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K-Ras4B lipoprotein synthesis: Biochemical characterization, functional properties, and dimer formation

Alexey Dementiev *,[1]

Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, 900 South Ashland Av., Chicago, IL 60607, USA

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ABSTRACT

K-Ras4B, a small GTPase and a key oncogene, plays a central role in the early steps of signal transduction from activated receptor tyrosine kinases by recruiting its downstream effectors to the cell membrane. Specific posttranslational modifications of K-Ras4B, including the addition of C-terminal farnesyl and methyl groups, mediate its proper membrane localization and signaling activity. The mechanism and molecular determinants underlying this selective membrane localization and molecular interactions with its many regulators and downstream effectors are largely unknown. Preparative amounts of the post-translationally processed K-Ras4B protein are necessary to carry out structural, functional, and cell biological studies of this important oncogene. In this work we describe a simple and efficient method for synthesis of milligram quantities of functionally active, fully processed K-Ras4B. Using this preparation, we observe K-Ras4B dimerization *in vitro*; this has not been observed previously and could be important for its activity, membrane anchoring, and translocation between different cellular membranes.

Introduction

Ras proteins are small membrane-associated GTPases that act as molecular switches to regulate cell growth, survival and differentiation by cycling between the active guanosine triphosphate (GTP)[2]-bound and the inactive guanosine diphosphate (GDP)-bound forms [1–3]. Ras activation allows these proteins to receive signals from different kind of receptors in responses to various growth factors and transmit it to a large number of downstream effectors [4,5]. Ras proteins are known to be oncogenic in a range of human cancers [6,7]. There are at least three Ras family genes in humans: *Harvey-ras, Neuroblastoma-ras* and *Kirsten-ras*; the latter produces two splice variants, a minor K-Ras4A variant and the major isoform, K-Ras4B, which is the one commonly mutated in cancer [8,9].

The hypervariable C-terminal region of K-Ras4B (HVR, consisting of 22 amino aid residues at its C-terminus) undergoes post-translational modifications by farnesylation, proteolysis, and methylation [10]. These modifications, as well as a stretch of eight positively charged lysine residues just upstream of the C-terminal farnesylated cysteine, determine the plasma membrane localization of K-Ras4B protein which is critical to its activity [11]. Membrane-bound K-Ras4B transduces the activation signal to the nucleus by engaging its downstream effectors that initiate the PI3K/Akt and MAPKs-mediatedpathways [12].

Over the past 30 years an extensive effort in studying Ras protein function was focused on the development of new effective therapeutics to antagonize aberrant signaling of K-Ras4B in human cancer. In particular, farnesyltransferase inhibitors (FTIs) were designed and many of them were promising as potential anti-cancer agents [13]. However, farnesyltransferase inhibitor strategy was hindered by alternative processing of K-Ras4B by geranylgeranyltransferase and off-target effects [14]. Other cancer therapeutic agents in combination with FTIs are currently used in clinical practice. For instance these include combined treatment with statins that inhibit cholesterol synthesis and osteoporosis drug bisphosphonates that inhibit farnesyl diphosphate synthase, cyclin-dependent kinase inhibitors and MEK kinase inhibitors [15–17]. Although the K-Ras4B inhibitory strategy was promising in preclinical trials and was partially successful in Phase II and Phase III clinical trials it is becoming clear that there are insufficient data on their efficiency in combination with other employed inhibitory approaches used. Therefore, the additional effort is required to

* Fax: +1-312-413-0353.
E-mail addresses: adement@uic.edu, seevich3326@hotmail.com
[1] Present address: 11751 Glenn Cr., Plainfield, IL 60585, USA.

[2] *Abbreviations used:* GTP, guanosine triphosphate; GDP, guanisine diphosphate; DDM, *n*-dodecyl-maltoside; NOG, *n*-octyl-glucopyranoside; DTAP, di-*tert*-amyl peroxide; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; EGS, ethylene glycol-bis(succinimidylsuccinate); sulfo-SMCC, sulfosuccinimidyl 4-[maleimidomethyl] cyclohexane1-carboxylate; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Fmoc, 9-fluorenylmethyl carbamate; RTK, receptor tyrosine kinase; MAPK, mitogen activated protein kinase pathway; PI3 K, phosphoinositide 3-kinase; Akt, serine/threonine kinase Akt; ERK, extracellular signal related kinase; Raf-1 RBD, Ras-binding domain of Raf-1.

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provide further insight into the mechanisms of K-Ras4B binding to the membrane. To attain these goals, preparative scale levels of fully processed K-Ras4B protein are required for these biochemical and structural studies.

Naturally processed Ras proteins can only be expressed and purified using mammalian and baculovirus expression systems with very low efficiency due to low fraction of posttranslationally modified Ras proteins in eukaryotic cells [18]. Bacterially expressed recombinant Ras protein was shown to be specifically lipidated *in vitro* by farnesyltransferase but the designed modification procedure and the purification protocol yielded only analytical amount of the lipidated protein [19,20]. More recently, the semi-synthetic experimental approaches seemed to be more versatile for expression of fully modified Ras proteins with higher yield [21,22]. The production of these modified proteins was based on a combination of bacterial expression techniques, lipopeptide synthesis and expressed protein ligation (EPL). In the case of K-Ras4B, the lipidated peptide corresponds to the hypervariable region of the protein and can be attached to the truncated version of K-Ras4B *via* intein-mediated protein splicing. The EPL approach requires expensive custom peptide synthesis and includes multiple steps of synthesis of protein substrates fused with relatively large intein domains which makes this approach time-consuming and often results in low ligation efficiency.

In this work we developed a novel strategy for obtaining milligram amounts of functionally active, fully modified K-Ras4B. This efficient chemoenzymatic approach consists of three main steps: bacterial expression of correctly folded N-terminal (residues 1–166) catalytic domain of K-Ras4B, chemical synthesis of the Cys-farnesyl-methyl ester modified C-terminal K-Ras4B peptide and transpeptidation of this C-terminal lipidated peptide to the catalytic domain by a sortase-mediated reaction. We use this preparation to reveal, for the first time, dimer formation by recombinant K-Ras4B protein under *in vitro* physiological conditions. We discuss the relevance of K-Ras4B dimerization to its biological function.

Experimental procedures

Materials

n-Dodecyl-maltoside (DDM), *n*-octyl-glucopyranoside (NOG) and DTAP, di-*tert*-amyl peroxide, a radical initiator were from Sigma–Aldrich (St. Louis, MO), farnesyl-L-cysteine methyl ester (Far–Cys) was from Enzo Life Sciences (Farmingdale, NY), sulfosuccinimidyl 4-[maleimidomethyl] cyclohexane1–carboxylate (sulfo-SMCC) were from Thermo Scientific (Rockford, Il), ethylene glycol-bis(succinimidylsuccinate) cross-linking reagent (EGS) was from Pierce (Rockford, IL), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were from Avanti Polar Lipids (Alabaster, AL), Raf-1 Ras-binding domain agarose beads (Raf-1 RBD agarose) were from Millipore (Temecula, CA), EnzChek phosphate assay kit was from Molecular Probes (Eugene, OR), monoclonal anti-C-K-Ras antibody (Ab-1) were purchased from Calbiochem (La Jolla, CA), Ni–NTA agarose was from Qiagen (Miami, Fl), Superdex 200 column (1 × 30 cm) and detection reagents for western blotting were from GE Healthcare (Piscataway, NJ), sortase A sample (700 μM stock protein concentration) bearing an N-terminal 6xHis-tag was generously provided by Doctor Klavs Dolmer (UIC, Chicago, I).

Protein expression and purification

The construct containing the DNA sequence for the first 166 N-terminal amino acid residues of human K-Ras4B was cloned into pET42a vector as described in [23]. This plasmid construct was used to generate a new construct (K-Ras(1–166)-sor) bearing the DNA sequence for the first 166 N-terminal residues of human K-Ras4B protein and downstream sequence for the sortase recognition site (LPETG) followed by Gly(6xHis)-tag using the QuikChange II site-directed mutagenesis kit (Stratagen).

The detailed procedures for K-Ras(1–166)-sor protein expression and purification were described elsewhere [23]. Breifly, BL21-A1 one shot cells (Invitrogen) transformed by the plasmid were grown in LB media at 22 °C, and the culture was induced as recommended by the manufacturer. The cells were harvested at 5 h after induction. The collected cells were lysed by sonication in 50 mM Na-phosphate buffer, pH 7.4, 50 mM NaCl, 2 mM β-mercaptoethanol, 2 mM $MgCl_2$, 50 μM GDP, 10 μg/ml DNase I and 0.2% (w/v) Triton-100 (30 ml of the buffer/bacterial cells collected from 1 L of the cultivated medium). The broken cells were centrifuged at 15,000*g* for 15 min, and the supernatant was discarded. The cell debris pellet was suspended in B-PER bacterial extraction reagent (Pierce, 25 ml of the reagent/cell pellet harvested from 1 L of the culture) supplemented with 1 mM β-mercaptoethanol and EDTA-free complete protease inhibitor cocktail tablets (Roche) followed by constant stirring at room temperature for 3 h. The extraction solution was centrifuged at 15,000*g* for 15 min, and the supernatant was dialyzed against 50 mM Tris–HCl buffer, pH 7.5, 300 NaCl, 10 mM imidazole, 0.5 mM β-mercaptoethanol, 1 mM $MgCl_2$ overnight at 4 °C. The dialyzed protein solution was incubated with 10% Ni–NTA agarose slurry for 2 h at room temperature, and the protein was eluted from the resin by a three-step imidazole gradient with elution steps of 50, 100 and 200 mM in the same buffer. The eluted protein was dialyzed against 50 mM Tris-citrate buffer, pH 6.5, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.5 mM β-mercaptoethanol and 25 μM GDP. The described expression and purification procedures provided the final yield of about 1 mg of pure K-Ras(1–166)-sor sample from 1 L of the cultivated medium. The protein concentration was determined using the Bradford method. SDS–PAGE analysis of the protein samples was performed on Novex 10–20% Tris–Glycine pre-cast mini gels (Invitrogen) under reducing conditions.

Synthesis of farnesyl-modified K-Ras4B protein by sortase-mediated ligation reaction

C-terminal 18-mer (GKEKMSKDGKKKKKKSKC) K-Ras4B peptide was synthesized by solid phase synthesis and lipid-modified by the sullfo-SMCC heterobifunctional crosslinker according to the following procedure: 70 μl of 74 mM farnesyl-cysteine methyl ether dissolved in dimethylsulfoxide was added to 2 mg sulfo-SMCC cross-linker sample (0.1 M final concentration) with the presence of 25% (w/v) *n*-octyl-glucopyranoside detergent (NOG) to produce a maleimide-activated farnesyl-cysteine at the first step of the reaction. The mixture was diluted approximately 4 times with 50 mM phosphate buffer, pH 7.5, in the presence of 5 mM DTAP reducing agent and 150 mM NaCl (conjugation buffer) followed by incubation at room temperature for 1.5 h. The reaction product was purified based on its limited solubility in aqueous buffers. Maleimide-activated farnesyl-cysteine was precipitated by the 15 times dilution with the conjugation buffer. The pellet was dissolved in 250 μl of 60% (v/v) ethanol and 20% (w/v) NOG. At the second step of the reaction, the activated farnesyl-cysteine was incubated with the C-terminal K-Ras4B peptide in conjugation buffer without DTAP agent at the final molar ratio of 5:1 (assuming quantitative synthesis of the activated farnesyl-cysteine at the first step) at room temperature for 12 h to create the C-terminal K-Ras4B peptide-farnesyl conjugate. The farnesylated peptide was purified by reversed-phase chromatography on a Nucleosil C-18 peptide column. The modified peptide concentration was