V. 2.4

6xHis MICAL-1 Protein Redox-CH domains

(Human recombinant)
Cat. # MIC01
Lot:
Upon arrival, store at 4°C (desiccated)
See datasheet for storage after reconstitution

Form:	Lyophilized yellow powder
Amount of material:	2 x 50 µg
Validated applications:	Actin Oxidation at Met 44, Met 47

Online Datasheet Contains
MIC01(V2.4) and MIC01-XL (V2.4)

Background Information

MICAL is an intracellular flavoprotein monoxygenase, conserved from insects to mammals, that functions as a catalyst for oxidation-reduction (redox) reactions [1,2]. Terman's group showed that MICAL interacts with F-actin and uses NADPH as a cofactor to oxidize actin at Met44 and Met47 [3] (see Figure 1). These studies were performed with a truncated version of the MICAL protein consisting of its Redox and CH domains, because mutagenesis studies identified these regions as being essential for actin oxidation [4].

Importantly, MICAL-mediated effects on actin were not occurring through a diffusible oxidant like H_2O_2, as reductants like DTT did not alter MICAL activity, and close proximity between MICAL and actin were necessary for oxidation [3]. MICAL oxidation is both physiological and enzymatically reversible by the MsrB family of methionine sulfoxide reductases [5,6]. Functionally, oxidation of Met44 has a profound effect on actin polymerization because the residue resides in the D-loop of subdomain 2 of the protein, which is critical for actin subunit contacts; thus, upon oxidation, Met44 becomes negatively charged and interferes with actin monomer-monomer interaction and promotes F-actin severing and depolymerization [7].

Regulation of actin oxidation at Met44/Met47 has been shown to destabilize F-actin in vivo [4] and to play a key role in a growing number of cellular processes, including, cytokinesis [10], axonal guidance, dendritic organization, synaptic development, heart and muscle development and cell viability [6].

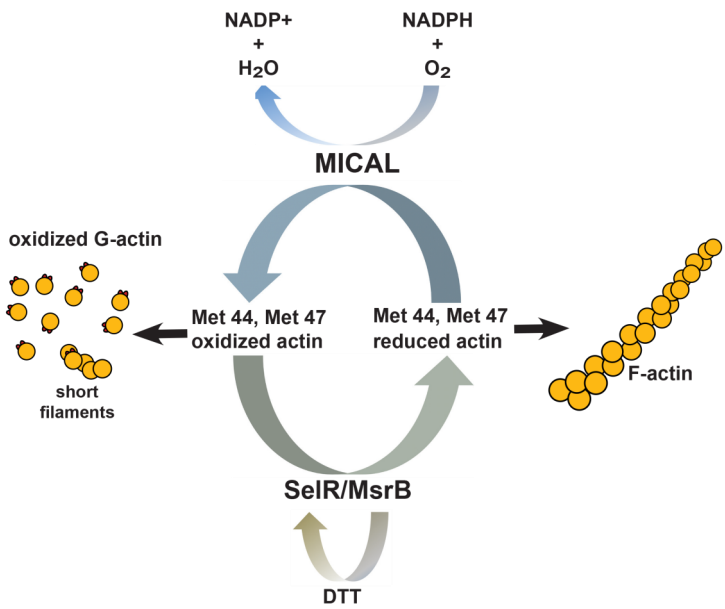

Figure 1. Actin Met 44 and Met 47 physiological redox system. A schematic diagram of the enzymes that control the physiological and reversible oxidation and reduction of methionines 44 and 47 of actin. Specifically, the MICAL family of proteins regulate oxidation of actin at Met 44 and Met 47. Conversely, the SelR/MsrB family of methionine sulfoxide reductase family specifically reduces actin at Met 44 and Met 47.

Material

Human MICAL1 protein (MIC01) [accession # Q8TDZ2 (MICA1_HUMAN)] has been produced and purified from a bacterial expression system. The recombinant protein is N-terminally 6x Histidine tagged and is comprised of the Redox and CH domains (aa: 1 -612) of the full length protein. MIC01 has an approximate molecular weight of 60 kDa. MIC01 is supplied as a yellow lyophilized powder.

Storage and Reconstitution

Shipped at ambient temperature. The lyophilized protein can be stored desiccated at 4°C for 6 months. For reconstitution, the product tube should be briefly centrifuged to collect the powder to the bottom of the tube.

Each tube of MIC01 contains 50 µg of protein. When reconstituted with 50 µl of water, the final buffer composition is 2 mM Tris pH 8.0, 20 mM NaCl, 1% sucrose, and 0.2% dextran. We recommend you make 10 µl aliquots, flash freeze, and store at –70°C.

When stored and reconstituted as described, MIC01 is stable for 6 months at –70°C.

Purity

Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 4-20% gradient tris-glycine gel. MICAL-1 protein was determined to be ≥ 90% pure (**Figure 2**).

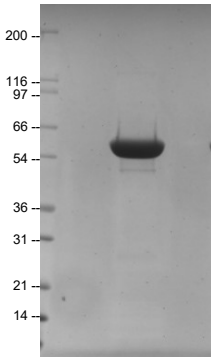

Figure 2. MICAL-1 Protein Purity Determination. A 10 µg sample of MICAL-1 protein was separated by electrophoresis in a 4- 20% tris-glycine gel and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein Assay Reagent (Cat. # ADV02). Mark12 standard molecular weight markers are from Invitrogen.

Activity

Effective MICAL-1 (MIC01) + NADPH oxidation of actin will reduce subtilisin A cleavage at M47/G48 by > 90%. See the next page for details.

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Evaluating MICAL Activity: Subtilisin A Limited Proteolysis Application

Rationale:
MICAL-1 (MIC01) is a protein that can specifically oxidize actin at amino acids M44 and M47 (β-actin nomenclature). It has been reported that M44 oxidation by MICAL is concomitant with M47 oxidation [3]. Subtilisin A is a protease that has been shown to specifically cleave actin between M47 and G48 [8,9]. When actin is oxidized at M47 by MICAL the efficiency of subtilisin A cleavage is significantly reduced [8]. Therefore, subtilisin A can be used in limited proteolysis assays to determine MICAL activity, and efficiency of M47 and M44 oxidation by evaluating cleaved versus uncleaved actin.

Expected Results:
Effective MICAL-1 (MIC01) + NADPH oxidation of actin will reduce subtilisin A cleavage at M47/G48 by > 90% under the conditions described below.

Reagents Needed:
MICAL (Cat. # MIC01): When used as described below, 50 μg will oxidize 2 ml of actin at 0.5 mg/ml
Rabbit skeletal muscle actin (Cat. # AKL99)
G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)
Precision red advanced protein assay (Cat. # ADV02)
2x polymerization buffer (5 mM Tris pH 7.5, 4 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)
NADPH (Cat. # N7505; Sigma)
Phenylmethylsulfonyl fluoride (PMSF)
Subtilisin A (Cat. # P5380; Sigma)

Method:
Day 1
1. Resuspend rabbit skeletal muscle actin (Cat. # AKL99) to 1.0 mg/ml with G-buffer (Cat. # BSA01) supplemented with 0.2 mM ATP and 0.5 mM DTT
2. Incubate the actin on ice for 1 h
3. Centrifuge the actin in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the supernatant to a new tube on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Add an equivalent volume of 2x polymerization buffer. The F-actin concentration should be 0.5 mg/ml (11.9 μM)
7. Incubate the actin at RT for 1 h
8. Resuspend one tube of MIC01 with 50 μl of water. The approximate concentration will be 1 μg/ul (16.67μM). This is your stock tube
9. Prepare NADPH to 100 mM stock; (100 mg NADPH in 1.2 ml of 0.01 M NaOH)
10. Prepare the following 4 samples in 1.5 ml centrifuge tubes as shown in Table 1
11. Leave samples at RT for 2 hours on your benchtop. This will allow MICAL to oxidize actin when NADPH is present
12. After oxidation, dialyze samples in G-buffer overnight.

Table 1: Composition of Experimental Samples for MICALOxidation

sample	Name	F-actin 11.9 μM	NADPH 100 mM stock	MIC01 16.67 μM stock
1	AKL99	500 μl	0	0
2	AKL99 + NADPH	500 μl	2 μl 400 μM Final	0
3	AKL99 + MIC01	500 μl	0	12 μl 0.4 μM Final
4	AKL99 + MIC01 + NADPH	500 μl	400 μM Final	0.4 μM Final

Table 2: Composition of Experimental Samples for Subtilisin Digestion

Sample	Name	Add Subtilisin	Add PMSF
1	ALK99	No	Yes
2	AKL99	Yes	Yes
3	AKL99 + NADPH	No	Yes
4	AKL99 + NADPH	Yes	Yes
5	AKL99 + MIC01	No	Yes
6	AKL99 + MIC01	Yes	Yes
7	AKL99 + Both	No	Yes
8	AKL99 + Both	Yes	Yes

Day 2
1. Centrifuge the samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
2. Determine the protein concentration
3. Make a 100 μl working stock at 0.1 mg/ml of samples 1-4. Use G-buffer for diluting samples
4. Aliquot 20 μl (2 μg actin) of each sample into individual 1.5 ml tubes. 2 aliquots per sample are needed for untreated versus subtilisin treated conditions: You should have the following 8 conditions as shown in Table 2
5. Before beginning subtilisin treatment boil a beaker of water. The water should be boiling when the subtilisin reaction is complete to inactivate the protease
6. Make a stock of subtilisin: 1 mg of subtilisin can be dissolved in 32 ml of 2 mM Tris pH 8.0/0.2 mM CaCl2 to make a 31.25 μg/ml stock. Aliquot and store at -70ºC.
7. Dilute a tube of subtilisin stock to 2 μg/ml with G-buffer.
   (*Subtilisin analysis: 1:200 w/w subtilisin to actin = 10 ng subtilisin / 2ug actin).*
8. Add 5 ul (10 ng) of subtilisin to treated condition for all 4 samples
   (*Subtilisin should be diluted and added to sample in less than 10 min*)
9. Treat actin samples with subtilisin for 15 min
10. During treatment make PMSF (10 mM: 1.74 mg PMSF per ml of isopropanol).
11. After 15 min of subtilisin treatment, stop the reactions by adding 1 μl of PMSF to each sample (1-8)
12. Add 5 μl of 5x reducing sample buffer to each sample (1-8)
    NOTE: Immediately boil samples once reducing buffer is added, as denaturing the actin sample allows trace activity of subtilisin A to rapidly cleave actin.
13. Immediately place samples into boiling water. Leave samples for 5 minutes
14. Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes.
15. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
16. Visualize with Coomassie staining. See Figure 3

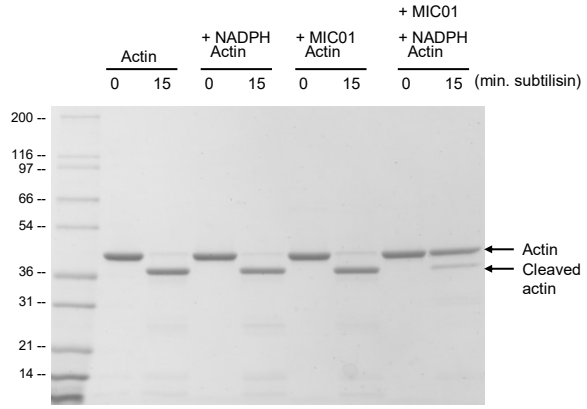

Figure 3. Subtilisin Assay on MICAL-1 Treated Actin. Actin (Cat. # AKL99) was diluted to 0.1 mg/ml (2.3 μM) and left untreated, or treated with NADPH (400 mM), MIC01 (0.076 μM) , or a combination of both (see method). 2 μg of each sample was then left untreated, or treated with subtilisin (1:200 w/w) for 15 min. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

Using MICAL as a research tool: Oxidized Actin Sedimentation Application

Rationale:

MICAL-1 (MIC01) is a protein that can specifically oxidize actin at amino acids M44 and M47 (β-actin nomenclature). When actin is oxidized at M44 its ability to polymerize is significantly diminished [3]. However, with increasing concentrations, oxidized actin can form augmented polymers [8]. Sedimentation assays can be used to measure the polymerization capabilities of untreated versus MICAL-oxidized actin.

Expected Results:

Effective MICAL-1 (MIC01) + NADPH oxidation of actin will reduce the % of actin that will polymerize relative to unoxidized actin in an actin concentration dependent manner as shown in Table 3

Table 3: Expected % actin polymer formed with unoxidized versus oxidized actin

	% polymer unoxidized actin	% polymer MIC01 oxidized actin
0.1 mg/ml actin	80-90%	40-50%
0.4 mg/ml actin	85-95%	68-78%

Reagents Needed:

MICAL (Cat. # MIC01): When used as described below, 50 μg is sufficient for 700 μl of 2x pol buffer + NADPH + MIC01 (see Table 4), equivalent to 6 polymerization assays.
Rabbit skeletal muscle actin (Cat. # AKL99)
G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)
Precision red advanced protein assay (Cat. # ADV02)
2x polymerization buffer (5 mM Tris pH 7.5, 4 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)
NADPH (Cat. # N7505; Sigma)

Method:

1. Resuspend rabbit skeletal muscle actin (Cat. #AKL99) to 1.0 mg/ml with G-buffer (Cat. # BSA01) supplemented with 0.2 mM ATP and 0.5 mM DTT
2. Incubate the actin on ice for 1 h
3. Centrifuge the actin in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the supernatant to a new tube on ice
5. Determine the protein concentration
6. Dilute actin to the following conc. 0.8 mg/ml and 0.2 mg/ml with G-buffer
7. Resuspend one tube of MIC01 with 50 μl of water. The approximate concentration will be 1 μg/ul (16.67μM). This is your stock tube
8. Prepare NADPH to 100mM stock; (100mg NADPH in 1.2 ml of 0.01 M NaOH)
9. Prepare Assay Polymerization Buffers as shown in Table 4
10. Prepare four microcentrifuge tubes labeled 1-4

Table 4: Composition of Assay Polymerization Buffers

Assay Polymerization Buffers	2X polymerization Buffer	NADPH 100 mM Stock	MIC01 16.67 μM Stock
2x pol buffer	250 μl	No	No
2x pol buffer + NADPH + MIC01	250 μl	1 μl 400 μM	18 μl 1.2 μM

Table 5: Composition of Experimental Samples

sample	name	0.2mg/ml actin	0.8 mg/ml actin	2x pol buffer	2x pol buffer +MIC01 + NADPH
1	AKL99 - 0.1 mg/ml	110 μl	0	110 μl	0
2	AKL99 + MIC01+ NADPH - 0.1 mg/ml	110 μl	0	0	110 μl
3	AKL99 - 0.4 mg/ml	0	110 μl	110 μl	0
4	AKL99 + MIC01 + NADPH - 0.4 mg/ml	0	110 μl	0	110 μl

11. Prepare the following samples in the microcentrifuge tubes as shown in Table 5
12. Transfer 200 μl of each sample to tubes compatible for ultracentrifugation.
    Accurate loading is important for tube balance
13. Incubate at room temperature for 1 h
14. Centrifuge at 100,000 g in an ultracentrifuge for 1.5 h
15. After centrifugation remove the top 180 μl of supernatant from each sample and place into new 1.5 ml tubes labeled S1-S4
    Leave the remaining supernatent, as the pellet may become detached if disturbed
16. Add 36 μl of 5x reducing sample buffer to tubes S1-S4
17. Resuspend pellets in 200 μl of 1x reducing sample buffer
18. Boil supernatant and pellet samples for 5 min
19. Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes
20. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
    Use 40 μl of supernatant and pellet for samples 1 and 2, and 10 μl of each for samples 3 and 4 which will result in equal actin concentration loading between the 0.1 mg/ml and 0.4 mg/ml samples
21. Visualize with Coomassie staining. See Figure 4

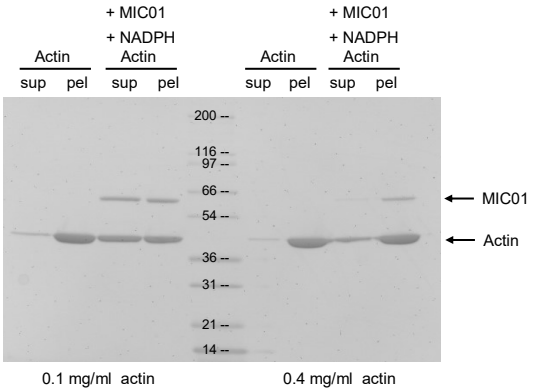

Figure 4. Actin Sedimentation +/- MICAL-1 Treatment. Actin (Cat# AKL99) was diluted to 0.2 mg/ml (4.6 μM) or 0.8 mg/ml (18.4 μM) (see method). Samples were then incubated with 2x polymerization buffer or 2x polymerization buffer supplemented with MIC01 + NADPH for 1 h at room temperatire. Samples were spun in an ultracentrifuge at 100,000 g for 1.5 h. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

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Using MICAL as a research tool: Oxidized Actin Polymerization Application

Rationale:

MICAL-1 (MIC01) is a protein that can specifically oxidize actin at amino acids M44 and M47 (β-actin nomenclature). When actin is oxidized at M44 its ability to polymerize is significantly diminished [3]. However, with increasing concentrations, oxidized actin can form augmented polymers [8]. Actin polymerization assays can be used to measure the polymerization capabilities of untreated versus MICAL-oxidized actin.

Expected Results:

Effective MICAL-1 (MIC01) + NADPH oxidation of actin will reduce >90% of pyrene labeled actin fluorescence signal at 1 hour when actin polymerization is performed at 0.05 mg/ml of pyrene-labeled actin.

Reagents Needed:

MICAL (Cat. # MIC01): When used as described below, 50 μg is sufficient for 700 μl of 2x pol buffer + NADPH + MIC01 (see Table 6), equivalent to 6 polymerization assays).

Pyrene-labeled rabbit skeletal muscle actin (Cat. # AP05)

G-buffer (Cat. # BSA01)

Precision red advanced protein assay (Cat. # ADV02)

2x polymerization buffer (5 mM Tris pH 7.5, 4 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)

NADPH (Cat. # N7505; Sigma)

Equipment Needed:

1. Fluorescence spectrophotometer with an excitation wavelength of 360 +/- 20 nm and an emission wavelength of 405 +/ - 10 nm or 420 +/- 20 nm. Cytoskeleton recommends the SPECTRAFluor Plus from TECAN Austria GmbH or Gemini from Molecular Devices Inc.
2. Black polystyrene 96 well assay plate (Costar, Cat. # 3915).

Method:

1. Dilute pyrene-labeled rabbit skeletal muscle actin (Cat. # AP05) to 0.45 mg/ml with G-buffer (Cat. # BSA01) supplemented with 0.2 mM ATP and 1 mM DTT. Leave on ice for 1 h to depolymerize actin oligomers
2. Centrifuge at 14,000 rpm at 4°C for 30 min to remove nucleating centers
3. Determine the protein concentration
4. Dilute pyrene-labeled actin (AP05) to 0.1 mg/ml with G-buffer
5. Resuspend one tube of MIC01 with 50 μl of water. The approximate concentration will be 1 μg/ul (16.67μM). This is your stock tube
6. Prepare NADPH to 100mM stock; (100mg NADPH in 1.2 ml of 0.01 M NaOH)
7. Prepare the following Assay Polymerization buffers as shown in Table 6
8. Pipet 100 μl of G-buffer into two wells (A1-2) (control samples)
9. Pipet 100 μl of the actin sample into eight wells (A3-10) of a black 96 well plate.
10. Place the 96 well plate into the fluorescent spectrophotometer and read the samples for 3 min to establish a baseline fluorescent measurement
11. After 3 min add 100 μl of Assay Polymerization Buffers into assigned wells (see Table 6)
12. Return the plate to the spectrophotometer and read the fluorescence every 30 s for 1 h
13. A typical polymerization fluorescent enhancement curve is shown in Figure 5

Table 6: Composition of Assay Polymerization Buffers

Assay Polymerization Buffers	2X polymerization Buffer	NADPH 100 mM Stock	MIC01 16.67 μM Stock	Add to Lanes: (step 11)
2x pol buffer	500 μl	0	0	A1-4
2x pol buffer + NADPH	220 μl	0.88 μl 400 μM	0	A5-6
2x pol buffer + MIC01	220 μl	0	15.8 μl 1.2 μM	A7-8
2x pol buffer + NADPH + MIC01	220 μl	0.88 μl 400 μM	15.8 μl 1.2 μM	A9-10

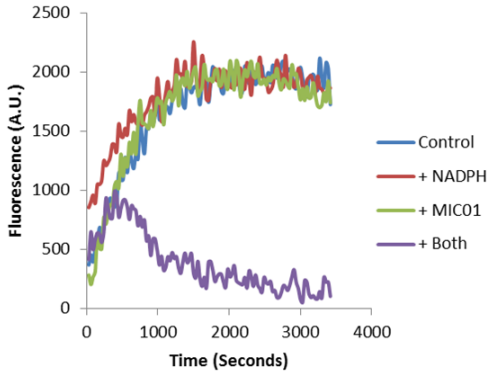

Figure 5. Actin Polymerization +/- MICAL-1 Treatment. Pyrene labeled-actin (Cat. # AP05) was diluted to 0.1 mg/ml (2.3 μM) (see method). Samples were then incubated with 2x polymerization buffer supplemented with nothing (labeled Control), NADPH alone (labeled +NADPH), MIC01 alone (labeled +MIC01), or the combination of both (labeled +Both). Upon actin polymerization fluorescence was detected with a spectrophotometer (see method below). A.U. = arbitrary units

References

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2. Wu H, Yesilyurt HG, Yoon J, Terman JR. The MICALs are a Family of F-actin Dismantling Oxidoreductases Conserved from Drosophila to Humans. *Sci Rep*. 2018;8(1):937, 10.1038/s41598-017-17943-5.
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5. Lee BC, Peterfi Z, Hoffmann FW, Moore RE, Kaya A, Avanesov A, et al. MsrB1 and MICALs regulate actin assembly and macrophage function via reversible stereoselective methionine oxidation. *Mol Cell*. 2013;51(3):397-404, 10.1016/j.molcel.2013.06.019.
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V. 2.4

6xHis MICAL-1 Protein Redox-CH domains

(Human recombinant)
Cat. # MIC01-XL
Lot:
Upon arrival, store at 4°C (desiccated)
See datasheet for storage after reconstitution

Form:	Lyophilized yellow powder
Amount of material:	1 x 1 mg
Validated applications:	Actin Oxidation at Met 44, Met 47

Background Information

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Importantly, MICAL-mediated effects on actin were not occurring through a diffusible oxidant like H_2O_2, as reductants like DTT did not alter MICAL activity, and close proximity between MICAL and actin were necessary for oxidation [3]. MICAL oxidation is both physiological and enzymatically reversible by the MsrB family of methionine sulfoxide reductases [5,6]. Functionally, oxidation of Met44 has a profound effect on actin polymerization because the residue resides in the D-loop of subdomain 2 of the protein, which is critical for actin subunit contacts; thus, upon oxidation, Met44 becomes negatively charged and interferes with actin monomer-monomer interaction and promotes F-actin severing and depolymerization [7].

Regulation of actin oxidation at Met44/Met47 has been shown to destabilize F-actin in vivo [4] and to play a key role in a growing number of cellular processes, including, cytokinesis [10], axonal guidance, dendritic organization, synaptic development, heart and muscle development and cell viability [6].

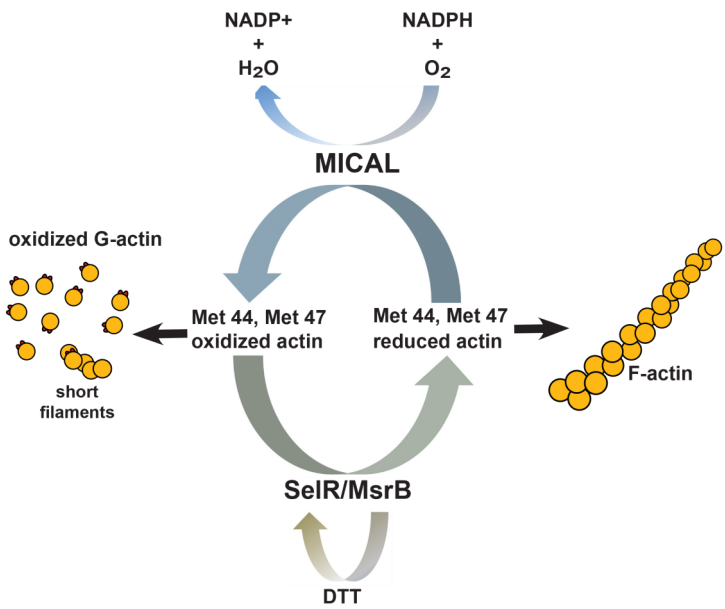

Figure 1. Actin Met 44 and Met 47 physiological redox system. A schematic diagram of the enzymes that control the physiological and reversible oxidation and reduction of methionines 44 and 47 of actin. Specifically, the MICAL family of proteins regulate oxidation of actin at Met 44 and Met 47. Conversely, the SelR/MsrB family of methionine sulfoxide reductase family specifically reduces actin at Met 44 and Met 47.

Material

Human MICAL1 protein (MIC01) [accession # Q8TDZ2 (MICA1_HUMAN)] has been produced and purified from a bacterial expression system. The recombinant protein is N-terminally 6x Histidine tagged and is comprised of the Redox and CH domains (aa: 1 -612) of the full length protein. MIC01 has an approximate molecular weight of 60 kDa. MIC01 is supplied as a yellow lyophilized powder.

Storage and Reconstitution

Shipped at ambient temperature. The lyophilized protein can be stored desiccated at 4°C for 6 months. For reconstitution, the product tube should be briefly centrifuged to collect the powder to the bottom of the tube.

Each tube of MIC01 contains 1 mg of protein. When reconstituted with 1 ml of water, the final buffer composition is 2 mM Tris pH 8.0, 20 mM NaCl, 1% sucrose, and 0.2% dextran. We recommend you make 10 µl aliquots, flash freeze, and store at –70°C.

When stored and reconstituted as described, MIC01 is stable for 6 months at –70°C.

Purity

Protein purity is determined by scanning densitometry of Coomassie Blue stained protein on a 4-20% gradient tris-glycine gel. MICAL-1 protein was determined to be ≥ 90% pure (**Figure 2**).

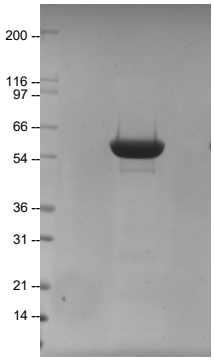

Figure 2. MICAL-1 Protein Purity Determination. A 10 µg sample of MICAL-1 protein was separated by electrophoresis in a 4- 20% tris-glycine gel and stained with Coomassie Blue. Protein quantitation was performed using the Precision Red™ Protein Assay Reagent (Cat. # ADV02). Mark12 standard molecular weight markers are from Invitrogen.

Activity

Effective MICAL-1 (MIC01) + NADPH oxidation of actin will reduce subtilisin A cleavage at M47/G48 by > 90%. See the next page for details.

Evaluating MICAL Activity: Subtilisin A Limited Proteolysis Application

Rationale:
MICAL-1 (MIC01) is a protein that can specifically oxidize actin at amino acids M44 and M47 (β-actin nomenclature). It has been reported that M44 oxidation by MICAL is concomitant with M47 oxidation [3]. Subtilisin A is a protease that has been shown to specifically cleave actin between M47 and G48 [8,9]. When actin is oxidized at M47 by MICAL the efficiency of subtilisin A cleavage is significantly reduced [8]. Therefore, subtilisin A can be used in limited proteolysis assays to determine MICAL activity, and efficiency of M47 and M44 oxidation by evaluating cleaved versus uncleaved actin.

Expected Results:
Effective MICAL-1 (MIC01) + NADPH oxidation of actin will reduce subtilisin A cleavage at M47/G48 by > 90% under the conditions described below.

Reagents Needed:
MICAL (Cat. # MIC01): When used as described below, 1 mg will oxidize 40 ml of actin at 0.5 mg/ml
Rabbit skeletal muscle actin (Cat. # AKL99)
G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)
Precision red advanced protein assay (Cat. # ADV02)
2x polymerization buffer (5 mM Tris pH 7.5, 4 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)
NADPH (Cat. # N7505; Sigma)
Phenylmethylsulfonyl fluoride (PMSF)
Subtilisin A (Cat. # P5380; Sigma)

Method:
Day 1
1. Resuspend rabbit skeletal muscle actin (Cat. # AKL99) to 1.0 mg/ml with G-buffer (Cat. # BSA01) supplemented with 0.2 mM ATP and 0.5 mM DTT
2. Incubate the actin on ice for 1 h
3. Centrifuge the actin in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the supernatant to a new tube on ice
5. Determine the protein concentration. We recommend using precision red advanced protein assay (Cat. # ADV02)
6. Add an equivalent volume of 2x polymerization buffer. The F-actin concentration should be 0.5 mg/ml (11.9 μM)
7. Incubate the actin at RT for 1 h
8. Resuspend one tube of MIC01 with 50 μl of water. The approximate concentration will be 1 μg/ul (16.67μM). This is your stock tube
9. Prepare NADPH to 100 mM stock; (100 mg NADPH in 1.2 ml of 0.01 M NaOH)
10. Prepare the following 4 samples in 1.5 ml centrifuge tubes as shown in Table 1
11. Leave samples at RT for 2 hours on your benchtop. This will allow MICAL to oxidize actin when NADPH is present
12. After oxidation, dialyze samples in G-buffer overnight.

Table 1: Composition of Experimental Samples for MICALOxidation

sample	Name	F-actin 11.9 μM	NADPH 100 mM stock	MIC01 16.67 μM stock
1	AKL99	500 μl	0	0
2	AKL99 + NADPH	500 μl	2 μl	0
3	AKL99 + MIC01	500 μl	0	12 μl
4	AKL99 + MIC01 + NADPH	500 μl	400 μM Final	0.4 μM Final

Table 2: Composition of Experimental Samples for Subtilisin Digestion

Sample	Name	Add Subtilisin	Add PMSF
1	ALK99	No	Yes
2	AKL99	Yes	Yes
3	AKL99 + NADPH	No	Yes
4	AKL99 + NADPH	Yes	Yes
5	AKL99 + MIC01	No	Yes
6	AKL99 + MIC01	Yes	Yes
7	AKL99 + Both	No	Yes
8	AKL99 + Both	Yes	Yes

Day 2
1. Centrifuge the samples in a microcentrifuge at 14,000 rpm for 30 min at 4°C
2. Determine the protein concentration
3. Make a 100 μl working stock at 0.1 mg/ml of samples 1-4. Use G-buffer for diluting samples
4. Aliquot 20 μl (2 μg actin) of each sample into individual 1.5 ml tubes. 2 aliquots per sample are needed for untreated versus subtilisin treated conditions: You should have the following 8 conditions as shown in Table 2
5. Before beginning subtilisin treatment boil a beaker of water. The water should be boiling when the subtilisin reaction is complete to inactivate the protease
5. Make a stock of subtilisin: 1 mg of subtilisin can be dissolved in 32 ml of 2 mM Tris pH 8.0/0.2 mM CaCl2 to make a 31.25 μg/ml stock. Aliquot and store at -70ºC.
6. Dilute a tube of subtilisin stock to 2 μg/ml with G-buffer.
   (*Subtilisin analysis: 1:200 w/w subtilisin to actin = 10 ng subtilisin / 2ug actin*)
5. Add 5 ul (10 ng) of subtilisin to treated condition for all 4 samples
   (*Subtilisin should be diluted and added to sample in less than 10 min*)
5. Treat actin samples with subtilisin for 15 min
6. During treatment make PMSF (10 mM: 1.74 mg PMSF per ml of isopropanol).
7. After 15 min of subtilisin treatment, stop the reactions by adding 1 μl of PMSF to each sample (1-8)
8. Add 5 μl of 5x reducing sample buffer to each sample (1-8)
   NOTE: Immediately boil samples once reducing buffer is added, as denaturing the actin sample allows trace activity of subtilisin A to rapidly cleave actin.
9. Immediately place samples into boiling water. Leave samples for 5 minutes
10. Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes.
11. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
12. Visualize with Coomassie staining. See Figure 3

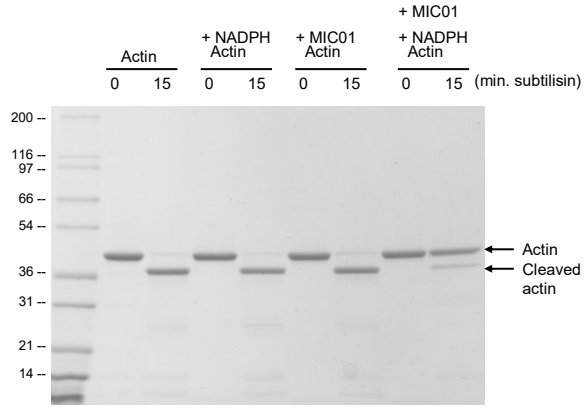

Figure 3. Subtilisin Assay on MICAL-1 Treated Actin. Actin (Cat. # AKL99) was diluted to 0.1 mg/ml (2.3 μM) and left untreated, or treated with NADPH (400 mM), MIC01 (0.076 μM) , or a combination of both (see method). 2 μg of each sample was then left untreated, or treated with subtilisin (1:200 w/w) for 15 min. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

Using MICAL as a research tool: Oxidized Actin Sedimentation Application

Rationale:

MICAL-1 (MIC01) is a protein that can specifically oxidize actin at amino acids M44 and M47 (β-actin nomenclature). When actin is oxidized at M44 its ability to polymerize is significantly diminished [3]. However, with increasing concentrations, oxidized actin can form augmented polymers [8]. Sedimentation assays can be used to measure the polymerization capabilities of untreated versus MICAL-oxidized actin.

Expected Results:

Effective MICAL-1 (MIC01) + NADPH oxidation of actin will reduce the % of actin that will polymerize relative to unoxidized actin in an actin concentration dependent manner as shown in Table 3

Table 3: Expected % actin polymer formed with unoxidized versus oxidized actin

	% polymer unoxidized actin	% polymer MIC01 oxidized actin
0.1 mg/ml actin	80-90%	40-50%
0.4 mg/ml actin	85-95%	68-78%

Reagents Needed:

MICAL (Cat. # MIC01): When used as described below, 1 mg is sufficient for 14 ml of 2x pol buffer + NADPH + MIC01 (see Table 4), equivalent to 127 polymerization assays.

Rabbit skeletal muscle actin (Cat. # AKL99)

G-buffer (Cat. # BSA01) (5 mM Tris pH 8.0, 0.2 mM CaCl2, 0.2 mM ATP, 0.5 mM DTT)

Precision red advanced protein assay (Cat. # ADV02)

2x polymerization buffer (5 mM Tris pH 7.5, 4 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)

NADPH (Cat. # N7505; Sigma)

Method:

1. Resuspend rabbit skeletal muscle actin (Cat. #AKL99) to 1.0 mg/ml with G-buffer (Cat. # BSA01) supplemented with 0.2 mM ATP and 0.5 mM DTT
2. Incubate the actin on ice for 1 h
3. Centrifuge the actin in a microcentrifuge at 14,000 rpm for 30 min at 4°C
4. Transfer the supernatant to a new tube on ice
5. Determine the protein concentration
6. Dilute actin to the following conc. 0.8 mg/ml and 0.2 mg/ml with G-buffer
7. Resuspend one tube of MIC01 with 50 µl of water. The approximate concentration will be 1 µg/ul (16.67µM). This is your stock tube
8. Prepare NADPH to 100mM stock; (100mg NADPH in 1.2 ml of 0.01 M NaOH)
9. Prepare Assay Polymerization Buffers as shown in Table 4
10. Prepare four microcentrifuge tubes labeled 1-4

Table 4: Composition of Assay Polymerization Buffers

Assay Polymerization Buffers	2X polymerization Buffer	NADPH 100 mM Stock	MIC01 16.67 µM Stock
2x pol buffer	250 µl	No	No
2x pol buffer + NADPH + MIC01	250 µl	1 µl 400 µM	18 µl 1.2 µM

Table 5: Composition of Experimental Samples

sample	name	0.2mg/ml actin	0.8 mg/ml actin	2x pol buffer	2x pol buffer +MIC01 + NADPH
1	AKL99 - 0.1 mg/ml	110 µl	0	110 µl	0
2	AKL99 + MIC01+ NADPH - 0.1 mg/ml	110 µl	0	0	110 µl
3	AKL99 - 0.4 mg/ml	0	110 µl	110 µl	0
4	AKL99 + MIC01 + NADPH - 0.4 mg/ml	0	110 µl	0	110 µl

11. Prepare the following samples in the microcentrifuge tubes as shown in Table 5
12. Transfer 200 µl of each sample to tubes compatible for ultracentrifugation.
    Accurate loading is important for tube balance
13. Incubate at room temperature for 1 h
14. Centrifuge at 100,000 g in an ultracentrifuge for 1.5 h
15. After centrifugation remove the top 180 µl of supernatant from each sample and place into new 1.5 ml tubes labeled S1-S4
    Leave the remaining supernatent, as the pellet may become detached if disturbed
16. Add 36 µl of 5x reducing sample buffer to tubes S1-S4
17. Resuspend pellets in 200 µl of 1x reducing sample buffer
18. Boil supernatant and pellet samples for 5 min
19. Briefly spin samples at 10,000 rpm to collect samples at the bottom of the tubes
20. Load a 4-20% tris-glycine gel and run at 170 V until the dye front reaches the end of the gel
    Use 40 µl of supernatant and pellet for samples 1 and 2, and 10 µl of each for samples 3 and 4 which will result in equal actin concentration loading between the 0.1 mg/ml and 0.4 mg/ml samples
21. Visualize with Coomassie staining. See Figure 4

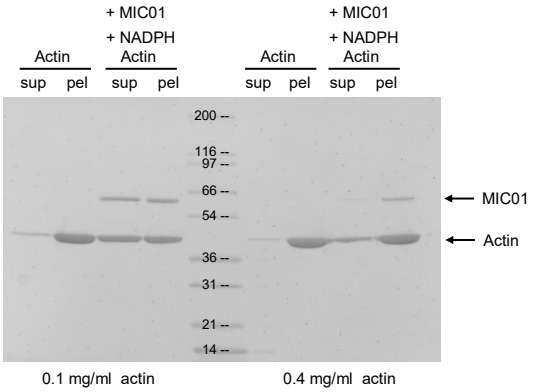

Figure 4. Actin Sedimentation +/- MICAL-1 Treatment. Actin (Cat# AKL99) was diluted to 0.2 mg/ml (4.6 µM) or 0.8 mg/ml (18.4 µM) (see method). Samples were then incubated with 2x polymerization buffer or 2x polymerization buffer supplemented with MIC01 + NADPH for 1 h at room temperatire. Samples were spun in an ultracentrifuge at 100,000 g for 1.5 h. Samples were then separated by SDS-PAGE and visualized with Coomassie staining.

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Using MICAL as a research tool: Oxidized Actin Polymerization Application

Rationale:
MICAL-1 (MIC01) is a protein that can specifically oxidize actin at amino acids M44 and M47 (β-actin nomenclature). When actin is oxidized at M44 its ability to polymerize is significantly diminished [3]. However, with increasing concentrations, oxidized actin can form augmented polymers [8]. Actin polymerization assays can be used to measure the polymerization capabilities of untreated versus MICAL-oxidized actin.

Expected Results:
Effective MICAL-1 (MIC01) + NADPH oxidation of actin will reduce >90% of pyrene labeled actin fluorescence signal at 1 hour when actin polymerization is performed at 0.05 mg/ml of pyrene-labeled actin.

Reagents Needed:
MICAL (Cat. # MIC01): When used as described below, 1 mg is sufficient for 14 ml of 2x pol buffer + NADPH + MIC01 (see Table 6), equivalent to 127 polymerization assays).
Pyrene-labeled rabbit skeletal muscle actin (Cat. # AP05)
G-buffer (Cat. # BSA01)
Precision red advanced protein assay (Cat. # ADV02)
2x polymerization buffer (5 mM Tris pH 7.5, 4 mM $MgCl_2$, 100 mM KCl, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT)
NADPH (Cat. # N7505; Sigma)

Equipment Needed:
1. Fluorescence spectrophotometer with an excitation wavelength of 360 +/- 20 nm and an emission wavelength of 405 +/ - 10 nm or 420 +/- 20 nm. Cytoskeleton recommends the SPECTRAFluor Plus from TECAN Austria GmbH or Gemini from Molecular Devices Inc.
2. Black polystyrene 96 well assay plate (Costar, Cat. # 3915).

Method:
1. Dilute pyrene-labeled rabbit skeletal muscle actin (Cat. # AP05) to 0.45 mg/ml with G-buffer (Cat. # BSA01) supplemented with 0.2 mM ATP and 1 mM DTT. Leave on ice for 1 h to depolymerize actin oligomers
2. Centrifuge at 14,000 rpm at 4°C for 30 min to remove nucleating centers
3. Determine the protein concentration
4. Dilute pyrene-labeled actin (AP05) to 0.1 mg/ml with G-buffer
5. Resuspend one tube of MIC01 with 50 µl of water. The approximate concentration will be 1 µg/ul (16.67µM). This is your stock tube
6. Prepare NADPH to 100mM stock; (100mg NADPH in 1.2 ml of 0.01 M NaOH)
7. Prepare the following Assay Polymerization buffers as shown in Table 6
8. Pipet 100 µl of G-buffer into two wells (A1-2) (control samples)
9. Pipet 100 µl of the actin sample into eight wells (A3-10) of a black 96 well plate.
10. Place the 96 well plate into the fluorescent spectrophotometer and read the samples for 3 min to establish a baseline fluorescent measurement
11. After 3 min add 100 µl of Assay Polymerization Buffers into assigned wells (see Table 6)
12. Return the plate to the spectrophotometer and read the fluorescence every 30 s for 1 h
13. A typical polymerization fluorescent enhancement curve is shown in Figure 5

Table 6: Composition of Assay Polymerization Buffers

Assay Polymerization Buffers	2X polymerization Buffer	NADPH 100 mM Stock	MIC01 16.67 µM Stock	Add to Lanes: (step 11)
2x pol buffer	500 µl	0	0	A1-4
2x pol buffer + NADPH	220 µl	0.88 µl	0	A5-6
2x pol buffer + MIC01	220 µl	0	15.8 µl	A7-8
2x pol buffer + NADPH + MIC01	220 µl	0.88 µl 400 µM	15.8 µl 1.2 µM	A9-10

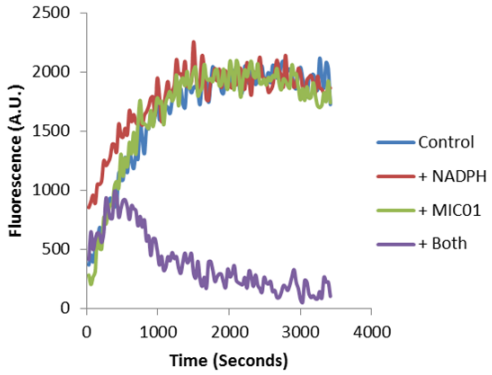

Figure 5. Actin Polymerization +/- MICAL-1 Treatment. Pyrene labeled-actin (Cat. # AP05) was diluted to 0.1 mg/ml (2.3 µM) (see method). Samples were then incubated with 2x polymerization buffer supplemented with nothing (labeled Control), NADPH alone (labeled +NADPH), MIC01 alone (labeled +MIC01), or the combination of both (labeled +Both). Upon actin polymerization fluorescence was detected with a spectrophotometer (see method below). A.U. = arbitrary units

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