

A Novel Method for the Production of Fully Modified K-Ras 4B

Tanmay S. Chavan*, James O. Meyer*, Luke Chisholm, Malgorzata Dobosz-Bartoszek, and Vadim Gaponenko

Abstract

Post-translational modifications in proteins play a major functional role. Post-translational modifications affect the way proteins interact with each other, bind nucleotides, and localize in cellular compartments. Given the importance of post-translational modifications in protein biology, development of methods to produce post-translationally modified proteins for biochemical and biophysical studies is timely and significant. At the same time, obtaining post-translationally modified proteins in bacterial expression systems is often problematic. Here, we describe a novel recombinant approach to prepare human K-Ras 4B, a protein that is post-translationally farnesylated, proteolytically cleaved, and methylated in its C-terminus. K-Ras 4B is a member of the Ras subfamily of small GTPases and is of interest because it is frequently mutated in human cancer.

The method relies on separate production of two structural domains—the N-terminal catalytic domain and the C-terminal peptide chemically modified with S-farnesyl-L-cysteine methyl ester. After the two domains are prepared, they are ligated together using the transpeptidase enzyme, sortase. Our procedure starts with the use of the plasmid of K-Ras 4B catalytic domain containing the sortase recognition sequence. After this, we describe the bacterial expression and purification steps used to purify K-Ras 4B and the preparation of the conjugated C-terminal peptide. The procedure ends with the sortase-mediated ligation technique. The produced post-translationally modified K-Ras 4B is active in a number of assays, including a GTP hydrolysis assay, Raf-1 binding assay, and surface plasmon resonance-based phospholipid binding assay.

Key words K-Ras 4B, Post-translational modification, Farnesylation, Sortase ligation, Expression, Purification, Cross-linking

1 Introduction

Approximately 0.5 % of all eukaryotic proteins undergo a form of post-translational modification known as prenylation [1]. Prenylation is an enzymatic reaction that results in addition of either a farnesyl or geranylgeranyl moiety by farnesyltransferase (FT) or geranylgeranyltransferase (GGT) enzymes, respectively. These prenyl groups

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facilitate protein–protein and protein–lipid interactions and regulate signal-transduction cascades.

Proteins modified with a farnesyl moiety include members of the Ras family of proto-oncogenes [2]. Ras proteins are small GTPases that play an essential role in many signaling processes. Three isoforms of classical Ras proteins have been discovered: H-, N-, and K-Ras. Ras proteins share a basic structure, comprising a nucleotide binding catalytic domain and a C-terminal hypervariable region (HVR), which harbors the farnesyl modification. The farnesyl group directs Ras proteins to specific locations in the cellular membrane where signaling is initiated [3, 4].

To study the details of farnesyl-mediated anchoring in the membrane and protein–protein interactions, sufficient amounts of modified proteins must be produced for *in vitro* experiments. While it is possible to prepare sizeable quantities of unmodified K-Ras [5], synthesis of fully modified K-Ras is far more problematic. It is hard to purify naturally modified K-Ras from mammalian cells because it has many interaction partners.

K-Ras 4B is a ubiquitously expressed splice variant of K-Ras. Its 3-step process of post-translational modification begins with the addition of a farnesyl group to a C-terminal CAAX sequence in the HVR, after which the AAX peptide is removed by Rce1 protease. Finally, the terminal cysteine is methylated by the isoprenylcysteine carboxyl methyltransferase (ICMT enzyme). The methylation step removes the C-terminal negative charge, renders the protein more hydrophobic, and aids in membrane binding [6, 7].

Recent progress in solid- and solution-phase procedures for preparation of synthetic lipidated peptides has allowed a more practical method to produce modified K-Ras 4B [8]. In this instance, the lipidated peptide corresponds to the K-Ras 4B HVR and can be attached to truncated K-Ras 4B via intein-mediated protein splicing or maleimidocaproyl-controlled ligation. This method requires custom production of a peptide with the post-translational modifications introduced during the peptide synthesis procedure.

Our method uses commercially available components for preparation of fully modified K-Ras 4B. To create the post-translationally modified HVR, we conjugate S-farnesyl-L-cysteine methyl ester to the isolated HVR peptide of K-Ras 4B. The bacterial transpeptidase, sortase, is subsequently used to ligate the modified HVR peptide to the truncated K-Ras 4B catalytic domain.

We demonstrate that the fully modified K-Ras 4B possesses GTPase activity, effector (Raf-1) binding capability and associates with membrane phospholipids. In conclusion, the approach that we describe to produce fully modified K-Ras 4B provides an opportunity to study the effect of post-translational modifications on protein–protein and protein–membrane interactions. The developed procedure can potentially be applied to other proteins that undergo similar post-translational modifications.

2 Materials

Prepare all solutions using ultrapure deionized water, with reagents stored at room temperature (21–25 °C) unless specified otherwise.

2.1 Bacterial Expression

1. Cloned K-Ras 4B catalytic domain construct with a C-terminal sortase recognition sequence “LPXTG,” where X is any amino acid (*see Note 1*), followed by a His-Tag, and two stop codons. We express this protein in a PET 42a vector (Novagen) stored at –20 °C.
2. One-shot BL 21AI cells (Invitrogen) stored at –80 °C.
3. Sterile SOC medium (Invitrogen, *see Note 2*) stored at 4 °C.
4. 50 mg/mL kanamycin sulfate stored at –20 °C.
5. LB cell medium: 25 g/L Luria Broth (LB)-Miller powder.
6. 10 % L-arabinose solution.
7. 100 % ethanol, molecular grade.
8. 1 M isopropyl β -D-1-thiogalactopyranoside (IPTG) stored at –20 °C.

2.2 Protein Extraction

1. First Extraction Buffer—Make one solution containing the following: 10 mM magnesium chloride (MgCl_2), 2 mM phenylmethylsulfonyl fluoride (PMSF) stored at 4 °C (Pierce), 50 $\mu\text{g/mL}$ DNaseI stored at –20 °C, 1 tablet of EDTA-free Complete (Roche Protease Inhibitor cocktail) per 50 mL of buffer volume, 10 mg lysozyme per 50 mL buffer volume, stored at –20 °C (*see Note 3*). Make the dilution in B-PER solution (Pierce), not water.
2. Second Extraction Buffer—Make one solution containing the following: 10 mM Tris-HCl pH 7.6, 50 mM KCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 2 mM β -mercaptoethanol (βME), 100 μM guanosine diphosphate (GDP) (*see Note 3*). Make the dilution in water.

2.3 Protein Purification

1. His-binding buffer containing glycerol: 20 mM Tris-HCl pH 7.6, 500 mM NaCl, 5 mM βME , 400 mL glycerol (*see Note 4*).
2. His-binding buffer: 0.5 M NaCl, 20 mM Tris-HCl pH 7.6.
3. His-wash buffer: 0.5 M NaCl, 10 mM imidazole (*see Note 5*), 20 mM Tris-HCl pH 7.6.
4. His-elution buffer: 1 M Imidazole, 20 mM Tris-HCl pH 7.6.
5. His-purification nickel-binding resin (Novagen). Store at 4 °C.
6. Disposable plastic columns (*see Note 6*).

**2.4 Farnesylation
Reaction for HVR
Peptide of K-Ras 4B
(for 500 μ L Reaction
Volume)**

1. 68 μ L of 73.6 mM S-farnesyl-L-cysteine methyl ester in DMSO.
2. 50 μ L of 50 % N-octyl glucopyranoside in dH₂O.
3. 381 μ L of phosphate buffer, pH 7.4: 137 mM NaCl, 2.68 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂ PO₄.
4. 1 μ L of 50 mM 4-dimethylaminopyridine (DTAP) stored at 4 °C.
5. 2 mg of Sulfo-SMCC crosslinker (Thermo Scientific).
6. Chemically synthesized HVR peptide (GKEKMSKDGKKK KKKSKC).

**2.5 Sortase-
Mediated Ligation**

1. 15 μ M sortase A (*see Note 7*).
2. 15 μ M K-Ras catalytic domain stored at 4 °C.
3. Tris-EDTA buffer pH 9: 50 mM Tris-HCl, 10 mM EDTA, 10 mM MgCl₂, 5 mM CaCl₂
4. 10 mM β ME.
5. 30 μ M HVR peptide of K-Ras conjugated with S-farnesyl-L-cysteine methyl ester using Sulfo-SMCC crosslinker.

**2.6 GTPase
Activity Assay**

1. 0.1 M GTP stored at -80 °C (*see Note 8*).
2. 10 μ M fully modified K-Ras 4B stored at 4 °C.
3. 1 mM 2-amino-6-mercapto-7-methylpurine riboside (MESG) stored at -20 °C.
4. 0.5 M EDTA, pH 8.
5. Purine nucleoside phosphorylase.
6. 1 M (NH₄)₂SO₄.
7. UV spectrophotometer.

**2.7 Raf-1
Binding Assay**

1. 0.1 M GTP γ S or GDP stored at -80 °C.
2. 0.25 M EDTA solution.
3. Raf-1 Ras-binding domain (RBD) agarose beads.
4. 1 M MgCl₂ solution.
5. HEPES buffer: 25 mM HEPES, 150 mM sodium chloride, 5 mM MgCl₂, 10 mM β ME, 5 % glycerol.
6. SDS loading buffer supplemented with 5 % β ME (710 mM).

**2.8 SPR Membrane
Binding Assay**

1. Running buffer: 50 mM Tris-citrate pH 6.5, 50 mM NaCl, 5 mM MgCl₂, 10 mM β -mercaptoethanol.
2. CM-5 SPR chip immobilized with phospholipid nanodiscs (e.g., DOPC, DPPC, DPPE, or a mixture of any of these) on one flow cell and ethanolamine on another to act as reference.
3. 2 M sodium chloride.
4. Biacore T100 SPR instrument.

3 Methods

3.1 Bacterial Expression

1. Place a single vial of one-shot BL21AI cells on ice and allow thawing. Add 1 μ L of K-Ras 4B PET 42a plasmid to vial. Keep vial on ice for 30 min.
2. Heat the vial to 42 °C for 45 s and return to ice for a further 2 min.
3. After this, add 350 μ L of sterile SOC medium to the cell vial and incubate with agitation at 37 °C and 250 rpm for 1 h.
4. Make a 350 mL solution of LB Broth supplemented with 350 μ L of 50 mg/mL kanamycin solution. Filter the solution through a 0.22 μ m pore-size membrane into a 1 L autoclaved conical flask.
5. After the SOC-cell culture has been incubated for 1 h, inoculate the 1 L LB Broth medium with the full 350 μ L of SOC-cell culture.
6. Incubate the cell culture at 37 °C and 250 rpm on the orbital shaker overnight.
7. Fill six 2,800 mL autoclaved conical flasks with 1 L of M9 medium in each [9]. Ensure that the medium has been filtered through a 0.22 μ m pore-size membrane and autoclaved beforehand.
8. Add kanamycin to each 2,800-mL flask to a final concentration of 50 μ g/mL.
9. Inoculate the 1 L of medium in each flask with 50 mL of the BL21AI/LB Broth culture and incubate at 37 °C, 250 rpm on an orbital shaker until the optical density (OD) measured at the wavelength of 600 nm reaches 0.6.
10. Induce cells by adding 200 μ L of 1 M IPTG, 40 mL of 10 % L-arabinose, and 20 mL of ethanol to each 2,800 mL flask.
11. Incubate the flasks at 18 °C for 22–24 h at 250 rpm on an orbital shaker.
12. Centrifuge cell culture at 14,000 $\times g$ for 15 min to form cell pellet. Discard supernatant. The cell pellet can be stored overnight at –80 °C if not used immediately.

3.2 Protein Extraction

3.2.1 First Extraction

1. Resuspend cell pellet in 25 mL of first extraction buffer per 1 L of cell culture. If pellet has been frozen, thaw it to room temperature first.
2. Incubate suspension at room temperature for 1 h with agitation.

3. Centrifuge suspension at $38,000 \times g$ for 35 min at 4 °C, then transfer supernatant (first extraction) to a separate vessel. Keep the pellets for the second extraction.
4. Add EDTA, KCl, β ME, and GDP to all of first extraction obtained, such that their final concentrations are 10 mM EDTA, 50 mM KCl, 5 mM β ME, and 100 μ M GDP.
5. Incubate the solution at room temperature for 2 h with agitation.
6. Dialyze first extraction in His-binding buffer containing glycerol overnight at 4 °C.

3.2.2 Second Extraction

1. Resuspend pellet from first extraction in 25 mL of second extraction buffer per 1 L of cell culture.
2. Incubate the suspension with agitation for 1 h then centrifuge at $38,000 \times g$ for 35 min.
3. Transfer supernatant (second extraction) to a separate vessel; the pellet can be discarded.
4. Dialyze the second extraction in His-binding buffer containing glycerol overnight at 4 °C.

3.3 Protein Purification (Use for Both First and Second Extractions Separately)

1. Prepare a disposable gravity column by washing with at least twice the total column volume of distilled water.
2. Add 4 mL of suspended nickel-binding resin to the column and allow storage ethanol to drain.
3. Wash the resin with at least 10 mL of His-binding buffer, then transfer resin to vessel containing the extraction.
4. Incubate the vessel at room temperature for 2 h under agitation.
5. Pour the extraction into the gravity column and wash with 500 mL of wash buffer in order to remove nonspecific binding to the nickel beads.
6. From the elution buffer stock, make 30, 60, 100, 200, and 1,000 mM imidazole elution buffers by diluting with His-binding buffer.
7. Elute the protein with elution buffer and collect the eluate in a falcon tube. Begin with 30 mL of 30 mM elution buffer followed by 20 mL of each remaining elution buffers in ascending order of imidazole concentration (*see Note 9*).
8. Run these purified fractions on an SDS-PAGE gel to confirm the presence of K-Ras catalytic domain.
9. Select the fractions with highest purity; these can then be pooled and dialyzed into Tris-EDTA buffer pH 9.

Figure 1 shows catalytic domain expressed and purified using this protocol on an SDS-PAGE gel developed with silver staining. The purity of the protein preparation is further confirmed using MALDI-TOF mass spectrometry (Fig. 2).

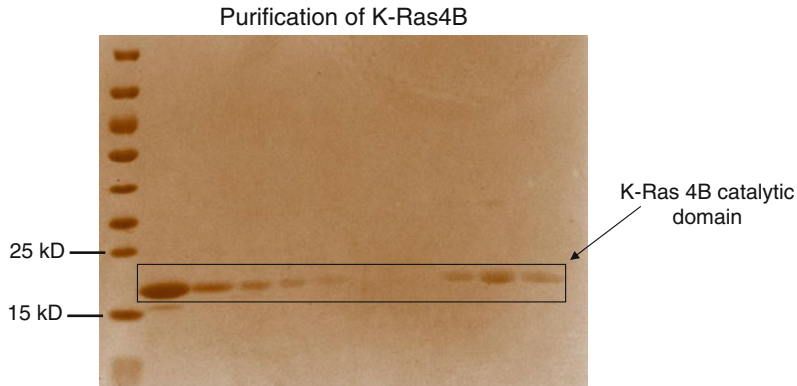


Fig. 1 Purification of K-Ras 4b. K-Ras 4B catalytic domain with N-terminal LPETG sequence and 6(His) tag, purified on a nickel column from the first extraction of 6 L of cell culture

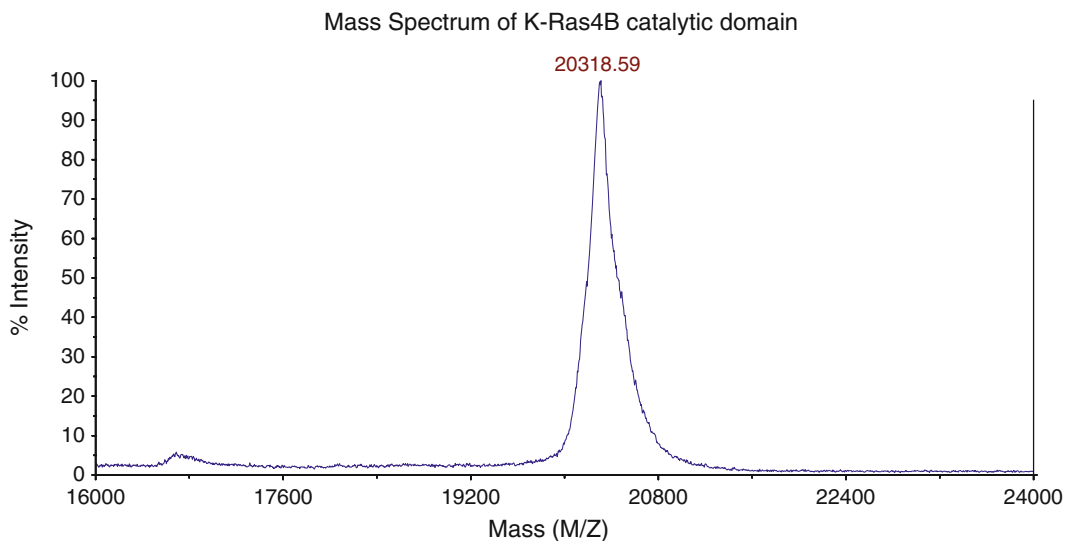


Fig. 2 Mass spectrum of K-Ras 4B catalytic domain. Mass spectrum of K-Ras 4B catalytic domain appended with N-terminal LPTTG sequence and 6(His) tag in 50 mM Tris-HCl pH 9. Sample analyzed using MALDI-TOF mass spectrometry

3.4 Farnesylation of Hypervariable Region of K-Ras4B

1. Take 68 μL of 73.6 mM S-farnesyl-L-cysteine methyl ester in 100 % dimethyl sulfoxide (DMSO) and add 2 mg of no-weigh sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC). Mix thoroughly till all of the Sulfo-SMCC dissolves.
2. Add 50 μL of 50 % (w/v) *N*-octyl- β -D-glucopyranoside to the above solution, followed by 381 μL of phosphate buffer, pH 7.4, and 1 μL of 50 mM di-tert-amyl peroxide (DTAP).
3. Incubate the reaction mix for 1.5 h and thereafter dilute to a final volume of 8 mL with phosphate buffer.

4. Centrifuge the solution at $3,000\times g$ for 30 min at 4 °C.
5. Discard the supernatant and dissolve the pellet in 150 μ L of 100 % ethanol followed by the addition of 100 μ L of 50 % (w/v) *N*-octyl- β -D-glucopyranoside.
6. Add the above solution to 2.009 mg of chemically synthesized HVR peptide dissolved in 750 μ L of 1 $\times$ phosphate buffer and incubate it at room temperature overnight.
7. Apply the reaction mix to a C-18 column and purify it by reversed phase high-performance liquid chromatography (RP-HPLC). Briefly, equilibrate the column with 90 % Buffer A (0.1 % trifluoroacetic acid) and 10 % Buffer B (90 % acetonitrile) before applying the sample. Thereafter, apply a linear gradient up to 80 % Buffer B. Elute the fractions at 66–68 % Buffer B and conduct a mass spectrometric analysis for purity.
8. Pool the pure fractions of farnesylated and methylated HVR and dry using Eppendorf Vacufuge.

3.5 Sortase-Mediated Ligation

1. Carry out reaction in 2 mL plastic microfuge tubes. To increase yield, carry out more individual reactions rather than directly scaling up the process.
2. Before proceeding with the reaction, dialyze K-Ras catalytic domain and sortase transpeptidase into pH 9 Tris–EDTA buffer (*see Note 10*).
3. Add 10 μ M K-Ras catalytic domain, 15 μ M sortase, 30 μ M F-HVR, 10 mM β ME, and 5 mM CaCl_2 to a 2 mL microfuge tube.
4. Place reaction mixture under agitation at room temperature for 48 h (*see Note 11*).
5. Halt the reaction by storing the reaction mixture at 4 °C. It is recommended that a sample of this is run on an SDS-PAGE gel to indicate the presence of reaction product (*see Note 12*).

Figure 3 shows the sortase-mediated ligation after 48 h at room temperature.

6. Separate the modified full-length recombinant K-Ras from the reaction mixture by equilibrating once again with His-nickel-binding resin (200 μ L/1 mL) for 1 h at room temperature and running through a gravity column. The product does not bind to the beads and can be immediately collected without need for elution.
7. To minimize the occurrence of nonspecific binding of the product to the nickel resin, it is recommended that NaCl is first added to the mixture to a concentration of 0.5 M before loading onto the column.

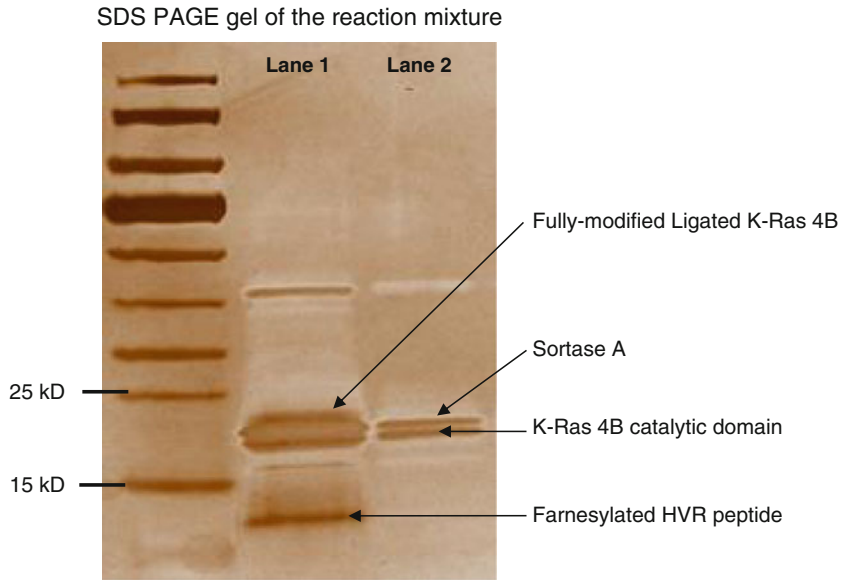


Fig. 3 SDS-PAGE gel of the reaction mixture. *Lane 1* shows the sortase-mediated ligation of K-Ras 4B catalytic domain after 48 h at room temperature. This reaction mixture contains modified product and sortase as well as unreacted HVR and catalytic domain. *Lane 2* shows sortase and K-Ras 4B catalytic domain

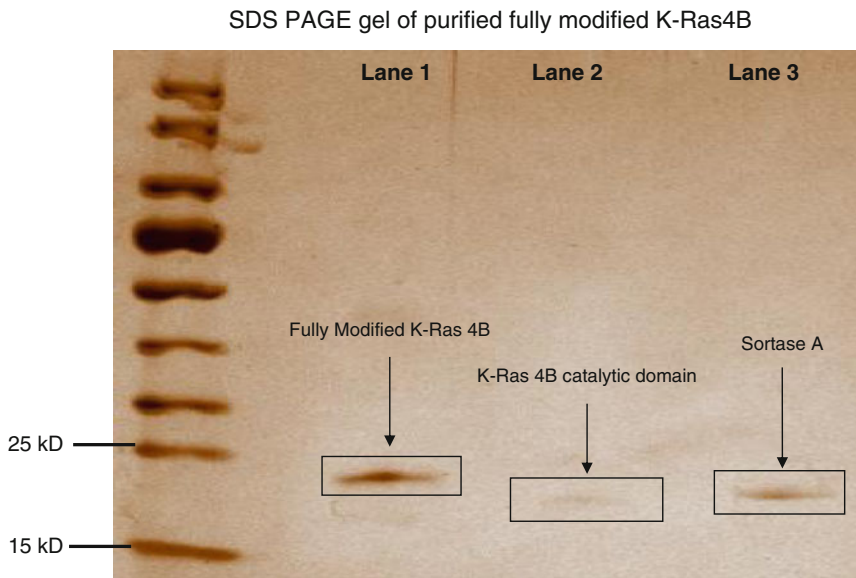


Fig. 4 SDS-PAGE gel of purified fully modified K-Ras4B. The figure shows the fully modified product after components of the sortase reaction were removed using nickel affinity purification. *Lane 1* contains fully modified K-Ras 4B. *Lanes 2* and *3* are controls with unreacted K-Ras 4B catalytic domain and sortase A as labelled

Figure 4 shows the fully modified K-Ras 4B construct purified from the sortase-mediated ligation reaction using this protocol.

Figure 5 shows the mass spectrum of the fully modified K-Ras 4B present in Fig. 4 (lane 1).

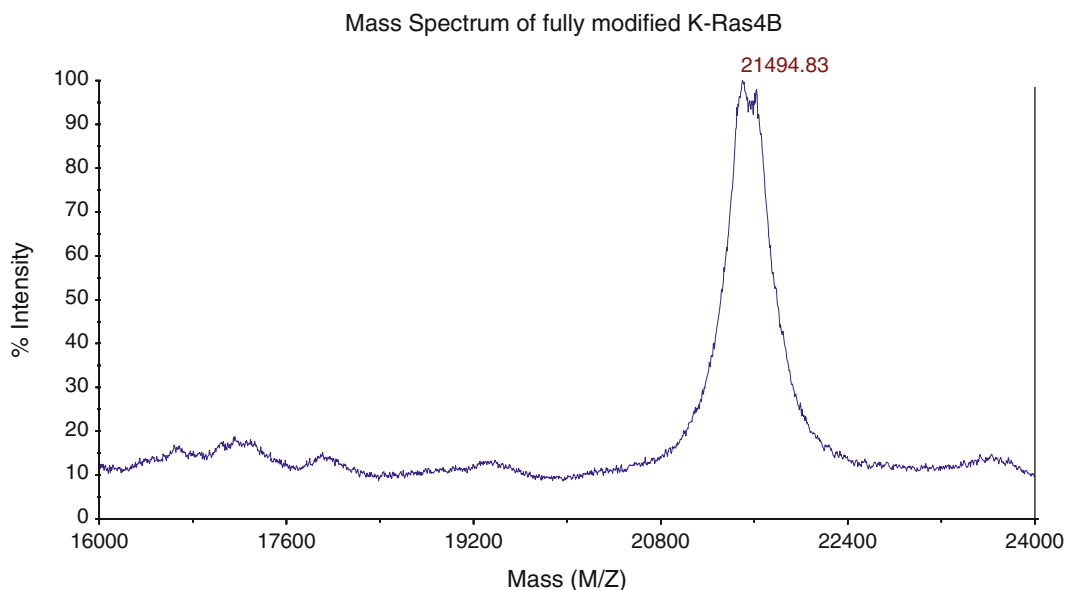


Fig. 5 The mass spectrum of fully modified ligated K-Ras 4B in Tris-EDTA buffer

3.6 GTP

Hydrolysis Assay

1. Prepare the reaction mixture for the sample: 10 μ M fully modified K-Ras 4B, 0.2 mM MESG, 1 unit/mL purine nucleoside phosphorylase, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1 mM EDTA, and 0.5 mM GTP. Fresh GTP should be added to both sample and control mixtures at the same time.
2. Prepare the control mixture with the same components of the reaction mixture excluding modified K-Ras, with the remaining volume made up using the K-Ras storage buffer (Tris-EDTA buffer pH 9). It is beneficial to make two volumes of the control reaction to blank the UV spectrophotometer prior to following the reaction.
3. Once these reaction mixtures are made up, immediately transfer samples to UV cuvettes (use quartz if available) and measure absorbance of the sample mixture against the control at 360 nm for at least 600 min. GTP should naturally hydrolyze in both cuvettes, though a far greater rate should be observed in the presence of an active GTPase.

Figure 6 shows the spectrophotometric recording of K-Ras 4B modified using the sortase-mediated ligation reaction as well as a positive control using the catalytic domain of K-Ras 4B.

3.7 Raf-1

Binding Assay

1. Carry out the GTP or GDP loading of protein samples by incubating them with 10 mM EDTA and 0.1 mM GTP γ S or 0.1 mM GDP, respectively. Incubate this mixture at room temperature for 1 h with agitation.

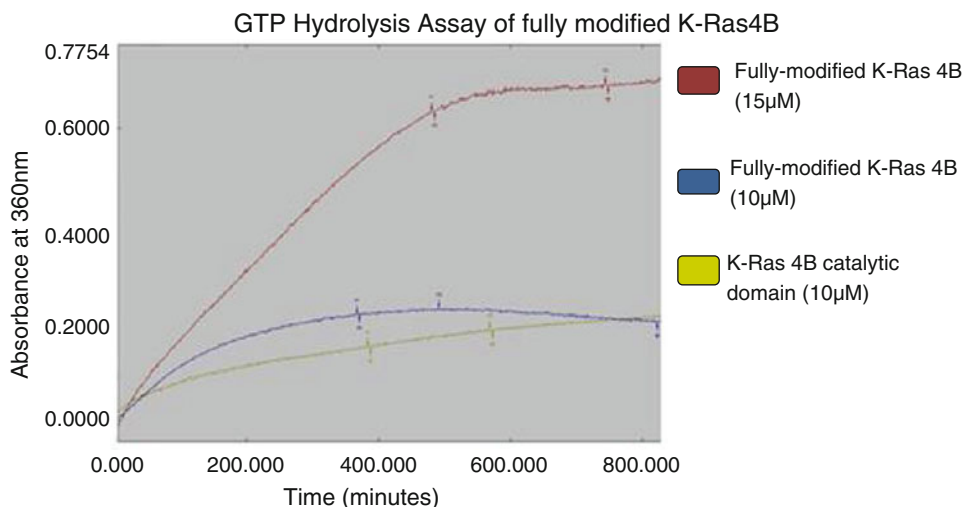


Fig. 6 GTP hydrolysis assay of fully modified K-Ras4B. Absorbance at 360 nm reflecting the release of inorganic phosphate as GTP is hydrolyzed to GDP. Free inorganic phosphate is absorbed by MESG which increases absorbance at 360 nm. Shown in *yellow* is the degree of GTP hydrolysis with 10 μ M K-Ras 4B catalytic domain in a 1 mL reaction. Shown in *blue* and *red* are GTP hydrolysis reactions with fully modified K-Ras 4B at 10 and 15 μ M concentrations, respectively

2. In order to stop the reaction, add MgCl_2 to a final concentration of 50 mM and three times the volume of HEPES buffer. Also add Raf RBD agarose resin to the mixture at this step and incubate it for 45 min at room temperature.
3. Wash the beads with HEPES buffer at least two times (*see Note 13*).
4. Add 20 μ L of HEPES buffer to the beads and 5 μ L of SDS loading dye and heat the reaction mixture at 90 $^{\circ}\text{C}$ for 7–10 min.
5. Centrifuge this mixture at $2,500\times g$ for 3 min and then load the supernatants on an SDS-PAGE gel.
6. Perform a western blot on the gel and probe it with an anti K-Ras antibody.

Figure 7 shows a western blot performed on samples after carrying out the Ras activation assay.

3.8 SPR Membrane Binding Assay

1. Apply the running buffer to the sensor chip containing phospholipids for equilibration (*see Note 14*).
2. To study the binding of modified and non-modified proteins, dissolve the protein samples to 2.4 μ M in the running buffer.
3. Apply the protein samples on the sensor chip. Use a flow rate of 10 μ L/min with a contact time of 240 s, a dissociation time of 240 s, and a regeneration time of 30 s.

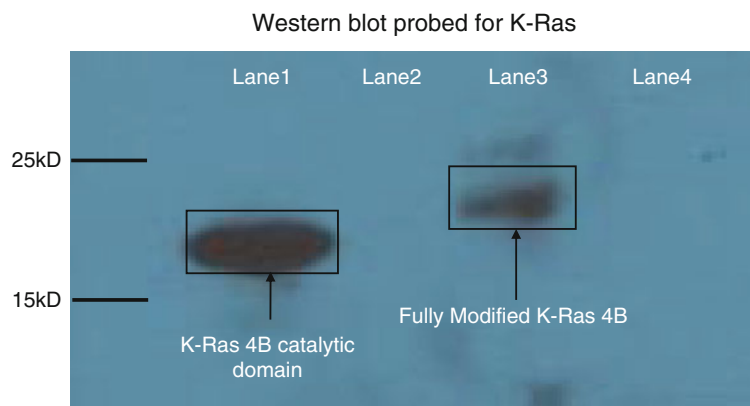


Fig. 7 Western blot probed for K-Ras. The western blot was run on GTP γ S-loaded K-Ras 4B catalytic domain (*Lane 1*), GDP-loaded K-Ras 4B catalytic domain (*Lane 2*), GTP γ S-loaded fully modified K-Ras 4B (*Lane 3*), and GDP-loaded fully modified K-Ras 4B (*Lane 4*) after carrying out the Ras activation assay. Since the GDP-loaded forms of K-Ras 4B do not bind to Raf, no band is seen in *lanes 2* and *4*. Since fully modified K-Ras 4B has a higher molecular weight as compared to its catalytic domain, it runs higher than the catalytic domain of K-Ras 4B

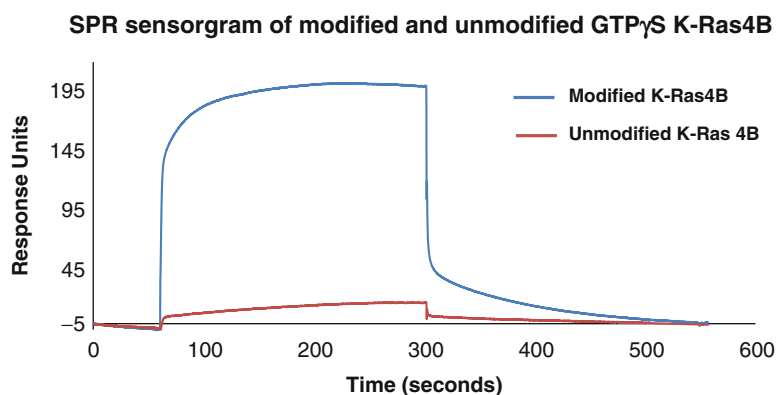


Fig. 8 SPR sensorgram of modified and unmodified GTP γ S K-Ras4B. Solutions of 2.4 μ M fully modified GTP γ S-loaded K-Ras 4B (*blue*) and 2.4 μ M unmodified K-Ras4B (*red*) were applied to immobilized DPPC nanodiscs at a flow rate of 10 μ L/min. Binding of the fully modified version of the protein leads to an increase in response units as compared to the unmodified protein

4. Use 2 M NaCl as the regeneration buffer to bring the response back to baseline before successive rounds of association and dissociation.
5. Use the affinity wizard to test the binding and dissociation of protein samples. Perform data analysis by plotting baseline corrected response units at equilibrium versus concentration of proteins for each cycle.

Figure 8 shows an SPR sensorgram comparing membrane binding abilities of the modified and unmodified K-Ras 4B.

4 Notes

1. The sortase recognition sequence is LPXTG where X is any amino acid with the exception of glycine or proline. Our constructs contain a threonine residue at this position.
2. Sterile SOC medium is packaged along with one-shot BL21AI cells from Invitrogen.
3. It is recommended to make the extraction buffers fresh every time.
4. 10 % glycerol is present in the His-nickel-binding buffer used to dialyze the K-Ras 4B catalytic domain but is not present in the His-nickel-binding buffer used to wash the nickel-binding resin. Glycerol serves as an osmolyte in the dialysis buffer and stabilizes the protein structure.
5. As imidazole is light sensitive, it is highly recommended that the elution and wash buffers in which it is present be stored in lightproof containers at 4 °C if they are to be kept for a prolonged period of time.
6. Purification can be done using an HPLC fitted with a His-nickel-binding resin cartridge and may yield more efficient purification. However, if this equipment is unavailable, good results can still be obtained using conventional gravity columns.
7. The sortase construct can be readily obtained from Addgene and transformed in standard competent *E. coli* cell lines [10].
8. GTP stocks must be fresh or stored at –80 °C and thawed to room temperature just prior to use. Once thawed, it must not be refrozen as GTP inherently hydrolyzes to GDP and inorganic phosphate making it unsuitable for hydrolysis assays.
9. Some K-Ras 4B may misfold during expression. Misfolded K-Ras 4B may have a higher nickel-binding affinity as compared to the correctly folded form, such that it will only elute at the highest concentrations of imidazole. If protein elution is observed only at low and high imidazole concentrations but not intermediates (100–200 mM), then the high imidazole fractions should be discarded as they are likely misfolded protein states.
10. The K-Ras 4B catalytic domain fractions purified for the sortase reactions should be dialyzed into Tris–EDTA buffer using dialysis cassettes and not buffer exchange columns. We have found that using these columns results in a significant loss of K-Ras 4B.

11. Some precipitation may be observed during the course of the reaction; if this becomes apparent, the precipitate should be separated using centrifugation with the reaction stalled by transferring to 4 °C. Then, proceed with the purification steps as described.
12. Protein yields can be relatively low for the reaction so it is recommended that silver staining be used to develop SDS-PAGE gels containing sortase reaction mixtures or purified product.
13. For washing the beads, centrifuge them at 2,500 × *g* for 3 min and discard the supernatant. Then, add new buffer and repeat the process.
14. Run all experiments at 25 °C on a Biacore T100 SPR instrument. For making the phospholipid nanodiscs, follow the protocol by Nath et al. [11].

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References

1. Cox AD, Der CJ (1992) Protein prenylation: more than just glue? *Curr Opin Cell Biol* 4:1008–1016
2. Casey PJ, Solski PA, Der CJ et al (1989) p21ras is modified by a farnesyl isoprenoid. *Proc Natl Acad Sci U S A* 86:8323–8327
3. Resh MD (2006) Trafficking and signaling by fatty-acylated and prenylated proteins. *Nat Chem Biol* 2:584–590
4. Hancock JF, Paterson H, Marshall CJ (1990) A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane. *Cell* 63:133–139
5. Campbell-Burk SL, Carpenter JW (1995) Refolding and purification of Ras proteins. *Methods Enzymol* 255:3–13
6. Trueblood CE, Boyartchuk VL, Picologlou EA et al (2000) The CaaX proteases, Afc1p and Rce1p, have overlapping but distinct substrate specificities. *Mol Cell Biol* 20:4381–4392
7. Clarke S, Vogel JP, Deschenes RJ et al (1988) Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proc Natl Acad Sci U S A* 85:4643–4647
8. Chen YX, Koch S, Uhlenbrock K et al (2010) Synthesis of the Rheb and K-Ras4B GTPases. *Angew Chem Int Ed Engl* 49:6090–6095
9. Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for enterobacteria. *J Bacteriol* 119:736–747
10. Mao H (2004) A self-cleavable sortase fusion for one-step purification of free recombinant proteins. *Protein Expr Purif* 37:253–263
11. Nath A, Atkins WM, Sligar SG (2007) Applications of phospholipid bilayer nanodiscs in the study of membranes and membrane proteins. *Biochemistry* 46:2059–2069

Ras Signaling

Methods and Protocols

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