Vimentin intermediate filaments modulate the motility of mitochondria

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ABSTRACT Interactions with vimentin intermediate filaments (VimIFs) affect the motility, distribution, and anchorage of mitochondria. In cells lacking VimIFs or in which VimIF organization is disrupted, the motility of mitochondria is increased relative to control cells that express normal VimIF networks. Expression of wild-type VimIF in vimentin-null cells causes mitochondrial motility to return to normal (slower) rates. In contrast, expressing vimentin with mutations in the mid-region of the N-terminal non-α-helical domain (deletions of residues 41–96 or 45–70, or substitution of Pro-57 with Arg) did not inhibit mitochondrial motility even though these mutants retain their ability to assemble into VimIFs in vivo. It was also found that a vimentin peptide consisting of residues 41–94 localizes to mitochondria. Taken together, these data suggest that VimIFs bind directly or indirectly to mitochondria and anchor them within the cytoplasm.

INTRODUCTION Mitochondria are the major source of metabolic energy, and they regulate intracellular calcium levels and sequester apoptotic factors (Nicholls and Budd, 2000). To meet the varying energy demands of different regions of the cytoplasm, mitochondria require an efficient transport and tethering system. In support of this idea, it has been shown that mitochondria are delivered to and sequestered in areas of the cell where metabolic requirements are high (Morris and Hollenbeck, 1993; Chada and Hollenbeck, 2003). Mitochondrial transport involves microtubule-dependent kinesins and dyneins and actin-dependent myosins. Frequently, all three types of motors are present on the surface of a single mitochondrion and work in concert to maintain the organelle’s correct intracellular localization (Hollenbeck and Saxton, 2005). Pathological conditions in neurodegenerative diseases are associated with disrupted transport of mitochondria in neural cells (Baloh, 2008).

There is evidence that the tethering of mitochondria involves intermediate filaments (IFs) (Linden et al., 1989; Leterrier et al., 1994; Wagner et al., 2003). In vertebrate cells, IFs form extensive interconnected cytoskeletal networks that extend throughout the cytoplasm (Goldman et al., 2008). Depending on the cell type, cytoskeletal IFs are assembled from one or more of a large multigene family of ~70 members (Herrmann et al., 2009). Each type of IF protein chain contains a highly conserved α-helical central rod domain flanked by variable, non-α-helical head and tail domains (Herrmann and Aebi, 2000). The rod domains are responsible for the assembly of the dimers (which are the building blocks of IF), and the head and tail regions are thought to be exposed on the surface of mature 10-nm IF, thereby providing potential binding sites for other proteins and organelles, such as mitochondria. In support of this supposition, IFs have been implicated in docking mitochondria in muscle cells (Milner et al., 2000; Stone et al., 2007), nerve cells (Straube-West et al., 1996; Wagner et al., 2003), and fibroblasts (Mose-Larsen et al., 1982; Summerhayes et al., 1983). There is also evidence that type III VimIFs and neurofilament heavy chain, a type IV IF protein, bind directly to mitochondria in vitro and that mitochondrial functions require IFs (Milner et al., 2000; Wagner et al., 2003; Tolstonog et al., 2005; Tao et al., 2009). Mitochondria can also associate with IF through interactions with the cytolinker, plectin (Reipert et al., 1999; Reznicek et al., 2003; Winter et al., 2008); and the depletion of...
plectin alters the shape of mitochondria, but has no impact on their motility (Winter et al., 2008).

Mutations in IF proteins that cause the disruption of IF networks also alter the morphology, distribution, and functions of mitochondria. For example, mutations in desmin IFs cause changes in the distribution and function of mitochondria in skeletal muscles and heart (Capetanaki, 2003), and a mutation in the neurofilament light chain that causes Charcot–Marie–Tooth disease results in the clustering of mitochondria in the cell bodies of neurons (Brownlees et al., 2002). In keratinocytes of patients with epidermolysis bullosa simplex, caused by mutations in keratins 5 and 14, there is an abnormal distribution of mitochondria (Uttam et al., 1996), and in keratinocytes expressing mutant keratins 8 and 18 there is enhanced susceptibility to apoptosis due to abnormalities in mitochondria (Gilbert et al., 2001). Morphological and functional changes in mitochondria have also been reported in vimentin-null fibroblasts (Tolstonog et al., 2001b).

In this study we demonstrate that mitochondria associate with the non-α-helical N-terminal domain of vimentin and that this binding inhibits their motility. Overall, the results show that VimIFs play essential roles in regulating both the localization and motility of mitochondria.

RESULTS

VimIFs inhibit the motility of mitochondria

The distribution patterns of mitochondria and VimIFs are similar in cultured fibroblasts. The network of long VimIFs in these cells encircles the nucleus and filaments extend radially toward the cell periphery. The mitochondria in these cells are also distributed throughout the perinuclear region, and they are frequently oriented parallel to the VimIFs (Figure 1). Although mitochondria are clearly associated with long VimIFs, they do not appear to associate with the vimentin squiggles or short IF present in the more peripheral cellular regions (Figure 1, B and C). The coalignment of mitochondria with VimIF could be explained either by their direct interaction or simply because the distribution of both depends on transport along microtubules that have a radial pattern in this cell type (Gyoeva and Gelfand, 1991; Rodionov et al., 1993; Prahlad et al., 1998).

To determine whether this colocalization is caused by an interaction between mitochondria and VimIFs, we analyzed mitochondrial distribution and motility in cells after perturbing the VimIF system. This analysis was accomplished by transfecting mouse 3T3 and MFT-6 fibroblasts with a dominant-negative mutant vimentin containing the N-terminal domain and the 1A α-helical domain of the central rod fused to enhanced green fluorescent protein (EGFP)-Vim[1–138]; EGFP-Vim[1–138] has been shown to disrupt VimIF organization (Kural et al., 2007; Chang et al., 2009). Under these conditions, the VimIF network in transfected cells is withdrawn from the cellular periphery and reorganized into an aggregate at the cell center (Figure 2A) (similar effects are seen in MFT-6 cells, not shown) (Kural et al., 2007; Chang et al., 2009; Mendez et al., 2010). Despite the fact that the microtubule system in these cells remains distributed throughout the cytoplasm (Figure 2C), the distribution of mitochondria is altered as shown by the clustering of these organelles in the central region of the cells where the majority of the reorganized VimIFs are
Figure 3: EGFP-Vim_{1-138} localizes to mitochondria. Cells (3T3) were transfected with plasmid pEGFP-Vim_{1-138} using FuGene 6 (Roche) and 24 h later were stained with MitoTracker Deep Red FM (Invitrogen; see Materials and Methods) (B) and then were fixed in methanol. (A) Epifluorescent image showing GFP localization. (C) Overlay of images in A and B. Bar, 10 μm.

An intact VimIF network alters mitochondrial motility. (A) MFT-6 cells transfected with pmCherry-Mito (left bar) or with pmCherry-Mito and pEGFP-Vim_{1-138} (right bar) using Maxifectin (DiaM). (B) MFT-6 cells (+/+); vimentin-null MFT-16 cells (−/−); vimentin null MFT-16 cotransfected with plasmids pVim and pEGFP-Vim (−/−, +Vim). Cells were also stained with MitoTracker Red CMXRos, and movements of fluorescently labeled mitochondria were recorded by time-lapse microscopy at 4-s intervals. Values are mean percentages of movements exceeding 0.2 μm/s ± SEM; n = number of cells, and, in brackets, the number of movements of mitochondria. Disrupting the intact VimIF network results in faster mitochondrial movements (A), whereas restoring the VimIF network in null cells slows mitochondrial movement (B).

The N-terminal domain of vimentin is involved in anchoring mitochondria

We next sought to determine whether the inhibitory effect of VimIF on rates of mitochondrial movements is passive, acting merely to obstruct their motility, or whether it results from the binding of VimIFs to mitochondria. In the latter case, we should be able to alter mitochondrial motility by mutating the interacting vimentin domain. To test this possibility, we expressed wild-type or mutated vimentin in vimentin-null MFT-16 cells. The design of the mutant forms of vimentin focused on deletions in the non-α-helical N-terminal domain (depicted in Figure 5A). The rationale for this approach is based on findings that numerous N-terminal deletions of vimentin retain their ability to assemble into IFs in vitro and in vivo (Herrmann et al., 1992; Shoeman et al., 2002). Figure 5B shows that the recombinant proteins are of the expected sizes, and that their expression level is low compared with endogenous vimentin in control cells. Each of these mutant vimentins assembles into VimIF networks in vimentin-null cells (Supplemental Figure S1) which are indistinguishable from the VimIF networks in control cells (Supplemental Figure S1A). Among these VimIF networks, only the form capable of interacting with mitochondria enabled mitochondria to move faster. By tracking the movements of individual mitochondria in mouse fibroblasts containing disrupted VimIF over time, we have determined that their motility increases by ∼70% (7.0 ± 0.7% vs. 4.0 ± 0.3% in nontransfected MFT-6 cells; see Figure 4A) (similar results were obtained with 3T3 cells; unpublished data). This finding suggests that normal arrays of VimIFs can restrict the movements of mitochondria. We also assayed the motility of mitochondria in vimentin-null MFT-16 fibroblasts (see Materials and Methods; Holwell et al., 1997). The proportion of fast movements of mitochondria in these cells is twice the proportion of fast movements of mitochondria in normal (vim^{+/+}) fibroblasts (9.0 ± 0.5% and 4.0 ± 0.3%, respectively) (Figure 4B; see Materials and Methods). Furthermore, expression of VimIF in vim-null cells (depicted in Supplemental Figure S1B) slows the rate of mitochondrial motility to a value similar to that seen in normal fibroblasts (4.0 ± 0.7%) (Figure 4B; see Materials and Methods). These results support the hypothesis that IFs modulate mitochondrial movements.

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requirements for sequences targeted to the outer mitochondrial membrane as it is moderately hydrophobic with two flanking regions containing positively charged amino acids (depicted in Figure 6A) (Rapaport, 2003); there is also a proline residue that has been shown to be required for the efficient targeting of some mitochondria-localized proteins (Allen et al., 2002). To determine whether proline is required for targeting to mitochondria, we transfected MFT-16 cells with vimentin P57R (Figure 5A). This mutant forms typical IF networks in MFT-16 cells (Supplemental Figure S1C), but it does not inhibit mitochondrial motility (Figure 5C).

We also determined whether an N-terminal fragment of vimentin containing the putative binding site is targeted to mitochondria when expressed in vimentin-null cells. For this we constructed a fusion protein formed with residues 41–94 of vimentin (2 amino acids shorter than the deletion fragment in construct VimΔ(41–96) due to the cloning procedure) and fluorescent protein Dendra2 (schematically depicted in Figure 6A). The results show that this chimeric protein strongly associates with mitochondria when expressed in vimentin-null cells (Figure 6B). In contrast, a similar fragment bearing the Pro-57 point mutation does not associate with mitochondria but distributes diffusely throughout the cytoplasm (Figure 6D). It therefore appears that the N terminus of vimentin is involved in the interaction of IFs with mitochondria and that Pro-57 is essential for this interaction.

Interaction of mitochondria with F-actin is mediated by vimentin IFs

There is evidence that the regulation of mitochondrial motility also involves actin. In support of this evidence, we recently demonstrated that the growth factor lysophosphatidic acid (LPA), acting through the small GTPase RhoA and its effector mDia1, inhibits mitochondrial motility (Minin et al., 2006). This inhibition is dependent on actin polymerization, as evidenced by the loss of inhibition following treatment with Latrunculin B (Lat B). To determine whether VimIFs modulate the association between mitochondria and F-actin, we examined the effects of LPA in vimentin-null cells. Following LPA treatment, the movements of mitochondria in normal fibroblasts are inhibited as previously described (Figure 7A). In contrast, the movements of mitochondria in LPA-treated, vimentin-null cells are not altered (Figure 7A). Furthermore, treatment with Lat B increases the motility of mitochondria in normal fibroblasts, but has no effect on the motility of mitochondria in vimentin-null cells (Figure 7B). These data suggest that VimIFs anchor mitochondria and link them to F-actin.

**DISCUSSION**

Only a small fraction of mitochondria at the periphery of the cell moves with relatively high speed (Trinczek et al., 1999; De Vos et al., 2003; Minin et al., 2006), whereas the majority of these organelles remain immobile or move slowly. The presence of immobile organelles is probably explained by their anchoring at sites in the cytoplasm where a local supply of ATP is required. Our results show that VimIFs modulate mitochondrial movements in cultured cells. Our results also show that the slower movements of mitochondria in the presence of VimIFs is not simply attributable to a passive hindrance of their motility (Kural et al., 2007), but rather is due to specific interactions of mitochondria with these cytoskeletal structures. This conclusion is based on our results showing that several vimentin mutants with altered N-terminal domains do not affect the motility of mitochondria, despite their ability to polymerize into VimIF networks.

Previous studies suggest two possible mechanisms by which mitochondria might bind to VimIFs: The binding could be mediated by an IF-associated protein, plectin isoform 1b, which has been shown to localize to mitochondria (Winter et al., 2008), and/or the N terminus of vimentin could be involved in a direct or indirect interaction with these organelles (Tolstonog et al., 2001a). In this study we show that plectin localizes to all of the mutant forms of vimentin (see, e.g., Supplemental Figure S2, D–F, showing colocalization of plectin antibody with Vimp57R-IFs and data in Gerashchenko et al., 2009). It is likely that plectin 1b binds to the mutant forms of vimentin used in this study, as they all contain the known plectin binding site (Reznicek et al., 2003). It appears therefore that direct interaction with vimentin is responsible for the inhibition of the mitochondrial movements. These data are further supported by the results of Winter and colleagues showing that plectin depletion does not alter mitochondrial motility (Winter et al., 2008).

Our results also demonstrate that it is the N-terminal domain of vimentin which interacts with mitochondria. Furthermore, the fragment of vimentin (residues 41–94) containing this putative binding site localized to mitochondria when expressed in vimentin-null cells. The proposed binding site meets the requirements for sequence targeted to the outer mitochondrial membrane as it is moderately hydrophobic with two flanking regions containing positively charged amino acids (depicted in Figure 6A) (Rapaport, 2003); there is also a proline residue that has been shown to be required for the efficient targeting of some mitochondria-localized proteins (Allen et al., 2002). To determine whether proline is required for targeting to mitochondria, we transfected MFT-16 cells with vimentin P57R (Figure 5A). This mutant forms typical IF networks in MFT-16 cells (Supplemental Figure S1C), but it does not inhibit mitochondrial motility (Figure 5C).

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This reasoning led us to propose that actin stress fibers are responsible for anchoring mitochondria at the cell periphery. Here we show that the disruption of F-actin by Lat B, or the induction of stress fibers by LPA, affects mitochondrial motility only in the presence of VimIFs. Thus, our results suggest that vimentin may act as an adapter between mitochondria and actin microfilaments. We therefore propose that mitochondria are associated with VimIFs directly through binding to the vimentin N terminus and/or indirectly by means of plectin (Winter et al., 2008), whereas the interaction of VimIFs with F-actin structures is mediated by plectin (see Figure 8; Svitkina et al., 1996; Wiche, 1998).

**MATERIALS AND METHODS**

**Cell culture**

Mouse 3T3 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Invitrogen) supplemented with...
Plasmids and transfection
Mitochondria in cells were tagged using pEGFP-Mito or pmCherry-Mito plasmid vectors that were prepared by modification of pEFFP-Mito (Clontech, Mountain View, CA). Plasmids pEGFP-Vim and pEGFP-VimΔ(1–138), which encode EGFP-tagged human vimentin and its N-terminal polypeptide (1–138 amino acids), respectively, were described previously (Yoon et al., 1998; Kural et al., 2007). Plasmids encoding unlabeled human vimentin and mCherry-tagged wild-type and mutated human vimentin were prepared by modification of the plasmid pEGFP-Vim using standard techniques. Plasmids encoding vimentin mutants retaining the ability to form IFs were made as described previously (Gerashchenko et al., 2009). Briefly, to make the plasmid encoding VimΔ(41–94) (Figure 5A), an inverted PCR was performed using the plasmid encoding human vimentin as a template and the primers AAGAAACACCGGCACCAACGAGA and CAGGCTGTAGGTCGCGGTT. The resulting product was ligated by its blunt ends to make the deletion mutant. Similarly, the plasmids encoding VimΔ(41–94), and VimΔ(45–70) (Figure 5A) were generated by using the primer pairs 1) GCTCGGCCGGCTCGCGGTGCCCGG and CCGGCTGCACCCAGCGTTG and 2) TAAGCTTATGG GCAGCGCGCTGCGCCCCA and AGTCGCGGC- P57R encoding vimentin with a Pro57Arg substitution was generated using a QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to the manufacturer’s protocol.

Transfection was performed using MaxiFect M (DiaM, Moscow, Russia), FuGene 6 (Roche, Pleasanton, CA), or Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. DNA (1 µg) was used for a 40-mm dish with 2 ml of medium. Cells were observed 16–20 h after transfection. For live cell analysis of mitochondrial movements in transiently transfected cells, the expression level of recombinant vimentin variants was monitored by fluorescence microscopy of IFs labeled by co-expressed EGFP- or mCherry-fused vimentin forms as described (Yoon et al., 1998), using cotransfection of plasmids encoding unlabeled vimentin forms with plasmids encoding their EGFP- or mCherry-tagged variants. Only cells with dense IF meshwork were taken for further analysis (see examples in Supplemental Figure S1). To obtain flat cells with better distributed mitochondria, sparse cultures were usually used. Sixteen to twenty hours post-transfection, 15 ng/ml MitoTrackerRed CMXRos or Deep Red FM (Invitrogen) was added to the culture medium and the cells incubated for 30 min before this medium was removed and replaced with fresh medium.

Immunofluorescence
IF staining in cell preparations was performed by indirect immunofluorescence using the mouse monoclonal antibody V9 (Chemicon International, Martinsried/Munich, Germany), the polyclonal rabbit antibody anti-vimentin (Helfand et al., 2003), or RVIM-AT (which reacts with murine vimentin [Avsyuk et al., 1997]), and FITC- or TRITC-labeled goat anti-mouse or goat anti-rabbit (The Jackson Laboratory, Bar Harbor, ME). Microtubules were stained using primary antibodies anti-α-tubulin (AbD Serotech, Raleigh, NC). Plectin was stained using antibody #446 (Wiche and Baker, 1982) and TRITC-labeled goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). Before staining, cells were either fixed with methanol (−20°C) or 4% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.1% Triton X-100 or, for better resolution of fine structures, first extracted with 1% Triton X-100 in 50 mM imidazole buffer, pH 6.8, containing 50 mM KCl, 0.5 mM MgCl2, 0.1 mM EDTA, 1 mM EGTA, and then fixed with 4% formaldehyde in PBS.

Live cell imaging
Cells were grown on glass coverslips to a density of 50–70% confluence, and coverslips were mounted in a chamber filled with DMEM supplemented with 10% FCS. Temperature was maintained at 37 ± 2°C with an Air Stream Incubator (Zeiss, Göttingen, Germany). Time-lapse epifluorescence microscopy was carried out on an Axiohot microscope (Zeiss) equipped with a Plan-Apochromat 63x 1.4 NA objective. Images were captured with a Micromax 782Y cooled CCD camera (Roper Scientific) driven by WinView32 software or with a AxioCam MRm camera (Zeiss) driven by AxioVision 4.6 (Zeiss) software. The frames were collected every 4 s with an exposure time of 0.5–1.0 s. To minimize phototoxic damage, a 100 W halogen lamp was used as a light source for fluorescent imaging of live cells.
SDS–PAGE and immunoblotting

Electrophoretic separation of proteins was performed using discontinuous 7.5% SDS–PAGE gels (Laemmli, 1970). For analysis of the expression of human vimentin and its mutants in transfected MFT-16 cells, samples of whole-cell protein extracts were run on gels and electroblotted onto nitrocellulose membrane (Hybond, GE Healthcare). Protein derived from whole-cell extracts of MFT-6 cells were also included in these analyses to show the level of endogenously expressed vimentin. The blots were stained with Ponceau S, blocked for 1 h in 5% goat serum in PBS containing 0.1% Tween 20, and subsequently incubated with 1 μg/ml of either V9 directed against human vimentin or RVIM-AT against mouse vimentin, in blocking solution for 1 h. After washing, the blots were incubated with horseradish peroxidase–conjugated goat anti–rabbit or anti–mouse immunoglobulin (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:5000 (vol:vol). Diaminobenzidine (Sigma, St. Louis, MO) was used to detect antibody binding. Tubulin was used as a loading control with anti–α-tubulin (DM1a; Sigma).

Quantitative analysis of mitochondrial motility

Image analysis was performed as described earlier (Minin et al., 2006) using an open source image analysis software, ImageJ (http://rswebw.nih.gov/ij). Because of the diverse morphology of mitochondria, we plotted the coordinates of one end for longer organelles and the center of mass for shorter ones. Mitochondrial motility was expressed in terms of the displacement distances and velocities. To define fast organelle movements, we applied a threshold of 200 nm/s. The values of the relative motility of mitochondria were expressed as mean percentages of fast movements in all recorded displacements ± SEM. In each experiment, 20–40 individual mitochondrial movements were analyzed in each of 10–15 cells. The significance of differences was estimated statistically by the paired-sample Student’s t test. Variability of the values calculated for different cells in the samples was analyzed by the same method and was insignificant.

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