission of the organelle.

Results

Depolarization Causes Drp1-Dependent Mitochondrial Fragmentation. We addressed the relationship between mitochondrial depolarization and shape in HeLa cells. We induced depolar-

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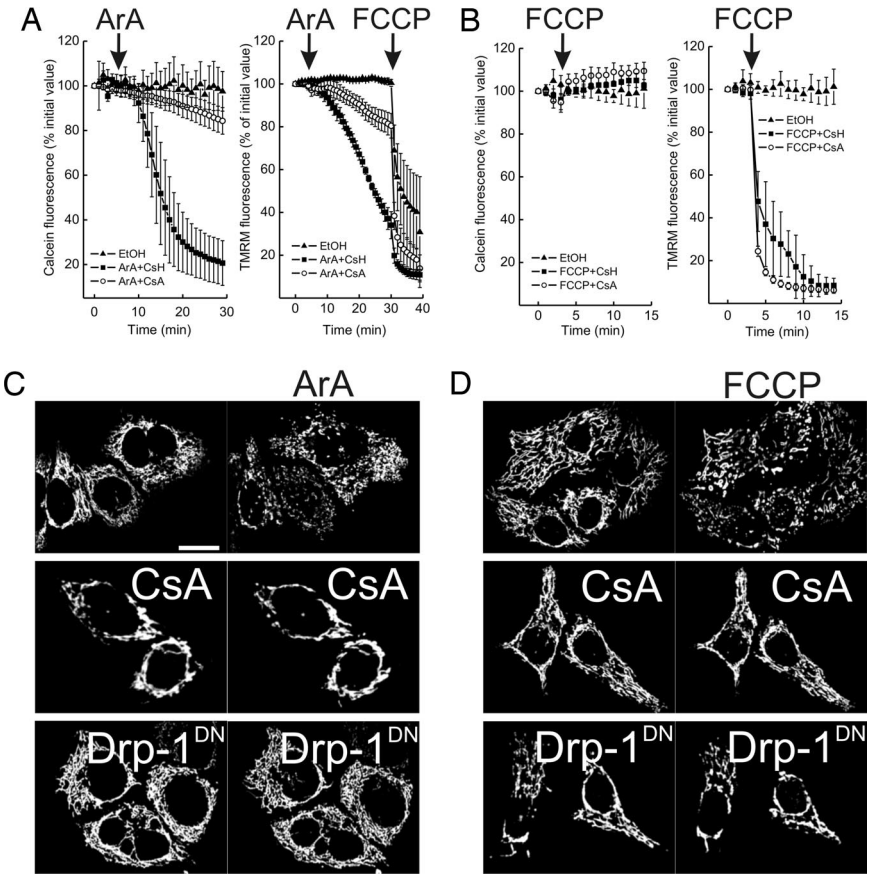

Fig. 1. Mitochondrial depolarization causes Drp1-dependent fission. (*A* and *B*) Changes in mitochondrial fluorescence of TMRM or calcein. HeLa cells loaded with calcein/Co^{2+} or with TMRM were treated where indicated (arrows) with 20 μM ArA (*A*) or 2 μM FCCP (*B*). Fluorescence intensities were normalized to the initial value for comparative reasons. Data represent mean ± SE of four independent experiments. Where indicated, cells were pretreated with CsA or with the inactive analogue CsH (18). (*C* and *D*) Representative confocal images of mtRFP fluorescence in HeLa cells before (left) or 30 min after (right) treatment with 20 μM ArA (*C*) or 2 μM FCCP (*D*). Where indicated, cells were pretreated with CsA or cotransfected with K38A Drp1 ($Drp1^{DN}$) (Scale bar, 20 μm).

ization by two means: (*i*) triggering the permeability transition (PT), which depends on the opening of an inner membrane channel permeant to solutes with a molecular mass <1500 Da (17); (*ii*) treating cells with a protonophore that dissipates the electrochemical gradient. The calcein staining/Co^{2+}-quenching technique allows direct imaging of PT *in situ*, while changes in mitochondrial fluorescence of tetramethyl rhodamine methylester (TMRM) permit to measure mitochondrial membrane potential (18). The lipid second messenger arachidonic acid (ArA) induced the expected decrease in mitochondrial fluorescence of both calcein and TMRM (Fig. 1*A*), documenting the occurrence of PT accompanied by long-lasting mitochondrial depolarization (18). Both events were prevented by Cyclosporine A (CsA), a calcineurin inhibitor that also desensitizes the PT pore via its mitochondrial receptor cyclophilin D (17) (Fig. 1*A*). Conversely, the protonophore carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone (FCCP) induced complete mitochondrial depolarization not accompanied by PT, as confirmed by the lack of calcein efflux from mitochondria and by the insensitivity to CsA of the drop in mitochondrial fluorescence of TMRM (Fig. 1*B*).

We next addressed the relationship between mitochondrial depolarization and shape. Real time confocal imaging of a mitochondrially targeted dsRed fluorescent protein (mtRFP) showed that ArA and FCCP caused complete fragmentation of the mitochondrial network within 15 min. In the case of FCCP this was preceded by the appearance of doughnut-like mitochondrial rings (Fig. 1 *C* and *D*). Of note, fragmentation relied on active Drp1 since it was completely blocked by expression of a Lys 38 to Ala mutant of Drp1 that acts as dominant negative ($Drp1^{DN}$, Fig. 1 *C* and *D*) (19). Thus, fragmentation induced by mitochondrial depolarization requires Drp1.

To address whether fragmentation was a consequence of depolarization *per se* or whether it required additional mitochondrial changes, such as PT, we decided to measure the effect of the PT inhibitor CsA on fragmentation by ArA and FCCP. As expected, CsA prevented fragmentation by the PTP inducer ArA (Fig. 1*C*), but unexpectedly also by FCCP (Fig. 1*D*), which does not induce PT and whose effects on mitochondrial membrane potential are insensitive to CsA (Fig. 1*B*).

Mitochondrial Fragmentation by Depolarization Depends on the Ca^{2+}-Dependent Phosphatase Calcineurin. These results suggested a role for CsA-sensitive pathways other than the PT in Drp1-dependent fragmentation of mitochondria induced by depolarization of the organelle and directed our attention to the Ca^{2+}-dependent cytosolic phosphatase calcineurin, inhibited by the CsA-cyclophilin A complex (20). Calcineurin is a heterodimeric protein composed of a catalytic subunit (CnA) that binds calmodulin and a regulatory subunit (CnB) that binds Ca^{2+}. It is involved in the transduction of a variety of Ca^{2+}-mediated signals, ranging from proliferation to death, to secretion of vesicles (20). We therefore reasoned that mitochondrial depolarization induced by ArA and FCCP could be associated with changes in cytosolic Ca^{2+} levels and therefore with the activation of calcineurin. The ratiometric dye Fura-2 showed a rapid increase in cytosolic Ca^{2+} after treatment with ArA or FCCP, followed by a sustained plateau (Fig. 2*A*). Emptying of ER Ca^{2+} stores completely abolished FCCP-induced Ca^{2+} transients and markedly inhibited those elicited by ArA (data not shown). Moreover, we found that TG-releasable Ca^{2+} peak was significantly reduced from 58 ± 4 to 27 ± 8 nM or to 39 ± 6 nM in cells treated with ArA or FCCP, respectively. Thus, mitochondrial depolarization by ArA and FCCP is associated with ER Ca^{2+} release. Together with the likely inhibition of the ATP dependent efflux pathways of the plasma membrane, this release

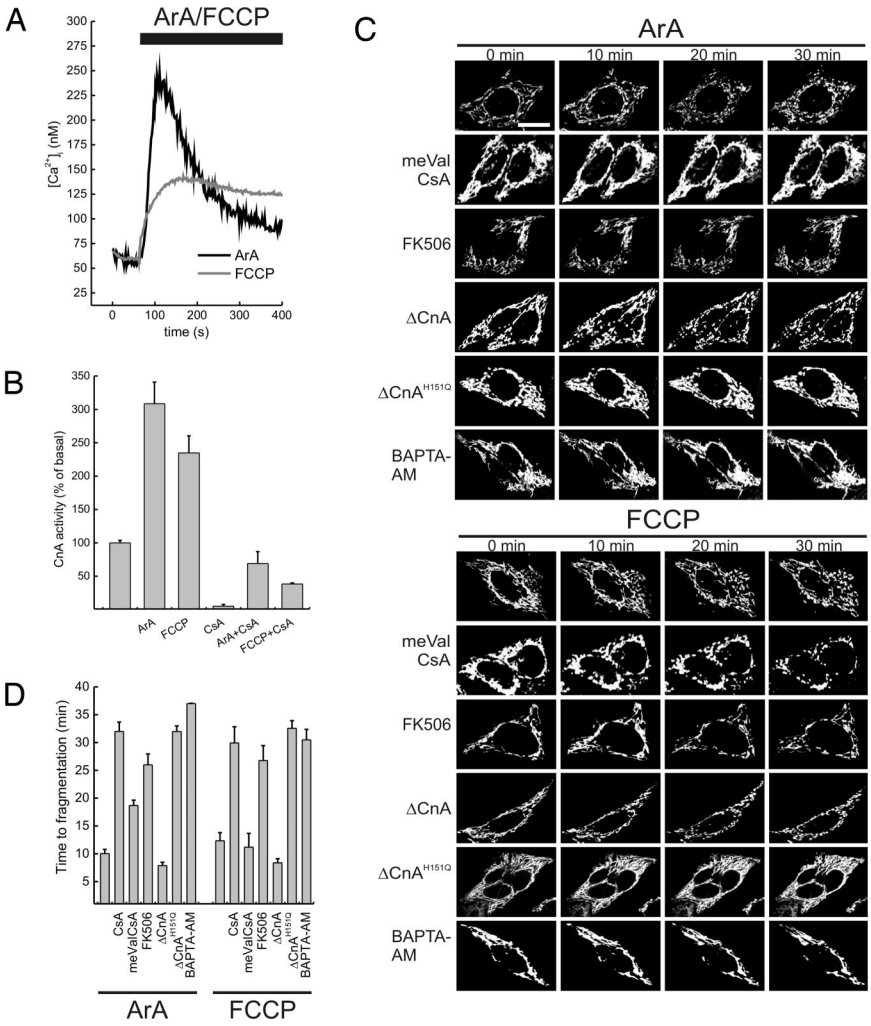

Fig. 2. Inhibition of calcineurin blocks mitochondrial fission by ArA and FCCP. (*A*) Representative traces of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) as measured by Fura-2 in HeLa cells treated where indicated with 20 μM ArA or 2 μM FCCP. (*B*) Calcineurin activity measured in cytosolic extracts of HeLa cells treated where indicated for 15 min with 20 μM ArA or 2 μM FCCP. Where indicated, cells were pretreated with 2 μM CsA for 30 min. Data are compared with the activity of untreated cells set as 100% and represent mean ± SE of four independent experiments. (*C*) Representative frames acquired at indicated times from real time confocal imaging of HeLa cells transfected with mtRFP. At t = 3 min, cells were treated as indicated with 20 μM ArA or 2 μM FCCP. Where indicated, cells were pretreated for 30 min with 2 μM mMeValCsA, 0.6 μM FK506 or 40 μM BAPTA-AM, or cotransfected with CnB plus ΔCnA or ΔCnA^{H151Q}. Bar, 20 μm. (*D*) Quantitative analysis of mitochondrial shape changes. Experiments were performed exactly as in (*C*). Morphometry was performed as described. Data represent mean ± SE of 8 different experiments. In each experiment, 20 cells were scored. In the case of BAPTA-AM, no fragmentation could be detected in the timeframe of the experiment.

results in a sustained cytosolic Ca^{2+} rise that is required for the activation of calcineurin (21). Accordingly, we recorded a 2- to 3-fold increase in calcineurin activity, which was, as expected, inhibited by CsA (Fig. 2*D*). This suggests that the effects of CsA on mitochondrial fragmentation could be related to the inhibition of calcineurin. To investigate the relative role of calcineurin in mitochondrial fragmentation, we turned to specific inhibitors of PT or calcineurin: the CsA analogue N-MethylVal-4-CsA (MeValCsA), a non-immunosuppressive analogue of CsA that binds to cyclophilins but not to calcineurin (22) and FK506, an immunosuppressive agent that blocks calcineurin via its receptor FKBP12 and has no effect on the PTP (23). FK506 greatly delayed fragmentation induced by both ArA and FCCP, while MeValCsA prevented fission only when caused by ArA (Fig. 2 *A–C*). Accordingly, MeValCsA but not FK506 inhibited depolarization by ArA [supporting information (SI) Fig. S1]. We next turned to a genetic approach to test the relationship between calcineurin and mitochondrial fission by ArA/FCCP. Expression of a constitutively active mutant of calcineurin lacking the CaM binding domain due to the introduction of a stop codon at position 392 (ΔCnA) increased fragmentation *per se*. It also accelerated fragmentation induced by both ArA and FCCP (Fig. 2 *E* and *F*). Conversely, a dominant negative mutant of calcineurin devoid of the CaM binding and the autoinhibitory domains and harboring an inactivating His-151 to Gln point mutation (ΔCnA^{H151Q}) (24) efficiently blocked depolarization-induced fragmentation (Fig. 2 *E* and *F*). Since calcineurin is activated in a Ca^{2+}-calmodulin dependent fashion, we explored whether fission by FCCP and ArA was dependent on Ca^{2+} by incubating cells with the membrane-permeant acetoxymethyl-ester of the Ca^{2+} chelating agent 1,2-bis (*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA). Chelation of intracellular Ca^{2+} completely blocked cytosolic Ca^{2+} rise (data not shown) and fission by ArA and FCCP (Fig. 2 *E* and *F*). In conclusion, these experiments show that mitochondrial depolarization is accompanied by the Ca^{2+}-dependent activation of calcineurin; and that inhibition of this phosphatase or chelation of intracellular Ca^{2+} blocks Drp1-dependent fission of the organelle.

Drp1 Is Dephosphorylated by Calcineurin in Response to Inducers of Mitochondrial Depolarization. How does calcineurin regulate Drp1-dependent mitochondrial fission? We reasoned that Drp1, similarly to dynamin I (25), could physically interact with calcineurin. Immunoprecipitation under mild detergent conditions in Ca^{2+}-free buffers showed that CnA indeed associates with Drp1 (Fig. 3*A*). Of note, a significant fraction of cyclophilin A was also immunoprecipitated by Drp1, suggesting that Drp1 could serve as a scaffold for both calcineurin and cyclophilin A. This possibility was confirmed when we found that CnA directly immunoprecipitated Drp1, but not cyclophilin A, as expected (Fig. 3*A*). The interaction between Drp1 and CnA was stimulated in cells treated with ArA (Fig. 3*B*) and FCCP (data not shown) and inhibited by the calcineurin inhibitors CsA and

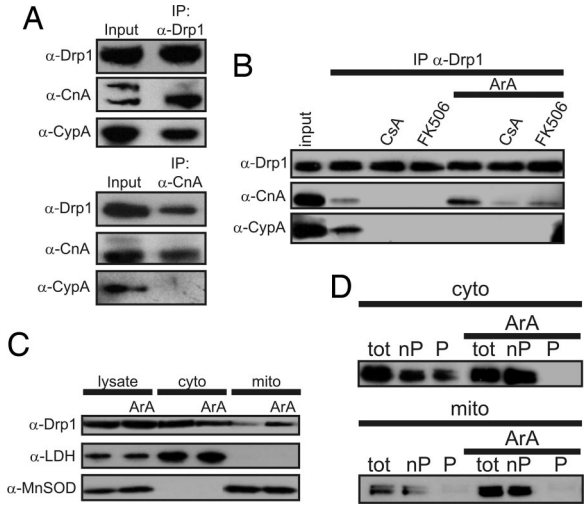

Fig. 3. Dephosphorylated Drp1 accumulates on mitochondria. (*A*) Equal amounts of HeLa cell proteins (100 μg) dissolved in CPBS were immunoprecipitated with the indicated antibodies and proteins in the lysates (Input, 1:5 dilution) and coprecipitated proteins were separated by SDS/PAGE and immunoblotted with the indicated antibodies. (*B*) Lysates from HeLa cells treated where indicated with 20 μM ArA or 2 μM FCCP for 15 min were prepared and equal amounts of protein (100 μg) dissolved in CPBS buffer were immunoprecipitated with an anti-Drp1 antibody. Coprecipitated proteins were analyzed by SDS/PAGE and immunoblotting with the indicated antibodies. Where indicated, cells were pretreated for 30 min with 2 μM mMeValCsA or 0.6 μM FK506. Input represents a 1:5 dilution of the total lysates. (*C*) Subcellular fractions of HeLa cells treated where indicated with 20 μM ArA for 15 min were prepared and equal amounts (40 μg) of protein from total, cytosolic and mitochondrial fractions were separated by SDS/PAGE and immunoblotted using the indicated antibodies. LDH, lactate dehydrogenase; MnSOD, Mn-dependent superoxide dismutase. (*D*) Experiments were preformed as in (*C*), except that cytosolic and mitochondrial fractions were loaded on a column binding phosphorylated proteins to separate phosphorylated and unphosphorylated proteins. Thirty μg of total (tot), phosphorylated (P) and unphosphorylated (nP) proteins from each fraction were separated by SDS/PAGE and immunoblotted using an anti-Drp1 antibody.

FK506 (Fig. 3*B*). This suggests that the interaction between calcineurin and Drp1 depends on the levels of Ca^{2+}, is stimulated by mitochondrial dysfunction, and is sensitive to the inhibition of calcineurin, similar to what was observed in the case of the dynamin I-calcineurin complex (25).

Previous reports showed that Drp1 can be reversibly phosphorylated, but whether this is associated with sustained changes in its GTPase activity is unclear (10, 13). A rate-limiting step in Drp1-mediated fission is its translocation to mitochondria, which could be in principle modulated by calcineurin. To verify this hypothesis, we measured by subcellular fractionation the amount of Drp1 associated with mitochondria. ArA (Fig. 3*C*) or FCCP (data not shown) induced an increase in mitochondrial levels of Drp1. Real time imaging experiments of Drp1-YFP translocation further confirmed that both FCCP (Fig. S2) and ArA (data not shown) stimulated the association of Drp1 with mitochondria before the occurrence of organellar fragmentation. We next evaluated whether ArA induced dephosphorylation of Drp1 and translocation of the dephosphorylated form to mitochondria. Immunoblotting of total lysates with an anti phospho-Serine 637 antibody indicated that after ArA treatment this residue becomes dephosphorylated (Fig. S3). We extended our analysis to the total phosphorylation status of the protein. A specific resin that selectively binds phosphorylated proteins allows quantification of the relative amount of dephosphorylated vs. phosphorylated Drp1 in subcellular fractions. We could identify discrete amounts of phosphorylated Drp1 in the cytosol of unstimulated cells, but no Drp1 bound to resin was retrieved in the cytosol of cells treated with ArA. Accordingly, mitochondrial Drp1 resulted, completely dephosphorylated, and its levels increased when organelles were isolated from cells treated with ArA (Fig. 3*D*). Immunofluorescence showed that a significant fraction of Drp1-YFP remained associated with microtubules (MT) after addition of ArA to cells treated with FK506 (Fig. S4). Moreover, real-time imaging confirmed that translocation of Drp1-YFP to mitochondria in response to ArA and FCCP is blocked by FK506 (data not shown). In conclusion, mitochondrial dysfunction is associated with dephosphorylation of Drp1 and the dephosphorylated form of Drp1 is retrieved in mitochondria. Translocation is prevented by blockage of calcineurin, suggesting that dephosphorylation is a key factor in controlling subcellular distribution of Drp1.

Subcellular Distribution of Drp1 Is Controlled by Phosphorylation at Ser-637. To verify the role of phosphorylation in subcellular distribution of Drp1 we generated mutants of a Drp1-YFP chimera mimicking constitutive phosphorylation (S→D) or dephosphorylation (S→A) of the two recently identified Drp1 phosphorylation sites, S616 and S637. While $Drp1^{S616}$-YFP mutants behaved in an almost superimposable manner to that of wt Drp1-YFP, mutagenesis of S637 had a major impact on subcellular localization and mitochondrial shape: $Drp1^{S637D}$-YFP was almost completely cytosolic, while the $Drp1^{S637A}$-YFP localized on mitochondria (Fig. 4 *A* and *B*). Accordingly, the $Drp1^{S616}$-YFP mutants displayed only marginal effects on steady-state mitochondrial morphology. We next investigated the relative role of each site on subcellular localization of Drp1 and on mitochondrial morphology by expressing Drp1-YFP chimeras mutated at both sites. This analysis confirmed that the phosphorylation status of S637 is dominant over that of S616: the double S616A, S637D mutant is mainly cytosolic and does not cause extensive mitochondrial fragmentation, while the double S616D, S637A mutant behaves exactly the opposite (Fig. 4 *A* and *B*). The effect on mitochondrial morphology of these mutants could reflect a differential ability to assemble into active oligomers. We therefore tested whether they retained normal oligomerization potential by chemical cross-linking and found no differences among the mutants described and with wt Drp1 (data not shown). Thus, the observed mitochondrial fragmentation seems to reflect the differential ability of the mutants to associate with mitochondria rather than their activity (10, 13) or their ability to oligomerize. We next measured translocation of the Drp1-YFP mutants to mitochondria induced by depolarization of the organelle. Real time confocal imaging experiments showed that while $Drp1^{S637A}$-YFP was already mostly mitochondrial, $Drp1^{S637D}$-YFP was retained in the cytoplasm, even when most of the wt and S616 mutants of Drp1 had translocated to mitochondria (Fig. S2). Accordingly, when we quantified the effect of overexpressed Ser mutants of Drp1 on FCCP and ArA induced mitochondrial fission, we found that the S637D mutant delayed fission in response to both, while S616D displayed no effect as compared with cells expressing wt Drp1. Accordingly, mitochondrial fragmentation was delayed in cells expressing the double S616A, S637D mutant (Fig. 4 *C* and *D*). It should be noted that residual fragmentation was observed also in cells expressing the S637D mutant as a consequence of the endogenous levels of Drp1. In conclusion, phosphorylation of residue S637 regulates translocation of Drp1 to mitochondria and therefore its ability to fragment the organelle.

Discussion

We have shown that two different inducers of mitochondrial depolarization cause Drp1-dependent fragmentation of the organelle. The cytosolic phosphatase calcineurin mediates a crucial step in this process, as shown by multiple lines of evidence.

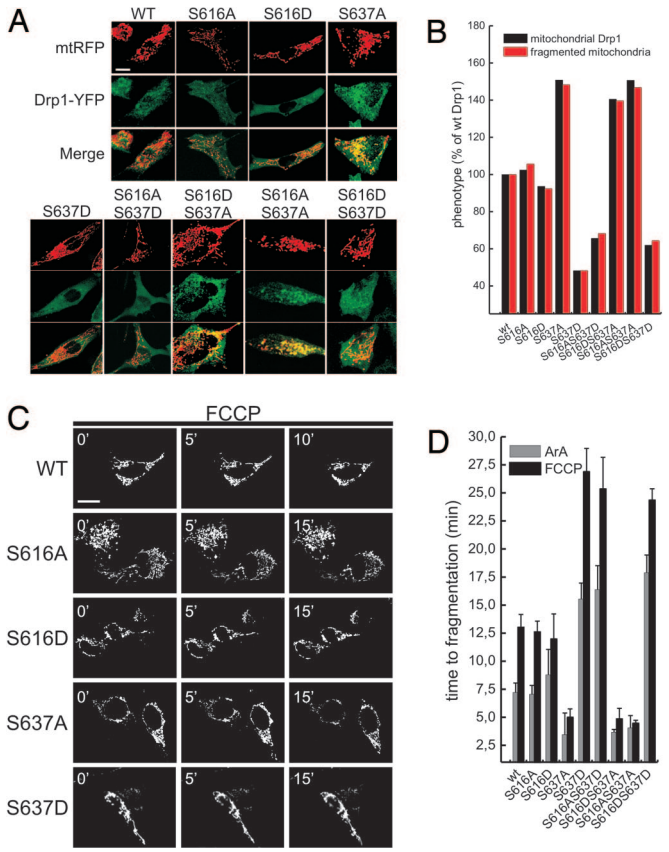

Fig. 4. Translocation of Drp1 to mitochondria is controlled by Serine 637. (*A*) Representative confocal images of HeLa cells cotransfected with mtRFP and the indicated Drp1-YFP mutants. Bar, 20 μm. (*B*) Analysis of subcellular localization of Drp1-YFP. Experiments were exactly as in (*A*). Classification of subcellular distribution of Drp1-YFP and mitochondrial morphometric analysis was performed as described in *SI Experimental Procedures*. Sixty to eighty cells were scored in each condition. (*C*) Representative frames acquired at indicated times from real time confocal imaging of HeLa cells cotransfected with mtRFP and the indicated mutants of Drp1-YFP. At t = 3 min, cells were treated with 2 μM FCCP. (*D*) Quantitative analysis of mitochondrial shape changes. Experiments were exactly as in C, except that after 3 min cells were treated with 20 μM ArA or 2 μM FCCP as indicated. Morphometric analysis was performed as described. Data represent mean ± SE of four different experiments. In each experiment, 10 cells were scored.

First, mitochondrial depolarization is associated with a sustained rise in intracellular Ca^{2+} levels, which in turn activate calcineurin. Second, fragmentation is sensitive to genetic and pharmacological inhibition of calcineurin. Third, Drp1 is retrieved in a complex with calcineurin and cyclophilin A. Fourth, depolarization results in calcineurin-dependent dephosphorylation of Drp1 and association of the dephosphorylated form with mitochondria. Fifth, site-specific mutagenesis shows that dephosphorylation of S637 regulates the translocation of Drp1 to mitochondria.

The overall shape of the mitochondrial network likely results from the integration of organellar and cytosolic cues. This is essential to integrate the remodeling of the reticulum during different phases of the cell cycle and to control the transport of the organelle (2). However, changes in mitochondrial bioenergetic parameters impinge on morphology of the organelle (3). Here, we link mitochondrial dysfunction to the recruitment of Drp1 on the organelle, mediated by its Ca^{2+}/calcineurin dependent dephosphorylation. The rise in cytoplasmic Ca^{2+} occurs independently of whether the depolarizing stimulus triggers PT. Thus, the determining factor is probably the severe depletion of ATP aggravated when the ATPase works in reverse as an attempt to maintain membrane potential of dysfunctional mitochondria (26). ATP depletion impairs plasma membrane and intracellular Ca^{2+} extrusion mechanisms to sustain the cytoplasmic Ca^{2+} rise. The ATPase inhibitor oligomycin, which does not induce severe ATP depletion or mitochondrial depolarization, does not increase cytoplasmic Ca^{2+} (data not shown). The reported effect of oligomycin on mitochondrial shape is therefore the likely consequence of Opa1 degradation (27): accordingly, overexpressed Opa1 inhibits it (data not shown). Mitochondrial fragmentation observed in several mitochondrial diseases can therefore result from inhibition of Opa1-dependent fusion (15), or from calcineurin/Drp1-dependent fission, when ATP levels drop to sustain the cytosolic Ca^{2+} plateau. For example, in NARP the ATPase is mutated and cannot hydrolyze ATP, while in MERRF the mutation affects the respiratory chain and the ATPase can work as an ATP consumer, trying to maintain membrane potential. Accordingly, dysregulation of Ca^{2+} signaling occurs only in MERRF (28) and MERRF mitochondria appear fragmented (15).

Our results suggest that calcineurin-dependent dephosphorylation of Drp1 primarily controls its association with mitochondria. Regulation of intracellular localization by reversible phosphorylation is a common theme in cell biology and calcineurin has been reported to be a key player in these processes, controlling translocation of NFaT to the nucleus and of the proapoptotic Bcl-2 family member Bad to mitochondria. During exocytosis, calcineurin couples the rise in presynaptic Ca^{2+} levels to translocation of dynamin I on nascent exocytotic vesicles (29, 30).

What is the relative role of S616 and S637 in controlling subcellular localization and function of Drp1? At least in cells in interphase (likely the main population in unsynchronized cultured cells) S637D dominates over S616A. Intriguingly, we found that the S616A mutant, mimicking dephosphorylation, promotes mitochondrial fission rather than fusion. It appears that the profusion effect of this mutation observed by Mihara and colleagues could be temporally restricted to mitosis (12), perhaps by other yet unknown posttranscriptional modifications on Drp1.

GTPase activity of recombinant Drp1 is greatly inhibited by PKA, yet the activity of Drp1 carrying the phosphomimetic S637D mutation is only slightly reduced (10). Thus, additional mechanisms should support the dramatic effect in mitochondrial morphology observed in cells expressing the S637 mutants. We show that Drp1 with phosphomimetic mutations on S637 is retained in the cytosol, providing an explanation for the reported effects on shape. Furthermore, the opposing effects of PKA and calcineurin on the same site likely serve as a switch to translate signals associated with Ca^{2+} changes of different strength, duration, and tone into different mitochondrial morphologies. During physiological (i.e., agonist evoked) Ca^{2+} signaling, activation of PKA can prevail over calcineurin mediated dephosphorylation of Drp1. Moreover, compartmentalization of both Ca^{2+} and cAMP signals could play a role in the local regulation of mitochondrial shape. Conversely, long lasting Ca^{2+} plateaus in the cytosol linked to full activation of calcineurin and to generalized fragmentation could for example account for the apoptotic mitochondrial fission.

Drp1 also participates in fission of endoplasmic reticulum and of peroxisomes (31), implying regulatory events that determine organelle selectivity. This could be achieved by posttranslational modifications orchestrated in the cytosol. Drp1 could be targeted to the organelle of interest by different spatiotemporal patterns of Ca^{2+} rises that are coupled to local calcineurin activation, an appealing possibility that deserves future investigation.

Materials and Methods

Imaging. Imaging of PTP opening and mitochondrial membrane potential in HeLa cells was performed as previously described (18). Data are graphed as percentage of the initial value for comparative reasons.

For confocal imaging, 10^5 cells seeded onto 24 mm-round glass coverslips were transfected as indicated and after 24 h were incubated in HBSS supplemented with 10 mM Hepes and placed on the stage of a Nikon Eclipse TE300 inverted microscope equipped with a spinning-disk PerkinElmer Ultraview LCI confocal system, a piezoelectric *z*-axis motorized stage (Pifoc, Physik Instrumente), and a Orca ER 12-bit CCD camera (Hamamatsu Photonics). Cells expressing mtRFP and/or Drp-1-YFP were excited using the 568-nm or the 440-nm line of the He-Ne laser (Perkin–Elmer) respectively with exposure times of 100 msec using a 60 $\times$ 1.4 NA Plan Apo objective (Nikon). Cells immunostained with TRITC- conjugated anti-tubulin antibody were excited with the 568-nm laser line.

For immunofluorescence, cells grown on 13-mm coverslips were fixed in 3.7% paraformaldehyde in PBS (20 min, 4 °C), permeabilized with 0.01% Nonidet-P40 (Gibco) and incubated with anti-tubulin (Santa Cruz Biotechnology) antibody (1:200). Cells were then stained with an isotype matched TRITC secondary antibody (Amersham, 1:200).

In time-course experiments, images were acquired every 10 sec for 40 min. Quantitative analysis of mitochondrial shape changes was performed by evaluating the time at which cells displayed fragmented mitochondria after addition of the inducer. Organelles were classified as fragmented when $>$50% of the total cellular mitochondria displayed a major axis <5 μm.

Localization of Drp1-YFP wt and variants was performed by classifying Drp1 as mitochondrial if $>$50% of YFP fluorescence colocalized with mtRFP fluorescence.

Ca^{2+} Measurements. Cytosolic Ca^{2+} measurements were performed exactly as previously described (32).

Calcineurin Activity Assay. Cells grown on 24-mm wells incubated in HBSS buffer were treated as indicated, washed several times with TBS buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl), resuspended in TBS, and lysed in CTBS buffer (6 mM CHAPS in TBS, pH 7.4). calcineurin activity was determined using an *in vitro* assay kit and following manufacturer instructions (Calbiochem). For comparative reasons, basal calcineurin activity in untreated cells was set to 100%.

Subcellular Fractionation, Immunoprecipitation, and Immunoblotting. Subcellular fractionation was performed as described in ref. 33.

For immunoprecipitation experiments, cells were lysed in CPBS buffer (6 mM CHAPS in PBS, pH 7.4). Lysates were incubated with the indicated antibody (1:50, 14 h, 4 °C) and the protein-antibody complex was precipitated by centrifugation after incubation with protein G-coated magnetic beads (Dynal, 2 h, 4 °C). The immunoprecipitated material was washed twice in CPBS and resuspended in SDS/PAGE loading buffer (NuPAGE), boiled, and loaded on 4–12% gels (NuPAGE).

For phosphorylation studies, cytosolic and mitochondrial fractions were loaded on a phosphoprotein binding column (Qiagen). Flow-through (unphosphorylated) and eluted (phosphorylated proteins) proteins were collected and concentrated and 30 μg of proteins were separated by 4–12% SDS/PAGE.

For immunoblotting, proteins were transferred onto polyvinylidene fluoride (Millipore) membranes and probed with the following antibodies: α-Drp1 (BD, 1:2000); α-calcineurin (BD, 1:1000), α-cyclophilin A (Upstate, 1:2000), α-LDH (Rockland, 1:2000), α-MnSOD (Stressgen, 1:3000), α-phosphoSer637Drp1 (10) (1:750). Isotype-matched, horseradish peroxidase-conjugated secondary abodies (Amersham) were used, followed by detection by chemiluminescence (Amersham).

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