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Characterizing F-actin disassembly induced by the Semaphorin signaling component Mical

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Abstract

The MICALs are a family of phylogenetically conserved cytoplasmic proteins that modulate various cellular behaviors and play critical roles in Semaphorin-Plexin signaling. Our recent results have revealed that the MICALs are an unusual family of actin regulatory proteins that use actin filaments (F-actin) as a direct substrate – controlling F-actin dynamics via stereospecific oxidation of conserved methionine (Met44 and Met47) residues within actin. In particular, the MICALs have a highly conserved flavoprotein monooxygenase (redox) enzymatic domain in their N-terminus that directly oxidizes and destabilizes F-actin. Here, we describe methods to characterize Mical-mediated F-actin disassembly using in vitro assays with purified proteins.

Keywords

MICALs; Plexin; oxidoreductase; F-actin disassembly; actin sedimentation; pyrene-actin

1. Introduction

Semaphorins (Semas) are one of the largest families of extracellular guidance cues and are well-known as repulsive cues that restrict axons from navigating into inappropriate areas (1–3). A body of work has revealed that Semas negatively regulate cell motility by disassembling the F-actin and microtubule cytoskeletons as well as by inhibiting cell-substrate interactions (3,4). Semas exert their effects through transmembrane receptors including Plexins (1,5). Plexins, in turn, deliver extracellular Sema signals into cells by interacting with well-known intracellular signaling molecules including small GTPases and kinases (3,5,6). Yet, how Semas-Plexins destabilize the F-actin and microtubule cytoskeletons as well as inhibit cell-substrate interactions is still poorly understood.

MICAL family proteins, which include one Drosophila Mical and three mammalian MICALs, are unusual cytosolic proteins that initially emerged as interacting partners with plexins (7). Each of the MICAL proteins contain from N to C terminal, a flavin adenine dinucleotide (FAD)-binding monooxygenase (redox) enzymatic domain, a calponin-homology (CH) domain, a LIM domain, a proline – rich region, and a sequence that

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resembles the α-like region of Ezrin-Radixin-Moesin (ERM) proteins (4,7). The MICALs are widely expressed in numerous tissues including the nervous system, thymus, lung, spleen, kidney, testis, liver, muscle, heart, fibroblasts and hematopoietic and fibroblast cell lines (4,8). The MICALs also play critical roles in at least some of these tissues including directing axon-axon repulsion (7,9,10), synaptogenesis (11), dendritic arborization (12), muscle formation (11), neurite outgrowth (13,14), heart development (14), axon targeting (15), growth cone collapse (16), cell viability (17), exocytosis (18), and other cell biological effects underlying morphology (e.g., (9,13,17,19–22)). Growing evidence also links MICALs to various pathological processes, including neuronal injury and regeneration (23), epilepsy (24), and cancer metastasis and progression (25–28).

To better understand the role and mechanisms of action of the MICALs in these different tissues and cellular events, it is critical to determine how the MICALs function at the molecular/biochemical level. Recently, we found that Mical uses F-actin as a direct substrate – employing its FAD-binding redox enzymatic domain and the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH) to posttranslationally oxidize the methionine 44 and 47 residues of actin and this oxidation results in F-actin severing and remodeling (9,20). Further analysis revealed that this Mical-mediated oxidation of actin is selectively reversed by the methionine sulfoxide reductase enzyme SelR/MsrB, which restores the normal polymerization properties of Mical-treated actin (10,21). Coupling this in vitro work to in vivo assays revealed that Mical and SelR regulate the stability of the actin cytoskeleton in different cells and during Semaphorin-Plexin signaling (9,10,20). Mical therefore is a critical negative regulator of the actin cytoskeleton that works downstream of Semaphorins-Plexins. Here, we provide methodologies to characterize Mical-mediated F-actin disassembly using purified proteins.

2. Materials

All solutions should be made with filtered deionized water (we use a sensitivity of 18MΩ·cm at 25°C) and analytical grade reagents and be sterilized by either autoclaving or filterization. Prepare and store all reagents at room temperature unless indicated otherwise.

2.1. Reagents for a pyrene-actin F-actin disassembly assay

1. Purified protein containing the active (redox) portion of Mical (such as $Mical^{redox}$ protein) (*see* Note 1).
2. 1M Tris-HCl, pH8.0: dissolve 121.1g Tris-base in 800ml H_2O. Adjust the pH to 8.0 by adding concentrated HCl at room temperature. Fill H_2O to 1000ml final volume and sterilize by filtering with 0.2μm filter or autoclaving.
3. 2M Tris-HCl, pH7.5: dissolve 242.2g Tris-base in 800ml H_2O. Adjust the pH to 7.5 by adding concentrated HCl at room temperature. Fill H_2O to 1000ml final volume and sterilize by filtering with 0.2μm filter or autoclaving.

[1]The $Mical^{redox}$ protein contains the redox domain of Mical (amino acids 44–531). For example, the appropriate portion of Mical (redox domain, amino acids 44–531) including 5' and 3' Bgl II sites and a stop codon at 3' end was amplified by PCR and inserted into the compatible BamH I sites of the pET28a bacterial expression vector (9). The resulting recombinant protein includes a N-terminal His_6-tag (9).

4. 0.5M $CaCl_2$: dissolve 55.5g $CaCl_2$ in 800ml H_2O and then adjust the volume to 1000ml. Autoclave and store at 4°C.

5. 100mM ATP (adenosine triphosphate): dissolve 0.78g ATP (disalt dehydrate) in 8ml H_2O and adjust the volume to 10ml. Sterilize by filtering with 0.2μm filter and store at −20°C.

6. 1M DTT: dissolve 1.5g DTT in 8ml H_2O and adjust the volume to 10ml and sterilize through 0.2μm filter.

7. 1M KCl: dissolve 74.55g KCl in 800ml H_2O and adjust the volume to 1000ml and autoclave.

8. 1M $MgCl_2$: dissolve 203.3g $MgCl_2 \cdot 6H_2O$ in 800ml H_2O and adjust the volume to 1000ml and autoclave.

9. 0.5M EGTA (Ethylene glycol tetraacetic acid), pH8.0: dissolve 190g EGTA in 800ml H_2O and adjust pH to 8.0 with concentrated NaOH. Adjust the volume to 1000ml and autoclave.

10. 10× general actin buffer (G-buffer): mix 500μl 1M Tris-HCl, pH8.0 and 40μl 0.5M $CaCl_2$ in 9.46ml H_2O.

11. 1× general actin buffer (G-buffer): mix 1ml 10× general actin buffer with 9ml H_2O to make 1× general actin buffer. Add 20μl 100mM ATP and 10μl 1M DTT immediately before use (final concentration is 5mM Tris-HCl, pH8.0, 0.2mM $CaCl_2$ and add 0.2mM ATP and 1mM DTT).

12. 10× actin polymerization buffer: mix 250μl 2M Tris-HCl, pH7.5, 5ml 1M KCl, 200μl 1M $MgCl_2$, 200μl 0.5M EGTA in 4.1ml H_2O. Add 200μl 100mM ATP and 50μl 1M DTT immediately before use (final concentration is 50mM Tris-HCl, pH 7.5, 500mM KCl, 20mM $MgCl_2$, 10mM EGTA, pH8.0 and add 2mM ATP and 5mM DTT).

13. 10mM NADPH: dissolve 0.0016668g NADPH in 200μL of 10mM Tris-HCl, pH8.0 (*see* Note 2).

14. Pyrene (pyreneidoacetamide)-labeled actin: purified actin can be labeled in the lab (29–31) or purchased pre-labeled (Cytoskeleton, Inc.).

15. Pyrene-labeled actin stock solution: 20mg/ml pyrene-labeled actin in 5mM Tris-HCl, pH 8.0, 0.2mM $CaCl_2$, 0.2mM ATP, 5% sucrose and 1% dextran. The actin protein can be snap frozen in 5μl aliquots in liquid nitrogen and stored at −70°C.

16. 96-well flat bottom black polystyrene plates

17. 1.7ml microcentrifuge tubes

18. Fluorescence spectrophotometer (SpectraMax M2, Molecular Devices).

[2]10mM NADPH stock solution can be stored in 20μl aliquots at −80°C for 6 months. Check that the solution is colorless, not yellowish, before use. If the NADPH stock solution is yellowish, a new fresh stock solution should be made.

2.2. Reagents for an actin sedimentation assay

1. 500 μl 8 × 34mm open-top thickwall polycarbonate centrifuge tubes (Beckman Coulter).
2. TLA-120.1 fixed angle ultracentrifuge rotor (Beckman Coulter).
3. Optima TLX table-top ultracentrifuge (Beckman Coulter).
4. 1M Tris-HCl, pH6.8: dissolve 121.1g Tris-base in 800ml H_2O. Adjust the pH to 6.8 by adding concentrated HCl with stirring at room temperature. Fill H_2O to 1000ml final volume and sterilize by filtering with 0.2μm filter or autoclaving.
5. 4× Laemmli sample buffer: 250mM Tris-HCl, pH6.8, 8% SDS, 40% glycerol, 0.032% bromophenol blue, 20% β-mercaptoethanol. Dissolve 4g SDS in 12.5ml of 1M Tris-HCl, pH6.8 and heat at 65°C until SDS completely dissolves. Then, cool down the solution and add 20ml glycerol and 0.016g Bromophenol blue. Add 10ml β-mercaptoethanol and adjust the volume to 50ml with H_2O. Aliquots and store at −20°C.
6. Filtered deionized water: prepare by purifying at a sensitivity of 18MΩ·cm at 25°C and filtering through a 0.2μm filter.
7. 1.5M Tris-HCl, pH 8.8 with sodium dodecyl sulfate (SDS): dissolve 181.17g Tris-base and 4g SDS in 800ml H_2O. Adjust the pH to 8.8 by adding concentrated HCl with stirring at room temperature. Fill H_2O to 1000ml final volume and sterilize by filtering with 0.2μm filter.
8. 0.5M Tris-HCl, pH 6.8 with SDS: dissolve 30.3g Tris-base and 2g SDS in 400ml H_2O. Adjust the pH to 6.8 by adding concentrated HCl with stirring at room temperature. Fill H_2O to 500ml final volume and sterilize by filtering with 0.2μm filter.
9. 10% ammonium persulfate (APS): dissolve 0.1g APS in 1ml H_2O. Store at 4°C for up to a week.
10. 10% SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel: Mix 1.7ml of 30% (w/v) acrylamide (ProtoGel, National Diagnostics), 1.25ml of 1.5M Tris-HCl, pH 8.8 with SDS, 50μl of 10% APS, 4μl of tetramethylethylenediamine (TEMED) with 2.05ml of H_2O to make a 10% resolving gel. Pour the resolving gel solution into assembled glass plates (Mini-PROTEAN Tetra Cell Systems, Bio-Rad) and solidify for at least 10mins. Mix 267μl of 30% (w/v) acrylamide (ProtoGel, National Diagnostics), 500μl of 0.5M Tris-HCl, pH 6.8 with SDS, 30μl of 10% APS, 2.5μl TEMED with 1.2ml H_2O to make a 4% stacking gel. Pour the staking gel onto the top of the solidified resolving gel and insert a comb (Mini-PROTEAN Tetra Cell Systems, Bio-Rad). Solidify for at least 5mins and then remove the comb and clean the wells to load samples (*see* Note 3).

[3]30% (w/v) acrylamide stock solution is neurotoxic. Wear gloves when handling.

11. 10× SDS-PAGE running buffer: dissolve 30.2g Tris-Base, 144g Glycine, 10g SDS in 800ml H_2O. Adjust the final volume to 1000ml. Dilute to 1× working concentration with H_2O before using.
12. Protein size marker: Precision™ Plus Protein All Blue Standards (Bio-Rad).
13. Coomassie gel staining solution: dissolve 2.5g Coomassie blue (Brilliant blue G-250, Fisher) in 500ml H_2O with 430ml methanol and 70ml acetic acid.
14. Coomassie gel destaining solution: mix 100ml acetic acid and 250ml methanol in 650ml H_2O.
15. 1.7ml microcentrifuge tubes
16. Vortexer
17. Parafilm
18. Mini-PROTEAN® Tetra Cell Systems vertical mini gel electrophoresis system (Bio-Rad)
19. Shaker
20. Scanner

3. Methods

3.1 Characterizing Mical^{redox} protein-mediated F-actin disassembly using pyrene-labeled actin

1. Set a fluorescence spectrophotometer to an excitation wavelength of 365nm and an emission wavelength of 407nm at 25°C.
2. Thaw four tubes of the pyrene-labeled actin stock solution and dilute to 1mg/ml by adding 400μl 1× G-buffer into 20μl of the pyrene-labeled actin stock solution (*see* Note 4).
3. Polymerize actin by adding 10μl of 10× actin polymerization buffer (0.25× final strength) and incubate at room temperature for 1hr while protecting from light.
4. Dilute the polymerized actin from Step 3 to 0.2mg/ml by adding 1.6ml of 1× G-buffer and mix gently by inverting the tubes 3–4 times (*see* Note 5).
5. Transfer 200μl of the actin solution from Step 4 into each well of a 96-well flat bottom black polystyrene plate. Place the 96-well flat bottom black polystyrene plate into a fluorescence spectrophotometer and shake for 5secs and read the samples once every 30secs for total 3mins to establish a peak fluorescent measurement for all samples (*see* Note 6).

[4]20μl pyrene-labeled actin is good for 8 reactions.
[5]This dilution initiates some F-actin disassembly due to a decrease of actin concentration. The rate of this spontaneous F-actin disassembly is the basal level of F-actin disassembly in the assays.
[6]If the fluorescence at zero point is very high, it is possible that ATP is old. Prepare a new stock solution of ATP and keep in −70°C before use.

6. Take out the plate and add 50nM purified Mical^{redox} protein (*see* Note 7) and 100μM NADPH co-enzyme (*see* Note 7) into the wells and return the plate to the spectrophotometer (*see* Note 8). Since FAD is purified with Mical^{redox} protein (it is non-covalently attached), there is no need to add additional FAD (*see* Note 9). Control groups are F-actin + Mical^{redox} and F-actin + NADPH and the experimental group is F-actin + Mical^{redox} + NADPH (*see* Note 7). Shake for 5secs and read the samples in each well once every 30secs for a total of 60mins. The depolymerization of pyrene-labeled actin will be observed as a decrease in the fluorescent signal (of the pyrene-actin) over time (Figure 1) (*see* Note 10).

7. Export the data from the fluorescence spectrophotometer and draw graphs using GraphPad Prism or Microsoft Excel. Normalize the zero points of all samples and present the depolymerization curve (Figure 1).

8. To visualize the composition of G-actin and F-actin in each reaction, an actin sedimentation assay can be performed (continue to 3.2 Actin sedimentation assay).

3.2 Characterizing Mical^{redox} protein-mediated F-actin disassembly using an actin sedimentation assay

1. Transfer 50μl of each different reaction (wells) of the pyrene-actin disassembly assay into polycarbonate centrifuge tubes.

2. Centrifuge at 156,565 × *g* for 20mins at room temperature.

3. Carefully transfer 50μl of supernatant into new 1.7ml microcentrifuge tubes and add 10μl of 4× Laemmli sample buffer and mix well (this tube will contain the actin that is present in the supernatant) (*see* Note 11).

4. In the polycarbonate centrifuge tubes from Step 3, add 50μl of filtered deionized water and pipet up and down several times and incubate on ice for 10mins to dissolve pelleted actin.

5. Cover the polycarbonate centrifuge tubes with parafilm tightly and vortex the tubes for 10secs and transfer the solution into new 1.7ml microcentrifuge tubes

[7]Highly pure Mical^{redox} protein should be used (9). Different concentrations of Mical can be used (we have performed these types of pyrene-actin experiments with concentrations of Mical protein ranging from 10nM to 1.2μM; e.g., (9, 10, 20)). Likewise, different concentrations of NADPH can be used (we have performed these types of pyrene-actin experiments with concentrations of NADPH ranging from 5μM to 400μM; e.g., (9, 10, 20)). It should also be remembered that Mical is not active without its co-enzyme NADPH, thus good controls for Mical's effects on F-actin are Mical^{redox} protein alone and NADPH alone.

[8]After adding purified Mical^{redox} protein and the NADPH co-enzyme into the wells, the plate should be immediately returned in the spectrophotometer for reading. If the fluorescence of the samples in the 96-well plate are not being read immediately, the reading for initial actin disassembly activity by Mical^{redox} protein will be missed.

[9]The color of purified Mical^{redox} protein is yellowish-orange (because of the FAD that is non-covalently bound with the purified Mical). The intensity of the color will increase with increasing concentrations of Mical/FAD. Mical^{redox} protein can be precipitated at high concentration; so it is a good idea not to concentrate over 2mg/ml. Also, remember that the Mical protein is not yellow in color, so if it becomes denatured/precipitates and "loses" its FAD molecule, the solution will remain yellow-orange but the Mical protein will no longer be in the solution.

[10]If highly pure Mical^{redox} protein is being used (9) but no activity is seen with purified Mical (i.e., a decrease in fluorescence in the well containing Mical^{redox} + NADPH), it is possible that NADPH is old. Prepare a fresh stock of NADPH.

[11]To avoid disturbing pellet on the tube bottom, carefully transfer only supernatant.

and add 10μl of 4× Laemmli sample buffer and mix well (this tube will contain the actin that is present in the pellet).

6. Boil the samples at 100°C for 5mins, briefly spin down the samples and load 15μl of each sample and a protein size marker into the wells of the 10% SDS-PAGE gel. Run the gel in a Mini-PROTEAN® Tetra Cell Systems vertical mini gel electrophoresis system or equivalent (*see* Notes 12–13).

7. Stain the gels for 1hr with Coomassie gel staining solution and destain the gels in Coomassie gel destaining solution for 1hr on a shaker and check the protein bands (*see* Note 14). Actin will be the major band (at ~43kDa). Mical^{redox} protein will migrate at ~57kDa. Actin in the supernatant represents depolymerized actin (G-actin) and actin in pellet represents polymerized actin (F-actin) (Figure 2).

8. After destaining the gels, scan the gels using a scanner and analyze the scanned images.

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[12]A prestained protein marker is useful for monitoring protein migration and separation while running a gel.
[13]Before loading samples, clean each well by pipetting or rinsing with a syringe to remove partially polymerized gel. Large wells can be used to prevent leaking of samples to adjacent wells when loading.
[14]To expedite gel staining/destaining process, the gel can be boiled for 30secs in staining solution or destaining solution using a microwave oven. Destain the gel several times with destaining solution until clear bands appear from a dark blue background.

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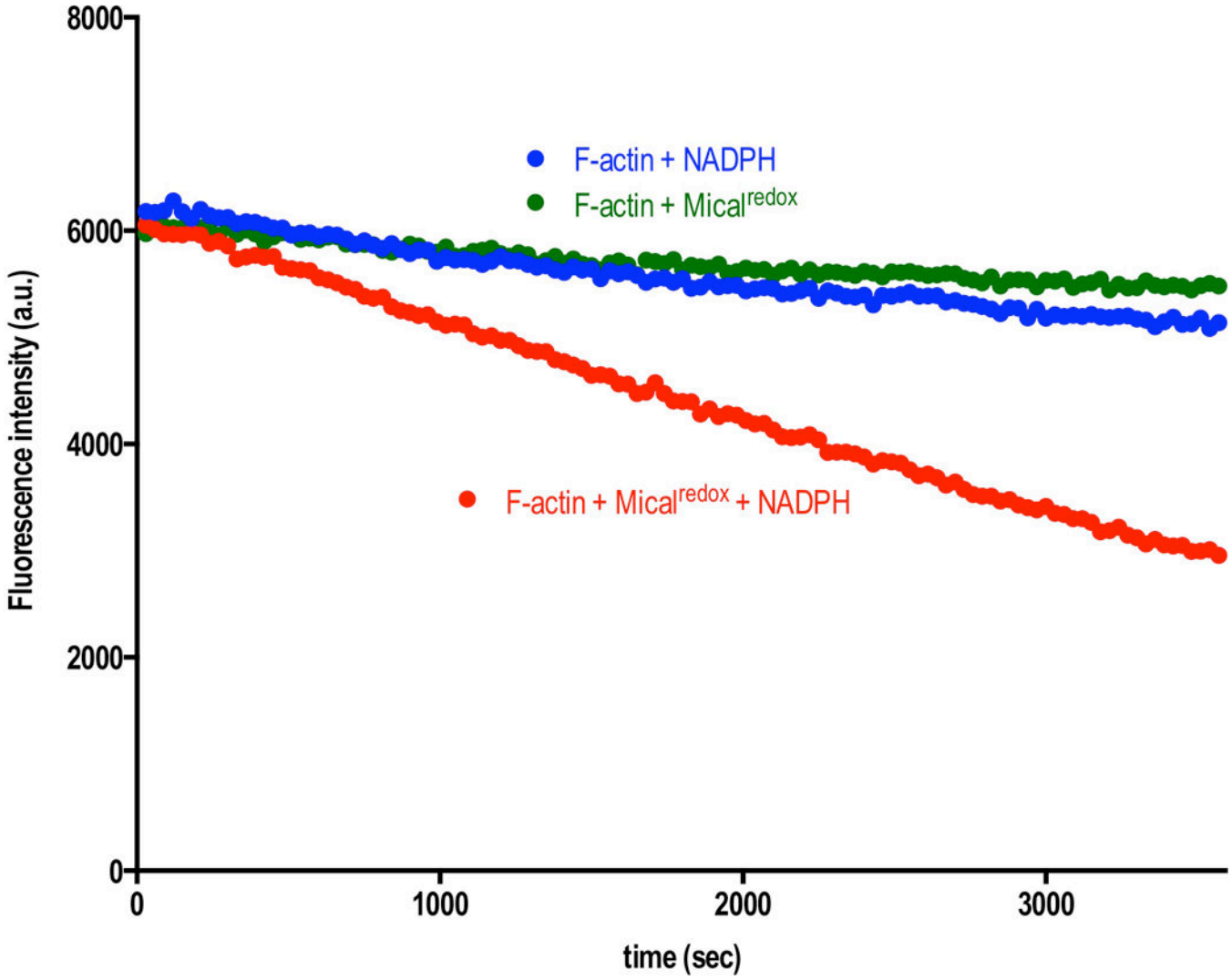

Figure 1. Analysis of purified Mical^{redox} protein-induced F-actin disassembly using pyrene-labeled actin

Pyrene-actin depolymerization assay, where the fluorescence of polymerized actin decreases as actin depolymerizes, reveals that purified Mical^{redox} protein induces actin depolymerization in the presence of its NADPH coenzyme.

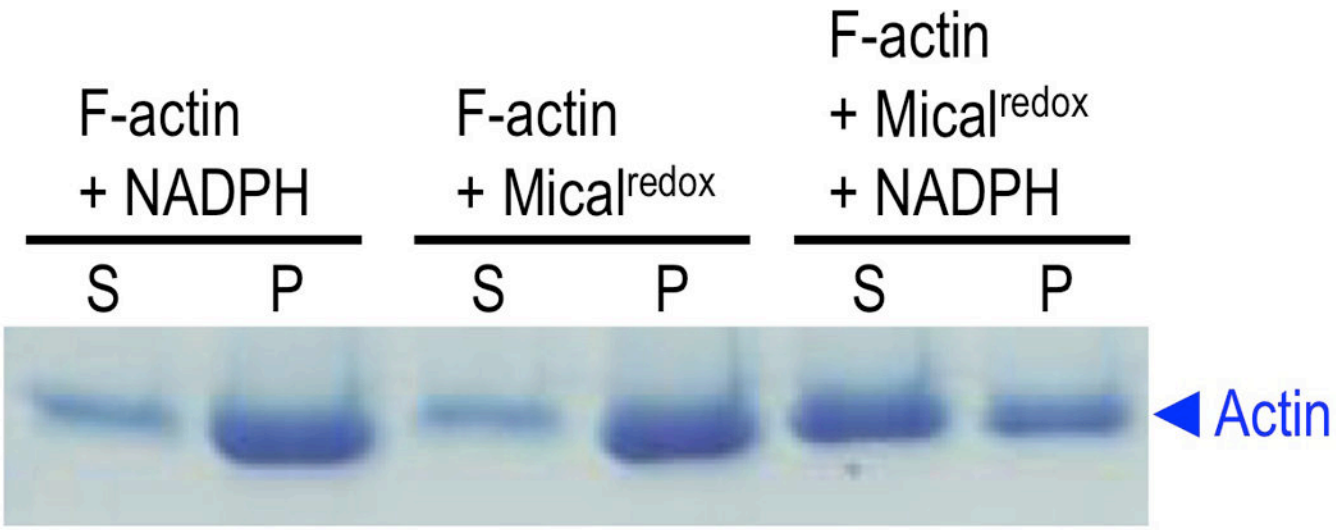

Figure 2. Analysis of purified Mical^{redox} protein-induced F-actin disassembly using an actin sedimentation assay

Actin sedimentation and Coomassie stained gels reveal that Mical^{redox} induces actin depolymerization in the presence of its NADPH coenzyme. Actin monomers (G-actin) are in the supernatant (S) and actin polymers (F-actin) are in the pellet (P).