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Solution NMR Studies of an Intrinsically Unstructured Protein within a Dilute, 75 kDa Eukaryotic Protein Assembly; Probing the Practical Limits for Efficiently Assigning Polypeptide Backbone Resonances

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This paper describes an efficient NMR strategy for assigning the backbone resonances of an intrinsically unstructured protein (IUP), p21-KID, bound to its biological target, Cdk2/cyclin A. In order to overcome the challenges associated with the high molecular weight (75 kDa) and low solubility of the ternary complex (0.2 mM), we used perdeuteration, TROSY, and high-sensitivity cryogenic NMR probes at high magnetic-field strengths (i.e. 16.4, 18.8 and 21.1 Tesla). p21-KID was also prepared by using specific amino acid isotope labels. Most importantly, we studied binary, subcomplexes that allowed resonance assignments to be made in stages. We show that subdomains of p21-KID folded within binary complexes into the same conformations as observed in the ternary, Cdk2/cyclin A complex. This is a general feature of IUPs, which often adopt highly extended conformations when bound to other proteins. This strategy is suitable for studies of IUPs within considerably larger biomolecular assemblies as long as the IUP can be uniformly and selectively isotope labeled.

Introduction

Intrinsically unstructured proteins (IUPs) or disordered protein segments are quite common in the proteomes of eukaryotic organisms.[1–4] Such proteins are frequently involved in cellular-regulatory functions, and their structural disorder confers functional advantages, including the ability to bind diverse targets by adopting multiple conformations.[5,6] In addition, protein flexibility has been shown to allow precise control of both the kinetics and thermodynamics of binding reactions,[7] and in turn to control biomolecular specificity.[8] Moreover, the sequential incorporation of unfolded monomers is a well-recognized mechanism of increasing the size and functional complexity of macromolecular assemblies.[9] Further insights into the functions of the very large number of IUPs in eukaryotic proteomes will be gained through study of their conformations both in the free state as well as within the biomolecular assemblies in which they exert their biological functions.

NMR spectroscopy has been used to study the structure and dynamics of protein assemblies in solution for decades. While structural information on protein assemblies is also available from X-ray crystallography, NMR studies in solution offer unique insights into the relationships between both structure and dynamics, and biomolecular function.[10–12] For assemblies comprised of individually folded proteins, a standard procedure is to first assign the resonances and determine the structure of one component in the free state and then to map the binding site for a second component by monitoring binding-induced chemical-shift changes. The resonance assignments and structure of the second component are determined independently, and the structure of the assembly can then be built up from the structures of the component parts. Limited, NMR-derived structural parameters for a protein assembly can then be used to determine its structure at modest resolution based on the structures of the components.[13] As assemblies become larger, it becomes more challenging to decipher the increasingly complex NMR spectra and obtain residue- and atom-specific structural information. However, methods such as HN TROSY[14] and methyl TROSY[15,16] allow high-resolution spectra to be obtained, resonance assignments to be established, and structure determination to be performed for protein systems with molecular masses up to ~80 kDa.[17] Unfortunately, this procedure, which relies on building up the structure of assem-

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blies from their component parts, cannot be applied in NMR studies of assemblies comprised of IUPs because their conformations in the native state are highly disordered and thus very different from those in the bound state. Structural information for IUPs in their functional form within assemblies and in solution must be gained through de novo NMR studies of intact protein assemblies. This unavoidably leads to studies of large molecular species and pushes the limits of NMR methodology and technology. Compounding these challenges, many eukaryotic IUPs and their assemblies exhibit limited solubility in vitro in aqueous buffers; this has limited the application of solution NMR in structural and dynamic studies.

NMR studies of IUPs in the free state are highly feasible because their inherent dynamics are associated with favorable relaxation properties and have been extensively reported.[18–26] Studies of IUPs within functional protein assemblies are more challenging due to the size limitations of solution NMR spectroscopy. To probe the limits of current approaches, we undertook solution NMR studies of a 75 kDa ternary protein complex that is comprised of an intrinsically unstructured protein, the kinase inhibitory domain (KID, 10 kDa) of the cell-cycle inhibitor, p21,[27] bound to cyclin-dependent kinase 2 (Cdk2, 35 kDa)[28] and cyclin A (30 kDa).[29] Here we report a strategy that combines existing methods and is suitable for studies of dilute protein solutions ($\geq$0.2 mM). This approach has allowed resonance assignments to be made for one IUP (p21-KID) within a large protein assembly. In order to overcome the technical challenges associated with the high molecular weight and low solubility (0.2 mM) of the ternary complex, we used perdeuteration,[30] TROSY,[14] and high-sensitivity cryogenic NMR probes[31,32] at high magnetic field strengths (i.e. 16.4, 18.8 and 21.1 Tesla). In addition, p21-KID was prepared by using specific amino acid isotope labels;[33,34] this allowed anchor points in sequential assignment patterns to be established. Most importantly, we studied binary subcomplexes; this allowed resonance assignments to be made in stages. Based on previous studies of p21-KID[5,35] and the related protein, p27-KID,[7,36] we postulate that p21-KID adopts a highly extended conformation when bound to Cdk2/cyclin A, with a compact, N-terminal domain contacting cyclin A (termed domain 1) and a longer, C-terminal domain contacting Cdk2 (termed domain 2). These features of the ternary complex made it possible to prepare binary subcomplexes comprised of p21-KID/Cdk2 and p21-KID/cyclin A, whose structures were relevant to that of the ternary complex. In the following, we describe the capabilities and limitations of our strategy for assigning ^{1}H, ^{13}C, and ^{15}N resonances for p21-KID within the 75 kDa ternary complex with Cdk2/cyclin A.

Results and Discussion

We used amino acid-specific ^{15}N labeling to augment triple-resonance,[34] sequential assignment methods in our studies of p21-KID/Cdk2/cyclin A. Seven different ^{15}N-labeled amino acids were introduced into p21-KID. Resonances for most ^{15}N-labeled amino acids were observed in 2D ^{1}H,^{15}N-TROSY spectra of the respective ternary complexes, including 6/8 leucines, 2/3 valines, 3/4 phenylalanines, 4/5 aspartic acids, 2/3 threonines, 1/1 isoleucine, and 1/1 tyrosine (see Supporting Information). It should be noted that, despite the presence of protons in the side chains of the ^{15}N-labeled amino acids, line widths were sufficiently narrow in 2D ^{1}H,^{15}N-TROSY spectra for resonances to be observed in reasonable acquisition times (24 to 36 h) on a 600 MHz spectrometer equipped with a noncryogenic probe. In addition, in all of the complexes studied herein, Cdk2 and cyclin A were used in their natural (unlabeled) isotopic form. The compatibility of unlabeled protein subunits within multiprotein assemblies with triple-resonance, TROSY-based NMR spectroscopy (vide infra) significantly reduces the costs of such studies.

We recorded 3D HNCA-TROSY[37] and HN(CO)CA-TROSY[38] spectra for ^{2}H/^{13}C/^{15}N-labeled p21-KID (tl-p21-KID) within the ternary complex. The concentration of the ternary complex was approximately 0.2 mM, and spectra were acquired for four days each by using an 800 MHz cryogenic probe. Even with reference to the 2D ^{1}H,^{15}N-TROSY spectra of ternary complexes with selectively ^{15}N-labeled p21-KID, analysis of these spectra allowed backbone resonance assignments to be made for only approximately $^{1}/_{3}$ of the residues in p21-KID. This stems from the ambiguous relationship between $^{13}C_{\alpha}$ chemical shifts and amino acid type,[39] and from the inability to differentiate intraresidue (i) and inter-residue (i–1) correlations in the HNCA spectrum. Resonances for 53 residues were observed in the HNCA spectrum; of these, 47 exhibited correlations for both i and i–1 residues, and the remaining six exhibited only i correlations (Figure 1). Poor S/N was observed in the HN(CO)CA spectrum due to the well-understood effects of C′ chemical-shift anisotropy at high magnetic-field strength;[37,38] Resonances for only 25 residues were observed, and 16 of these were also observed in the HNCA spectrum (Figure 1).

We found that the 2D ^{1}H,^{15}N-TROSY spectrum of tl-p21-KID bound to Cdk2 exhibited many of the resonances observed in that of the ternary complex (compare Figure 2E and C) and that both of these spectra were very different from that of p21-KID in the free, disordered state (Figure 2B). These data provide strong evidence that domain 2 of p21-KID (Figure 2A) adopts the same conformation when bound to Cdk2 as when bound to Cdk2/cyclin A. An advantage of studying the p21-KID/Cdk2 binary complex was that it was soluble at a significantly higher concentration (0.4 mM) than was the ternary complex (0.2 mM). This allowed additional triple-resonance NMR experiments to be performed. In particular, we recorded a 3D HNCACB-TROSY[38] spectrum for p21-KID within this 45 kDa complex using a cryogenic probe operating at 700 MHz in 2.0 days. In this spectrum, most residues within domain 2 exhibit i and i–1 correlations for both $^{13}C_{\alpha}$ and $^{13}C_{\beta}$ resonances (Figure 1). Analysis of the HNCACB spectrum for the p21-KID/Cdk2 binary complex, and HNCA and HN(CO)CA spectra of p21-KID/Cdk2/cyclin A (Figure 1), with reference to 2D ^{1}H,^{15}N-TROSY spectra of selectively ^{15}N-labeled p21-KID within ternary complexes, allowed most resonances of domain 2 of p21-KID to be unambiguously assigned (Figure 2C and E). Correlations used to establish sequential resonance assignments for

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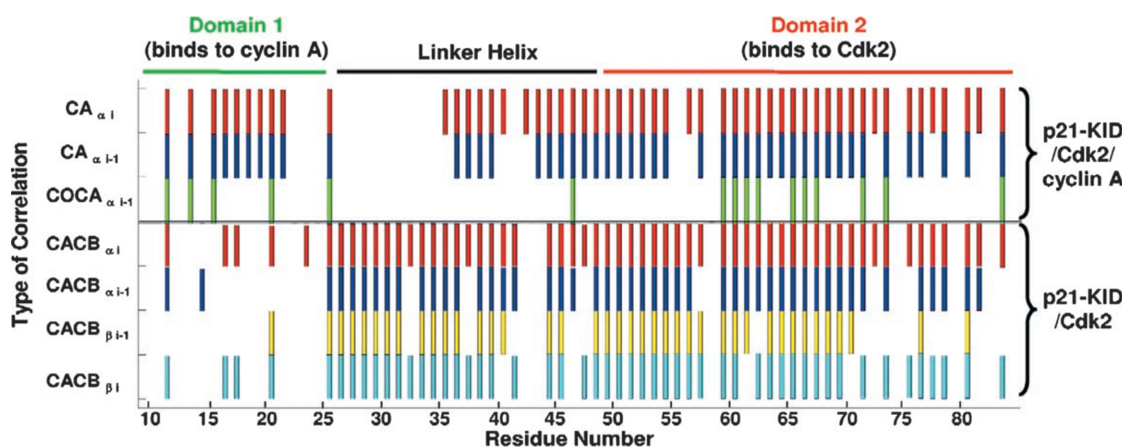

Figure 1. Establishing resonance assignments for $^{2}H/^{13}C/^{15}N$-p21-KID (tl-p21-KID) within protein complexes that regulate cell division. Correlations observed in 3D HNCA and HN(CO)CA spectra of the tl-p21-KID/Cdk2/cyclin A ternary complex (75 kDa) and in the HNCACB spectrum of the tl-p21-KID/Cdk2 binary complex (45 kDa). Correlations in the HNCA spectrum to residues i and i–1 are indicated by red and blue bars, respectively. A correlation in the HN(CO)CA spectrum to residue i–1 is indicated by a green bar. Correlations between C_α resonances in the HNCACB spectrum to residues i and i–1 are indicated by red and blue bars, respectively, and correlations between C_β resonances to residues i and i–1 are indicated by cyan and yellow bars, respectively.

domain 2 of p21-KID in complexes with Cdk2/cyclin A and Cdk2 are shown in the Supporting Information.

Only a small segment of p21-KID makes contact with cyclin A (domain 1, Figure 2A) and, therefore, only a relatively small number of resonances are shifted to new positions when p21-KID is bound to cyclin A (compare Figure 2D and B). These resonances were also observed in the 2D $^{1}H,^{15}N$-TROSY spectrum of the ternary complex (compare Figure 2D and E). Through these simple comparisons, we readily identified resonances of residues within domain 1 of p21-KID in the binary (with cyclin A) and ternary complexes. These were assigned through analysis of the 3D HNCA and HN(CO)CA spectra for the ternary complex (Figure 2D and 2E). However, lengthy 3D NMR experiments with the p21-KID/cyclin A binary complex were hampered by poor solubility and a tendency of cyclin A to aggregate and precipitate over time.

Despite facing significant NMR technical challenges and biochemical difficulties, we assigned 76% of the backbone resonances (and 42% of $^{13}C_\beta$ resonances) of p21-KID within the complex with Cdk2/cyclin A. Relatively few observed resonances remain unassigned (2/60 residues) and resonances were not observed for several residues in the linker helix segment (7 residues), possibly due to exchange broadening.

Conclusion

In summary, we present an efficient approach for making resonance assignments for IUPs within multiprotein assemblies at low concentration ($\geq$0.2 mM). Of course, this approach is also suitable for making assignments of natively folded proteins within assemblies. The system under study here has an aggregate mass of 75 kDa, but the methodology is likely to be useful in studies of complexes that are considerably larger. For studies focused on IUPs, the IUP component of a complex must be prepared in extensively isotope-labeled form which, currently, is most feasible in bacteria. In our experience, eukaryotic IUPs often are expressed at high levels in bacterial inclusion bodies and, since they lack tertiary structure, can often be extracted from the insoluble fraction and purified at high yield in bioactive form by using denaturing conditions.[5,19,40] Importantly, our results also show that not all components of complexes involving IUPs need to be isotope labeled; this allows nonbacterial expression systems to be utilized for other components. Studies of isotope-labeled IUPs within otherwise unlabeled complexes allows detailed characterization of the structure and dynamics of the IUP in the bounds state and comparison of these results with those of the disordered, free state. Of course, information on the structure of other components within multiprotein assemblies is desirable, when they can be prepared in isotope-labeled form. While selective and nonselective isotope labeling of an IUP is essential in these studies and the availability of high-field magnets enhances the S/N in NMR spectra, the use of cryogenic probes, with significantly improved S/N over room-temperature NMR probes, is the recent development that makes studies of this type possible. Now that resonance assignments for p21-KID are nearly complete, we will move forward with further studies that will allow detailed structural and dynamic analysis of this cell-cycle regulator in its biologically active form, in solution.

Experimental Section

Expression and purification of proteins: Full-length human Cdk2 and truncated human cyclin A (residues 173–432 of human cyclin A) were expressed in *E. coli* and purified by using established procedures.[7] cDNA for p21-KID (residues 9–84 of human p21, 76 amino acids) was subcloned into pET24a (Novagen), and the protein was expressed in His-tagged form in *E. coli* BL21(DE3) at 37 °C by using isopropyl-β-D-thiogalactopyranoside (IPTG).[41] His-tagged p21-KID was purified by using Ni^{2+}-affinity chromatography (Amersham-Pharmacia resins) and HPLC (C_4 column, Vydac, Hesperia, CA).[5] The purity and identity of each protein sample was confirmed by SDS-PAGE and MALDI-TOF mass spectrometry. Isotope-labeled samples of p21-KID were prepared in 3-(*N*-morpholino)propane sulfonic acid MOPS-based minimal medium[42] enriched with

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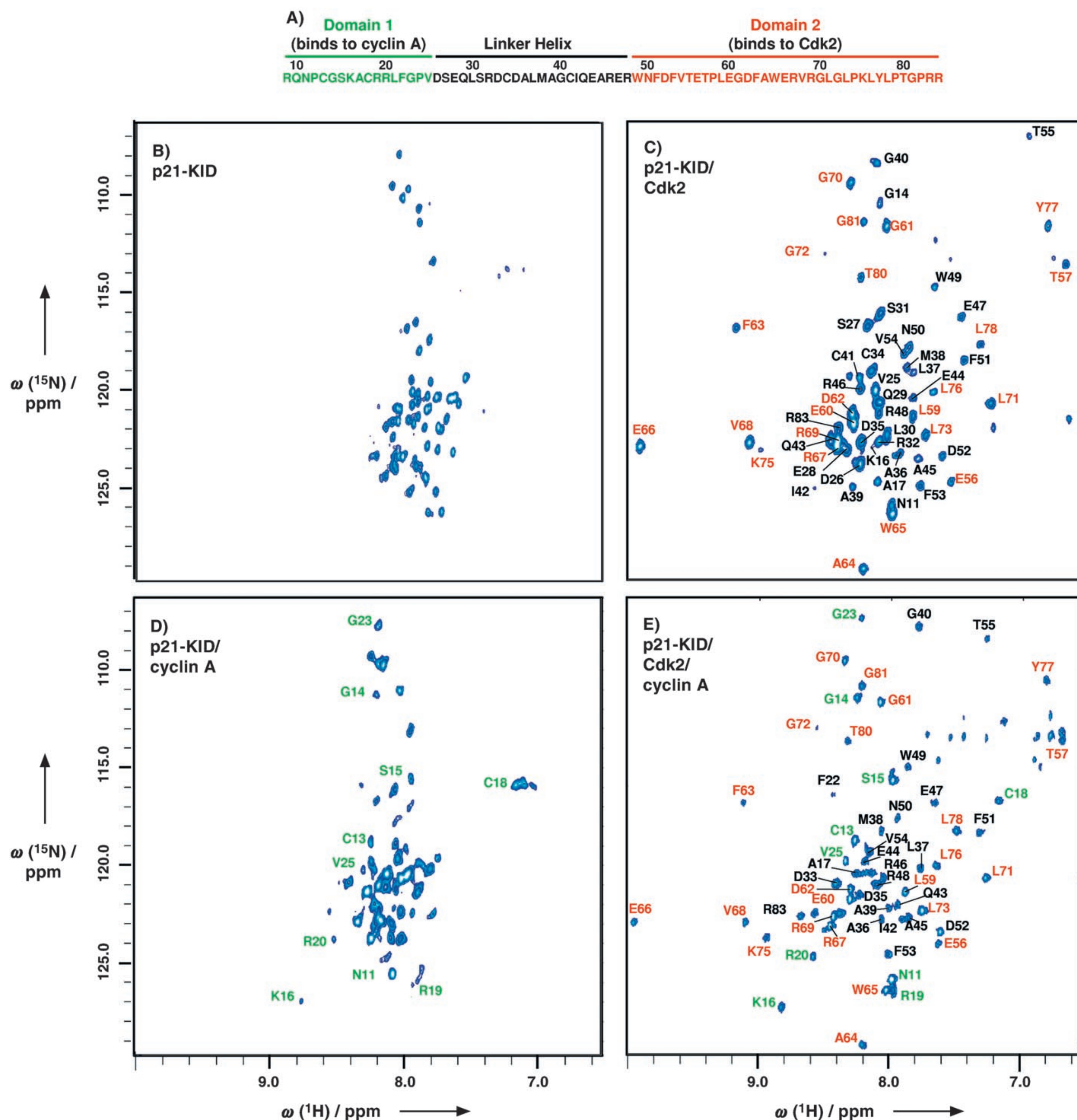

Figure 2. 2D TROSY spectra of binary complexes (C and D) were used to assign resonances for tl-p21-KID in the ternary complex (E). A) The sequence of p21-KID: domains 1 (green) and 2 (red) bind independently to cyclin A and Cdk2, respectively. B) Native p21-KID is disordered in solution. 2D TROSY spectra of tl-p21-KID bound to C) Cdk2, D) cyclin A, and E) Cdk2/cyclin A were used to identify interacting residues. Residues of p21-KID that bind cyclin A are green in (D) and (E), and those that bind Cdk2 are red in (C) and (E).

isotope-labeled compounds. $^{15}NH_4Cl$, glycerol, and ^2H_2O were used for $^2H/^{15}N$-labeled p21-KID. $^{15}NH_4Cl$, $^{13}CH_3{}^{13}CO_2^-$, and ^2H_2O were used For $^2H/^{13}C/^{15}N$-labeled p21-KID. To produce p21-KID labeled at specific amino acids with ^{15}N, an auxotrophic *E. coli* strain DL39-(DE3)[34] was cultured in MOPS-based minimal medium supplemented with amino acids. One or two ^{15}N-labeled amino acids were included (leucine, valine, phenylalanine, aspartic acid, threonine, isoleucine or tyrosine).

NMR studies: The NMR buffer in all studies was potassium phosphate (20 mM), pH 6.5, arginine (50 mM), ^2H_2O (8% *v*/*v*), DTT (5 mM) and sodium azide (0.02% *w*/*v*). All three complexes (p21-KID/Cdk2, p21-KID/cyclin A, p21-KID/Cdk2/cyclin A) were isolated by using size-exclusion chromatography (Superdex 200, Amersham-Pharmacia) in HEPES buffer followed by exchange into NMR buffer by ultrafiltration (Centricon units, Amicon, Billerica, MA) to final concentrations of 0.4 mM, 0.2 mM and 0.2 mM, respectively. All NMR experi-

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ments were performed at 35 °C. 2D ^{1}H,^{15}N-TROSY spectra for residue-specific ^{15}N-labeled p21-KID in ternary complexes were recorded on a Varian Inova 600 MHz spectrometer with acquisition times of 24 to 36 h. For both binary complexes (p21-KID/Cdk2 and p21-KID/cyclin A), 2D ^{1}H,^{15}N-TROSY spectra were acquired with ^{2}H/^{15}N-labeled p21-KID on a Bruker Avance 800 spectrometer (Memphis, Tennessee). The 2D ^{1}H,^{15}N-TROSY spectrum for the ternary complex (^{2}H/^{15}N-labeled p21-KID/Cdk2/cyclin A) was acquired on a Bruker Avance 900 spectrometer (Frankfurt, Germany) equipped with cryogenic probe. The 3D HNCACB-TROSY[38] spectrum for the ^{2}H/^{13}C/^{15}N-labeled p21-KID/Cdk2 binary complex was recorded on a Bruker Avance 700 MHz spectrometer (Fällanden, Switzerland). Backbone resonance assignments for p21-KID in ternary complexes were determined through analysis of 3D HNCA-TROSY[37] and HN(CO)CA-TROSY[38] spectra, recorded for ^{2}H/^{13}C/^{15}N-labeled p21-KID with a Bruker Avance 800 MHz spectrometer (Fällanden, Switzerland). For all 3D experiments, cryogenically cooled probes were used to increase sensitivity. Spectra were processed by using NMRPipe software[43] and analyzed by using Felix software (Accelerys). For all spectra, the ^{1}H dimension was referenced to external TSP, and the ^{13}C and ^{15}N dimensions were referenced indirectly by using the appropriate ratios of gyromagnetic ratios.[44]

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