

Autoinhibitory Interaction in the Multidomain Adaptor Protein Nck: Possible Roles in Improving Specificity and Functional Diversity[†]

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ABSTRACT: Nck is a functionally versatile multidomain adaptor protein consisting of one SH2 and three SH3 domains. In most cases, the SH2 domain mediates binding to tyrosine-phosphorylated receptors or cytosolic proteins, which leads to the formation of larger protein complexes via the SH3 domains. Nck plays a pivotal role in T-cell receptor-mediated reorganization of the actin cytoskeleton as well as in the formation of the immunological synapses. The modular domain structure and the functionality of the individual domains suggest that they might act independently. Here we report an interesting intramolecular interaction within Nck that occurs between a noncanonical yet conserved (K/R)x(K/R)RxxS sequence in the linker between the first and second SH3 domain (SH3.1/SH3.2) and the second SH3 domain (SH3.2). Because this interaction masks the proline-rich sequence binding site of the SH3.2 domain, the intramolecular interaction is self-inhibitory. This intramolecular interaction could, at least partially, explain the remarkable specificity of Nck toward proteins with proline-rich sequences. It may prevent nonspecific low-affinity binding while keeping the site available for high-affinity bivalent ligands that can bind multiple sites in Nck. This indicates that Nck does not simply adopt a “beads on a string” architecture but incorporates a higher-order organization for improved specificity and functionality.

Noncatalytic tyrosine kinase (Nck)¹ is a 47 kDa adaptor protein, consisting of three N-terminal SH3 domains and a single SH2 domain at the C-terminus (1–3). There are two human proteins that share the designation Nck: Nck1/Nck α and Nck2/Nck β (also known as Grb4). The Nck1 and Nck2 isoforms exhibit a high degree of sequence homology, being 68% identical and 79% similar over their entire protein sequences. The modular architecture of the Nck protein provides a scaffold for a variety of protein–protein interactions, and more than 60 interaction partners have been reported. In most cases, the SH2 domain mediates binding to tyrosine-phosphorylated receptors or cytosolic proteins, which leads to the formation of larger protein complexes via SH3 domains (1, 2).

Nck is functionally quite versatile. For instance, Nck is known to be involved in cellular activation, reorganization of the actin cytoskeleton, cell movement, and responses to cell stress. In T-cells,

Nck plays a pivotal role in T-cell receptor (TCR)-mediated reorganization of the actin cytoskeleton as well as in the formation of immunological synapses (1, 2). Upon activation of T-cells, Nck is recruited to a membrane proximal site via interaction with tyrosine-phosphorylated SLP76, providing a scaffold for the Wiskott-Aldrich syndrome protein (WASP)-dependent actin remodeling machinery (2–5). Nck is also directly recruited to the CD3 ϵ component of TCR in an activation-dependent manner (6). However, the roles of the CD3 ϵ –Nck interaction are disputed (1, 6–9).

Each SH3 domain exhibits remarkable specificities, with identified interaction partners having clear preferences for particular SH3 domains in Nck (1, 2, 10). These specificities allow Nck to have multiple simultaneous interactions with different binding partners. For example, WASP binding occurs through the third SH3 domain (SH3.3) (5), whereas the WASP interacting protein (WIP) (11) and p21-activated kinase (Pak1) (12, 13) bind to the second SH3 domain (SH3.2) (2–5). The first SH3 domain (SH3.1) interacts with CD3 ϵ in a noncanonical fashion (6, 7, 9). Recent determinations of the structures of isolated Nck domains (14–19) as well as the structures of the Nck–CD3 ϵ and Nck–PINCH1 complexes (9, 20) provide insights into the ligand binding specificities of individual domains. However, the functions and specificities of the multidomain adaptor protein might not be fully elucidated by the sum of those of the individual domains. Recent interactome analysis showed that a significant number of proteins that interact with individual isolated SH3 and SH2 domains were not identified in the analysis using full-length Nck (10). On the other hand, it is also known that a Nck construct that has all SH3 domains showed stronger binding than isolated individual

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¹Abbreviations: Nck, noncatalytic tyrosine kinase; Nck-FL, full-length Nck; SH3.1, first SH3 domain; SH3.2, second SH3 domain; SH3.3, third SH3 domain; TCR, T-cell receptor; WASP, Wiskott-Aldrich syndrome protein; WIP, WASP interacting protein; Pak1, p21-activated kinase; PRS, proline-rich sequence; JM, juxtamembrane.

domains for some of the binding partners (21). Still, the basis of the specificity, in the context of the full-length protein, has not been addressed.

Here we report an interesting intramolecular interaction within the Nck2 molecule, identified by NMR structural analysis of full-length Nck and its various deletion mutants. The analysis revealed that an intramolecular interaction occurs between a conserved noncanonical (K/R)x(K/R)RxxS sequence in the SH3.1/3.2 linker and the SH3.2 domain of Nck2. This interaction masks the proline-rich sequence (PRS) binding site of the SH3.2 domain and thus works as a self-inhibitory mechanism. The PRS of two physiologically identified binding partners, SOS1 (22, 23) and Pak1 (12, 13), exhibited much lower affinities (by ~5-fold) in the presence of the intramolecular interaction. Thus, the intramolecular interaction might work as a safety switch keeping the site available only for high-affinity stable interactions that can overcome the intramolecular interaction. Our identification of this higher-order structure in Nck might help in the understanding of the functional versatility of this multidomain adaptor protein.

EXPERIMENTAL PROCEDURES

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. All stable-isotope-labeled materials were acquired from Cambridge Isotope laboratories (Cambridge, MA). Nck2_{71–90} (NH₂-⁷¹LKDTLGLGKTRRKTSARDAS⁹⁰-CONH₂), SOS1 (NH₂-¹¹⁴⁶EVPPPPVPPRR¹¹⁵⁷-CONH₂), and Pak1 (NH₂-⁹QDKPPAPPMRN¹⁹-CONH₂) peptides were purchased from Tufts University Core Facility (Boston, MA).

Expression and Purification of Nck Proteins. The human Nck2 gene was purchased from Open Biosystems (Huntsville, AL). The primers used to amplify the genes are listed in the Supporting Information. The PCR products were ligated to a modified pET28b vector, pET28P, or pET30b vector at *Nde*I and *Xho*I sites following the manufacturer's instructions. The pET28P vector has a His₁₀ tag and the PreScission Protease (GE Healthcare Biosciences, Pittsburgh, PA) sequences between the *Nco*I and *Nde*I sites. The pET30 vector was used to make C-terminal polyhistidine-tagged constructs. The pET28P vector was used to make N-terminal polyhistidine-tagged constructs, in which the polyhistidine tag can be cleaved by the PreScission Protease. The C-terminal deletion constructs were produced from the N-terminal polyhistidine-tagged full-length Nck2 construct by introduction of a stop codon (TGA). QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) was used to make the C-terminal deletion constructs as well as point mutants of Nck2. Either BL21(DE3) or Rosetta2(DE3) cells were used as host strains for expression. All the clones were verified by DNA sequencing.

All proteins were expressed in *Escherichia coli* by induction with IPTG at a final concentration of 1 mM. Overexpressed proteins in the soluble fraction were then purified using Ni-NTA affinity chromatography using standard procedures. N-Terminal polyhistidine-tagged constructs were then cleaved by the PreScission Protease. All the proteins were subjected to gel filtration chromatography using a Superdex-75 column (GE Healthcare Biosciences). For the uniformly ¹⁵N- and ¹³C-labeled samples, the cells were cultured in [¹⁵N,¹³C]M9 medium containing 8.5 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 0.5 g/L NaCl, 2 mM MgCl₂, and 0.1 mM CaCl₂, which is supplemented with 1 g/L ¹⁵NH₄Cl and 2 g/L ¹³C glucose as the nitrogen and carbon sources, respectively.

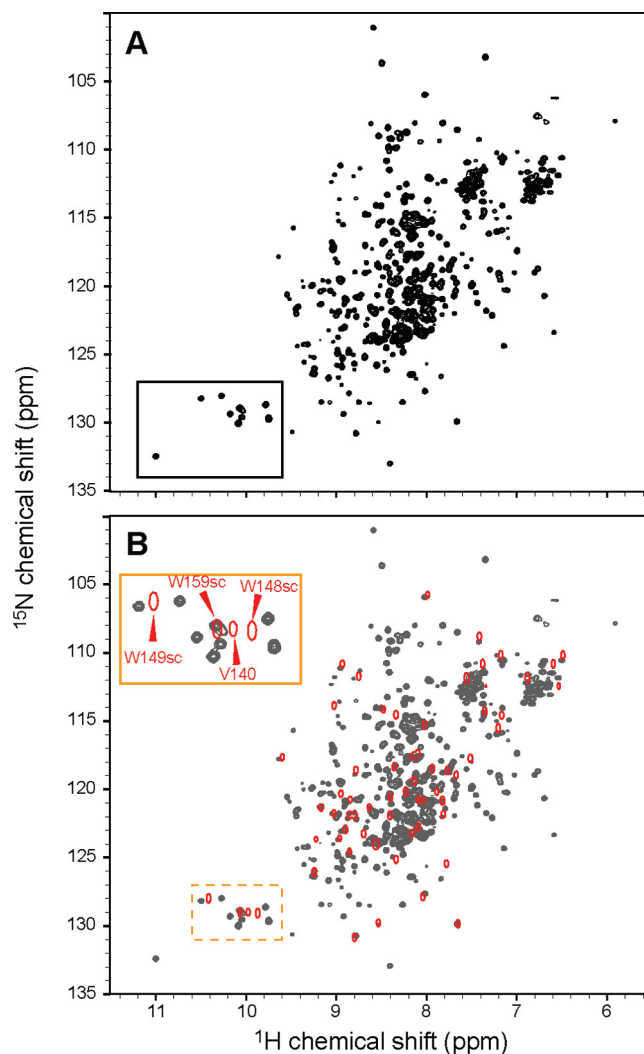


FIGURE 1: Comparison of ¹H–¹⁵N HSQC spectra of Nck-FL and isolated SH3.2. (A) ¹H–¹⁵N HSQC spectrum of 100 μM [U-²H, ¹⁵N]Nck-FL. (B) ¹H–¹⁵N HSQC spectrum of 100 μM [U-¹⁵N]Nck SH3.2 (red contour) overlaid with that of [U-²H, ¹⁵N]Nck-FL (gray). The region containing the tryptophan indole signals is enlarged in the inset, and the assignments are indicated (sc stands for Trp side chain indole signals).

NMR Experiments. All experiments were performed on a Bruker Avance 500 spectrometer equipped with a cryogenic probe at 25 °C unless otherwise indicated. All the Nck protein samples were prepared in 10 mM sodium phosphate buffer (pH 6.8) with 100 mM NaCl and 1 mM DTT. Spectra were processed using XWINNMR (Bruker) and analyzed with Sparky (24). The resonance assignments for individual domains as well as deletion constructs of Nck (Δ169 and 70–168) were made with HNcocaN and HNcocaNH experiments (25), and standard triple-resonance experiments (26).

RESULTS

Although the modular domain structure of Nck protein suggests that its individual domains might be independent, there is some evidence of functional cross-talk between structural elements in the full-length Nck2 (Nck2-FL) molecule (10, 21). In exploring the possibility of domain–domain or domain–linker interactions in Nck, we compared the ¹H–¹⁵N HSQC spectrum of Nck2-FL with those of the isolated individual SH3 domains under the same experimental conditions (Figure 1 and Figure S1 of the Supporting

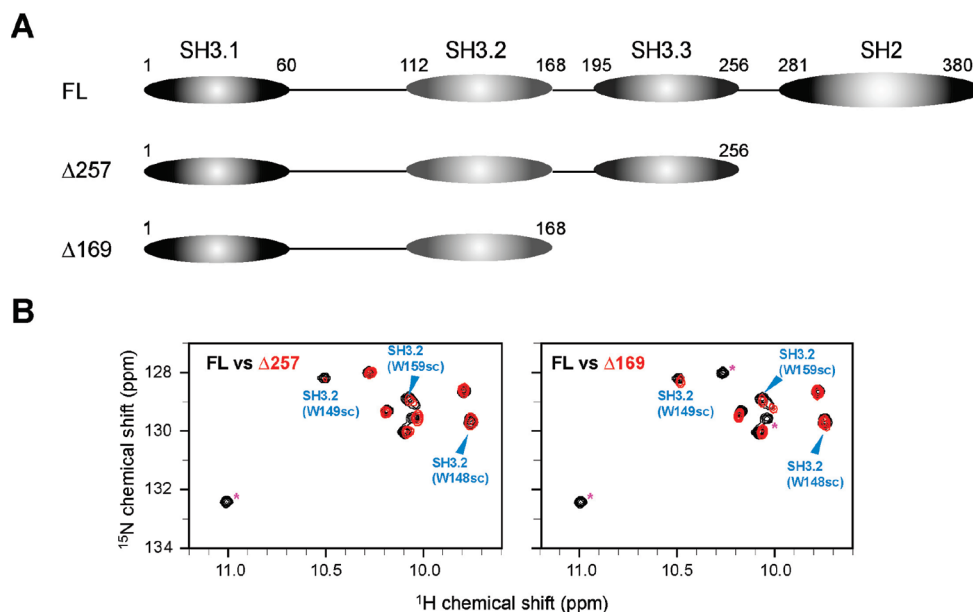


FIGURE 2: C-Terminal deletion constructs of Nck2. (A) Schematic representation of C-terminal deletion constructs of Nck2. (B) Comparison of the ^1H – ^{15}N HSQC spectrum between $100\ \mu\text{M}$ [^2H , ^{15}N]Nck-FL and $100\ \mu\text{M}$ [^2H , ^{15}N]Nck $\Delta 257$ (left, red contour) or [^2H , ^{15}N]Nck $\Delta 169$ (right, red contour). The region containing the tryptophan indole signals is shown. Arrowheads with assignments indicate the tryptophan indole signals from SH3.2 domains. Signals from the SH2 domain are indicated with an asterisk in the $\Delta 257$ spectrum. In the $\Delta 169$ spectrum, SH2 and SH3.2 domain signals are indicated with asterisks.

Information). As there are ~ 100 residues of linker regions in the Nck2-FL protein, the central part of the spectrum did not exhibit many isolated peaks (Figure 1A). Nevertheless, it was possible to compare the peak positions of the well-isolated peaks and those of the tryptophan side chains. As shown in the expanded spectrum in Figure S1, the peaks of the Trp indole side chains in isolated SH3.1 and SH3.3 are not much different from those in the full-length protein. However, the tryptophan side chain resonances from SH3.2 have positions quite different from the positions of those in Nck2-FL (Figure 1B). Since Trp148 in SH3.2 is a critical residue for the binding to a PRS, the SH3.2 domain seems to have a different environment when it is in the full-length protein as compared to the isolated state.

One immediate possible explanation would be the presence of the intramolecular interaction between SH3.2 and an internal PRS. Although the sequence of the full-length Nck2 protein was analyzed for such an element, no region was discovered that has the canonical PxxP motif in the protein. Therefore, we conducted a thorough search of the site for intramolecular SH3.2 interaction by means of a series of deletion mutants. As the first step in identifying the intramolecular binding site, we examined the possibility that Nck2 SH3.2 interacts with the C-terminal half of the Nck2 protein. Two different C-terminal deletion constructs, $\Delta 169$ and $\Delta 257$, in which C-terminal side of the protein is deleted at the C-terminal border of the second and third SH3 domains, were made (Figure 2A). The presence of intramolecular interaction was examined by signal distribution in the Trp indole regions. However, even the shortest construct, $\Delta 169$, which has only the SH3.1 and SH3.2 domains, showed almost the same chemical shifts as intact Nck for the Trp residues of SH3.2, indicating that no SH3.2 interaction exists in the C-terminal half of Nck (Figure 2B).

Subsequently, we investigated possible interaction sites in the SH3.1 domain and the SH3.1/SH3.2 linker by deleting the N-terminus of Nck2 $\Delta 169$ (Figure 3A). The deletion of the SH3.1 domain (Nck2_{60–168}) still yielded the same spectra (Figure 3B,

top), excluding the involvement of SH3.1 in the domain–domain interaction. It rather indicates that the SH3.2 binding site would have to be in the 41-amino acid SH3.1/SH3.2 linker (residues 60–110). Comparison of the sequence of human Nck2 with those of other Nck proteins showed that the N-terminal half of the SH3.1/SH3.2 linker is highly conserved (residues 60–91, 61% identical among Nck proteins), which implies the possible importance of this region in Nck proteins (Figure 3C). Although deletion of the first 10 amino acids (Nck2_{70–168}) did not affect the signals from the SH3.2 domain residues (Figure 3B, middle), deletion of the conserved region (Nck2_{90–168}) caused significant changes in the SH3.2 signals (Figure 3B, bottom).

Figure 3D shows the location of the residues that were shifted with the deletion of the conserved region of residues 70–89 (red spheres). Interestingly, the shifted residues are found at the binding site for PRS. A PRS usually binds to the surface formed by the RT and n-SRC loops, and it perfectly overlaps with the binding residues for the region of residues 70–89. It is also worth noting that this intramolecular interaction sequence has no Pro residues or other known SH3 binding motifs. Thus, the interaction might represent a new mode of interaction involving SH3 domains. Although there are some differences in chemical shifts between Nck2_{90–168} and the isolated SH3.2 domain, those residues are mostly in the proximity of the N-terminus of the domain (Figure 3D, yellow spheres), which might reflect the local environmental change caused by the loss of N-terminal proximal sequences. This assumption is further corroborated by the fact that Nck2_{90–168} showed the same chemical shifts as Nck2_{100–168} (data not shown).

To confirm that the chemical shift changes observed at the PRS binding site actually reflect direct interaction between the sequence of residues 70–89 and the SH3.2 domain, we then synthesized a peptide corresponding to residues 71–90 of Nck2 and titrated it to the isolated SH3.2 domain. As shown in Figure 4A, titration of the 71–90 peptide to the SH3.2 domain induced chemical shift changes, most of the titrated signals having moved

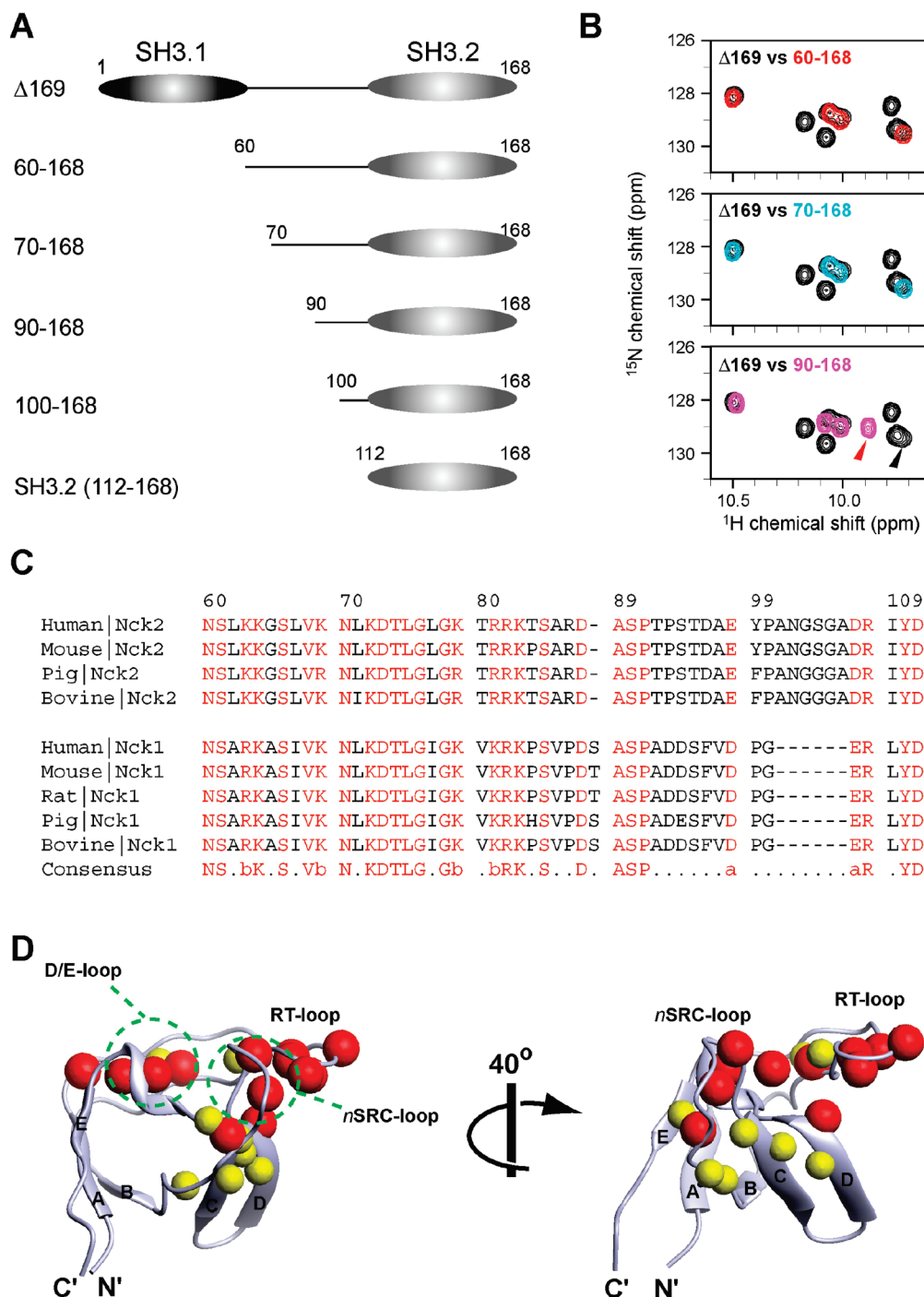


FIGURE 3: N- and C-terminal deletion constructs of Nck2. (A) Schematic representation of the N- and C-terminal deletion constructs of Nck2. (B) Comparison of the ^1H - ^{15}N HSQC spectra between 100 μM [^{15}N]Nck2 $\Delta 169$ and 100 μM [^{15}N]Nck2₆₀₋₁₆₈ (top, red contour), [^{15}N]Nck2₇₀₋₁₆₈ (middle, cyan contour), or [^{15}N]Nck2₉₀₋₁₆₈ (middle, pink contour). The region containing the tryptophan indole signals is shown. The peak positions of W148 indole, which shifted significantly between Nck2 $\Delta 169$ and Nck2₉₀₋₁₆₈, are indicated with arrowheads that have same color with its spectrum. (C) Sequence alignment of human, mouse, rat, pig, and bovine Nck proteins in the SH3.1/SH3.2 linker regions. (D) Mapping of the residues that are shifted by the deletion of the residues 70–89 (red) or 90–110 (yellow). Residues that are shifted >0.05 ppm in Nck2₇₀₋₁₆₈ with respect to 90–168 are represented as red spheres. Yellow spheres are for the residues shifted >0.05 ppm in Nck2₉₀₋₁₆₈ with respect to isolated SH3.2. Residues shifted >0.05 ppm in both cases are represented with red spheres. Chemical shift changes are normalized by the term $[(\delta\text{N}/5)^2 + (\delta\text{H})^2]^{1/2}$, where δN and δH are chemical shift changes in nitrogen and proton dimensions, respectively. Although Asn112 was also shifted >0.05 ppm in Nck2₉₀₋₁₆₈ with respect to isolated SH3.2, the residue is not shown in Figure 4D because it is not defined in the determined structure. The solution structure of the isolated SH3.2 domain, which contains residues 114–170 (Protein Data Bank entry 2FRW), was used for mapping (16).

toward the chemical shifts of Nck2₇₀₋₁₆₈. This indicates that the external peptide can reproduce the intramolecular interaction. The residues perturbed by the 71–90 peptide overlap well with those shifted by the deletion of residues 70–89 (Figure 4B). The result confirms that the intramolecular binding site for SH3.2 consists

of residues 71–90 in Nck2. The K_D for this *trans* interaction is very weak (~ 1.7 mM), as estimated from the concentration dependence of the chemical shift changes (Figure 4C).

To further identify the hot spot of this interaction, we created mutants of Nck2₇₀₋₁₆₈ at every position in the region of residues

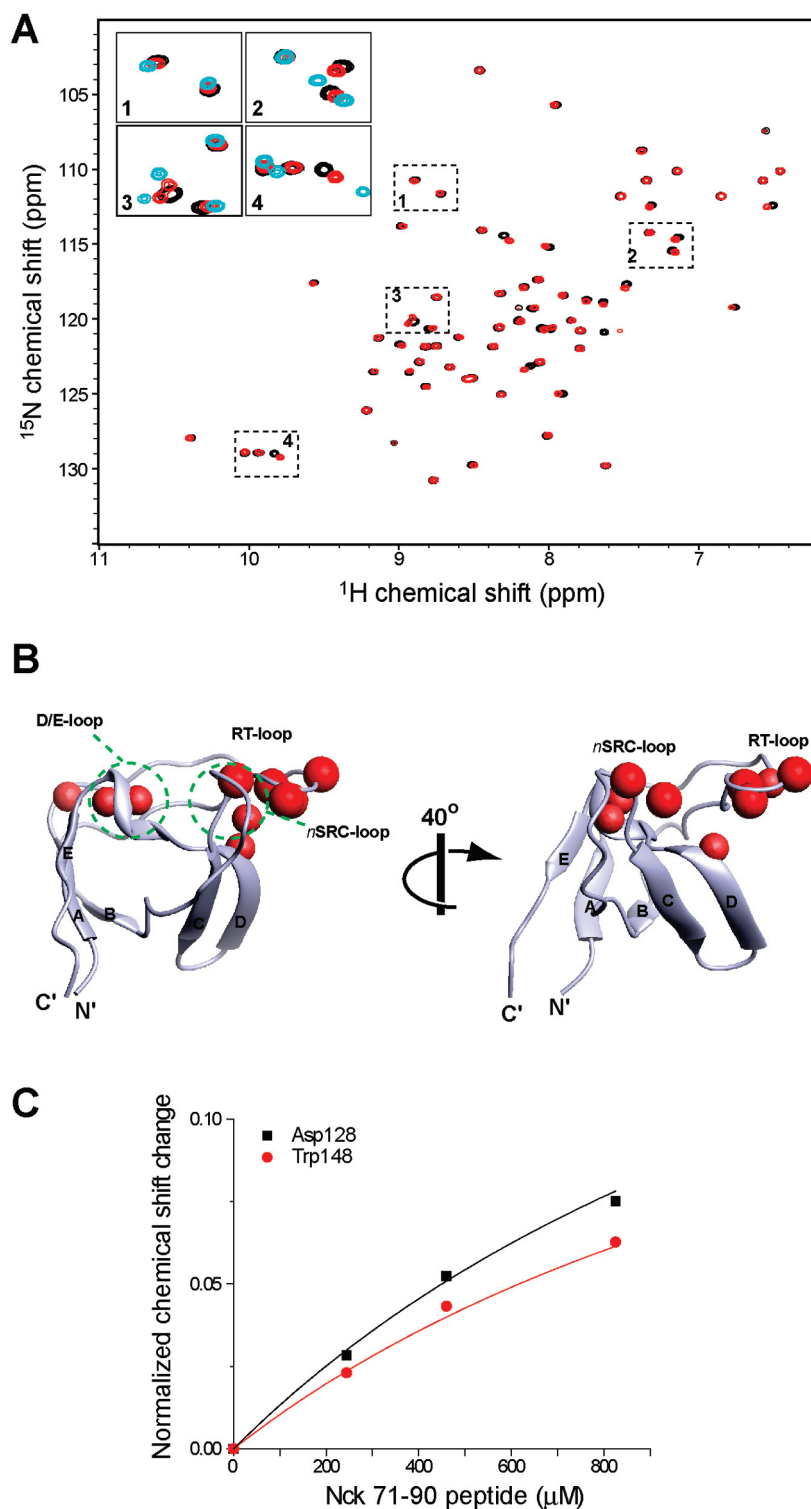


FIGURE 4: Titration of Nck_71–90 peptide to the isolated SH3.2 domain. (A) Comparison of the ^1H – ^{15}N HSQC spectra of 100 μM $[\text{U}-^{15}\text{N}]$ Nck SH3.2 with (red) or without (black) 800 μM Nck2_70–90 peptide. Four regions in the HSQC spectrum are enlarged as insets. In the inset, the ^1H – ^{15}N HSQC spectrum of 100 μM $[\text{U}-^{15}\text{N}]$ Nck2_70–168 is also colored cyan. (B) Mapping of the residues that are shifted by titration of Nck_71–90. Residues that are shifted > 0.2 ppm with titration of 800 μM 71–90 peptide are represented as red spheres. Chemical shift changes are normalized by the term $[(\delta\text{N}/5)^2 + (\delta\text{H})^2]^{1/2}$, where δN and δH are chemical shift changes in nitrogen and proton dimensions, respectively. The structure and orientation of the molecules are the same as in Figure 3. (C) Concentration-dependent chemical shift changes induced by the Nck2_71–90 peptide. Chemical shift changes for Asp128 and Trp148 with fast exchange are shown. The maximum chemical shift change is assumed to be the difference between Nck2_70–168 and isolated SH3.2.

70–89. Some of these exhibited intact intramolecular interaction and gave spectra very similar to that of the wild type, as with L75A (Figure 5A, top). However, as represented by R82A, several residues showed disrupted interaction exhibiting peak patterns similar to those of isolated SH3.2 (Figure 5A, bottom). The analysis

identified a stretch of seven residues containing a noncanonical KxRRKxS sequence in the first and second SH3 domain (SH3.1/SH3.2) linker as the hot spot of this intramolecular interaction (Figure 5B). In addition, the D128A mutation in the acidic RT loop and the W148A mutation of the Trp residue that are known

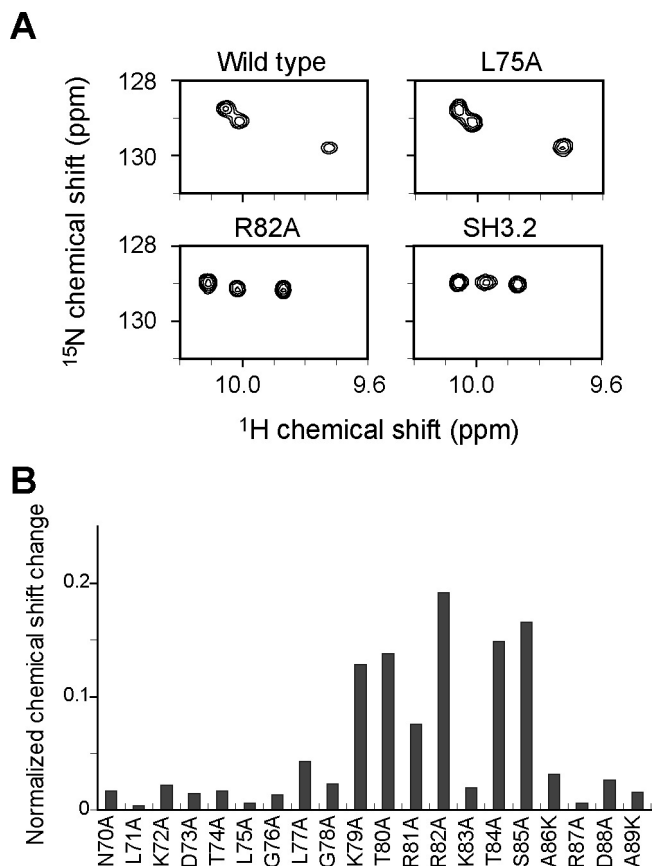


FIGURE 5: Mutation analysis of Nck2_{70–168}. (A) Comparison of ¹H–¹⁵N HSQC spectra. Tryptophan indole regions of Nck2_{70–168} wild type (top left), L75A (top right), and R82A (bottom left) and the isolated SH3.2 domain (bottom right) are shown. (B) Normalized chemical shift changes of Trp148 indole signals in the Nck2_{70–168} mutants. Chemical shift changes are normalized as described in the legend of Figure 4.

to be important for SH3–PRS interaction disrupted the intramolecular interaction (Figure S3 of the Supporting Information). This further supports the notion that the basic KxRRKxS sequence binds to the acidic RT loop, overlapping with the PRS recognition site.

As mentioned above, the intramolecular interaction occurs at the PRS recognition site in the Nck2 SH3.2 domain. Thus, we next tested if the intramolecular interaction masks the PRS recognition site in the SH3.2 domain and, thereby, inhibits the interaction with its ligands. SOS1 (23) and Pak1 (12, 13) are both known to bind to the Nck SH3.2 domain. We synthesized two class II PRS peptides from the sequences of SOS1 (1146-EVPVPPVPPRR-1157) and Pak1 (9-QDKPPAPPMPRN-19) and compared their binding to the isolated SH3.2 domain and Nck2_{70–168}. The SH3.2 domain showed chemical shift perturbation patterns with fast-to-intermediate exchange kinetics upon binding to the SOS1 (Figure 6A) and Pak1 peptides (data not shown), and submillimolar affinities (Figure 6B,C). As expected, the two PRS peptides bind to the typical canonical PRS recognition site in the SH3.2 (112–168) domain (data not shown). In comparison, the K_D values of these peptides with respect to Nck2_{70–168} were greatly increased by 4.7-fold in both cases (K_D values shown in Figure 6B,C). These results show that the intramolecular interaction is inhibitory with respect to ligand binding. Both peptides were still able to bind to Nck2_{70–168} at much higher concentrations (Figure 6A and Figure S3 of the

Supporting Information). In addition, the direction of the chemical shift changes with the SOS1 peptide matched perfectly with that of the isolated SH3.2 domain with a saturating peptide concentration (Figure 6A, inset). This indicates that the PRS peptides can replace the intramolecular interaction.

DISCUSSION

Using NMR structural analysis of full-length Nck2 as well as a variety of its deletion mutants, we identified an unusual intramolecular interaction within the Nck2 molecule. It occurs between a noncanonical 79-KTRRKTS-85 sequence in the SH3.1/3.2 linker and SH3.2 of Nck2. The stretch of the sequence is mostly conserved except for Thr80 (Val in Nck1) and Thr84 (Pro or His in Nck1 as well as Pro in mouse Nck2). Considering the high degree of homology between the SH3.2 domains, this intramolecular interaction appears to be a common characteristic shared by Nck family proteins. However, as a simple mutation of Thr84 in Nck2 to Pro (Nck1 type mutation) or Ala disrupted the interaction, we cannot exclude the possibility that the intramolecular interaction is a unique feature of the Nck2 molecule (Figure 5).

The interaction occurs at the PRS binding site in SH3.2 and is self-inhibitory. Attempts to determine the structure of Nck2_{70–168} were not successful, due to the fact that ¹H–¹⁵N correlations were not observed in a HSQC spectrum for residues 83–87. However, we were able to identify NOEs between the intramolecular interaction site and the Trp148 indole (see Figure S5 of the Supporting Information). Because the cross-peaks perfectly match the NOEs that are observed for the main chain amide resonance of R82, which forms a part of the intramolecular interaction motif, it is likely that Trp149 H^{ε1} is in the proximity of the amide proton of R82. We tried to find conditions under which those missing signals in HSQC can be observed, by varying the temperature (10–35 °C) and/or lowering the salt concentration (the NaCl concentration was lowered to 0 mM from 100 mM), which turned out to be unsuccessful. In addition, residues 79–82 showed, on an average, only 30% signal intensities compared with the SH3.2 domain signals. This indicates the dynamic nature of the intramolecular interaction. This conclusion is also supported by the very weak affinity ($K_D \sim 2$ mM) of the 71–90 peptide for the isolated SH3.2 domain. It is also possible that the intramolecular interaction site adopts multiple conformations. The dynamic intramolecular interaction allows the two physiological binding partners containing PRS, SOS and Pak1, to replace the inhibitory intramolecular interaction at higher concentrations. Intramolecular interactions between SH3 domains and PRS have been reported for several proteins and proven to be functionally important (27–30). Still, to the best of our knowledge, this report is the first case of an SH3 intramolecular interaction with a noncanonical sequence devoid of prolines, which are generally required for SH3 domain interaction. It is worth noting that the sequence also lacks obvious hydrophobic sequence patterns, which are known to be important for PRS interactions as well as basic interactions that are supposed to mimic the PRS interactions. Nevertheless, it would be difficult to detect this kind of interaction by sequential analysis.

Besides the PRS sequences, the Nck1 SH3.2 domain is known to bind to the 45-residue juxtamembrane (JM) domain of EGFR, which is not a typical PxxP proline-rich motif (17). More specifically, NMR analysis identified that a basic region extending from Arg647 to Thr654 (647-RHIVRKRT-654) of EGFR binds to Nck1 SH3.2 with a K_D value of 80 μM. Although the EGFR JM domain peptide is not identical to the intramolecular interaction

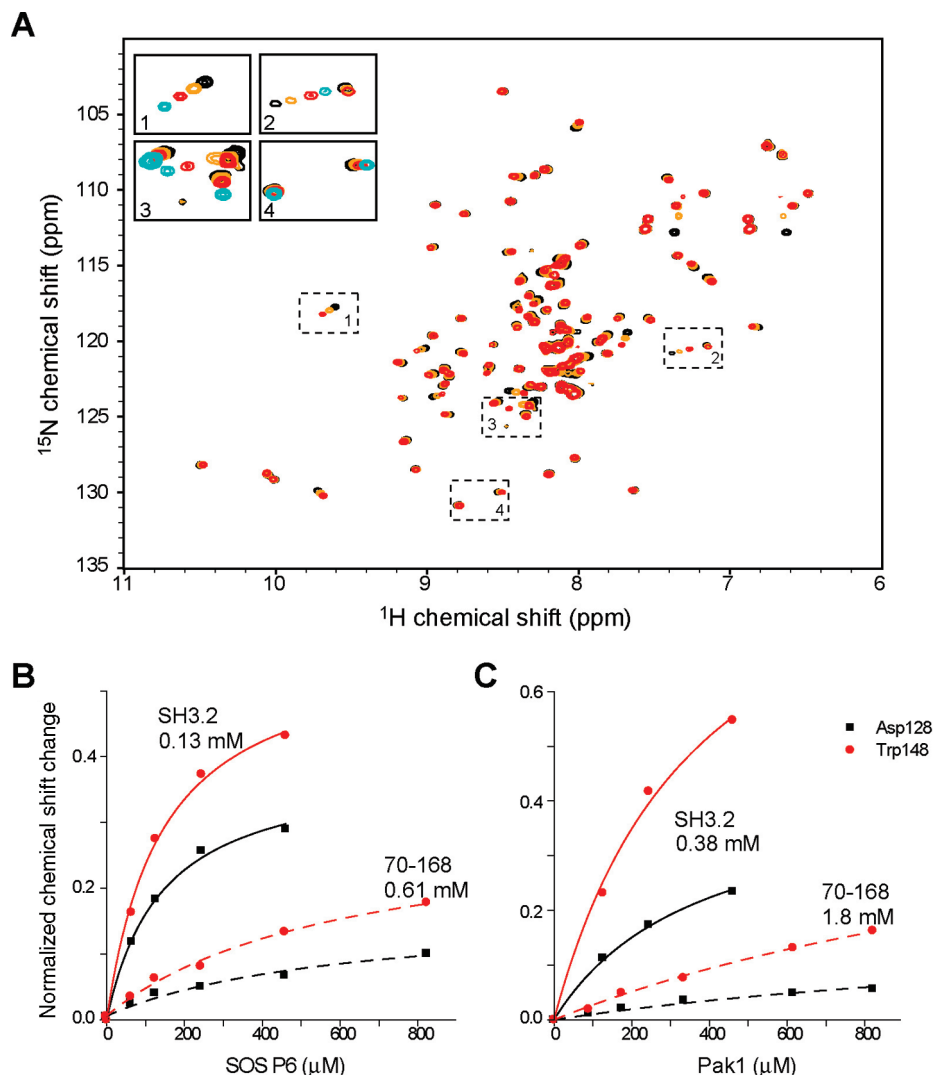


FIGURE 6: Titration of the PRS peptides with the isolated SH3.2 domain. (A) Titration of the SOS1 peptide with 130 μM [$U\text{-}^{15}\text{N}$]Nck 70–168. The ^1H – ^{15}N HSQC spectrum of the free state (black) or with 130 μM (orange) and 830 μM SOS peptide (red). The four dotted regions in the HSQC spectrum are enlarged and shown as insets. In the insets, the ^1H – ^{15}N HSQC spectrum of 130 μM [$U\text{-}^{15}\text{N}$]Nck SH3.2 with 830 μM SOS peptide is colored cyan. (B and C) Concentration-dependent chemical shift changes induced by (B) SOS and (C) Pak1 peptides. Chemical shift changes in ppm are normalized as described in the legend of Figure 4. Chemical shift changes for Asp128 and Trp148 with fast exchanges are shown. K_D values that were calculated from the concentration-dependent chemical shift changes are also shown.

site of Nck2, these peptides share a highly basic nature characterized by a cluster of three consecutive basic residues. This indicates, along with a very acidic electrostatic potential on the SH3 domain surface, the importance of electrostatic interaction in the intramolecular interaction.

Although not extensively analyzed in this paper, the SH2 domain also seems to be in a different environment in intact Nck as compared with the isolated condition (Figure S6A of the Supporting Information). Since the deletion of the first two SH3 domains did not interfere with the SH2 domain signals and isolation of SH3.3 did not change the distribution of those signals, it can be assumed that the SH2 domain is most likely to interact with the SH3.3/SH2 linker (Figure S6B). It is quite interesting to point out that some of the SH2 residues that show different chemical shifts between the excised domain and Nck-FL are situated in the AB loop, which is responsible for the recognition of phosphorylated Tyr (Figure S6C). Thus, similarly to SH3.2, the SH2 domain might also have intramolecular interaction that can modulate ligand binding. The presence of intramolecular modulation in the SH2 domain has also been pointed out in the

screening analysis, aimed at identifying the binding partners of the individual domains using mass spectroscopy (10). However, by the time of our study, the region responsible for the intramolecular interaction had yet to be identified.

In Tec family kinases, PRS is involved in an intramolecular interaction with an adjacent SH3 domain, which prevents the Itk SH3 domain and the proline-rich region from interacting with their protein ligands, Sam68 and Grb-2, respectively (27). As indicated in the paper, with the presence of an intramolecular interaction, the ligand binding affinity can be specifically and temporally regulated by post-translational modification such as phosphorylation. The conserved Ser85 residue of Nck at the intramolecular interaction site is predicted to be a phosphorylation site for 90 kDa ribosomal protein S6 kinase (p90-RSK) and/or protein kinase C (PKC) (31). Because both of the kinases become upregulated by activation of the TCR signaling pathway, Nck function can also be regulated by phosphorylation of Ser85 at the intramolecular interaction site. Because the electrostatic interaction between the basic intramolecular interaction site and the acidic surface of SH3.2 is considered to be the driving force of

the interaction, phosphorylation at the interaction site is most likely to be energetically disfavoring the intramolecular masking, thus opening up SH3.2 to its ligands. Indeed, the Ser85Glu mutation disrupted the intramolecular interaction as judged from the ^1H – ^{15}N HSQC spectrum of the mutant (Figure S3 of the Supporting Information).

It is also possible that this intramolecular interaction with the linker region improves the specificity of the SH3.2 domain and the Nck molecule per se. A protein, which only has the SH3.2 interaction site, would not be favored by the Nck molecule, since it requires additional energy to replace intramolecular masking in trans. On the other hand, a bivalent SH3.2 ligand, which has an additional binding site in other domains in Nck or outside of the PRS recognition site in the SH3.2 domain, could overcome the intramolecular interaction more easily in cis fashion, using the additional interaction as an anchor. It is worth noting that some of the Nck ligands, including Pak1 and SOS1, are known to bind to Nck-FL much more tightly than the individual SH3 domain of Nck, which suggests that a cooperative interaction is necessary for tight complex formation (10, 12, 21–23). For example, Pak1 can bind both SH3.2 and SH3.3 domains. It is likely that Pak1 binds first with the SH3.3 domain which is already available and replaces SH3.2 masking for tighter binding. On the other hand, an errant nonspecific ligand with low affinity would not be able to access the SH3.2 domain.

In conclusion, we provide evidence of the presence of a higher-order structure in Nck. The intramolecular interaction occurs between the basic (K/R)x(K/R)RxxS sequence in the SH3.1/3.2 linker and the acidic SH3.2 domain of Nck. Although the function of the intramolecular interaction has to be addressed in vivo, its dynamic nature as well as the presence of a conserved Ser residue shows new features of Nck in improving its specificity and functional maneuverability.

SUPPORTING INFORMATION AVAILABLE

List of primers for used to amplify the Nck2 genes, comparison of ^1H – ^{15}N HSQC spectra of Nck2-FL and isolated domains of Nck2 (SH3.1 and SH3.3), additional mutation data, titration of the Pak1 peptide to Nck2_{70–168}, and evidence of intermolecular NOEs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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