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ARTICLE

^{1}H, ^{15}N, and ^{13}C resonance assignments of the intrinsically disordered SH4 and Unique domains of Hck

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Abstract

Hematopoietic cell kinase (Hck) is an important signaling enzyme and a potential drug target for HIV infections and Bcr/Abl-chronic myeloid leukemia. The protein shares the same SH4–Unique–SH3–SH2–kinase multi-domain architecture as the other eight members of the Src family of non-receptor tyrosine kinases. These enzymes are often found anchored to the intracellular side of the membrane via lipidation of the SH4 domain and are integral components of signaling cascades localized at the cell surface. Despite the detailed structural information available for the SH3, SH2, and kinase domains of Hck, the intrinsically disordered nature of the SH4 and Unique domains has resulted in a lack of information for this important region of the protein that is responsible for membrane association. Here, we report the ^{1}H, ^{15}N and ^{13}C chemical shifts of the Hck SH4–Unique domains at pH 4.5.

Keywords Src family kinase · Hck · Intrinsically disordered protein

Biological context

Protein kinases are enzymes that catalyze the transfer of the γ-phosphate of ATP to an amino acid side chain (phosphorylation) in specific proteins. Phosphorylation is used to activate numerous signaling pathways within cells, leading to cell proliferation, differentiation, migration, and metabolic changes. The activity of kinases must be tightly regulated for proper cellular function. It is therefore not surprising that the human genome encodes over 500 protein kinases to execute this task in a controlled fashion. Among these, a group of non-receptor tyrosine kinases termed the Src family kinases (SFKs) has received considerable attention for the central role its members have played in the development of the field of tumor virology and subsequently in defining our current understanding of the molecular mechanisms of carcinogenesis. The SFKs are comprised of nine highly conserved signaling proteins (Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr, and Yrk) with a similar regulatory mechanism. All of the SFKs function as molecular switches whose catalytic activity can be modulated in response to specific cellular signals, such as growth factors or cytokines binding to membrane-bound receptor proteins.

Structurally, all nine members of the SFKs share a common multi-domain topology. The well-studied core of the protein is comprised of two regulatory "Src homology" domains (SH3 and SH2) and a kinase domain (SH1) capable of transferring phosphate from ATP to tyrosine residues. Preceding the core is an intrinsically disordered region of low sequence conservation referred to as the "Unique" domain and a small membrane-anchoring SH4 domain located at the N-terminus that can be myristoylated or palmitoylated (Resh 1994). Interactions between the SH2, SH3 and kinase domains of SFKs have led to paradigm defining descriptions of interdomain allosteric regulation and represent a promising test case for structure based drug design. The SH4-U is often omitted from this picture, as it is typically removed in constructs designed for crystallization because of its intrinsically disordered structure. Recent NMR studies on Lck (Kim et al. 2003) and Src (Maffei et al. 2015), however, suggest that this region is capable of mediating lipid–protein as well as inter- and intramolecular protein–protein interactions, and plays key roles in regulating the function of the kinases.

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Hematopoietic cell kinase (Hck) is a member of the SFKs notable for its critical role in Bcr/Abl-chronic myeloid leukemia and its interactions with the HIV-1 accessory protein Nef (Poh et al. 2015). The two dominant isoforms of human Hck are referred to as p59Hck and p61Hck. The longer p61 isoform contains an additional 21 residues at the N-terminal end (MGGRSSCEDPGCPRDEERAPR), and is derived from an alternate start codon of the same mRNA. Both isoforms may be myristoylated at the N-terminus but p59Hck can also be palmitoylated on Cys3. The variable acylation is thought to be a major determinant in localizations within the cell: p59Hck is mainly associated with the plasma membrane and p61Hck with lysosomal membranes. In at least one case the tumorigenic activity of Hck requires cooperative action of both isoforms, and despite their similarity, p59Hck and p61Hck are differentially localized and trigger distinct phenotypes when activated. Thus, a fundamental understanding of the SH4-U region is likely to lead to the discovery of new intermediate states and alternate strategies for inhibition of kinase activity for drug development. Here we present the NMR chemical shift assignments of both the p61 and p59 isoforms of $Hck_{SH4\text{-}U}$ at low pH.

Methods and experiments

Cloning, expression and purification of $Hck_{SH4\text{-}U}$

A synthetic gene fragment encoding residues 1–79 of human p61Hck was cloned into the NdeI and SalI sites of a modified pET28a vector containing an N-terminal TEV-cleavable His6 tag. The bases encoding the last glycine of the TEV site and the NdeI site were deleted by site-directed mutagenesis so that TEV cleavage of the expressed protein would yield residues 2–79 of p61Hck. To generate $p59Hck_{SH4\text{-}U}$ the bases encoding residues 2–22 of p61Hck were deleted from the Hck.p61.4U construct by site-directed mutagenesis to produce the Hck.p59.4U expression plasmid.

$Hck_{SH4\text{-}U}$ expression and purification

A single colony of transformed BL21(DE3) *Escherichia coli* was used to inoculate a 60 mL starter culture of ^{13}C ^{15}N labeled M9 minimal medium (U-^{13}C glucose, $^{15}NH_4Cl$) containing 50 µg/mL kanamycin and a small aliquot of a resuspended multi-vitamin tablet in ddH_2O (approximately 1/10th of a CVS Spectravite tablet per liter of growth medium). Following overnight growth in a shaking incubator set to 37 °C and 250 rpm, the cultures were scaled up by transferring 40 mL of the starter growth into 1 L of fresh medium. When an OD_{600} of 0.8 was reached, the culture was transferred to a 4 °C cold room for 20 min, then grown at 18 °C and 250 rpm for an additional 20 min prior to induction with 1 mM IPTG. Cells were harvested after 20 h of growth post induction and frozen at − 80 °C for up to 1 month.

To purify $Hck_{SH4\text{-}U}$, cells were resuspended in 30 mL of lysis buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 5% glycerol, and 20 mM imidazole) with three EDTA-Free Pierce Protease Inhibitor Mini Tablets. The cells were lysed by three passes at 15,000 psi in an Emulsiflex C5 homogenizer, and the lysate was cleared by centrifugation at 130,000×*g* for 50 min. The resulting supernatant was passed over a POROS-20 MC column charged with Ni^{2+} from which the protein was eluted with lysis buffer containing 500 mM imidazole. The His-tag and imidazole were removed by incubation of the protein with TEV protease during dialysis overnight at 4 °C against 20 mM Tris, pH 8.0, 100 mM NaCl, 5% glycerol, 0.05 mM EDTA, and 1 mM DTT. The resulting sample was then passed over Ni–NTA Agarose to remove TEV and any remaining His-tagged $Hck_{SH4\text{-}U}$. As a last step, the samples were exchanged into 50 mM sodium acetate with 5 mM TCEP at pH 4.5, and then separated from any remaining contaminants using a Superdex 200 column on an ÄKTA FPLC. Typical yield for ^{15}N ^{13}C labeled $p61Hck_{SH4\text{-}U}$ was 14 mg and $p59Hck_{SH4\text{-}U}$ was 1.6 mg/L culture as estimated using an extinction coefficient of $\varepsilon_{280} = 1.49\ mM^{-1}$ for both constructs (the only chromophore in the reduced sample is not included in the truncation). Owing to the high potential for error associated with having only one Tyr for determining concentrations spectrophotoscopically, the extinction coefficient of $p61Hck_{SH4\text{-}U}$ was confirmed using a Pierce BCA Protein Assay Kit. SDS-PAGE and ESI–MS were used to confirm the identity and purity of the samples. $p59Hck_{SH4\text{-}U}$ samples appeared to be more prone to aggregation, so attempts were made to purify the protein at higher pH (10 mM Tris, 150 mM NaCl pH 8.0), but this did not result in significant increases in yield. For NMR data collection, pure fractions were concentrated and brought up to 5% ($p59Hck_{SH4\text{-}U}$) or 10% D_2O ($p61Hck_{SH4\text{-}U}$). Samples were stable for two or more weeks in Shigemi tubes sealed with Parafilm.

NMR spectroscopy

$p61Hck_{SH4\text{-}U}$

Data were collected on a 900 MHz Bruker Avance II equipped with a TCI cryoprobe and a 600 MHz Bruker Avance III HD with a TXI probe. ^1H–^{15}N HSQC, CBCA(CO)NH, HNCACB, HNCO, HN(CA)CO, HNCA, HN(CO)CA, HBHACONH, ^1H–^{15}N TOCSY–HSQC and ^1H–^{15}N NOESY–HSQC experiments were collected in order to make chemical shift assignments. Additionally, the high number of proline residues in the construct (10 of 78) prompted the collection of ^{13}C detect "protonless" ^{13}C–^{15}N CON, CACO and CBCACON data sets.

p59Hck$_{SH4-U}$

Data were collected on a 600 MHz Bruker Avance III HD with TXI probe. ^{1}H–^{15}N HSQC, CBCACONH, HNCO, and HBHACONH were collected in order to make chemical shift assignments. Additionally non-uniform sampling (NUS) was used to collect ^{1}H–^{15}N TOCSY–HSQC, HNCA, HNCACB, and HN(CA)CO data sets.

^{1}H chemical shifts were referenced to DSS at 25 °C. Indirect referencing was applied with DSS Ξ values to obtain ^{15}N and ^{13}C shifts. NMR data were processed with NMRPipe (Delaglio et al. 1995) and MDD (Jaravine et al. 2006), and assigned with SPARKY (Goddard and Kneller 2008) and PINE (Bahrami et al. 2009).

Assignments and deposition

Buffer and temperature screens demonstrated that p61Hck$_{SH4-U}$ backbone amide proton chemical exchange rates were sufficiently slow at pH 4.5 and 25 °C to produce high quality NMR spectra. Addition of TCEP to the sample further improved the spectra (Fig. 1a) and the protein was stable enough in 50 mM acetate to facilitate detailed study by NMR under reducing conditions. In addition to the standard triple resonance experiments, the high number of proline residues in the construct (10 of 78) prompted the collection of ^{13}C detect "protonless" data sets to aid in assignment (inset Fig. 1a). Following data collection, peak information was submitted to the PINE server (Bahrami et al. 2009) to generate preliminary assignments, which were then corrected manually. In all, the chemical shifts of 576 atoms were assigned, comprising 98.9% of the possible N, H, C′, C_{α}, C_{β}, H_{α}, and H_{β} resonances, including 67 out of 67 backbone amides.

Comparison of the ^{1}H–^{15}N HSQC of p59Hck$_{SH4-U}$ (Fig. 1b) with that of p61Hck$_{SH4-U}$ indicated that a majority of the peaks had little to no perturbation upon removal of the first 21 amino acids. Therefore, only a subset of the triple resonance experiments (those containing only the i to i − 1 connectivities) was necessary to verify and complete the vast majority of the assignments. NUS was used to collect three additional data sets in order to confirm the assignments and provide chemical shifts for residues preceding prolines. For p59Hck$_{SH4-U}$, the chemical shifts of 411 atoms were assigned corresponding to 96.9% of the possible N, H, C′, C_{α}, C_{β}, H_{α}, and H_{β} resonances, including 49 out of 49 backbone amides.

The anticipated intrinsic disorder of these constructs (Amata et al. 2014) was evident in the low chemical shift dispersion in the ^{1}H dimension (Fig. 1) and was verified by circular dichroism (data not shown). The structural propensities on a residue-by-residue basis were investigated further with the software SSP (Marsh et al. 2006). The output from this program assigns scores to each residue indicating the fraction of conformers in the disordered ensemble with either α or β-structure, where an SSP score of + 1 reflects fully formed α-helical structure, and a score of − 1 reflects fully formed β-structure. Figure 2 shows the SSP scores for p61Hck$_{SH4-U}$ and p59Hck$_{SH4-U}$. The data for the two constructs return values that are nearly identical for the overlapping regions, with most values in both constructs fluctuating between 0.2 and 0.0, indicating a slight preference for α-helical structure. One region, spanning residues 45–52, is the only segment in the constructs that has a notable

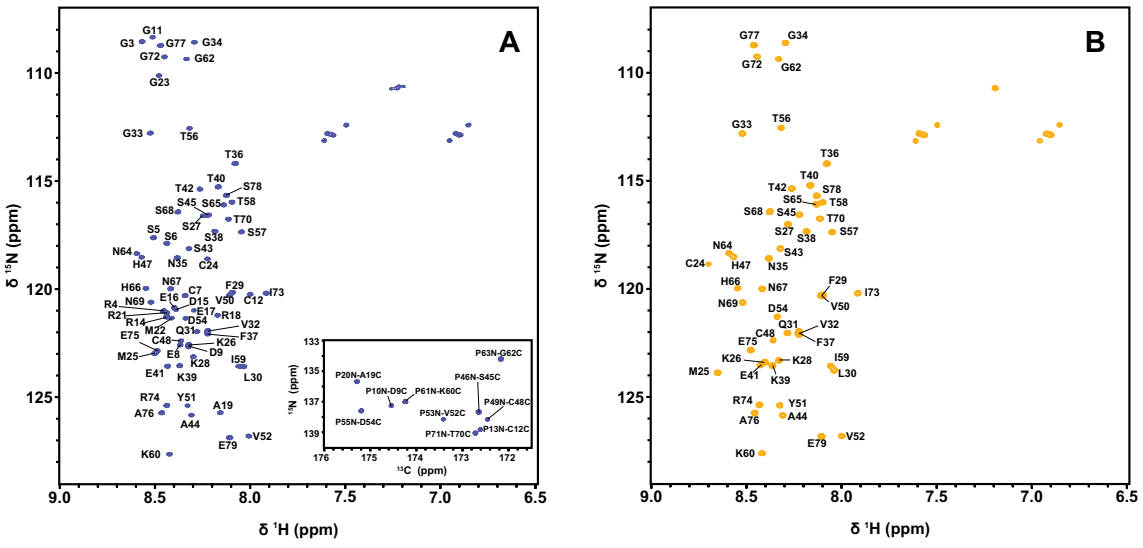

Fig. 1 Assigned ^{1}H–^{15}N HSQC spectra of **A** p61Hck$_{SH4-U}$ at 21.1 T (with the proline region of ^{13}C–^{15}N CON spectrum shown in the inset), and **B** p59Hck$_{SH4-U}$ at 14.1 T. All spectra were collected at 25 °C at pH 4.5 in 50 mM sodium acetate

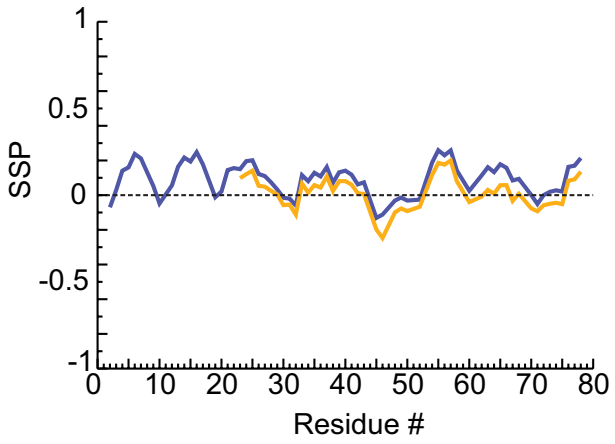

Fig. 2 SSP scores for p61Hck$_{SH4\text{-}U}$ (blue) and p59 Hck$_{SH4\text{-}U}$ (orange) suggest that the overlapping regions of the two isoforms have similar solution properties and that the 21 amino acid extension does not substantially perturb the structural propensities of the remainder of the construct

preference for β-structure. The close agreement for the overlapping regions of the two constructs suggests that the removal of the first 21 amino acids has little influence on the solution properties of the remainder of the Unique domain, and suggests little to no long range interactions under these conditions. Studies are underway to further characterize the solution and lipid-bound properties of these constructs. Chemical Shifts have been deposited in the Biological Magnetic Resonance Data Bank under 27553 (p59Hck$_{SH4\text{-}U}$) and 27554 (p61Hck$_{SH4\text{-}U}$).

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