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Expression and Purification of FLAG-Rab3A Phosphorylated at pThr86

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Eve Napier[1], Amir Khan[1]

[1]School of Biochemistry and Immunology, Trinity College Dublin, Dublin 2, Ireland

AKhanLab

Amir Khan
Trinity College Dublin

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We use this protocol and it's working

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Abstract

Leucine Rich Repeat Kinase 2 (LRRK2) phosphorylates a subset of Rab GTPases at a conserved Ser/Thr residue in their switch 2 region. MST3 kinase phosphorylates Rab GTPases at the same site and has previously been used to phosphorylate Rab8 and Rab10. This protocol describes a method to produce milligram amounts of FLAG-tagged Rab3A (residues 18-190, Q81L) phosphorylated at Thr86.

Materials

Cells
Competent BL21(DE3)

Plasmids - ordered from Genscript
pET28a(+)-FLAG-Rab3a (18-190, Q81L)
pET28a(+)-MST3

Buffers

- Extraction buffer: 20 mM Tris-HCl pH 8, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$
- Wash buffer: 20 mM Tris-HCl pH 8, 300 mM NaCl, 40 mM imidazole, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$
- Elution buffer: 20 mM Tris-HCl pH 8, 300 mM NaCl, 200 mM imidazole, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$
- Low salt ion exchange buffer: 10mM NaCl, 10mM MES pH 5.2, 1mM DTT, 5 mM $MgCl_2$
- High salt ion exchange buffer: 1M NaCl, 10mM MES pH 5.2, 1mM DTT, 5 mM $MgCl_2$
- Phosphorylation buffer: 50mM Tris-HCl pH 7.5, 150mM NaCl, 10mM $MgCl_2$
- 10% Phostag-SDS PAGE: 1.8ml Acrylamide (40% v/v), 3.3ml ddH20, 1.8ml separating buffer, 52.5µl Phos-tag reagent (5mM)(FUJIFILM Wako), 5.25µl $MnCl_2$ (100mM), 35µl 10% APS, 7µl TEMED
- Coomassie Stain: 10% acetic acid, 40% ethanol, 0.5% brilliant blue powder (Fisher)
- Destain solution: 10% acetic acid, 40% methanol

Equipment

- AKTA basic / AKTA purifier (Cytiva)
- Mono Q 5/50 GL ion exchange column, (GE Healthcare)
- Shaking incubators (Infors: set at 18/37°C, 180 rpm)
- Gravity flow columns (Bio-Rad Econocolumn) and suitable laboratory stand
- Sonicator (Branson Sonifier 250)
- SDS-PAGE system (ATTO)

Reagents

- Sterile LB and 2xYT media
- Kanamycin (30 µg/ml stock, sterile filtered)
- IPTG (1M stock, sterile filtered)
- Bradford assay solution (Quick Start 1x, Bio-Rad #5000205)
- 10 IU Thrombin aliquots (Cytiva 27-0846-01)
- Ni-agarose resin (Thermo Scientific)

Protocol

1 pET28a(+)-FLAG-Rab3a (18-190, Q81L) and pET28a(+)-MST3 were expressed and purified following the general steps with modifications noted.

Transformation of plasmid into competent cells

2 Mix 10-20ng of pET28a(+)-FLAG-Rab3a (18-190, Q81L) plasmid with 100µl competent BL21(DE3) cells and incubate on ice for 30 minutes.

3 Heat shock the mixture for 3 minutes at 37°C and recover on ice for 2 minutes.

4 Add 1ml sterile LB media and incubate at 37°C, 800rpm for 45 minutes.

5 Plate 50µl of mixture onto LB Agar plates supplemented with 30µg/ml kanamycin prewarmed at 37°C and incubate overnight at 37°C.

6 Select a colony and grow in 2ml sterile LB media supplemented with 30µg/ml kanamycin until OD600 0.6-0.8 is reached.

7 To prepare a glycerol stock, add 700µl culture to 300µl 50% glycerol and freeze at -80°C.

Overnight Culture

8 Add scraping of glycerol stocks to 10ml sterile media and incubate overnight at 37°C, 180rpm

> Note
>
> Overnight cultures should be prepared at 1:50 final expression volume e.g., 20 ml of overnight culture to inoculate 1L of media.

Inoculation and Induction of protein expression

9 Add 30µg/ml Kanamycin to 1L sterile 2xYT and inoculate with overnight culture.

Note

MST3 must be expressed in LB media.

10 Incubate at 37°C, 180 rpm (120-180) for 1.5-2 hours.

11 Check the absorbance of a sample at 600nm.

Note

After OD600 reaches ~0.2 it doubles every 20-25 minutes.

12 Once OD600 reaches 0.6-0.8, move the flask to 18°C and continue shaking for ~30 minutes.

13 Add 0.5 mM IPTG and continue to incubate overnight at 18°C, 180 rpm.

Note

MST3 should be induced with 0.1mM IPTG.

Bacterial cell collection and lysate preparation

14 Decant the bacterial culture into 500ml centrifuge tubes and centrifuge at 3500 x g for 10 mins at 4°C.

15 To purify directly, add ~25ml of cold extraction buffer supplemented with 10mM β-mercaptoethanol and 5mM $MgCl_2$ and vortex to dislodge the cell pellet, creating a suspension.

Note

All buffers should be supplemented with 5mM $MgCl_2$ when purifying FLAG-Rab3A and MST3.

Note

Alternatively, cell pellets can be stored at this stage by resuspending in 25ml PBS, centrifuging at 4000 x g for 10 mins at 4°C and freezing at -20°C.

16 Transfer suspension to a Dounce homogeniser and gently move the pestle up and down until all clumps are removed.

17 Sonicate the lysate on ice at the following settings: cycle 30%, output 4-5, 2 minutes timer (Branson Sonifier 250). Repeat sonication 3 times, leaving 1 minute between sonication rounds.

18 Transfer lysate to centrifuge tubes, balance and centrifuge at 20,000 x g for 45 minutes, at 4°C.

Purification using Ni-agarose

19 Add 5ml of resuspended Ni-agarose (50% slurry in extraction buffer) to a gravity flow column, creating a bed volume of 2.5ml.

20 Wash column with 2ml extraction buffer and let flow through until ~0.5cm liquid left on top of resin.

21 Load supernatant onto column and discard the flow through.

22 Wash with 5-10 volumes of extraction buffer until no more protein comes off the column.

Note

To test if there is protein coming through the column, 30µl of Bradford reagent is added to parafilm and 3µl of flow through added - if protein is present, Bradford turns blue.

23 Apply 2-5 columns of wash buffer to the column.

Note

Wash buffer should be used with extreme caution when purifying MST3 as it is easily washed off the column.

24 To elute the bound His-tagged protein, the Ni-agarose is resuspended in 2 columns volumes of elution buffer and the flow through collected on ice.

25 Continue to wash with elution buffer until all bound protein has been eluted (approximately 8-15ml of flow through containing the desired protein is collected).

Dialysis and cleavage of His-tag

26 To remove the His-tag from His-FLAG-Rab3A, it first must be dialysed into a buffer containing less imidazole. MST3 is left uncleaved however it is also dialysed into a buffer containing less imidazole which is more appropriate for phosphorylation.

27 1L of extraction buffer is prepared with 1mM DTT rather than β-mercaptoethanol.

28 Eluted protein is added to a dialysis tube cut to an appropriate length suspended in the buffer and incubated with gentle stirring for ~1 hr at 4°C.

29 Thrombin protease is added to FLAG-Rab3A in the dialysis tube (2 × 10 IU per tube) and incubated overnight at 4°C.

30 Transfer cleaved protein out of the dialysis tube.

30.1 At this stage MST3 can be used for phosphorylation reactions without further purification (Figure 1). If desired it can be concentrated in an Amicon® Ultra centrifugal filter.

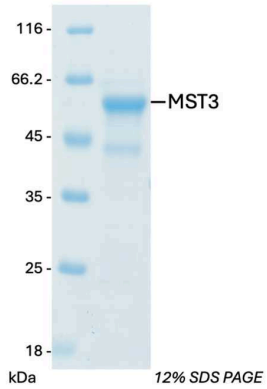

Figure 1: Coomassie stained 12% SDS PAGE gel showing purified MST3 kinase

31 Add 3 ml 50% Ni-agarose resin to the tube and incubate on rotator for 15 minutes, 15 rpm, 4°C.

32 Transfer Ni-agarose and cleaved protein into a gravity flow column and allow resin to settle.

33 Open stopcock and collect flow through containing cleaved protein on ice.

34 Wash column with 1-2ml extraction buffer to wash off additional cleaved protein.

35 Add protein to an Amicon® Ultra centrifugal filter and centrifuge at 4000 x g at 4°C in 10 minute increments until desired concentration is reached.

Note

Samples can be taken throughout purification process for analysis by SDS PAGE to monitor progress. A 15µl sample should be mixed with 4X SDS loading buffer and boiled at 96°C for 5 minutes.

Phosphorylation of FLAG-Rab3A

36 To phosphorylate the Rab protein, approximately 8mg of purified FLAG-Rab3A are incubated with 2mg purified MST3 and 2mM ATP and the volume made up to 4ml with

phosphorylation buffer.

Note

ATP is prepared as a 100mM stock and should be adjusted to pH >7.5.

37 Incubate the mixture for 40 minutes at room temperature on a floor roller.

38 Add to an Amicon® Ultra centrifugal filter, topped up with low salt ion exchange buffer and centrifuged at 4000 x g at 4°C for approximately 10-15 minutes until a volume of 1.5-2ml is reached.

39 Top up the centrifugal filter with low salt ion exchange buffer and centrifuge until a volume of 3-4ml is reached.

40 Load the protein onto a Mono Q 5/50 GL ion exchange column, connected to an AKTA basic or AKTA purifier and equilibrated in low salt ion exchange buffer at 4°C at a flow rate of 0.5ml/min.

41 Elute protein in 0.5ml fractions with a 0-35% gradient from low-to-high salt buffer.

42 Fractions containing phospho-FLAG Rab3A are identified by Phostag gel electrophoresis and pooled (Figure 2).

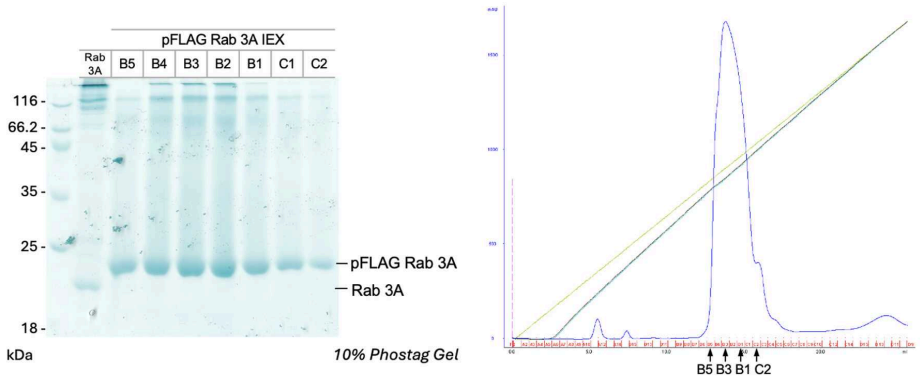

Figure 2: Ion Exchange of phospho-FLAG Rab3A and Coomassie stained 10% Phostag Gel with IEX fractions

Phostag gel electrophoresis

43 Prepare a 10% Phostag separating gel with 1.8ml Acrylamide (40% v/v), 3.3ml ddH20, 1.8ml separating buffer, 52.5µl Phos-tag reagent (5mM)(FUJIFILM Wako), 5.25µl MnCl2 (100mM), 35µl 10% APS, 7µl TEMED.

44 Add approximately 7ml to the gel cast, top with 1ml isopropanol and leave to set for 1 hour.

45 Make a stacking gel, add to the gel cast along with a comb and set for 30 minutes.

46 Samples are prepared for gel analysis by mixing 15µl of protein with 5µl 4X SDS loading buffer and boiling at 96°C for 5 minutes. 10 MnCl2 is then added.

47 Load 5µl of samples onto the gel and run at 90V for 30 minutes followed by a further 2-2.5 hours at 100V.

48 Stain the gel by Coomassie staining for 1 hour followed by destaining until bands are clearly visible (Figure 2).