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Positively charged residues within the MYO19 MyMOMA domain are essential for proper localization of MYO19 to the mitochondrial outer membrane

Jenci L. Hawthorne[#], Prachi R. Mehta[#], Pali P. Singh[#], Nathan Q. Wong, and Omar A. Quintero

Department of Biology, University of Richmond, VA 23173

[#] These authors contributed equally to this work.

Abstract

Myosins are well characterized molecular motors essential for intracellular transport. MYO19 copurifies with mitochondria, and can be released from mitochondrial membranes by high pH buffer, suggesting that positively-charged residues participate in interactions between MYO19 and mitochondria. The MYO19-specific mitochondria outer membrane association domain (MyMOMA) contains ~150 amino acids with a pI ~9, and is sufficient for localization to the mitochondrial outer membrane. The minimal sequence and specific residues involved in mitochondrial binding have not been identified. To address this, we generated GFP-MyMOMA truncations, establishing the boundaries for truncations based on sequence homology. We identified an 83-amino acid minimal binding region enriched with basic residues (pI ~ 10.5). We sequentially replaced basic residues in this region with alanine, identifying residues R882 and K883 as essential for mitochondrial localization. Constructs containing the RK882-883AA mutation primarily localized with the endoplasmic reticulum (ER). To determine if ER-associated mutant MyMOMA domain and mitochondria-associated wild type MyMOMA display differences in kinetics of membrane interaction, we paired FRAP analysis with permeabilization activated reduction in fluorescence (PARF) analysis. Mitochondria-bound and ER-bound MYO19 constructs displayed slow dissociation from their target membrane when assayed by PARF; both constructs displayed exchange within their respective organelle networks. However, ER-bound mutant MYO19 displayed more rapid exchange within the ER network than did mitochondria-bound MYO19. Taken together these data indicate that the MyMOMA domain contains strong membrane-binding activity, and membrane targeting is mediated by a specific, basic region of the MYO19 tail with slow dissociation kinetics appropriate for its role(s) in mitochondrial network dynamics.

Keywords

mitochondria; myosin; organelle transport; outer membrane

Introduction

Cellular physiology and homeostasis require proper localization and function of intracellular resources, including mitochondria. Defects in Mfn2, a protein involved in mitochondrial fusion, are one cause of the peripheral neuropathy Charcot-Marie-Tooth subtype 2A [Baloh et al. 2007; Kuhlbaumer et al. 2002; Zuchner et al. 2004], and defects in the fusion factor OPA1, result in in autosomal dominant optic atrophy [Alexander et al. 2000]. Like many membrane-bound organelles and intracellular structures, mitochondrial localization, activity, and dynamics depend on cytoskeletal interactions. Mitochondrial association with intermediate filaments increases mitochondrial membrane potential, likely impacting ATP-generation [Chernoivanenko et al. 2015]. In *S. cerevisiae*, mitochondrial transport into the developing bud has been shown to be actin-dependent [Boldogh et al. 2003; Boldogh et al. 2001; Higuchi et al. 2013], and recent reports demonstrate a role for actomyosin in mitochondrial fission in mammalian cells [Korobova et al. 2014; Korobova et al. 2013].

Microtubule- and actin-based motors have been identified on mitochondria, and are involved in mitochondrial dynamics. Axonal mitochondrial transport has been shown to be both actin- and microtubule-dependent. Treatment with actin- or microtubule-destabilizers disrupts filament dynamics altering mitochondrial movement [Ligon and Steward 2000; Morris and Hollenbeck 1995], and mitochondrial positioning in response to growth factors [Chada and Hollenbeck 2004]. Mitochondrial transport in cultured myocytes was also shown to be actin- and microtubule-dependent [Iqbal and Hood 2014]. Specific motors in the kinesin family, such as KIF1B β and KIF5B, have been shown to associate with mitochondria [Jellali et al. 1994; Leopold et al. 1992; Nangaku et al. 1994; Tanaka et al. 1998]. In *Drosophila*, motors primarily responsible for anterograde and retrograde fast axonal transport of mitochondria have been shown to be kinesin-1 and dynein, respectively [Pilling et al. 2006]. Actin-based motors have also been shown to be involved in axonal mitochondrial transport [Pathak et al. 2010]. Myosin classes that associate with the mitochondria or that have been shown to be involved in mitochondrial dynamics include myosin II [Korobova et al. 2014; Lalwani et al. 2008], myosin V [Altmann et al. 2008], and myosin VI [Kelleher et al. 2000].

Class XIX myosins directly interact with mitochondria [Quintero et al. 2009], and have recently been identified in species ranging from vertebrates to unicellular holozoans [Sebe-Pedros et al. 2014]; this class was lost from lineages leading to flies and roundworms [Odriontz and Kollmar 2007]. MYO19 contains a plus-end directed, actin-activated ATPase [Lu et al. 2014] with mechanochemical characteristics suggesting involvement in mitochondrial transport or positioning [Adikes et al. 2013]. RNA interference approaches have identified a role for MYO19 in mitochondrial inheritance during mitosis, where depletion of MYO19 leads to asymmetric mitochondrial inheritance into daughter cells, as well as to defects in cytokinesis [Rohn et al. 2014]. MYO19 has also been shown to be involved in responses to cellular stress, such as glucose starvation [Shneyer et al. 2016]. Mitochondrial binding is mediated by the MYO19-specific mitochondrial outer membrane association (MyMOMA) domain--a sequence of approximately 150 amino acids well-conserved within the class but lacking sequence homology to other known myosin cargo-binding domains or mitochondrial binding domains [Quintero et al. 2009]. GFP-tagged MYO19 expression constructs containing the MyMOMA domain localize to mitochondria,

whether N-terminally or C-terminally tagged. The biochemical properties mediating the MYO19-mitochondria interaction have not been well-characterized *in vivo*.

Here we report that MYO19 mitochondrial-binding is dependent on the MyMOMA region and is partially electrostatic in nature. Informatic analysis indicates that a sub-region of the MyMOMA domain is well-conserved within class-XIX myosins, and expression of GFP-labeled truncations indicates that the sub-region is necessary for mediating mitochondrial binding. Mutational analysis indicates that a specific set of well-conserved, basic amino acid residues are essential for mitochondrial targeting, but not for membrane-binding. We also report that although MYO19 does not appear to exchange readily with soluble cellular pools, it does exchange dynamically within the mitochondrial network.

Results and Discussion

MYO19 binding to the mitochondrial outer membrane depends on an electrostatic interaction

To determine the mechanism of the MYO19-mitochondrial interaction, we isolated mitochondria-enriched fractions via differential centrifugation of HeLa and B16F1 cell lysates [Horie et al. 2002]. We then treated mitochondria-enriched fractions with either buffer containing detergent (Triton-X100), or high pH buffer followed by a second medium-speed centrifugation (Figure 1A). Treatment with detergent should disrupt membranes--peripheral and transmembrane proteins would remain in the supernatant following centrifugation [Horie et al. 2002; McBride et al. 1992]. Both endogenous MYO19 and the transmembrane mitochondrial outer membrane protein, porin, remained in the supernatant following detergent treatment. On occasion some MYO19 signal remains associated with the pellet following detergent extraction even though the porin is completely solubilized. We hypothesize that this detergent-resistant fraction may be due to some MYO19 forming aggregates and precipitating, rather than remaining membrane-associated. Treatment with high pH buffer would disrupt charge-based interactions, but should not result in the release of transmembrane proteins. MYO19 remained in the supernatant following 100mM Na₂CO₃ treatment and a medium-speed centrifugation step, indicating that the interaction between MYO19 and the mitochondrial outer membrane is partially electrostatic in nature (Figure 1B).

A central region within the MyMOMA domain is well conserved and is sufficient for mitochondrial localization

We used sequence alignment and informatics approaches to identify regions within the MYO19 tail potentially involved in charge-dependent binding to mitochondria. We aligned the MyMOMA domains from thirteen vertebrate MYO19 orthologs using Clustal Omega [Sievers et al. 2011] in order to identify regions of strong and weak sequence homology. We also identified regions with a large number of positively charged amino acids, and chose to focus attention on specific positively charged amino acids that were well-conserved across species (Figure 1C). A central region within the MyMOMA domain corresponding to amino acids 853-935 of the human protein is well conserved and is enriched for positively-charged residues. 10 of the 83 residues in the human sequence are either arginine or lysine. Seven of

those charged residues are conserved between the mouse and human sequence, and five residues are conserved between the zebrafish and human sequence. These data suggest that the amino acid 853-935 region of the human MYO19 sequence could mediate charge-based interactions, such as binding to mitochondrial membranes.

To test this hypothesis, we generated three GFP-tagged MyMOMA truncations based on identification of drop-offs in sequence homology: T1 (824-852), T2 (853-935), and T3 (936-970) (Figure 1D). We then expressed these constructs in HeLa cells, and assayed for colocalization with Mitotracker CMXRos-labeled mitochondria (Figure 2A). Of the three constructs generated, only GFP-T2 showed obvious co-localization with mitochondria. Similar results were obtained when constructs were transfected into B16F1 cells (Supplemental Figure 1A). We quantified mitochondrial localization by calculating the “mito/cyto ratio” for each experimental condition—the ratio of mitochondria-localized GFP fluorescence to cytosolic GFP-fluorescence. Ratios greater than one indicate a higher concentration of GFP label on the mitochondria than in the cytosol (Supplemental Figure 1B and 1C). Both full-length GFP-MyMOMA and GFP T2 displayed ratios greater than 1, while GFP-T1 and GFP-T3 displayed ratios of approximately 1 (Figure 2B).

Mutation of amino acids 882 and 883 disrupts mitochondrial localization

To determine if the positively charged amino acids within the T2 sequence contribute to mitochondrial binding, we mutated 8 of the 10 positively charged residues in the T2 region to alanine. We chose to mutate the 7 amino acids that were conserved between mouse and human orthologs at positions R855, R882, K883, R915, K923, R927, and K928. We included an eighth residue, R932, as it was part of a cluster of positively charged residues at the C-terminus of T2. The construct containing all eight alanine substitutions did not display mitochondrial localization, but instead showed a reticular pattern (Figure 3A). To identify which residues were responsible for mitochondrial localization, we sequentially mutated individual positively charged residues to alanine in GFP-T2 truncation and again assayed for mitochondrial localization. In two instances (RK882-883 and RK927-928), two positively-charged amino acids appear next to each other in sequence. In those instances both positively charged amino acids were mutated to alanine in combination. All versions of GFP-T2 with alanine single- or double-substitutions localized to mitochondria, except for the RK882-883AA construct (Figure 3A, Supplemental Figure 1A). Although no longer mitochondria-localized, GFP-T2 RK882-883AA was not diffuse, displaying a similar reticular pattern to the construct containing 8 alanine substitutions. Cotransfection with the endoplasmic reticulum label DsRed2-ER revealed that GFP-T2 RK882-883AA localized to the endoplasmic reticulum (Figure 3B).

Other studies have demonstrated a shift in membrane-specificity of other proteins associated with internal membranes. Microsomal cytochrome *b₅* normally localizes to the endoplasmic reticulum, but the addition of two positively charged amino acids to the C-terminus of its membrane localization domain shifts the localization to the mitochondrial outer membrane [Borgese et al. 2001]. Conversely, mutation of positively-charged residues proximal to the mitochondrial-binding domain of outer mitochondrial membrane cytochrome *b₅* resulted in localization of the mutant proteins to the endoplasmic reticulum [Kuroda et al. 1998].

Additionally, naturally occurring splice variants of VAMP-1 differ in the amount of positive charge in their membrane-localization domains and localize to either the endoplasmic reticulum or the mitochondrial outer membrane, dependent on the presence of positively charged residues [Lan et al. 2000].

The mito/cyto ratio for GFP-T2 RK882-883AA was lower than that for GFP-T2, but greater than 1, suggesting some mitochondrial localization (Figure 3C). Detectable mitochondrial localization could be due to a low level of direct mitochondrial binding. Alternatively, mitochondria and endoplasmic reticulum structures are often in close proximity [Friedman et al. 2011], and as our imaging approaches employed wide-field epifluorescence microscopy techniques we could not distinguish between those possibilities. Introduction of the RK882-883AA mutation into GFP-tagged MyMOMA also resulted in strong colocalization with the endoplasmic reticulum (Figure 3A). Although occasionally cells with ER localization were observed, when the RK882-883AA mutation was introduced into full-length GFP-MYO19, the majority of transfected cells displayed fluorescence that was more diffuse than it appeared in the other constructs with RK882-883 mutated (Figure 3A). This could be due to possible interactions between the MYO19 tail and the MYO19 motor domain, as a number of other myosins are regulated in their motor activity and cargo-binding through interactions between their motor and tail domains [Spink et al. 2008; Trybus and Lowey 1984; Wang et al. 2004].

Wild type and mutant MyMOMA-T2 bind a subset of positively charged lipids in vitro

Mitochondrial membranes contain a variety of acidic phospholipid species [Daum and Vance 1997; de Kroon et al. 1997] including cardiolipin [Hovius et al. 1990] and phosphoinositides [Rosivatz and Woscholski 2011; Watt et al. 2002]. Acidic lipids have the potential of mediating charge-based interactions with the MYO19 MyMOMA domain. To examine potential interactions between the amino acids encoded in the T2 construct and acidic phospholipids, we purified GFP-tagged MyMOMA T2 from bacteria for *in vitro* lipid blot binding assays. The constructs contained 6x-His, GFP, and FLAG tags (Supplemental Figure 2A). Through the combination of immobilized cobalt-affinity chromatography and FLAG-affinity chromatography we were able to generate samples where GFP-T2 and GFP-T2 RK882-883AA were the major protein present in their separate purifications (Supplemental Figure 2B). Both constructs demonstrated the ability to bind some, but not all, acidic phospholipids *in vitro*, including phosphatidic acid, PIP, PIP2 and PIP3 (Figure 3D). Binding to the mitochondria-specific lipid cardiolipin was not apparent with either construct. Similarly purified FLAG-GFP-6xHis did not detectably bind any of lipid species (data not shown). These data suggest that other regions of the T2 sequence besides RK882-883 are involved in membrane-binding, and that acidic lipid binding likely does not contribute to mitochondrial membrane targeting by the MyMOMA domain.

Current predictive informatic approaches do not identify the specific mechanism mediating the MYO19/mitochondria interaction

We previously demonstrated that GFP-tagged truncations of MYO19 containing the MyMOMA domain localized to mitochondria whether GFP-attachment was N-terminal or C-terminal [Quintero et al. 2009]. The lack of an obvious N-terminal signal recognition

sequence [Neupert and Brunner 2002], the fact that additional GFP sequence did not block mitochondrial localization, and the observation that endogenous MYO19 can be released from mitochondrial membranes via high pH treatment all suggest that the mechanism of MYO19 binding is not via a transmembrane insertion [Borgese et al. 2003][Allen et al. 2002].

Just as sequence homology (or lack of homology) between multiple MYO19 orthologs allowed us to identify the T2 region as a putative mitochondrial binding region, pairing informatics-based structural prediction across multiple MYO19 orthologs assists in prediction of possible structures and mechanisms for mitochondrial outer membrane binding. We were unable to identify any putative structural domains within the MyMOMA domain using the Pfam [Finn et al. 2010], nor intrinsically disordered membrane binding domains [Brzeska et al. 2010] using DISOPRED. PSIPRED [Jones 1999] and Phyre² secondary structure predictions [Kelley et al. 2015] suggest that amino acids orthologous to RK882-883 of the human sequence are all contained within an α -helix that agrees closely with helix predictions made by PSIPRED. Analysis of these putative helices using HeliQuest [Gautier et al. 2008] indicated that the amphipathic nature of the predicted helices varied species to species (Supplemental Figure 3A) and that the consensus sequence resulted in an α -helix with amphipathic nature, and possibly representing a monotopic membrane insertion (Supplemental Figure 3B).

Recently, Shneyer et al. demonstrated that synthetic peptides consisting of amino acids 858-883 of human MYO19 bound strongly to synthetic liposomes with a lipid composition similar to mitochondria, and that a synthetic peptide containing the same amino acids and the RK882-883SS mutation bound to synthetic ER-mimicking liposomes *in vitro* [Shneyer et al. 2016]. They also predicted that the sequence N-terminal of residue 882 could fold into a membrane anchor consisting of a monotonically inserted α -helix. Taken together these data may indicate a one or two α -helix motif may be present within the MyMOMA domain that targets the protein to membranes. Although suggestive of a possible membrane interaction, the possibility remains that some of the MyMOMA interaction with mitochondrial membranes may be mediated protein-protein interactions. The lack of structural information and obvious sequence homology which would be required to fully characterize the properties of the MYO19/mitochondria interaction remains a challenge for identifying molecular interactions that underlie MYO19 localization to mitochondria. However, many mitochondrial outer membrane proteins contain similar patterns of basic residues necessary for mitochondrial anchorage such as TOMM20 [Likic et al. 2005], Miro [Fransson et al. 2006], and SPIRE [Manor et al. 2015].

Transient kinetic analysis and steady-state kinetic analysis suggest that MYO19 exchanges minimally with soluble pools but is dynamic within the mitochondrial network

Although we may not know the specific molecular mechanism, we were able to determine some of the kinetic properties of the MYO19/mitochondria interaction *in vivo*. We previously showed that MYO19 displayed slow exchange with cytosolic pools as assayed by FRAP analysis [Quintero et al. 2009], where the immobile fraction was approximately 0.6

and the time to 50% maximal recovery was approximately 250 seconds, which was a similar immobile fraction and slower recovery than the mitochondrial matrix label, GFP-Mito.

First, we wanted to examine whether the T1 and T3 sequences might contribute to the longevity of MYO19-mitochondria interaction even though these regions could not localize to mitochondria on their own. GFP-T1 and GFP-T3 did not previously show appreciable mitochondrial binding (Figure 2), but the possibility existed that mitochondrial binding could have been masked by diffuse cytoplasmic fluorescence. We chose to address this question using permeabilization-activated reduction in fluorescence (PARF) analysis [Singh et al. 2016]. By permeabilizing the plasma membrane with a low dose of digitonin, cytosolic pools of GFP-label undergo a large-scale dilution, establishing a non-equilibrium condition and favoring dissociation events. By quantifying the fluorescence loss from mitochondrial structures over time, we were able to compare the dissociation kinetics for our GFP-labeled proteins of interest. In addition, we used the mitochondrial outer membrane protein mchr-TOMM20 [Likic et al. 2005], and the ER-localization signal of cyto b_5 made to mitochondria-localize by the addition of two positively charged residues, GFP-Cyto b_5 RR [Borgese et al. 2001], as controls (Supplemental Figure 4A, Table 1). mchr-TOMM20 showed minimal dissociation from mitochondrial membranes as the immobile fraction was large (0.92 ± 0.02). More of the GFP-Cyto b_5 RR fluor dissociated from mitochondrial membranes over the time-course of the experiment (immobile fraction = 0.59 ± 0.10) compared to mchr-TOMM20. We observed that GFP-Cyto b_5 RR transfected cells often had a more obvious initial soluble fluorescence pool that was not associated with mitochondria. This pool was lost from the cell quickly after permeabilization as can be seen by the initial fast-phase of fluorescence loss (Supplemental Figure 4A). The slow phases of mchr-TOMM20 and GFP-Cyto b_5 RR fluorescence loss were more similar in magnitude after the soluble pool of GFP-Cyto b_5 RR was lost. When assayed by PARF analysis, there was no indication that either GFP-T1 or GFP-T3 was binding to mitochondria as their fluorescence loss was quite rapid (Figure 4A, Table 1, Supplemental Movie 1). Fluorescence loss for GFP-T1 and GFP-T3 was similarly rapid to what has been previously reported for cytosolic GFP [Singh et al. 2016]. We believe that the “bump” in the GFP-T1 curve may be due to the delayed loss of label leaving the nucleus and diffusing through the region of interest (ROI).

Next, we undertook PARF analysis to determine if the RK882-883AA mutation disrupted the kinetics of membrane binding in addition to disrupting the specificity of membrane binding of the T2 region. For GFP-MyMOMA, GFP-T2, and GFP-T2 RK882-882AA, the loss of fluorescence consisted of an initial fast drop in fluorescence followed by a slower loss of fluorescence (Figure 4A, Table 1, Supplemental Movie 2). We attribute the initial fast loss of fluorescence to loss of soluble signal. For GFP-T2 RK882-883AA it was more difficult to select regions-of-interest containing only the membrane of interest, and we suspect that the larger initial drop in fluorescence for the mutant construct is due to a larger contribution of soluble pool within the region of interest. It is also possible that movement of ER membranes out of the region of interest as the network rearranged following plasma membrane permeabilization could have contributed to a larger fast-phase contribution compared to the wild type constructs. Since the slow-phase loss rates are essentially parallel for all three membrane-bound constructs, our interpretation is that the apparent dissociation from membrane-bound pools occurs at nearly the same rate for all three constructs. All of

the membrane-bound constructs had a large immobile fraction, indicating little loss of membrane-bound material over the four-minute time course of the experiment; this suggests that although the RK882-883AA mutation disrupts proper membrane localization, the membrane-binding ability of the mutant construct has not been appreciably altered. This finding is in agreement with the recent report from Shneyer et al. based on *in vitro* liposome binding assays [Shneyer et al. 2016]. These data also support the hypothesis that the T1 and T3 regions do not appreciably contribute to the MyMOMA/mitochondria binding interaction as there was minimal difference in the rate of fluorescence loss for the slow-phase dissociation of GFP-T2 compared to GFP-MyMOMA.

Based on our PARF analysis, we predicted that GFP-T2 bound to mitochondria would exchange little with other pools, but we had not determined if the construct would be dynamic within the mitochondrial network, and rates of exchange with soluble pools are not an indication of dynamics within an organelle network. To address this question, we utilized FRAP analysis examining the exchange dynamics of GFP-T2 bound to mitochondria in specific cellular situations: networked mitochondria which were visually connected to other mitochondria outside of the photobleached ROI, isolated mitochondria which were not visibly connected to other mitochondria outside of bleached ROI, and ROI that contained both networked and isolated mitochondria (Figure 4B-C, Table 2, Supplemental Movie 3). Isolated mitochondria recovered little fluorescence as illustrated by a large immobile fraction (0.76 ± 0.02 , $n = 10$ cells), while networked mitochondria could be seen to recover fluorescence from the other structures outside of the bleached ROI, and had a smaller immobile fraction ($t_{1/2} = 99 \pm 57$ s, 0.51 ± 0.09 , $n = 17$ cells) than isolated organelles (Figure 4B-C, Table 2, Supplemental Movie 3). Compared to the isolated mitochondria, the networked mitochondria and mixed ROI displayed a larger variation. This is likely due to the variability in connectivity between the mitochondria in the ROI and outside of it. ER-bound GFP-T2 RK882-883AA recovered quickly from photobleaching (Figure 4B-C, Table 2, Supplemental Movie 4). The FRAP kinetics within the ER network for GFP-T2 RK882-883AA ($t_{1/2} = 10.5 \pm 2.5$ s, immobile fraction = 0.0 ± 0.1 , $n = 13$ cells) were similar in magnitude to those observed for nuclear envelope proteins resident in the ER [Zuleger et al. 2011]. In addition to being faster than the kinetics of the wild type mitochondria-associated MYO19 constructs, the kinetics of GFP-T2 RK882-883AA were also faster than our two control mitochondria outer membrane proteins, GFP-Cyto**b**₅RR and mchr-TOMM20 (Supplemental Figure 4B-C, Table 2, Supplemental Movies 5 and 6 respectively).

The PARF and FRAP data indicate that the membrane binding domain for MYO19 is contained within amino acids 853-935. Mutational analysis indicates that this region contains a structure capable of interacting similarly with both mitochondrial and endoplasmic reticulum membranes. Although the mutant construct was mislocalized to the ER, the dissociation kinetics had not changed detectably, but the mobility within the organelle network was faster than for the wild-type construct. The difference in internetwork mobility of the ER-bound mutant GFP-T2 compared to the wild-type mitochondria-bound GFP-T2 suggests that there may be properties of the mitochondrial membranes that are limiting the dynamics of the GFP-T2 within the organelle network. These could include direct interactions with other protein complexes within the membrane, or indirect limitations of diffusion due to the existence of structures generally limiting mobility of all species

within the plane of mitochondrial membranes. It is also possible that because of the geometry of the mitochondrial network (less interconnected) differs when compared to the ER network (more continuous and reticular), bleached mitochondria within the ROI have less access to other pools of labeled protein with which to exchange than does the ER within the bleached ROI. Taken together, the results from our kinetic analyses suggest that the amino acid residues RK882-883 play a central role in specificity of mitochondrial localization for the MyMOMA domain, but not in mediating the binding to membranes.

Considering the MYO19/mitochondria interaction in the context of MYO19 function

The data presented indicate that although the membrane binding region of the MyMOMA domain shares some characteristics with transmembrane proteins anchored in the mitochondrial outer membrane, MYO19 localization to the outer membrane is likely not via a transmembrane mechanism, since it can be displaced from mitochondrial membranes by incubation in carbonate buffer. Although MYO19 may interact with acidic phospholipids *in vitro*, acidic lipid interactions do not determine the identity of the membranes to which MYO19 is bound *in vivo* since wild type and mutant versions of the membrane binding domain both bind to a number of acidic phospholipids, though each localizes to different organelles within the cell. Additionally, while sequence homology indicates that the residues mediating mitochondria-binding are well-conserved within class-XIX MyMOMA domains, it has proven difficult to identify homologous structures in other proteins with similar functions based on sequence. Taken together, these data suggest that MYO19 localization to the mitochondrial outer membrane may be mediated through a mechanism not yet described in the literature. It remains to be seen whether positively charged amino acids are recognized by cytosolic machinery involved in placing MYO19 on/in the mitochondrial membrane, or by a receptor/binding partner within the mitochondrial membrane which recruits MYO19 to its endogenous location [Borgese et al. 2007].

What is clear is that amino acids 882 and 883 are involved in targeting MYO19 to the mitochondrial membrane through an unknown mechanism, and that the MyMOMA domain, while tightly associated with mitochondrial membranes, remains dynamic within the mitochondrial network. It is possible that localization of MYO19 protein to sites requiring MYO19 activity may depend on intra-organelle mobility and subsequent concentration of MYO19 at such privileged sites. In other instances, localization of membrane resident proteins has been shown to be influenced by the curvature of the membranes to which they are attached [Bozic et al. 2015; McMahon and Boucrot 2015]. As mitochondria often move with a highly curved leading end, it would be worth investigating if membrane curvature plays a role in MYO19-mediated mitochondrial motility. It remains to be seen how mobility of MYO19 within the mitochondrial network relates to efficient mitochondrial network dynamics and homeostasis.

Materials and Methods

Sequence analysis

The MYO19 amino acid sequences from a variety of vertebrates including mammals (human: NP_001157207.1, mouse: NP_079690.2, elephant: XP_003414411.1), marsupials

(Tazmanian devil: ENSSHAP00000004843, grey short-tailed opossum: XP_003340270.1), a monotreme (duck-billed platypus: XP_007671112.1), birds (zebrafinch: XP_002196706.1, chicken: XP_415895.4), an amphibian (*Xenopus laevis*: NP_001089346.1), a reptile (anole: XP_003230080.1), and fish (zebrafish: XP_001920083.4, coelacanth: ENSLACP00000019811, and pufferfish: ENSTRUP00000016436) were manually trimmed to the c-terminal sequence containing the third IQ motif and containing the MyMOMA domain. Sequences were aligned using Clustal Omega with standard settings [Sievers et al. 2011].

Plasmids

peGFP-C1, peGFP-N3, eGFP-Mito, and DsRed-ER were obtained from Clontech. GFP-Cyto b_5 RR [Borgese et al. 2001] was a gift from N. Borgese. mchr-TOMM20 was a gift from Michael Davidson (Addgene plasmid # 55146).

Cloning of MYO19 tail truncations and mutants by PCR mutagenesis

Human MYO19 tail corresponding to amino acids 824-970 was cloned into pEGFPN3 (Clontech) via megaprimer PCR mutagenesis [Geiser et al. 2001]. EGFP-tagged non-overlapping truncations corresponding to amino acids 824-852, 853-935, and 936-970 were also inserted into pEGFP-N3 via megaprimer PCR mutagenesis. Briefly, forward and reverse primers with sequence overlapping the target plasmid at the 5' end and overlapping the insert sequence at the 3' end were used to generate a PCR product that contained the insert flanked by sequence from the target vector (the megaprimer) using PFU Ultra II DNA polymerase (Agilent). The purified megaprimer was then used in a mutagenesis PCR reaction with the target plasmid as template and PFU Ultra II as the polymerase. The reaction was digested with DpnI (New England Biolabs) prior to transformation into XL-1 Blue bacteria (Stratagene). Insertion was verified by sequencing. Smaller base pair changes were generated via site-directed mutagenesis PCR using PFU Ultra II DNA polymerase. The primers used in this study are listed in Supplemental Table 1.

Cell Culture and transfection

HeLa cells [Scherer et al. 1953] and B16F1 cells [Fidler 1975] were maintained in DMEM high-glucose media supplemented with 10% FBS, 50 units/mL penicillin, and 50 μ g/mL streptomycin (growth media) at 37°C in a humidified atmosphere of 5% CO₂. Cells were passaged using 0.25% trypsin-EDTA. For imaging experiments, HeLa cells were grown on 22mm glass coverslips (#1.5). B16F1 cells were grown on glass coverslips coated with 10 μ g/ml laminin in PBS for 2h.

Cells were transfected with Lipofectamine 2000 (Life Technologies) using the manufacturer's protocol. DNA was diluted to 6.7 μ g/ml in Optimem without media or antibiotics in a final volume of 150 μ L. 4 μ L of Lipofectamine was diluted into 150 μ L of Optimem without serum or antibiotics and then mixed with the DNA dilution. Complexes were allowed to form for 5 minutes at room temperature. The entirety of the DNA/reagent mix was added drop-wise to a well of a 6-well plate. Cells were used for experimentation 18 to 30 hours after transfection.

Mitochondrial Isolation by differential centrifugation

HeLa cells were plated in culture flasks with fresh growth media overnight. Cells were trypsinized and then centrifuged at $600 \times g$ for 10 minutes. The pellet was resuspended in HES (10mM HEPES, pH 7.4, 2mM EDTA, 10% Sucrose) with protease inhibitors (10 μ g/mL aprotinin, 10 μ g/mL leupeptin and 1mM PMSF) to a concentration of 4×10^6 cells/ml. Samples were homogenized with 50 passes of a Dounce homogenizer using Pestle B. Lysates were centrifuged at 10 minutes at $600 \times g$, 4°C (pellet-P1). The supernatant (S1) was split into three equal samples for treatment and spun at $12,000 \times g$, 4°C for 15 minutes (P2). The pellets were resuspended in the appropriate buffers (HES with 1% Triton-X100, HES with 100mM Na₂CO₃, or HES) and incubated at 4°C for 30 minutes. Samples were centrifuged again at $12,000 \times g$, 4°C for 15 minutes. The supernatant from the control (HES) sample (S2), was centrifuged at $120,000 \times g$, 4°C for 15 minutes. Pellets were resuspended in a volume equal to the supernatant from which they were generated, and 5x Laemelli sample buffer (310mM Tris-HCl pH 6.8, 10% SDS, 50% Glycerol, 2.5% Bromophenol Blue, 7.5% β -Mercaptoethanol) was added to all samples prior to boiling at 95°C for 5 minutes.

Western blotting

Samples were loaded onto a 4-12% Bis-Tris NuPAGE gels and then transferred to nitrocellulose. Transfer was verified by Ponceau staining (0.5% Ponceau Red-S, 2% Acetic acid in H₂O). Membranes were blocked in TBST (50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1% Tween-20) with 5% powdered milk (blocking buffer) for 1 hour, incubated with primary antibody diluted in blocking buffer for 1h, and washed in TBST. Membranes were exposed to the appropriate secondary antibody diluted in blocking buffer for 1 hour, and washed in TBST prior to incubation with Bio-Rad Clarity reagent and exposure to film. Films were scanned in using a Canon CanoScan 9000F with a transparency adaptor. MYO19 was detected in HeLa cells using 0.3 μ g/ml chicken anti-human MYO19 [Quintero et al. 2009] and 80ng/ml donkey anti-chicken HRP (Jackson Immuno Research). MYO19 was detected in B16F1 samples using 0.17 μ g/ml rabbit anti-mouse MYO19 [Rohn et al. 2014] and 40ng/ml donkey anti-rabbit HRP (Jackson Immuno Research). Porin was detected in all samples using 5ng/ml mouse anti-human porin (LifeTechnologies, A31855), and 10 ng/ml donkey anti-mouse HRP (Jackson Immuno Research).

Fixing and staining of transfected cells

Transfected cells were incubated with 200nM Mitotracker CMXRos or Deep Red (Life Technologies) diluted in growth media 20 minutes under normal growth conditions and then washed 3×5 min with fresh growth media. Coverslips were then briefly washed with warm PBS and fixed with 4% paraformaldehyde in PBS for 10 minutes. Cells were permeabilized with 0.5% Triton-X100 in PBS for 5 minutes, and then stained with DAPI (75 nM) in PBS for 20 minutes followed by 4×10 min washes with PBS. In some instances Alexa647 phalloidin (6.6 nM) was included in the DAPI staining buffer. Coverslips were mounted on slides in PBS with 80% glycerol and 0.5% n-propyl gallate and sealed with nail polish.

Image Acquisition

Images were acquired using an Olympus IX-83 microscope outfitted with a PLAN APON 60x/1.42NA DIC objective, an EXFO mixed gas light source, Sutter filter wheels and shutters, a Hamamatsu ORCA-Flash 4.0 V2 sCMOS camera, and Metamorph imaging software. For fixed cell imaging, z-stack images (0.2µm steps) were captured sequentially using the Sedat Quad filter-set (Chroma), and exposure times maintained constant within an experimental data set. For live-cell imaging, time-lapse images were captured at an interval of one frame every two seconds.

Fixed-cell quantitative image analysis

Fluorescent micrographs were used to determine the ratio of organelle-localized fluorescence to cytoplasm-localized fluorescence (Mito/Cyto ratio). The integrated density of in-focus organelles, cytosol and background were measured with 4x4 pixel boxes using FIJI [Schindelin et al. 2012]. To calculate the Mito/Cyto ratio, integrated density of the background regions were subtracted from the integrated density of both the organelle-localized and the cytosol regions, and then the ratio of organelle fluorescence to cytosol fluorescence calculated. For each cell measurement, the ratio was calculated from five separate regions within one cell and then averaged. A ratio with a value greater than 1 indicates that the signal is more concentrated in the organelle region than in the cytosol region.

Permeabilization-activated reduction in fluorescence (PARF) analysis

PARF analysis was carried out as previously described [Singh et al. 2016]. Transfected HeLa cells were imaged in an open-top Rose chamber [Rose et al. 1958] with 300µL KHM buffer (pH 7.4, 110 mM potassium acetate, 20 mM HEPES, 2 mM MgCl₂) at the rate of one frame every 2 seconds. After the first 20 frames, digitonin was added (t=0) for a final concentration of 25µM. FIJI was used to calculate reduction in fluorescence by selecting regions of cells not including the nuclei and measuring the average fluorescence intensity at each frame, and then calculating the fluorescence intensity relative to the frame prior to permeabilization (t = 0). Data from multiple experiments were averaged together and the mean gray value relative to t = 0 was plotted over time and fit to a double exponential function of the form $y = a + b * e^{(-ct)} + d * e^{(-ft)}$. The half-life ($t_{1/2}$) was calculated using the exponential decay function calculating the value of t when y = 0.5.

Fluorescence recovery after photobleaching (FRAP) analysis

Transfected HeLa cells were imaged closed Rose chamber filled with Optimem without phenol red containing 50 units/mL penicillin, and 50µg/mL streptomycin. In some instances, the cells were stained with 4nM Mitotracker CMXRos for 10-20 minutes prior to imaging. Images were collected on an Olympus FV1200 laser scanning confocal microscope outfitted with a PLAN APON 60x/1.4NA objective at a frame-rate of 1 frame every 3 seconds. After the first 10 frames, regions of interest were illuminated for photobleaching at high laser power for 1 second. FIJI was used to calculate fluorescence recovery by selecting regions of interest and measuring the average fluorescence intensity at each frame, and then calculating the fluorescence intensity relative to the frame prior to photobleaching (t = 0s). Data from

multiple experiments were corrected for photofading due to imaging [Applewhite et al. 2007], averaged together, and the mean gray value relative to $t = 0$ was plotted over time and fit to the function $y = a*(1 - e^{-bt}) + c$. The half-life ($t_{1/2}$) was calculated using the exponential decay function calculating the value of t when $y = 0.5$. Immobile fraction was calculated by determining the asymptote being approached.

Protein Purification

Rosetta 2 (DE3)pLysS supercompetent bacteria were transformed with expression constructs. Cultures were grown to ~ 0.6 OD₆₆₀ in Terrific Broth containing 100 μ g/ml carbenicillin and 30 μ g/ml chloramphenicol, and then induced to express by the addition of IPTG to a final concentration of 1mM. Cells were grown at 16°C overnight. The bacteria were then pelleted by centrifuging at $8,000 \times g$ for 20 minutes at 4°C, resuspended in 20 mL 1x PBS, repelleted at $4500 \times g$ for 30 minutes at 4°C, and snap-frozen in liquid nitrogen after the removal of supernatant. Pellets were resuspended in lysis buffer (50mM Tris-HCl pH 8.0, 500mM NaCl, 0.5% Triton, 0.5% deoxycholate, 10mM imidazole, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 1mM PMSF) and sonicated with a Branson Digital Sonifier 450 sonicator (67% duty-ratio, 50% power, for 30 seconds). Lysozyme was added to a final concentration of 0.1mg/ml and the suspension rocked for 20 minutes at 4°C. The supernatant was cleared of insoluble debris by centrifugation at $25,000 \times g$ for 20 minutes at 4°C. Talon beads (Clontech) were then added to the supernatant and rocked for 2 hours at 4°C. The mixture poured into a column and the beads allowed to collect. The bed was washed 3 times with 5mL of wash buffer (50mM Tris-HCl, 200mM NaCl, 0.05% Triton, 10mM Imidazole, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 1mM PMSF, 2.5% glycerol, pH 8), and then eluted with 250mM imidazole diluted in wash buffer. FLAG resin (Sigma) was added to the collected eluate and rocked at 4°C overnight. The bead mixture was then poured into a column and washed with FLAG wash buffer (50mM Tris pH 8, 200 mM NaCl, 0.05% Triton, 2 mM imidazole, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 1 mM PMSF, 2.5% glycerol), and then eluted with FLAG elution buffer (50mM Tris pH8, 200 mM NaCl, 0.05% Triton, 2 mM imidazole, 10 μ g/mL aprotinin, 10 μ g/mL leupeptin, 1 mM PMSF, 2.5% glycerol, 100 μ g FLAG peptide/mL) and collected in fractions. A Bradford assay was done on each preparation to determine the concentration of protein, and SDS-PAGE followed by Coomassie staining to verify enrichment of the expected protein species.

Lipid Blots

Nitrocellulose membrane strips spotted with membrane lipids (Echelon) were incubated in PBS with 3% bovine serum albumin and 0.1% Tween-20 (blocking buffer) for 1 hour at room temperature, with gentle shaking. Blots were then incubated with 0.5 μ g/ml purified protein in blocking buffer for 1 hour. After washing with PBS 3×10 minutes, blots were incubated with 30ng/ml chicken anti-GFP antibody (Aves) for 1h at RT with gentle shaking. Blots were washed with PBS and then incubated with 50ng/ml donkey anti-chicken HRP antibody for 1h at RT. Following washing, blots were visualized using Clarity reagent (Bio-Rad) in combination with autoradiography film. Blots included the following lipids: triglyceride (trigly), diacylglycerol (DAG), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (cardio), phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP),

phosphatidylinositol-4,5-bisphosphate (PIP2), phosphatidylinositol-3,4,5-triphosphate (PIP3), cholesterol (chol), sphingomyelin (sphng), and 3-sulfogalactosylceramide (sulfa).

Quantification, analysis, and statistics

Both Metamorph (Universal Imaging) and FIJI were used for image analysis. Exponential fits for PARF and FRAP analysis were performed using Kaleidagraph. Data were compared by Tukey analysis using Minitab 12 software. Data points are expressed as means $\pm$ S.E. of the mean. Parameters calculated from exponential fits are expressed as mean $\pm$ the SEM-adjusted fit [Singh et al. 2016], by generating curve fits of the mean + SEM and mean – SEM, and reporting the error as the difference between the value calculated from the mean fit and the value calculated from the SEM-adjusted fit. All images were prepared for publication using FIJI, Metamorph, Photoshop, or some combination of these software packages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MYO19 co-isolates with mitochondria-enriched fractions and the interaction is partially charge-based

A) Schematic diagram of mitochondrial isolation by sequential differential centrifugation. “S” indicates supernatant and “P” indicates pellet. B) MYO19 (109kD) tracks with mitochondria-enriched fractions, as indicated by the presence of the integral mitochondrial outer membrane protein, porin/VDAC (37kD). To determine possible mechanisms of MYO19 binding, P2 pellets were resuspended in either HES buffer with 1% Triton X-100 or 100mM Na₂CO₃, pH 11. Incubation in buffer that permeabilized mitochondrial membranes or that altered the pH released MYO19 from mitochondria, while only permeabilization released porin. C) A multiple sequence alignment of 13 vertebrate MYO19 MyMOMA domains indicates regions of homology with respect to positive charge. Human MYO19 is highlighted in grey, and amino acid positions with positively charged residues in the human sequence are highlighted in black with white text. Amino acid number corresponding to the human sequence is noted. D) This sequence alignment revealed regional differences in conservation across the tail domain, and was used to generate three non-overlapping truncations of the tail: T1 (824-852), T2 (853-935), and T3 (936-970) constructs.

Figure 2. MYO19 tail MyMOMA domain contains a conserved region that is essential for mitochondria localization

A) GFP-MyMOMA (824-970) and GFP-Myo19 T2 (853-935) colocalize with Mitotracker-labeled mitochondria, in HeLa cells. T1 (824-852) and T3 (936-970) do not localize to mitochondria. Scale bar is 10 μ m. All images were captured under identical imaging conditions. Overlays contain the GFP-label (green), Mitotracker label (red), and phalloidin label (blue). B) Quantification of mitochondrial localization by calculation of the mito/cyto ratio indicates that GFP-MyMOMA and GFP-T2 show concentration of fluorescence signal on the mitochondria when compared to cytosol. (* $p < 0.05$, Tukey analysis, $n_{\text{MyMOMA}} = 24$ cells, $n_{\text{T1}} = 12$, $n_{\text{T2}} = 23$, and $n_{\text{T3}} = 11$). Error bars represent 90th and 10th percentiles; the box top, middle, and bottom indicate the 75th, 50th, and 25th percentile respectively. The symbol (.) indicates the sample mean.

Figure 3. Elimination of positively charged residues in the T2 region of human MYO19 alters mitochondrial association

A) Mutation of 8 positively charged residues within the MYO19 T2 results in the loss of mitochondrial localization. Mutant versions of GFP-MYO19 T2 (R855A, R915A, K923A, RK927-928AA, and R932A) colocalize with Mitotracker-labeled mitochondria in HeLa cells while GFP-T2 RK882-883AA fails to localize to mitochondria. Introducing the RK882-883AA mutation into GFP-MyMOMA domain or full-length GFP-MYO19 also results in disrupted mitochondrial localization. Scale bar is 10 μ m. B) The RK882-883AA version of GFP T2 colocalizes with DsRed2-ER labeled tubules. C) Quantification of mitochondrial localization by calculation of the mito/cyto ratio indicates that the RK882-883AA mutant version of GFP-T2 has a decreased mito/cyto ratio when compared to wild-type GFP-T2. (* $p < 0.0001$, t-test, $n_{T2} = 24$ cells and $n_{RK882-883AA} = 26$). Error bars represent 90th and 10th percentiles; the box top, middle, and bottom indicate the 75th, 50th, and 25th, percentile respectively. The symbol (•) indicates the sample mean. D) Binding of purified GFP-T2 and GFP-T2 RK882-883AA mutant protein to membrane lipid strips

indicates that both constructs binds to a subset of negatively charged membrane lipids (acidic lipids shown in red). Neither construct bound the mitochondria-specific acidic lipid, cardiolipin. Lipid strips were processed at the same time and developed on the same piece of film and are representative of three experimental trials. Blots included the following lipids: triglyceride (trigly), diacylglycerol (DAG), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (cardio), phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), phosphatidylinositol-4,5-bisphosphate (PIP2), phosphatidylinositol-3,4,5-triphosphate (PIP3), cholesterol (chol), sphingomyelin (sphng), and 3-sulfogalactosylceramide (sulfa).

Figure 4. Kinetic analyses of GFP-T2 and GFP-T2 RK882-883AA mutant indicate differences in exchange kinetics

A) PARF analysis of GFP-MyMOMA, GFP-T1, GFP-T2, GFP-T3, and the GFP-T2 RK882-883AA mutant indicates that all three constructs containing the T2 region show slow apparent dissociation and immobile fractions greater than 0.6. GFP-T1 and GFP-T3 show fast exchange with no obvious immobile fraction. A summary of calculated PARF kinetic parameters can be found in Table 1. B) When examined by FRAP analysis, fluorescence recovery of GFP-T2 in isolated mitochondria is minimal with an immobile fraction = 0.76 ± 0.02 , while recovery in mitochondria connected to the network of mitochondria outside of the bleached region is faster ($t_{1/2} = 99 \pm 57$ s, immobile fraction = 0.51 ± 0.09). GFP-T2 RK882-883AA mutant bound to the ER displays rapid exchange kinetics with essentially no immobile fraction ($t_{1/2} = 10.5 \pm 2.5$ s, immobile fraction = 0.0 ± 0.07). A summary of calculated FRAP kinetic parameters can be found in Table 2. C) Individual frames from Supplemental Movies 3 (GFP-T2) and 4 (GFP-T2 RK882-883AA) demonstrating the slower recovery of fluorescence in networked (blue box) versus isolated mitochondria (red box). The RK882-883AA mutant version of GFP-MYO19 T2 recovers quickly from photobleaching. Yellow boxes indicate the bleached region, scale bar represents $10 \mu\text{m}$, and time is indicated in seconds. Bleaching at $t = 0$ s.

Table 1

PARF kinetic analysis for proteins used in this study

Protein and condition	$t_{1/2}$	Immobile fraction	R	Cells	Experiments
mchr-TOMM20	-	0.92 ± 0.02	0.936	50	11
GFP-Cyto b_5 RR	-	0.59 ± 0.10	0.994	12	5
GFP-MyMOMA	-	0.72 ± 0.02	0.988	32	8
GFP-T1	4.28 ± 0.73	0.02 ± 0.02	0.980	38	6
GFP-T2	-	0.73 ± 0.03	0.990	47	11
GFP-T3	3.81 ± 0.45	0.02 ± 0.02	0.993	23	5
GFP-T2, RK882-883AA	-	0.56 ± 0.03	0.992	30	6

Table 2

FRAP kinetic analysis for proteins used in this study

Protein and condition	$t_{1/2}$	Immobile fraction	R	Cells
mchr-TOMM20	-	0.66 ± 0.08	0.991	7
GFP-Cyto b ₅ RR	221 ± 57	0.46 ± 0.01	0.994	12
GFP-T2, mixed	-	0.59 ± 0.05	0.960	16
GFP-T2, networked mito.	99 ± 57	0.51 ± 0.09	0.982	17
GFP-T2, isolated mito.	-	0.76 ± 0.02	0.978	10
GFP-T2, RK882-883AA	10.5 ± 2.5	0.0 ± 0.07	0.973	13