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Backbone and sidechain ^1H , ^{15}N and ^{13}C assignments of the KSR1 CA1 domain

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Abstract

The backbone and side chain resonance assignments of the murine KSR1 CA1 domain have been determined based on triple-resonance experiments using uniformly [^{13}C , ^{15}N]-labeled protein. This assignment is the first step towards the determination of the three-dimensional structure of the unique KSR1 CA1 domain.

Keywords

KSR1; CA1; MAPK; scaffold protein

Biological context

Kinase Suppressor of Ras (KSR) proteins are scaffolds that regulate the activation of the ser/thr kinase ERK, a mitogen activated protein kinase (MAPK) (Yu et al 1998). A variety of extracellular signals, including growth factors and cytokines, trigger the ERK MAPK pathway, which regulates important cellular responses such as proliferation and differentiation (Boulton et al 1991; Ng et al 2001). The strength and duration of the ERK signal modulates the resulting cellular response and is controlled by a host of regulatory proteins, including kinases, phosphatases, and scaffolding proteins (for a review, see Ebisuya et al 2005). Of the ERK-specific scaffolding proteins, KSRs are the best studied. These multidomain proteins consist of an unique N-terminal domain (CA1), an unstructured proline-rich region (CA2), a small atypical cysteine-rich domain (CA3) required for membrane localization, an unstructured serine/threonine-rich region (CA4) containing an ERK-specific binding motif, and a pseudokinase domain (CA5) that binds MEK (MAPK/ERK Kinase) and Raf (MEK Kinase) (Morrison et al 2003). By binding all three components of the ERK MAPK module—Raf, MEK, and ERK—KSR1 effectively increases the local concentration of activating kinases relative to ERK, thereby driving efficient ERK activation. Additionally, recent evidence suggests that KSR1 may play a direct, non-catalytic role in the activation of Raf (Rajakulendran et al 2009).

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The unique CA1 domain of KSR1 is highly conserved in all KSR proteins except for *C. elegans* KSRs (Clapron et al 2007). Immunoprecipitation experiments with both mouse (McKay et al 2009) and fly (Roy et al 2002) KSR1 proteins show that the CA1 domain is required for stable binding of Raf. Specifically, mutation of conserved leucine and arginine residues (L56G and R57S in mouse) in the CA1 domain disrupts the ability of KSR1 to pull down B-Raf (McKay et al 2009). However, our knowledge of the molecular mechanism of KSR1 regulation of the ERK MAPK module is lacking. Biochemical and biophysical investigation of the nature of the interaction between KSR1 and Raf, MEK, and ERK is necessary for fully understanding the role of KSR1 as a scaffold and as an ERK regulatory protein. Structural studies of the CA1 domain will not only offer additional information regarding its role as a potential Raf-binding domain, but will also reveal novel information on the overall function of this unique domain.

As an essential first step towards the structural and functional characterization of the unique KSR1 CA1 domain, we report here the complete backbone and side chain chemical shift assignment of the murine KSR1 CA1 domain.

Methods and experiments

The codon-optimized open reading frame of the KSR1 CA1 domain (residues 25–170) was synthesized by DNA2.0 (Menlo Park, CA) and cloned into a bacterial expression vector, pJexpress411. The protein was engineered to have an N-terminal expression, purification, and TEV-cleavage tag (MGSDKIH HHHHHENLYFQG).

The expression vector was transformed into BL21 (DE3) Star cells (Invitrogen) and cultures were grown to an optical density (OD₆₀₀) of 0.8 at 37C with shaking at 250 rpm. Protein expression was induced by the addition of 1 mM isopropylthio--D-galactoside (IPTG), and the cultures were grown for an additional 16 hours at 18C with shaking at 250 rpm. Freshly transformed cells were grown in LB medium containing selective antibiotics. To prepare uniformly ¹³C/¹⁵N-labeled and ¹⁵N-labeled protein, bacterial cultures were grown in M9 minimal medium containing selective antibiotics and 4 g/l [¹³C]-D-glucose and/or 1 g/l ¹⁵NH₄Cl (Cambridge Isotope Laboratories, Cambridge, MA) as the sole carbon and nitrogen sources, respectively.

The cells were harvested by centrifugation, resuspended in ice-cold lysis buffer (50 mM Tris pH 7.0, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100, and EDTA-free protease inhibitor tablets (Roche)), and lysed by high-pressure cell homogenization (Avestin C3 Emulsiflex). The bacterial lysate was clarified by centrifugation at 16000 x g for 30 minutes at 4C. After filtration, the supernatant was loaded onto a HisTrap HP column (GE Healthcare) equilibrated with 50 mM Tris, pH 7.0, 5 mM imidazole, and 500 mM NaCl, and the His₆-tagged protein was eluted with a 5–500 mM imidazole gradient over 60 ml. Purified fractions were then pooled and digested with tobacco etch virus (TEV) protease overnight at 4C while being dialyzed against 50 mM Tris, pH 7.0, and 500 mM NaCl. Cleaved protein was further purified using Ni²⁺-NTA immobilized metal affinity chromatography followed by size exclusion chromatography (Superdex 75 26/60 (GE Healthcare) equilibrated in 20 mM NaPO₄, pH 6.5, 100 mM NaCl, 0.5 mM TCEP) to a

purity of >98% and a final yield of ~70 mg protein/ L culture. Purified protein was concentrated to 2 mM. For long-term storage, the protein was flash frozen in liquid nitrogen and stored at -80°C .

NMR spectroscopy

All NMR measurements were performed at 298 K. The sequence-specific backbone assignment was obtained from the following experiments performed on a Bruker AvanceII 500 MHz spectrometer: 2D [^1H , ^{15}N] HSQC, 2D [^1H , ^{13}C] HSQC, 3D HNCA, 3D HNCACB, 3D CBCA(CO)NH, 3D (H)CC(CO)NH, 3D HBHA(CO)NH, 3D HNCO, 3D HN(CA)CO and a 3D ^{15}N -resolved [^1H , ^1H] NOESY spectrum. The side chain assignment of all aliphatic residues was obtained from the 3D HC(C)H-TOCSY recorded on a Bruker AvanceII 800 MHz spectrometer. The aromatic side chains were assigned based on 2D [^1H , ^1H] NOESY (Bruker AvanceII 800 MHz), 2D [^1H , ^1H] TOCSY, and 2D [^1H , ^1H] COSY spectra (both Bruker AvanceII 500 MHz) acquired with a KSR1 CA1 sample dissolved in 100% D_2O solution after complete H/D exchange of all labile protons. The NMR spectra were processed with Topspin 1.3 or 2.1 (Bruker, Billerica, MA) and analyzed using the CARA software package (www.nmr.ch).

Assignment and data deposition

Assignments were obtained for more than 97% of the backbone nuclei ($^{13}\text{C}\alpha$, $^{13}\text{C}\text{O}$, $\text{H}\alpha$, ^{15}N , and H_N) and 98% of side chain $^{13}\text{CH}_n$ moieties. Of the 145 expected backbone amide N-H pairs (4 prolines), 141 were identified; the missing ones corresponding to the N-terminal three-residue cloning artifact and Cys60 (Figure 1). All side chain resonances were assigned, except for the $^{13}\text{CH}_2$ of Trp127, where accurate assignment was uncertain, as well as side chain NH_N moieties of arginines, lysines, and the N-terminal histidine. Figure 2 shows the secondary structure propensity (ssp) scores (Marsh et al. 2006) based on $\text{C}\alpha - \text{C}\beta$ chemical shift values. KSR1 CA1 domain exhibits positive deviations from random coil chemical shift values, indicating an exclusively α -helical structure. All chemical shifts were deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number 17045.

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Figure 1.

(A) Cartoon of the full-length KSR1 protein. KSR1 consists of five conserved areas (CA1-5) shown in dark gray. Structured regions are depicted as thick cylinders, and unstructured regions, as predicted by IUPRED, as thin cylinders. The CA1 domain is a unique, well-folded domain at the N-terminus of KSR1. (B) Fully annotated 2D [^1H , ^{15}N] HSQC spectrum of the KSR1 CA1 domain recorded at 298 K on a Bruker AvanceII 800 MHz spectrometer. Assigned residues are labeled with the residue name (single letter code) and number in the sequence. NH₂ groups of glutamine and asparagine side chains are indicated by a solid line –, tryptophan side chains are indicated by an asterisk *, and arginine NH_N moieties are indicated by a cross †. (C) Secondary structure propensity (SSP) scores for

residues 25–170 of the KSR1 CA1 domain. The RefDB random coil database was used in the calculation. The α -helical regions are indicated by gray cylinders.