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TITLE:

Fully Processed Recombinant KRAS4b: Isolating and Characterizing the Farnesylated and Methylated Protein

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SUMMARY:

Prenylation is an important modification on peripheral membrane binding proteins. Insect cells can be manipulated to produce farnesylated and carboxymethylated KRAS4b in quantities that enable biophysical measurements of protein-protein and protein-lipid interactions

ABSTRACT:

Protein prenylation is a key modification that is responsible for targeting proteins to intracellular membranes. KRAS4b, which is mutated in 22% of human cancers, is processed by farnesylation and carboxymethylation due to the presence of a 'CAAX' box motif at the C-terminus. An engineered baculovirus system was used to express farnesylated and carboxymethylated KRAS4b in insect cells and has been described previously. Here, we describe the detailed, practical purification and biochemical characterization of the protein. Specifically, affinity and ion exchange chromatography were used to purify the protein to homogeneity. Intact and native mass spectrometry was used to validate the correct modification of KRAS4b and to verify nucleotide binding. Finally, membrane association of farnesylated and carboxymethylated KRAS4b to liposomes was measured using surface plasmon resonance spectroscopy.

INTRODUCTION:

Posttranslational modifications play a key role in defining the functional activity of proteins. Modifications such as phosphorylation and glycosylation are well established. Lipid modifications are less well characterized, however. It is estimated that as much as 0.5% of all cellular proteins may be prenylated[1]. Prenylation is the transfer of a 15-carbon farnesyl or a 20-carbon geranylgeranyl lipid chain to an acceptor protein containing the CAAX motif[2]. Prenylated proteins have been implicated in the progression of several human diseases including premature aging[3], Alzheimer's[4], cardiac dysfunction[5], choroideremia[6], and cancer[7]. The small GTPases, HRAS, NRAS, and KRAS[1], nuclear laminins, and the kinetochores CENP-E and F are farnesylated proteins under the basal condition. Other small GTPases, namely RhoA, RhoC, Rac1, cdc-42, and RRAS are geranylgeranylated[8], whereas RhoB can be farnesylated or geranylgeranylated[9].

The small GTPase KRAS4b functions as a molecular switch, essentially transmitting extracellular growth factor signaling to intracellular signal transduction pathways that stimulate cell growth and proliferation, via multiple protein-protein interactions. There are two key aspects of KRAS4b biochemistry that are essential for its activity. First, the protein cycles between an inactive GDP and an active GTP bound state whereby it actively engages with effectors. Second, a C-terminal poly-lysine region and a farnesylated and carboxymethylated cysteine direct the protein to the plasma membrane, enabling recruitment and activation of downstream effectors. Mutant KRAS4b is an oncogenic driver in pancreatic, colorectal, and lung cancer[10], and as such, therapeutic intervention would have a huge clinical benefit. Production of authentically modified recombinant protein that is farnesylated and carboxymethylated would enable biochemical screening using KRAS4b in combination with membrane surrogates such as liposomes or lipid nanodiscs[11,12].

Farnesyl transferase (FNT) catalyzes the addition of farnesyl pyrophosphate to the C-terminal cysteine in the CAAX motif in KRAS4b. After prenylation, the protein is trafficked to the endoplasmic reticulum (ER) where the Ras converting enzyme (RCE1) cleaves the three C-terminal residues. The final step in processing is methylation of the new C-terminal farnesylcysteine residue by the ER membrane protein, isoprenylcysteine carboxyl methyltransferase (ICMT). Expression of recombinant KRAS4b in E. coli results in the production of an unmodified protein. Previous attempts to produce processed KRAS4b have been limited due to insufficient yields for structural or drug screening experiments or have failed to recapitulate the native full-length mature protein[13,14]. The protocol presented here utilizes an engineered baculovirus-based insect cell expression system and purification method that generates highly purified, fully processed KRAS4b at yields of 5 mg/L of cell culture.

Careful protein characterization is essential to validate the quality of recombinant proteins prior to embarking on structural biology or drug screening studies. Two key parameters of fully processed KRAS4b are validation of the correct prenyl modification and the availability of the farnesylated and carboxymethylated C-terminus (FMe) for interaction with membrane substitutes or lipids. Electrospray ionization mass spectrometry (ESI-MS) of the KRAS4b-FMe was used to measure the molecular weight and confirm the presence of the farnesyl and carboxymethyl modifications. Native mass spectrometry, where samples are sprayed with

nondenaturing solvents, was used to demonstrate that KRAS4b-FMe was also bound to its GDP cofactor. Finally, surface plasmon resonance spectroscopy was used to measure the direct binding of KRAS4b-FMe with immobilized liposomes.

PROTOCOL:

1. Protein purification

1.1. Prepare buffers A–H, as seen in **Table 1**.

[Place **Table 1** here]

1.2. Prepare the purification materials (see **Table of Materials**): protease inhibitor cocktail without EDTA or other chelators; immobilized metal affinity chromatography (IMAC) column; cation exchange chromatography (CEX) column; 0.45 µm syringe filter; 2 M imidazole (pH = 7.5); ultracentrifuge capable of 100,000 x *g*; high speed benchtop centrifuge capable of 4,000 x *g*; centrifugal filter units (10 KDa NMWL); spectrophotometer capable of reading at 280 nm; His6-tobacco etch virus (TEV) protease; and chromatography instrumentation capable of mixing two buffer solutions, applying solutions to columns, creating gradient elutions from column, and collecting fractions from columns.

1.3. Thaw cells from ~2 L of insect cell culture previously stored at -80 °C or use freshly harvested cells. See Gillette et al. 2019[15] for details about the Tni.FNL insect cell line and expression protocols. Carefully resuspend the cells with 200 mL of Buffer A with added 1:200 v/v protease inhibitor without introducing air or creating foam.

NOTE: Step 1.3 and subsequent steps (except as noted) should be performed at room temperature (~22 °C). It is important that the sample be homogeneous to allow complete lysis in the next step.

1.4. Lyse cell pellet in a microfluidizer at 7,000 psi for two passes or in an equivalent lysis system. Alternative methods of lysis have not been explored.

1.5. Clarifying insect cell lysates is problematic due to the presence of compounds that clog filters. Thus, ultracentrifugation (100,000 x *g* for 30 min at 4 °C) is very important. A white lipid residue on the surface of the supernatant should be avoided and can be removed or reduced using cotton swabs. Filter the decanted supernatant through a 0.45 µm PES filter. Use the clarified sample immediately or store at -80 °C.

NOTE: The residue blocks filters quickly and many filters may be necessary. Failure to reduce the amount of this material will lead to high column back pressure in subsequent steps.

1.6. Determine the volume of the clarified lysate and adjust the sample to 35 mM imidazole by the addition of 2 M imidazole stock. Load the sample at 3 mL/min onto a 20 mL IMAC column

(HisPrep FF 16/10 column) pre-equilibrated in Buffer B for three column volumes (CVs).

1.7. Although the target protein is typically quantitatively adsorbed to the column, it is best to collect the column flow for subsequent analysis. Wash the column until the Abs_{280} reaches a steady baseline (<100 milliabsorbance units [mAU]) with Buffer B at 3 mL/min for 20 mL (1 CV) then for ~3 CV at 4 mL/min for the remainder of the wash step. Typically, 60–80 mL of Buffer B is needed to achieve baseline absorbance.

1.8. Elute the protein with a 400 mL (20 CV) gradient from Buffer B to Buffer C while collecting 8 mL fractions. Typically, the target protein elutes in the first half of the gradient (**Figure 1A**; not all later fractions are shown).

1.9. Analyze protein fractions using SDS-PAGE/Coomassie staining. Pool peak fractions and dialyze the pool overnight against 2 L of Buffer D at 4 °C.

NOTE: Low pH is critical to ensure protein binding in the next step. However, the pH change during this dialysis occurs slowly, and this is a convenient step for overnight incubation. Alternative buffer exchange strategies (e.g., higher buffer concentration to speed the pH change) have not been investigated. The protein will begin to slowly precipitate over time at lower pH and lower salt concentrations and a small amount of precipitation is normal during this step. Because of this, we avoid exchanging the buffer to the 100 mM NaCl necessary for binding to the next column during dialysis. Rather, an NaCl concentration of 200 mM is used for dialysis and the protein is diluted to 100 mM (see below) immediately prior to application to the column. We have not investigated if this precipitation is specific to KRAS4b, but the protein that does precipitate has been confirmed as KRAS4b-FMe. For this reason, we suggest using the higher NaCl concentration in the dialysis buffer for any protein of interest as a precaution until the stability of the target protein in the binding buffer can be determined.

1.10. Remove the sample from dialysis and centrifuge at 4,000 x *g* for 10 min to remove any precipitate. The final dialyzed, clarified sample at this point is still often hazy but can be applied without further processing onto the subsequent CEX column.

1.11. Prepare a 20 mL cation exchange column (see **Table of Materials**) by washing with three CVs of Buffer G, then with three CVs of Buffer F.

NOTE: While we have not meticulously evaluated other resins, several colleagues have found poor resolution with resins other than SP Sepharose High Performance resin.

1.12. Dilute 20 mL of the dialyzed sample to a final NaCl concentration of 100 mM by adding 20 mL of Buffer E and apply the diluted sample to the cation exchange column.

1.13. Continue to load the column by adding freshly diluted samples prepared as described above to the load tube as the previous dilution is nearing the end of the load.

NOTE: Diluting the entire sample at once to 100 mM NaCl has resulted in significant loss of target protein due to precipitation.

1.14. Wash the column to baseline Abs_{280} with Buffer F. This typically requires 3 CV. The protein is eluted from the column during a 400 mL (20 CV) gradient from Buffer F to 65% Buffer G, collected in 6 mL fractions (**Figure 1B**).

1.15. Continue washing the column for an additional 1.5 CV (65% Buffer G) once the gradient is completed. Choose positive fractions based on both SDS-PAGE/Coomassie blue stain analysis and inspection of the UV trace of the chromatogram.

NOTE: The UV trace typically contains five peaks. Beginning at lower salt concentrations, the initial peak is usually unprocessed and/or proteolyzed protein. The second and third peaks are a mixture of two forms of processed protein: the second peak is primarily a farnesylated intermediate (FARN), while the third is primarily the fully processed FMe protein of interest. Peaks four and five represent His6-MBP-KRAS4b fusion proteins (all of the protein is in this format at this point, as no TEV digestion has occurred). These are not N-acetylated at the N-terminus and are farnesylated (peak four) and farnesylated and carboxymethylated (peak five) at the C-terminus. As peak two and peak four will produce identical proteins after tag removal (i.e., KRAS4b-FARN) these can be pooled at this stage if desired. Similarly, peak three and peak five can be combined to produce a single lot of KRAS4b-FMe (**Figure 1B**, **Figure 2**). However, it is advised that this pooling only be done when the nature of the protein in the separate peaks has been confirmed.

1.16. Digest the protein pooled in step 1.15 with His6-TEV protease (~200 µg of protease/mL of sample.

NOTE: We make His6-TEV protease using the plasmid and protocols available from Addgene (https://www.addgene.org/92414). Dialyze the TEV digest against Buffer A (10 kDa MWCO dialysis tubing with a minimum of 40 mL of Buffer A per mL of sample) for 2 h at room temperature and then overnight at 4 °C.

1.17. After digestion and dialysis, load the protein directly to a 20 mL IMAC column equilibrated with Buffer A at 3 mL/min.

NOTE: Collect 7 mL fractions during this entire chromatography, because the target protein has a low affinity for the column but might not be entirely bound to the resin. Wash the column at 3 mL/min with Buffer A for a total of 3 CVs or until a baseline absorbance is reached.

1.18. The target protein is eluted with a 5 CV gradient of Buffer C from 0%–10%, collecting 7 mL fractions. As a precaution, additional bound proteins can be eluted with 100% Buffer C. Positive fractions are identified after analysis by SDS-PAGE (**Figure 1C**). Typically, the target protein elutes at a low imidazole concentration (i.e., in the 0%–10% Buffer C gradient) but sometimes elutes in the flow-through or column wash.

1.19. Dialyze the final pool against Buffer H. Determine the protein concentration by spectrophotometry at 280 nm.

NOTE: Be sure to account for the presence of the nucleotide when determining the extinction coefficient to use for this calculation.

1.20. The protein can be concentrated to ~2–5 mg/mL using a centrifugal filter unit (10 K NMWL) without significant protein loss. Filter with a 0.22 µM syringe filter. Measure the solution absorbance at A_{280} to calculate the final protein concentration (**Figure 1D**). Snap freeze aliquots in liquid nitrogen and store frozen samples at -80 °C.

NOTE: The protein purified is bound to GDP. KRAS4b-FMe can be exchanged into GppNHp using previously published protocols[16].

2. Sample preparation for intact mass analysis and native mass analysis

2.1. Sample preparation for intact mass analysis

2.1.1. Thaw protein sample on ice prior to analysis. For intact mass analysis, dilute the protein to 0.1 mg/mL in 20 mM ammonium bicarbonate (pH = ~8.4). For native mass analysis, dilute the protein to a concentration of 0.1 mg/mL in 50 mM ammonium acetate (pH = ~7.5). Vortex each sample for 5–10 s, then centrifuge at 12,500 x *g* for 1 min to pellet any solid material in the solution. Remove 10–20 µL of each supernatant and transfer to a glass autosampler vial. Cap each vial tightly to prevent contamination or evaporation.

NOTE: For either intact mass analysis or native mass analysis, the sample may be desalted prior to analysis using a 10 kDa MWCO cellulose membrane filter to buffer-exchange into the final dilution buffer.

2.2. Preparation of the extended mass range (EMR) mass spectroscopy for mass analysis

NOTE: Prior to running any analysis on the mass spectrometer, make sure that it has been recently calibrated. Also, always wear gloves and other personal protective equipment as necessary to avoid any harmful chemicals and to avoid contaminating samples and solvents. Calibration is done using a calibration solution obtained commercially and a 2 mg/mL cesium iodide solution prepared in-house.

2.2.1. Open the analysis software and load the appropriate instrument method file. For intact mass analysis of KRAS4b proteins, suitable starting parameters are as follows: positive mode of detection; three microscans, resolution = 70,000; AGC target = $3e^6$; maximum integration time = 200 ms; and a scan range = 70–1,800 mass-to-charge ratio (m/z). For native mass analysis of KRAS4b proteins, suitable starting parameters are as follows: positive mode of detection; 10 microscans, resolution = 17,500; in-source collision-induced dissociation (CID) = 20 eV; AGC

target = $3e^6$; collision energy (CE) = 20; maximum integration time = 200 ms; and a scan range = 500–10,000 m/z.

2.3. Preparation of chromatographic solvents and column

2.3.1. Prepare the solvents necessary for intact mass analysis. Solvent A is water with 0.1% formic acid. Solvent B is 80% acetonitrile and 0.1% formic acid in water.

2.3.2. Prepare the solvents necessary for native mass analysis. Solvent A is 0.1 M ammonium acetate in water and Solvent B is water.

2.3.3. After the appropriate solvents are prepared, purge the system so that the tubing is filled with the appropriate solvents and all air bubbles are removed from the lines.

2.3.4. Install the appropriate column (a reverse-phase column for intact mass analysis and a size exclusion column for native mass analysis). For intact mass analysis, the flow rate is 0.5 mL/min, and the column temperature (using a column heater) is 50 °C. Equilibrate the column for 15–20 min. For native mass analysis, the flow rate is 0.25 mL/min.

NOTE: Always monitor the column back-pressure to ensure that it does not rise above the upper limit, which could indicate a clogged line or that the column matrix is compromised. Replace the column if necessary.

2.4. Sample analysis

2.4.1. Place the prepared protein sample in the glass autosampler vial into the appropriate vial rack in the LC systems autosampler. Create a sample(s) list in the software program with the appropriate instrument method for the desired analysis. Once the sample list is complete, click the **Play** button to start the analysis.

2.4.2. Inject 1–2 µL of sample per analysis, with a water blank before and after each sample, to ensure no carry-over is present.

NOTE: The intact mass analysis is very different than the native mass analysis and requires very different instrument method settings. This is because with the intact mass analysis, the protein is “relaxed” in the presence of an organic solvent, and thus is capable of accepting more charges. It is easier to deconvolute and resolve the isotopic distribution of the charge states. Native mass analysis provides a narrow charge distribution of the protein ions, and based on the protein, requires different voltage and collision energy settings.

2.5. Data analysis and protein deconvolution

2.5.1. Export the spectrum of the sample to software where it can be transformed to the de-convoluted spectrum that will display the intact mass (or native mass) of the protein. The intact

mass will have an isotopic peak distribution due to the high abundance of charged spectral peaks. The native mass will typically have very few peaks due to the low abundance of the charged spectral peaks (**Figure 3D**).

2.5.2. Expand the mass-to-charge ratio (m/z) range of the peak d to confirm that the measured mass is consistent with the predicted mass. Occasionally, due to the addition of charges to the protein, there may be a slight variation between the expected molecular weight (MW) and the observed MW. Also, as with many mass spectrometry analyses, adducts such as sodium can contribute to the appearance of additional peaks in the data, even with carefully desalted samples. Lower abundant peaks with an m/z that is lower than expected are sometimes observed. This is most likely due to a neutral loss, which is the loss of a functional group due to the ionization process.

NOTE: As expected, there will be differences between the intact mass and the native mass peaks. The intact mass display will show the protein's expected MW. It will not have the extra MW of the attached nucleotide or other modifications. However, the native mass peak will show the protein with any added nucleotide.

3. Validation of KRAS4b-FMe binding to liposomes

3.1. Membrane liposomes preparation

3.1.1. Prepare a buffer consisting of 20 mM HEPES (pH = 7.3), 150 mM NaCl, and 1 mM TCEP.

3.1.2. Liposome preparation materials include 25 mM 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 10 mM 1-palmitoyl-2-oleoyl-glycero-3-phospho-L-serine (POPS) stocks in chloroform; a lipid extruder set with a holder and heating block; argon gas and liquid nitrogen; an ultrasonic bath; glass screw thread vials with PTFE foam liners; and a plate reader capable of dynamic light scattering detection.

3.1.3. Thaw lipid stocks in chloroform at room temperature for 30 min. Prepare 1 mL of 5 mM 70:30 POPC: POPS liposomes by aliquoting 106 µL of 25 mM POPC (stock) and 118 µL of 10 mM POPS (stock) into a glass vial.

NOTE: Do not aliquot lipids with a plastic tip, because the chloroform could dissolve certain plastic tips.

3.1.4. Dry lipids under a steady stream of argon gas. Slowly rotate the glass vial when applying the argon gas to form a thin layer of dry film.

3.1.5. Put samples under a vacuum lyophilizer overnight to remove excess chloroform.

3.1.6. Reconstitute the lipids by adding 1 mL of buffer to the dried film and vortex the mixtures for 5 min. Hydrate the lipid mixtures by rocking for 1 h at 25 °C.

NOTE: The sample will be very turbid upon addition of the buffer to the dried film layer of lipids.

3.1.7. Perform five freeze/thaw cycles by alternating placing the sample vial between an ice-water (4 °C or lower) and a warm water bath (25 °C or higher).

3.1.8. Place the sample vial into the ultrasonic bath and sonicate the sample for about 0.5 h or until the sample becomes semitransparent.

3.1.9. Extrude the samples using the lipid extruder set. Assemble the extrusion kit as shown in the manufacturer's instructions. Extrude the samples using 0.1 µm filter paper. Load the samples into one glass syringe and attach it to one end of the extrusion apparatus. Add an empty syringe on the other end of the extrusion apparatus. Push back and forth 10–20x until your samples become fully transparent.

3.1.10. Spin the final samples for 30 min at 20,000 x *g* to remove any large aggregates.

3.1.11. Use dynamic light scattering (DLS) to determine the size and homogeneity of the liposomes (optional). Place 30 µL of the liposomes into a 384 well plate reader. Spin the plate at 1,000 x *g* for 30 min. Place the plate into a plate reader and collect 10 light scattering measurements.

3.2. Charactering KRAS4b-FMe binding to liposomes via surface plasmon resonance (SPR)

3.2.1. Assemble the required materials: an SPR instrument; sensor chip L1; 7 x 14 mm vial tubes; and CHAPS.

3.2.2. Prepare a buffer containing 20 mM HEPES (pH = 7.3), 150 mM NaCl, 1 mM TCEP. Prepare a 2:1 dilution series of KRAS4b-FMe from 60 µM–0.06 µM in a total volume of 200 µL (10 titrations total). Transfer the final diluted samples into vial tubes (see **Table of Materials**), place the vial tubes into a rack, and insert the samples into the SPR instrument.

3.2.3. Insert the L1 sensor chip into the SPR instrument. Attach the buffer and prime the system with buffer for 7 min.

3.2.4. Set up an automated method as follows:

3.2.4.1. Set the instrument temperature to 25 °C.

3.2.4.2. Activate the sensor chip L1 by rinsing it with two 1 min injections of 20 mM CHAPS dissolved in water at 30 µL/min flow rate.

3.2.4.3. Perform start-up cycles of ten 1 min injections of running buffer to hydrate the chip surface.

3.2.4.3.1. Deposit the liposomes onto the sensor chip L1 by injecting the liposomes onto the flow cell (FC)-2 of the L1 chip with 2 min injections at 5 µL/min. Aim for a capture level of at least ~3,000 response units (RUs).

NOTE: Increase the injection time until you achieve the appropriate capture level.

3.2.4.4. Inject three cycles of buffer over both FC-1 and FC-2 with 1 min injections at 30 µL/min.

3.2.4.4.1. Inject the various KRAS4b-FMe titrations from the lowest to the highest concentration series. Inject the proteins using 1 min injections for the association phase and 2 min injections for the dissociation phase at 30 µL/min flow rate over both FCs.

3.2.4.4.2. Regenerate the sensor chip L1 by rinsing it with two 1 min injections of 20 mM CHAPS at 30 µL/min.

3.2.5. Fit the data using the SPR evaluation software using a single site binding model to obtain apparent binding affinities (see the Discussion section for an explanation of data fitting).

REPRESENTATIVE RESULTS:

One of the largest variables in the protocol is the amount of expressed target protein (His6-MBP-tev-KRAS4b). This protocol was developed using an isolate from a *Trichoplusia ni* cell line, Tni-FNL[17], adapted for suspension growth and weaned from serum. Given the wide range of results reported across the various insect cell lines with the baculovirus expression system, it is advisable that Tni-FNL be used, at least initially, to produced KRAS4b-FMe.

A dark protein that migrates to ~65 kDa should be obvious in the clarified lysate as well as the elution fractions (**Figure 1A**). The fusion protein should be one of the two most prominent stained bands in the lysate with the other being the co-expressed FNTA/B that comigrates at ~48 kDa.

Within the purification, the CEX step is critical for two reasons: 1) it serves to reduce the extent of proteolysis as the hypervariable region (HVR) is quite susceptible, and 2) it enriches for the fully processed protein as indicated in the notes for this step of the protocol. It is, however, the most complicated part of the protocol. This is because the processed protein tends to precipitate at lower salt concentrations, even fused to MBP. Fortunately, this is not an all-or-none phenomenon, and it also takes place somewhat slowly over time. The protocol takes advantage of this by dialyzing to 200 mM NaCl prior to the CEX step. At this concentration, the precipitation was not as significant as at 100 mM. Therefore, the protocol is designed to limit the length of time the protein is in a buffer of this salt concentration. The mixing of small aliquots of the CEX column load with equal volumes of zero-salt buffer immediately prior to column loading limits the exposure (in terms of time) to low salt conditions and thus limits precipitation. **Figure 2A** depicts the most typical result from the CEX step, with the most prominent being peak 3, which contains predominantly KRAS4b-FMe. **Figure 2B** depicts elution profiles that have been observed to give a sense of the variability of the procedure's outcome. Because these can sometimes be

misleading, it is prudent to create pools of all peaks and store these at -80 °C until the peak of interest has been fully purified and passed quality tests.

As noted in the protocol, the use of HiPrep SP Sepharose High Performance columns seems to be critical in achieving the separation observed in **Figure 2A**. While it is not yet clear why peak three frequently harbors farnesylated-only KRAS4b in addition to the desired farnesylated and carboxymethylated KRAS4b, the poor resolution others have reported with alternative CEX resins (personal communication) suggests this phenomenon is not solely a result of ionic interactions.

Typical yields ranged from 1–6 mg/L, with 3–5 mg/L achieved frequently (>90%, n > 50). Intact mass analysis using ESI-MS confirmed the precise molecular mass of the proteins and thereby the relative proportion of farnesylation and/or carboxymethylation (**Figure 3**). While typical final lots contained some detectable KRAS4b-FARN, this proportion was less than 15% in terms of peak height from this analysis. **Figure 3A** shows representative data for KRAS4b that is not prenylated expressed in E. coli, **Figure 3B** shows KRAS4b-FMe eluted in peaks 3 and 5 from CEX, and **Figure 3C** shows KRAS4b-Farn eluted in peak 2 from CEX.

The mass of the KRAS4b bound to GDP was also determined for these samples using native mass analysis (**Figure 3D**). Exchanging the samples into ammonium acetate ensured a "softer" ionization and the native complex stayed intact, providing confirmation of the nature of the nucleotide bound to KRAS4b.

To validate that farnesylation and carboxymethylation are required for KRAS4b-FMe to bind to membranes, we measured the interaction of KRAS4b-FMe to liposomes via SPR. As shown in **Figure 4**, KRAS4b-FMe bound to the liposomes while unprocessed KRAS4b did not, thereby demonstrating that processed KRAS4b-FMe is required for interaction with membranes.

FIGURE AND TABLE LEGENDS:

Figure 1: SDS-PAGE gels of the purification process. (**A**) IMAC capture from lysate. FNTA/B are the dark bands migrating at ~48 kDa and the His6-MBP-tev-KRAS4b is the dark band migrating at ~67 kDa. M = protein molecular weight ladder; T = total protein; L = clarified lysate/column load; F = column flow through. Fractions are eluted with an increasing imidazole concentration gradient. (**B**) CEX fractions labelled 1–5 correspond to the peak fractions from the elution peaks shown in the chromatogram of **Figure 2**. (**C**) TEV digestion and second IMAC. C = pool from CEX step pre-TEV cleavage; L = load sample post-TEV cleavage. The species of interest are labeled. (**D**) One and five micrograms of final KRAS4b-FMe protein. The gels depicted are representative results from multiple productions.

Figure 2: CEX chromatography elution profile. (**A**) The typical CEX elution profile has four to five major peaks. Aside from peak 1, which typically is a proteolyzed form of unprocessed KRAS4b lacking the four C-terminal amino acids, the four peaks numbered 2 through 5 in the panel result from variability in the processing of the N- and C-termini of the protein, with progressively more positively charged molecules eluting later in the gradient (see step 1.15). (**B**) Additional examples of CEX elution profiles.

Figure 3: Intact and native mass spectrometry analysis of purified KRAS4b proteins. Intact mass spectrum and deconvoluted peak analysis results for (**A**) unprocessed KRAS4b 2-185, predicted MW = 21,064; (**B**) KRAS4b 2-185-FMe, peaks 3 and 5 from CEX, predicted MW = 21,281; (**C**) KRAS4b 2-185-Farn, peak 2 from CEX, predicted MW = 21,267. (**D**) Native mass deconvolved peak analysis for E. coli expressed KRAS4b 2-185 (i), KRAS-FMe 2-185 (ii), and KRAS-Farn 2-185 (iii), all in the GDP-nucleotide bound state.

Figure 4: KRAS4b-FMe binding to liposomes via SPR. Size distribution of 70:30 POPC:POPS liposomes obtained by DLS. (**A**) DLS data show an average diameter of ~200 nm for the liposomes with an 18% polydispersity. (**B**) SPR binding sensorgrams of 60–0.6 µM KRAS4b-FMe to 70:30 liposomes captured onto a sensor L1 chip. (**C**) Fit of the steady state binding isotherms derived from the SPR data provided an apparent KD of 1 µM of KRAS4b-FMe binding to liposomes. The binding response is normalized by dividing by the surface capture level of the liposomes. (**D**) SPR binding kinetics of 60–0.6 µM KRAS4b-2-185 to 70:30 liposomes captured onto a sensor L1 chip.

DISCUSSION:

As noted in the Representative Results section, the most critical step during the purification is the handling of the sample during the time it is in lower salt. Limiting the time that the sample is exposed to less than 200 mM NaCl will help reduce precipitation and increase sample yield. Interpretation of the results of the CEX can be difficult if the profile does not match the expectations (see **Figure 2**). Until the protocol has become routine, it is advised that the CEX elution fractions that are not taken forward be stored at -80 °C until the intact mass analysis has been completed to ensure that the proper material was taken forward. Outside of the parameters noted above for the CEX step, the protocol is relatively easy to modify at the lysis and IMAC steps. It is also worth noting that there is no size exclusion chromatography (SEX) separation step in the protocol. This is omitted due to losses we have observed during early method development (unpublished results). It is noteworthy that these losses are not observed when the protein is in complex with nanodiscs, suggesting the loss in the absence of lipids is due to hydrophobic interaction between the protein and the resin.

This method represents a large increase in both the quantity (>10x higher) and quality (in comparison to bona fide processed KRAS4b expressed in human cells) that has been reported in the literature[14,18,19]. The yield of 3–5 mg/L has enabled structural biology efforts that require high protein requirements[16,20]. Additional posttranslational modifications for other members of the RAS family as well as additional posttranslational modification in general are currently being investigated.

For intact mass analysis of KRAS4b, a concentration of 0.1 mg/mL works well. Lower concentrations can be used due to the ability of the relaxed protein configuration to accept charges. However, in native mass analysis, where the protein is in its native folded conformation, there are lower mass-to-charge ratios and higher concentrations are required. Thus, using lower concentrations of protein results in a decrease of the signal and produces less reliable results. The advantage of native mass spectrometry is that the buffer is compatible with retaining the

complex of nucleotide bound to KRAS4b. Therefore, it is possible to confirm the nucleotide-bound forms of the proteins (**Figure 3D**). As with any mass spectrometry analysis, neutral losses (such as a methyl group) are observed (see minor species with a loss of 18 in intact mass analysis, **Figure 3A–C**). In native mass spectrometry analysis, protein adducts with Na^+ or K^+, present after extensive buffer exchange, can be observed. This is evident in the minor peaks of +23 in **Figure 3D**, panels i-iii).

Our SPR data (**Figure 4**) show that the fully processed form of KRAS4b is required for recapitulating KRAS4b-membrane interaction in vitro. A major advantage of using the L1 chip to capture the liposomes in our SPR experiments is that the L1 chip surface is dextran coated and modified with lipophilic groups[21], thus allowing for direct capture of the liposomes without any further modification. SPR is a powerful technique to study ligand-ligand interactions providing information on stoichiometry and binding affinity. Sensorgrams that work properly should have an association phase that reaches a steady state. This maximal binding response (R_{max}) provides an estimate of the stoichiometry for the interaction. The dissociation rate should follow a single exponential decay, assuming a 1:1 binding interaction[22]. However, proteins binding to a membrane surface usually occur with more complex stoichiometries[23] and multiple affinities that result in sensorgrams with more complex kinetics. We have not managed to fit the kinetics to a multisite binding model. However, the equilibrium binding isotherms can be fit using commercial evaluation software to provide an apparent equilibrium binding affinity (K_D). Regardless, our SPR data clearly differentiates between how unprocessed KRAS4b and KRAS4b-FMe bind to liposomes. Thus, SPR still provides a useful tool in deciphering protein-lipid interactions.

Collectively, using SPR we demonstrate that fully processed KRAS4b-FMe is required for membrane binding. Production of the fully farnesylated and carboxymethylated form of KRAS4b using this approach provides the quantity and quality of material necessary for structural biology[20], biophysical characterization of membrane interactions[23], and screening studies against the KRAS4b-FMe:RAF complex in the presence of lipid bilayers.

ACKNOWLEDGMENTS:

We acknowledge cloning and expression support from Carissa Grose, Jen Melhalko, and Matt Drew in the Protein Expression Laboratory, Frederick National Laboratory for Cancer Research. This project has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government

DISCLOSURES:

The authors have nothing to disclose.

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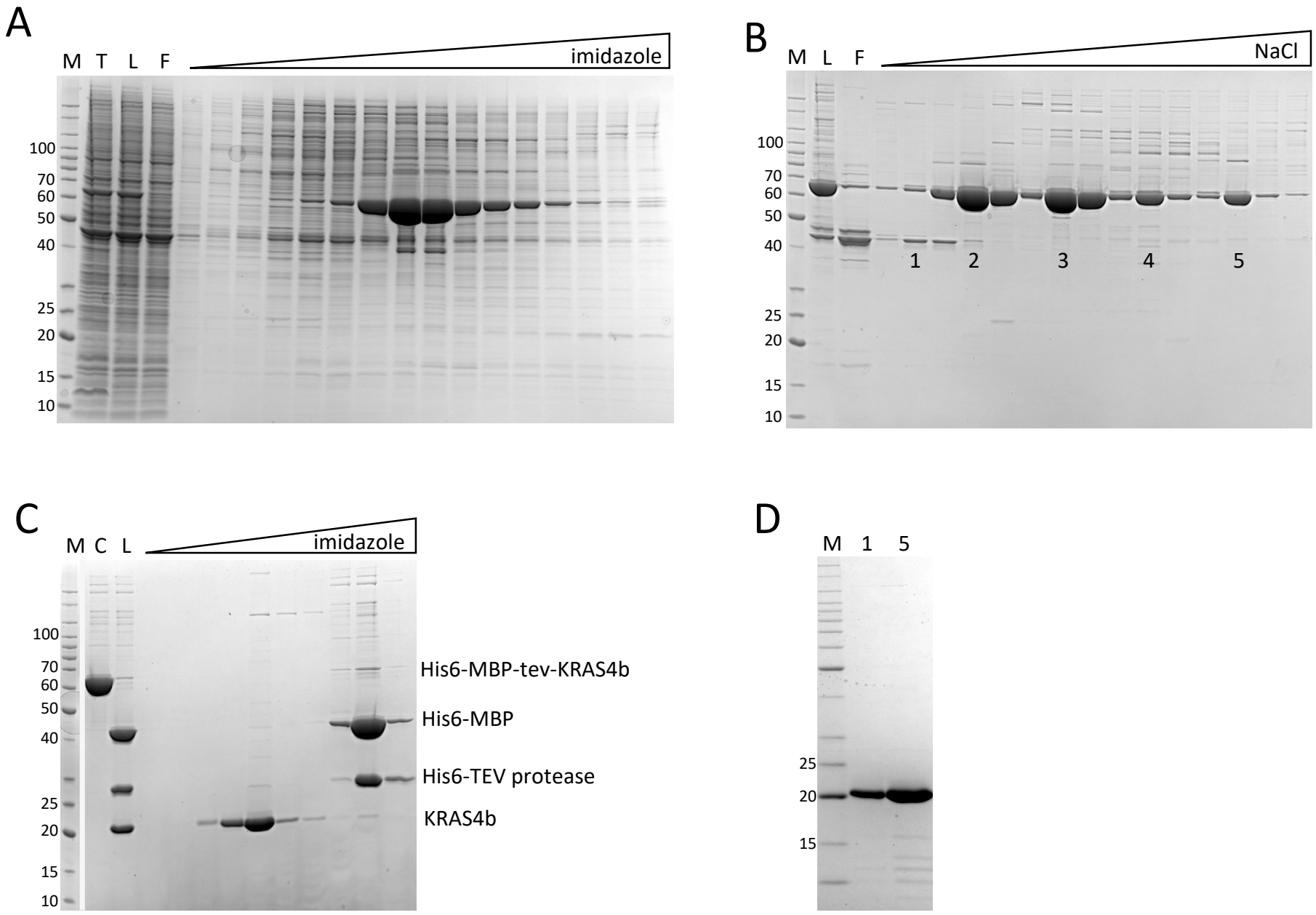
620 recruitment of the GTPase KRAS to the plasma membrane. *Journal of Biological Chemistry.* **294**,
621 2193–2207 (2019).
622

Figure 1



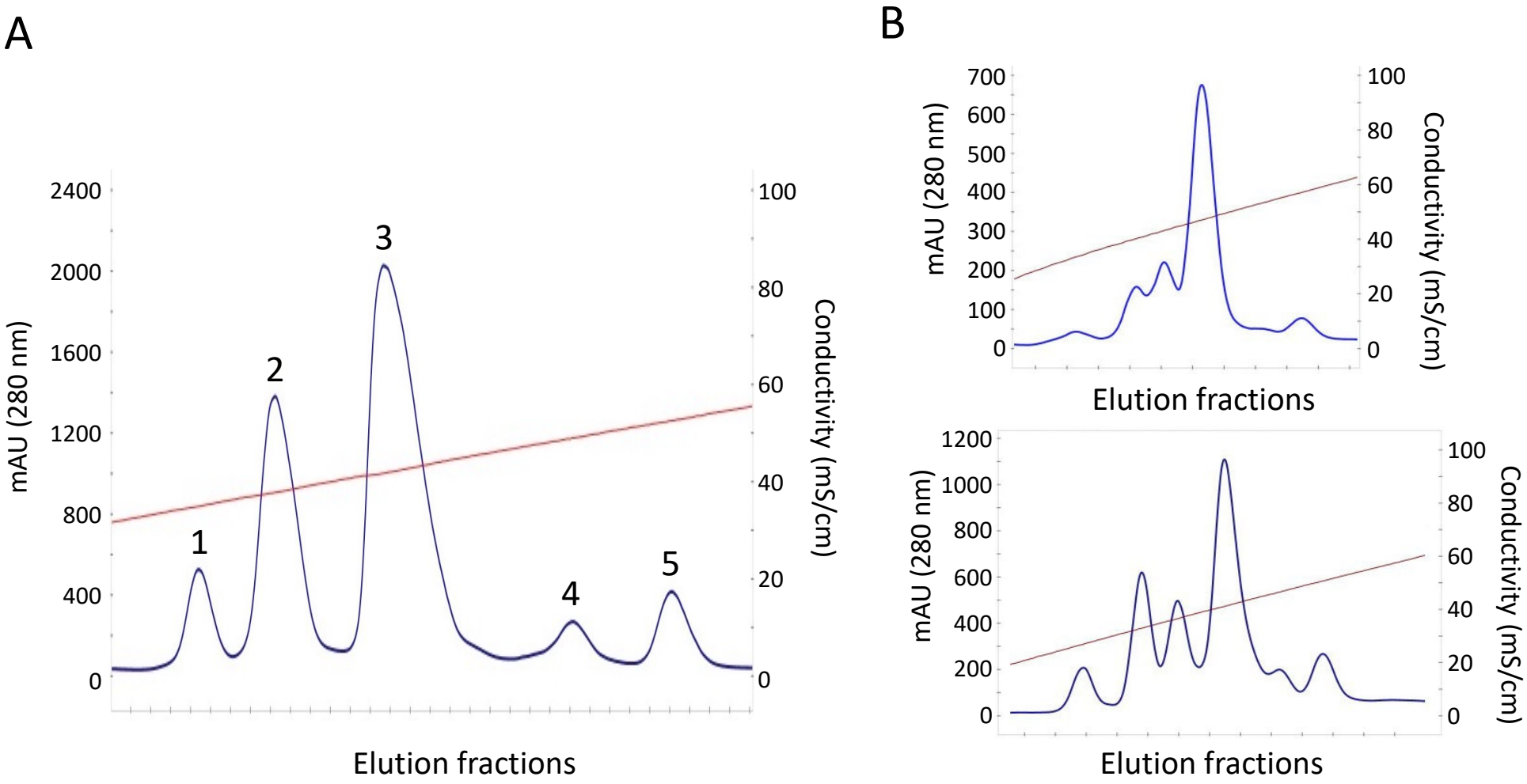
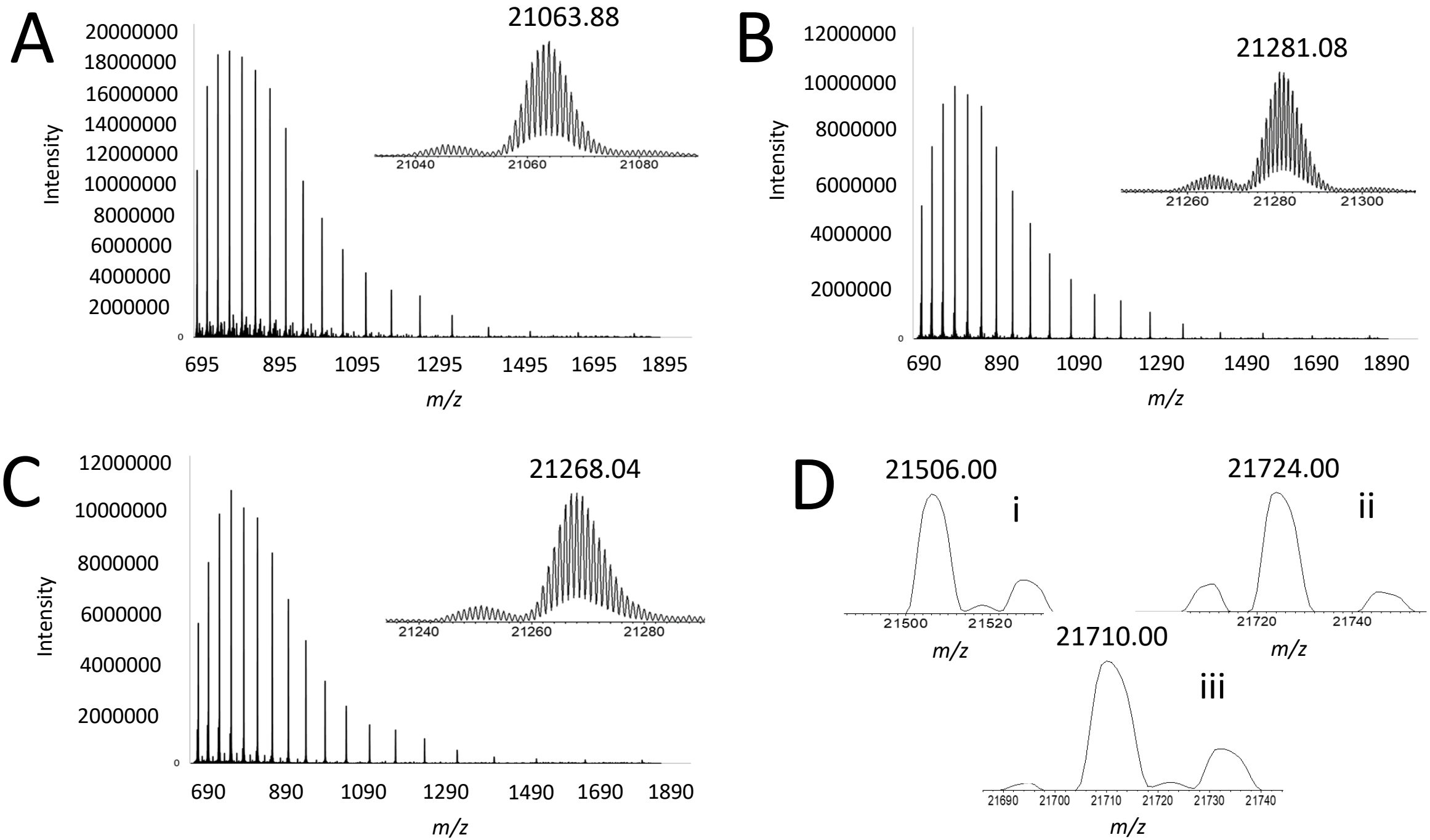


Figure 3



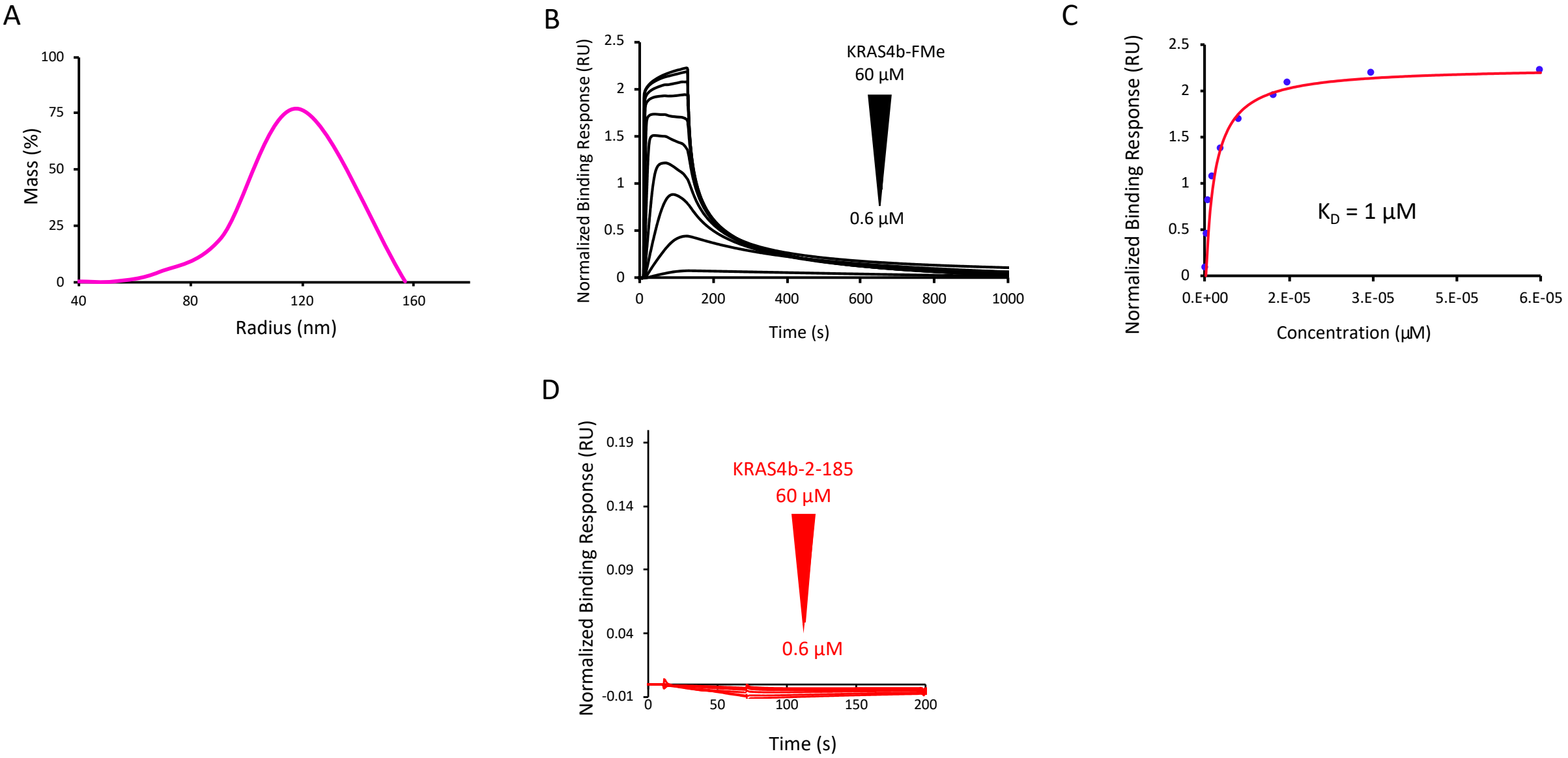


Table 1

Buffer solution	Buffering agent (all 20 mM)	pH	NaCl (mM)	imidazole (	$MgCl_2$
A	HEPES	7.3	300	-	5
B	HEPES	7.3	300	35	5
C	HEPES	7.3	300	500	5
D	MES	6.0	200	-	5
E	MES	6.0	-	-	5
F	MES	6.0	100	-	5
G	MES	6.0	1000	-	5
H	HEPES	7.3	300	-	1

TCEP

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1

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1.8 mL Safe-Lock Tubes, Natural	Eppendorf	22363204	
11 mm Cl SS Interlocked Insert Autosampler Vials	Thermo Scientific	30211SS-1232	
1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC)	AVANTI POLAR LIPIDS	850457	purchase as liquid stocks in chloroform
1-palmitoyl-2-oleoyl-glycero-3-phospho-L-serine (POPS)	AVANTI POLAR LIPIDS	840034	purchase as liquid stocks in chloroform
5427R Centrifuge	Eppendorf		
Acetonitrile, HPLC Grade	Fisher Chemical	A998-1 1L	
Ammonium Acetate	Sigma-Aldrich	09689-250g	
Argon gas	Airgas	ARUP	
Assay Plate 384	CORNING	3544	
Biacore T200 Instrument	GE Healthcare		
Blue Snap-It Seals, T/S	Thermo Scientific	C4011-54B	
Branson Ultrasonic Bath	Thermo Fisher	15-336-1000	
Cation Exchange Chromatography (CEX) column	GE Healthcare Life Sciences	29018183	HiPrep SP Sepharose High Performance
CHAPS	Sigma	C3023	
Dyna Pro Plate Reader	Wyatt Technologies		
Exactive Plus EMR Mass Spectrometer	Thermo Scientific		
Formic Acid	Sigma-Aldrich	F0507-500Ml	Use Reagent Grade or better
Gilson vials 7x14 mm Tubes	GE Healthcare	BR-1002-12	
Glass screw thread vials with PTFE foam liners	Scientific Specialities	B69302	
High speed/benchtop centrifuge	Thermo Fischer Scientific	05-112-114D	capable of up to 4,000 xg
His6-Tobacco Etch Virus (TEV) protease	Addgene	92414	Purified as per Raran-Kurussi *et al.* (2017) Removal of Affinity Tags with TEV Protease. In: Burgess-Brown N. (eds) Heterologous Gene Expression in E.coli. Methods in Molecular Biology, vol 1586. Humana Press, New York, NY

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Immobilized Metal Affinity Chromatography (IMAC) column	GE Healthcare Life Sciences	28-9365-51	HisPrep FF 16/10
In-House Water Supply, Arium Advance	Sartorius Stedim		Resistivity of 18 MΩ0-cm
Lipid extruder set with holder	AVANTI POLAR LIPIDS	610023	
Liquid nitrogen	Airgas	NI-DEWAR	
M110-EH microfluidizer	Microfluidics		
MabPac RP UHPLC Column, 4 um, 3.0 x 50 mm	Thermo Scientific	088645	
MabPac SEC-1 Column, 5 um, 300 Å, 2.1 x 150 mm	Thermo Scientific	088790	
MagTran software	Thermo Scientific		
Methanol, HPLC Grade	VWR Chemicals	BDH20864.400	
NGC Chromatography System	BioRad	78880002	NGC Quest^{TM} 100 Chromatography system
Protease Inhibitor Cocktail without EDTA or other chelators	Millipore Sigma	P8849	
Rubber Caps type 3	GE Healthcare	BR-1005-02	
Series S Sensor Chip L1	GE Healthcare	29104993	
Spectrophotometer	Thermo Fischer Scientific	13-400-519	Absorbace at 280nm
Ultra-15 Centrifugal Filter Units, 10K NMWL	Millipore Sigma	UFC901008	PES membrane
Ultracel 10K MWCO Ultra 0.5 mL Centrifuge Filters	Amicon	UFC501024	
Ultracentrifuge	Beckman Coulter	Optima - L80K	capable of 100,000 x*g*
Vanquish UHPLC (Pump, Column Hearter, and LC System)	Thermo Scientific		
Vortex Genie 2	Fisher	12-812	
Water, HPLC Grade	Sigma-Aldrich	270733-1L	May use in-house water source (see below)
Whatman GD/XP PES 0.45 μm syringe filter	GE Healthcare - Whatman	6994-2504	
Xcalibur QualBrowser	Thermo Scientific		proteomics software

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sponsored by the National Cancer Institute

9/21/2019

Dear Dr., Steindel,

Thank you for response to our manuscript submission. Below is highlighted all of the editor's comments and our responses. In addition, we have revised lines 107-110, 115-118, 127-133, 136-148, 168-178, 188-190, and 200-203 (original submission numbering) to minimize textual overlap with a previous book chapter. Finally, we have identified a section of the manuscript that we feel is a key step in the protein purification and should be featured in the videography.

Reviewer #2:
Minor Concerns:
1 The authors are using 'KRAS' and 'KRAS4b' interchangeably throughout the text. For example, 'KRas' is used in lines 45, 63, and 243, whereas 'KRas4b' is used in lines 67, 74, and 78. These are only handful of examples. Please be consistent, since KRas can also represent KRas4A.

- All KRAS has been replaced with KRAS4b

2 Figures do not have figure numbers. Makes difficult to read.

- Unsure of this issue – all figures have numbers

3 The strain of insect, Trichoplusia ni, is listed in line 393. This should come earlier such as in Intro or Protocol section.

- The following sentence was inserted ealier in the mthods, line 118. "See Gillette *et al*. (2019) for details about the Tni.FNL insect cell line and expression protocols that are beyond the scope of this report."

4 Abstract (line 31) – KRASb4 should be KRAS4b.

- Corrected

5 Abstract (line 23) - The authors state KRas4b is mutated in 25% of human cancers. The authors should provide a citation for this %. Please make sure this '25%' is K-Ras-mutated cancer, not pan-Ras-mutated cancer.

- This has been modified to "KRAS4b, which is mutated in 22% of human cancers" and Prior et al, Cancer Research 2012, vol 72 p 2457-67

6 Line 41/42: NRas and KRas also can be geranylgeranylated when farnesylation is inhibited. The authors should include "under the basal condition" at the end of 'farnesylated protein' in line 42.

- "under basal conditions" was inserted

7 Line 73: "(FMe)" should be included after "farnesylated and carboxymethylated C-terminus" since the authors do not describe what "FMe" stands for.

- This has been corrected

8 Line 495: Re-write the sentence. "This does not hold not true for native mass analysis.."

- This sentence has been written to clarify. "However, in native mass analysis where the protein is in its native folded conformation there are lower mass to charge ratios and higher concentration are required."

9 The table showing Material/Equipment/Comments/Description is broken in PDF version.

- This has been fixed

Reviewer #3:
Minor Concerns:
1. Please clarify the apparent affinity for KRAS-FMe binding to liposomes, as Figure 4 shows 1uM, where as the text in the legend states 1 mM.

- This has been corrected to 1uM

2. It would be nice to include a step in the purification for nucleotide exchange to produce the active GTP (or more stable GMPPNP analogue) bound protein too, especially as the future potential application mentioned (screening against KRAS:Raf complex) would require the protein to be in the active state.

- This is an important point. However, the authors feel that providing an additional method for the nucleotide exchange would make the protocol too long. To address the reviewers concern we have inerted the following statement "1.21 Note the purified protein is bound to GDP. KRAS4b-FMe can be excahnged into GppNHp using previously published protocols[16]." At the end of the purification protocol. If the editors would prefer, we can include an additional section outlining the exchange protocol.

3. Would inserting the L1 chip and allowing buffer to flow over the surface to hydrate the chip for a defined time period prior to activation improve liposome immobilization levels?

- We clarified by inserting the following statement “Perform start-up cycles of ten 1-minute injections of running buffer to hydrate the chip surface.” This is part of our routine protocol and we have not tested the effect on immobilization levels when omitting this step

4. Please clarify what the SPR response is normalized to. It would be nice to note aspects to look out for in the sensorgrams to indicate data quality (eg. reaching steady state, expected Rmax) or note why this is not possible (eg. complicated stoichiometry or liposomes not having a uniform molecular weight for RMax calculations). It would also be good to refer readers at section 3.2.5 to the discussion section to note why the 1:1 binding model may be inappropriate. Has a multi-site binding model been tested?

- We added a more detailed explanation of the SPR data and our approach to fitting the sensorgrams. “SPR is a powerful technique to study ligand-ligand interactions providing information on stoichiometry and binding affinity. Well behaved sensorgrams should have an association phase that reaches a steady state, this maximal binding response (Rmax) provides an estimate of the stoichiometry for the interaction. The dissociation rate should follow a single exponential decay, assuming a 1:1 binding interaction[22]. However, proteins binding to a membrane surface usually occur with more complex stoichiometries[23] and multiple affinities that result in sensorgrams with more complex kinetics. We have not been successful in fitting the kinetics to a multi-site binding model. However, the equilibrium binding isotherms can be fit using commercial evaluation software to provide an apparent equilibrium binding affinity (K_D).”

Sincerely,

Andy Stephen Ph.D.

Principal Scientist,

RAS Biochemistry and Biophysics

Frederick National Laboratory for Cancer Research