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# Expression, purification, and characterization of soluble K-Ras4B for structural analysis

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ABSTRACT

A p21 GTPase K-Ras4B plays an important role in human cancer and represents an excellent target for cancer therapeutics. Currently, there are no drugs directly targeting K-Ras4B. In part, this is due to the lack of structural information describing unique features of K-Ras4B. Here we describe a methodology allowing production of soluble, well-folded K-Ras4B for structural analysis. The key points in K-Ras4B preparation are low temperature expression and extraction of K-Ras4B from the insoluble fraction using a nucleotide loading procedure in the presence of $Mg^{2+}$ and citrate, a low affinity chelator. Additionally, a significant amount of K-Ras4B could be extracted from the soluble fraction. We show that recombinant K-Ras4B is monomeric in solution. Excellent NMR signal dispersion suggests that the protein is well-folded and is amenable to solution structure determination. In addition, using phospholipid bilayer nanodiscs we show that recombinant K-Ras4B interacts with lipids and that this interaction is mediated by the C-terminal hypervariable region.



## Introduction

Ras proteins are small plasma membrane associated GTPases that act as signal tranducers between cell surface receptors and several intracellular signaling cascades. These molecules regulate cell survival, proliferation, motility, and cytoskeletal organization. Over 150 small GTPases sharing 40–50% of sequence homology comprise the Ras superfamily [1]. The four classical p21 Ras proteins are H-Ras, N-Ras, and the splice variants K-Ras4A and K-Ras4B, of which K-Ras4B is more abundant in most tissues. Ras proteins are mutated in up to 30% of human malignancies of epithelial origin [2]. However, greater than 85% of all Ras mutations are found in K-Ras [3]. Thus, among Ras proto-oncogenes K-Ras appears to be the "preferred" target for activating mutations in human malignancies.

The classical p21 Ras proteins are highly homologous and activate very similar signaling pathways. Ras proteins contain several functional domains including a nucleotide binding domain, an effector binding domain, a nucleotide exchange factor binding domain, and a C-terminal domain that is responsible for membrane localization. The N-terminal 166 amino acids comprising the catalytic domain of Ras exhibit 95% of sequence identity. Only the C-terminal hypervariable region is significantly different among Ras proteins displaying less than 15% of sequence homology. Unlike other Ras isoforms, the hypervariable region of K-Ras4B contains a polylysine region and a farnesyl group. Other Ras isoforms are palmitoylated in addition to the farnesyl modification.

Although H-Ras has been well characterized using NMR [4] and X-ray crystallography [5], K-Ras4B received significantly less attention from structural biologists. Thus far, only one crystal structure of GTP-γ-S-loaded catalytic domain of K-Ras4B has been solved (Y. Tong et al., unpublished results); and that appears to be identical to the structure of the GTP-γ-S-loaded catalytic domain of H-Ras. The overall fold of the Ras catalytic domain is defined by five helices arranged around five beta-strands. Five regions of Ras proteins participate in nucleotide binding. Nucleotide binding specificity is determined by interactions between conserved D119 and A146 residues with guanine nucleotides. Additional interactions are important for stabilization of the GTP-bound structure. The key role of $Mg^{2+}$ in GTP binding is supported by the fact that $Mg^{2+}$ ligands are highly conserved and are part of the effector loop region, also known as Switch I (residues 30–40) that is important for interactions with GTPase activating proteins. A water molecule attacks the phosphate group of GTP resulting in GTP hydrolysis. GTP hydrolysis initiates a conformational change into the inactive GDP-bound form involving the effector loop (residues 32–36) [6]. Guanine nucleotide exchange factors interact with the Switch II region (residues 98–108) and are responsible for replacement of GDP with GTP [7].

The structural differences between H-Ras-GDP and H-Ras-GTP are mostly found in the Switch I and Switch II regions [8]. The amide groups of residues 60 and 61 form hydrogen bonds with the γ-phosphate of GTP. Similar backbone-phosphate interactions are found in the Switch I region. Structural analysis of oncogenic

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mutants at positions 12, 13 and 61 of H-Ras revealed very minor changes in protein structure [5]. The mutations disrupt the catalytically active constellation of residues, alter the p$K_a$ of the GTP γ-phosphate, and hinder GTP hydrolysis [9].

Despite the very high degree of homology, studies suggest that each Ras isoform functions in a unique and radically different way from the other Ras proteins in normal physiological processes as well as in pathogenesis. For instance, K-Ras4B knockout but not H-Ras is embryonic lethal [10]. Differential activation of effector proteins by K-Ras4B versus H-Ras is widely known [11–13]. For example, K-Ras4B, as compared to H-Ras, activates Rac signaling more efficiently as demonstrated using membrane ruffling and cell migration assays [11]. Nucleotide exchange factor Ras-GRF1 preferentially activates H-Ras over K-Ras and N-Ras [14]. Another nucleotide exchange protein RasGRP2 activates K-Ras and N-Ras but not H-Ras [15], while mSos1 prefers H-Ras over K-Ras [13]. Although differences in subcellular localization of Ras proteins may account for some of the functional variation [16], inherent biochemical differences may also exist. For example, Sur8, RassF2, and calmodulin specifically interact only with K-Ras4B *in vitro* [17–19]. One possible explanation for the binding specificity and differential activation of signaling pathways is the influence of the hypervariable C-terminal region. In fact, the hypervariable region of H-Ras may transiently interact with the catalytic domain and induce perturbations in structure and dynamics propagating throughout the whole protein [4]. Based on our recent analysis of K-Ras4B binding to calmodulin, we propose that the hypervariable region of K-Ras4B also interacts with the catalytic domain in the GDP-bound form [20]. The hypervariable region of K-Ras4B may induce changes in the whole K-Ras4B protein creating a structurally and dynamically unique system with features that could be used for drug design. This underscores the importance of detailed structural characterization of full-length K-Ras4B.

To facilitate the discovery of therapeutic agents specifically targeting K-Ras4B in human cancer, we wish to determine the three-dimensional structure of full-length K-Ras4B and to understand how the hypervariable region helps K-Ras4B to achieve its signaling specificity. To this end, we have expressed and purified full-length K-Ras4B for structural analysis. In addition, using nuclear magnetic resonance (NMR)[1] and size-exclusion chromatography we demonstrate that K-Ras4B is a well-folded monomeric protein in solution and is amenable for structure determination. Moreover, using a novel bilayer nanodisc technology [21] we show that the recombinant K-Ras4B is capable of interaction with phospholipids primarily through the hypervariable region.

## Experimental procedures

### *Protein expression, purification, and activity*

Cloning of human K-Ras4B into the pET42a bacterial expression vector was performed as previously described [20]. A C-terminal 6× histidine tag was added to the K-Ras4B sequence during cloning to ease protein purification and to reduce the known C-terminal proteolytic degradation [4,22]. To optimize the production of recombinant K-Ras4B, BL21-AI cells (Invitrogen) containing the K-Ras4B gene were induced and grown in M9 media [23] containing 0.4 M NaCl at 37 °C for 5–8 h and 18 °C for 15 h, with shaking at 250 rpm. For both conditions the cells were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.2% arabinose at 37 °C and an OD of approximately 0.6. The temperature of the shaker-incubator was reset to 18 °C to allow for gradual cooling of the cultures. The induction time was 15 h. The final OD (just before harvesting) was 1.4 and 1.75 for the 37 °C and 18 °C cultures, respectively. One milliliter of cell culture was harvested before and after induction to assess the induction of K-Ras4B expression. Another tested condition included addition of 2% ethanol during induction and cell growth for 22–28 h. Cells were harvested by centrifugation and stored at −80 °C overnight. The first extraction of K-Ras4B was performed using B-PER bacterial extraction reagent (Pierce) with 10 mM $MgCl_2$, 50 μg/mL DNaseI, 2 mM phenylmethanesulfonyl fluoride (PMSF), 1–2 tablets of EDTA-free complete (Roche), and 10 mg of lysozyme. The cells were lysed for approximately 1 h at room temperature to allow for both thawing and efficient lysis. The cell debris was removed by centrifugation for 30 min at 18,000 rpm in an SS34 rotor at 4 °C. The supernatant was supplemented with 0.1 mM GDP, 20 mM sodium citrate, 50 mM KCl, and 5 mM β-mercaptoethanol and incubated at room temperature for one hour with stirring. Additional extraction of K-Ras4B from the pellet was performed with 10 mM Tris/HCl pH 7.6, 20 mM Na-Citrate, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM GDP, and 2 mM β-mercaptoethanol. The suspension was incubated on an orbital shaker for 1 h, and then spun in a centrifuge the same way as the first lysate. Soluble K-Ras4B was dialyzed into His-Bind 1× Binding Buffer (Novagen) with 10% glycerol. Recombinant K-Ras4B from each of the two extractions was loaded separately onto $Ni^{2+}$-charged His-Bind resin (Novagen) by incubation for 2 h at room temperature. The slurry was loaded into a disposable column (Bio-Rad) and was washed extensively with 1× His-Bind Binding Buffer (no imidazole or glycerol) and with 1× His-Bind Wash Buffer (Novagen) containing 10 mM imidazole. K-Ras4B was eluted using a gradient from 60 mM to 1 M imidazole. The maximum elution was achieved at 200 mM imidazole. The purity of K-Ras4B preparations was analyzed by SDS–PAGE electrophoresis and the pure fractions were pooled. Pure K-Ras4B was dialyzed into the storage buffer containing 50 mM Tris-citrate pH 6.5, 50 mM NaCl, 5 mM $MgCl_2$, 0.01 mM GDP and 10 mM β-mercaptoethanol for storage. A pressurized stir-cell was used to concentrate protein solutions. Similar protocols were used to prepare truncated K-Ras4B (1–166) (K-Ras4B$_{tr}$). An SDS–PAGE gel was run to check for induction of expression and the purity of the first and second extractions from the two temperature conditions. 1.04 mg of whole cell pellet from the 18 °C and 37 °C cultures and the final insoluble pellet from the second extractions were mixed with 4X Laemlli gel loading buffer, heated for 7 min at 80 °C and loaded on the SDS–PAGE gel. The total protein concentration for each of the purified first and second extractions from both the 18 °C and 37 °C cultures was normalized to 25 μM. Eighteen microliters of each protein sample was mixed with 6 μL of 4X Laemlli gel loading buffer and heated for 7 min at 80 °C. Twenty microliters of each protein sample was loaded on the gel and run for 55 min at 180 V. The gel was developed using Coomassie stain.

Gel filtration of purified K-Ras4B was performed using the Superdex-75 column to assay for the presence of impurities and possible oligomerization of K-Ras4B. MALDI-TOF mass-spectrometry was also done on $^{15}N$ enriched protein to check for impurities and possible post-translational modifications of K-Ras4B. The activity of purified K-Ras4B was assayed as described previously [20] using the Ras Activation Assay Kit (Millipore). To calculate the total possible yield, K-Ras4B was also extracted using a previously published protocol that uses urea for extraction under denaturing conditions in addition to the soluble fraction [24].

### *NMR experiments*

$^{15}N$ enriched K-Ras4B was expressed, purified and concentrated as described above except that $^{14}NH_4Cl$ was replaced with $^{15}NH_4Cl$

[1] *Abbreviations used:* NMR, nuclear magnetic resonance; IPTG, isopropyl β-D-1-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; SPR, surface plasmon resonance; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine.