The binding interaction between the Nck2 SH2 domain and the phosphorylated ephrinB initiates a critical pathway for the reverse signaling network mediated by Eph receptor-ephrinB. Previously, the NMR structure and Tyr phosphorylations of the human ephrinB cytoplasmic domain have been studied. To obtain a complete story, it would be of significant interest to determine the structure of the Nck2 SH2 domain that shows a low sequence identity to other SH2 domains with known structures. Here, we report the determination of the solution structure of the human Nck2 SH2 domain and investigate its interactions with three phosphorylated ephrinB fragments by NMR spectroscopy. The results indicate that: 1) although the human Nck2 SH2 domain adopts a core tertiary fold common to all SH2 domains, it owns some unique properties such as a shorter C-terminal helix and unusual electrostatic potential surface. However, the most striking finding is that the C-terminal tail of the human Nck2 SH2 domain adopts a short antiparallel β-sheet that, to the best of our knowledge, has never been identified in other SH2 domains. The truncation study suggests that one function of the C-terminal tail is to control the folding/solubility of the SH2 domain. 2) In addition to [Tyr(P)304]ephrinB2301–322 and [Tyr(P)316]ephrinB2301–322, here we identified [Tyr(P)330]ephrinB2324–333 also capable of binding to the SH2 domain. The detailed NMR study indicated that the binding mechanisms for the three ephrinB fragments might be different. The binding with [Tyr(P)304]-ephrinB2301–322 and [Tyr(P)316]ephrinB2301–322 might be mostly involved in the residues over the N-half of the SH2 domain and provoked a significant increase in the backbone and side chain dynamics of the SH2 domain on the microsecond-millisecond time scale. In contrast, binding with [Tyr(P)330]ephrinB2324–333 might have most residues over both halves engaged but induced less profound conformational dynamics on the μs-ms time scale.

Signaling transduction pathways usually start with the transmembrane receptor proteins, and subsequently the signal is further transmitted into intracellular space via protein-protein interactions between the cytoplasmic domains of the receptors and downstream intracellular binding partners. The transmembrane Eph receptors, the largest family of receptor tyrosine kinases containing 14 members, plays an essential role in the development of multicellular organisms by functioning at the interface between pattern development and morphogenesis, such as axon guidance, cell migration, segmentation, and angiogenesis (1–5). So far eight mammalian ephrins have been identified, which can be divided into two structural and functional families, ephrinA and ephrinB. EphrinB and their Eph receptors are all membrane-anchored proteins and are unique in their ability to transmit signals bidirectionally, thus mediating contact-dependent cell-cell interactions for guidance and assembly of cells (1–7). Recently, the Eph-ephrinB-mediated signaling network has been implicated in learning and memory formation (8), neuronal regeneration (9–10), pain processing (11), and differential expressions of ephrinB were also correlated with tumorigenesis (12).

The cytoplasmic tail of the ephrinB proteins plays an integral role in mediating reverse signaling by interacting with intracellular protein binding partners (13, 14). In particular, the interaction between the cytoplasmic region of ephrinB and the SH2 1 domain of the Nck2 or Grb4 adapter protein initiates downstream signaling pathways regulating cytoskeleton assembly and remodeling (7, 14). Nck adapter family consists of two members, namely Nck1 and Nck2 (15–17). The human Nck2 is a 380-residue protein only comprised of three N-terminal SH3 and one C-terminal SH2 modular domains, thus called adapter proteins (15, 18). The Nck adapter proteins serve as a pivotal point to connect the cellular surface receptors down to the multiple intracellular signaling networks in a “Tyr(P) → SH2/SH3 → effector” manner (17). Briefly, the Nck SH2 domain binds to the phosphorylated cytoplasmic domain of the transmembrane receptors, whereas its SH3 domains recruit proline-rich effector proteins to tyrosine-phosphorylated kinase or their substrates. It has been proposed that Nck proteins are extensively involved in organization of actin cytoskeleton, cell movement, or axon guidance (16–17). Recently, we have determined the NMR structure of the ephrinB2 cytoplasmic subdomain, and showed that in the absence of tyrosine phosphorylation, the 22-residue motif identified for binding with the Nck2 SH2 domain adopted a well packed β-hairpin structure with high conformational stability (19). Particularly, it has been demonstrated that phosphorylation of any of the three tyrosine residues in the 22-residue motif dramatically abolished its well packed β-hairpin structure and peptides with either Tyr304 or Tyr316 phosphorylated were able to bind to the human Nck2 SH2 domain (20).
However, structural information about the other half of the ephrinB-Nck2 interaction story, namely the structure of the Nck2 SH2 domain, is still lacking. In particular, sequence alignment indicated that less than 40% identity was found between the Nck SH2 domains with other groups of SH2 domains (15,21). As illustrated in Fig. 1, the C-terminal residues that were usually considered not to be the core part of the SH2 domains have even lower sequence homology with other SH2 domains with the three-dimensional structure determined. To this end, it would be of significant interest to determine the structure of the Nck2 SH2 domain to gain deeper insight into its binding interactions with phosphorylated ephrinB. Therefore, the present study aimed to determine the three-dimensional structure of the human Nck2 SH2 domain, as well as to investigate its binding interactions with three phosphorylated ephrinB peptides by use of NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Cloning and Expression of the Full-length and Truncated Human Nck2 SH2 Domains—The DNA fragment encoding the full-length SH2 domain corresponding to residues 283 to 380 of the human Nck2 protein was subcloned into a modified pET32a (Novagen) vector with S-tag and thioredoxin genes removed with BamHI and XhoI restriction sites by using two primers (forward) 5′-GGGGGATCCAGAGATGTTAGTAC-TACGGG-3′ and (reverse) 5′-GGGCTCGAGTCACTGCAGGGCCCTGAC-GCG-3′. The truncated human Nck2 SH2 domain with C-terminal residues 367 to 380 deleted was subcloned into the same vector using the same restriction sites as above by replacing the reverse primer with 5′-CCGCTCGAGTCAGTGAAGATGGGCCGCTT-3′. The DNA sequences of the constructs were confirmed by automated sequencing.

The recombinants were expressed in BL21(DE3) Escherichia coli cells induced by 0.5 mM isopropyl 1-thiogalactopyranoside overnight at 20 °C. The full-length SH2 domain was found to be soluble after overexpression but the truncated one was all found in the inclusion body. Therefore, the full-length SH2 domain protein was purified with nickel-nitrilotriacetic acid resin (Qiagen) under native conditions, whereas the truncated one was purified under the denaturing condition. Both the dialysis and fast dilution methods were used to refold the truncated SH2 domain. The preparation of the isotope-labeled full-length proteins followed similar procedures except for growing the cells in M9 medium with addition of (15NH4)2SO4, [13C]glucose for 15N/13C double labeling, respectively (22). However, structural information about the other half of the ephrinB-Nck2 interaction story, namely the structure of the Nck2 SH2 domain, is still lacking. In particular, sequence alignment indicated that less than 40% identity was found between the Nck SH2 domains with other groups of SH2 domains (15,21). As illustrated in Fig. 1, the C-terminal residues that were usually considered not to be the core part of the SH2 domains have even lower sequence homology with other SH2 domains with the three-dimensional structure determined. To this end, it would be of significant interest to determine the structure of the Nck2 SH2 domain to gain deeper insight into its binding interactions with phosphorylated ephrinB. Therefore, the present study aimed to determine the three-dimensional structure of the human Nck2 SH2 domain, as well as to investigate its binding interactions with three phosphorylated ephrinB peptides by use of NMR spectroscopy.

NMR Assignments and Structure Modeling—Heteronuclear NMR experiments were performed as described previously (22,23) on an 800 MHz Bruker Avance spectrometer equipped with pulse field gradient units or on a 500 MHz Bruker Avance spectrometer equipped with an actively shielded cryoprobe and pulse field gradient units. The NMR spectra acquired for both backbone and side chain assignments included 13N-edited HSQC-TOCSY, HSQC-NOESY, as well as triple-resonance experiments HNCA, CBCA(CO)NH, HNCO, (H/C- C(CO)NH, H(CO)NH, and HCCH-TOCSY. NOE restraints were derived from 13N- and 15N-NOESY spectra acquired both on 500 and 800 MHz spectrometers. The full-length and truncated Nck2 SH2 domains were expressed in BL21(DE3) Escherichia coli cells induced by 0.5 mM isopropyl 1-thiogalactopyranoside overnight at 20 °C. The full-length SH2 domain was found to be soluble after overexpression but the truncated one was all found in the inclusion body. Therefore, the full-length SH2 domain protein was purified with nickel-nitrilotriacetic acid resin (Qiagen) under native conditions, whereas the truncated one was purified under the denaturing condition. Both the dialysis and fast dilution methods were used to refold the truncated SH2 domain. The preparation of the isotope-labeled full-length proteins followed similar procedures except for growing the cells in M9 medium with addition of (15NH4)2SO4, [13C]glucose for 15N/13C double labeling, respectively (22). The purified protein was immediately dialyzed against 20 mM sodium phosphate buffer (pH 6.8) in the presence of 5 mM dithiothreitol, and then concentrated for NMR experiments. The presence of the His tag was confirmed by SDS-PAGE analysis.

After many rounds of refinement, a final set of unambiguous NOEs, hydrogen bond restraints were utilized for structure calculations with a simulated annealing protocol implemented by the CNS program (28–30). The 10 lowest-energy structures accepted by CNS protocol were selected for detailed analysis by use of the graphic software MolMol (31). The NMR structures of the human Nck2 SH2 domain were deposited in the Protein Data Bank, with the PDB ID of 1Z3K.

Binding with Three Phosphorylated EphrinB Fragments—In addition to the two phosphorylated ephrinB2 fragments, [Tyr(P)304]-ephrinB2301–322 and [Tyr(P)316]ephrinB2301–322 identified previously (20), here we found that the 10-residue C-terminal tail with Tyr330 phosphorylated (designated as [Tyr(P)324]ephrinB2324–333) was also able to bind to the human Nck2 SH2 domain.

For characterization of the binding interactions between the SH2 domain and Tyr-phosphorylated ephrinB2 fragments, one-dimensional 1H and two-dimensional 1H-15N HSQC spectra of the 15N-labeled SH2 domain were acquired at a protein concentration of ~100 μM in the absence or presence of three phosphorylated ephrinB2 fragments at a molar ratio of ~1:1.5 (SH2/peptide). The perturbed residues were assigned by superimposing the two HSQC spectra in the absence and presence of the peptides.

RESULTS

Protein Cloning and Expression of the Full-length and Truncated SH2 Domains—Previously, we failed to obtain a sample with a concentration high enough for three-dimensional NMR experiments because of the limited solubility of the human Nck2 SH2 domain prepared by thrombin cleavage of the glutathione S-transferase fusion protein (20). In the present study, we cloned and expressed the His-tagged version of the SH2 domain whose solubility was largely enhanced, thus allowing the generation of protein samples with a concentration of ~500 μM suitable for acquiring all NMR experiments for structure determination. In contrast, the truncated SH2 domain with the C-terminal last 14 residues deleted (Fig. 1) was totally insoluble and all accumulated in the inclusion body under different expression conditions. Refolding attempts by either dialysis or fast dilution all failed. This result indicated that although the 14-residue tail of the human Nck2 SH2 domain was usually considered not to be part of the SH2 core domain (15), it did play a critical role in controlling the folding or solubility of the Nck2 SH2 domain.

NMR Assignments and Secondary Structures—The human Nck2 SH2 domain consisted of 98 residues. Except for four residues Arg4, Gly6, Gln34, and Glu86 backbone assignments were successfully achieved for all other non-proline residues as shown in Fig. 1. Side chain carbon assignments were also derived from examination of the HSQC-based hydrogen-deuterium exchange experiment.

A set of manually assigned unambiguous NOE restraints together with dihedral angle restraints predicted by the TALOS program (26) based on five chemical shift values (15N, Ca, Cα, CO, and Ha) was used to calculate initial structures of the human Nck2 SH2 domain by the CYANA program (27). With the availability of the initial structure, more NOE cross-peaks in the two NOESY spectra were automatically assigned by the CYANA program followed by manual confirmation. After many rounds of refinement, a final set of unambiguous NOEs, hydrogen bond restraints were utilized for structure calculations with a simulated annealing protocol implemented by the CNS program (28–30). The 10 lowest-energy structures accepted by CNS protocol were selected for detailed analysis by use of the graphic software MolMol (31). The NMR structures of the human Nck2 SH2 domain were deposited in the Protein Data Bank, with the PDB ID of 1Z3K.

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For characterization of the binding interactions between the SH2 domain and Tyr-phosphorylated ephrinB2 fragments, one-dimensional 1H and two-dimensional 1H-15N HSQC spectra of the 15N-labeled SH2 domain were acquired at a protein concentration of ~100 μM in the absence or presence of three phosphorylated ephrinB2 fragments at a molar ratio of ~1:1.5 (SH2/peptide). The perturbed residues were assigned by superimposing the two HSQC spectra in the absence and presence of the peptides.
completed except for the four above residues based on analyzing CCCONH and HCCH-TOCSY spectra. In total, around 90% of side chain protons were assigned. In particular, all hydrophobic residues including five Ala, eight Val, eight Leu, and three Ile residues were unambiguously assigned.

Fig. 2 presents the difference between the Cα and Cβ conformational shifts of the human Nck2 SH2 domain, which was accepted as a good indication of the secondary structure pattern (32). Fig. 2c shows the NOE patterns to further define the protein secondary structures (33). Based on these data, it appears that the major part of the protein shared the conserved secondary structure patterns common to all SH2 domains. However, a close examination suggested that the C-terminal tail unique for the Nck SH2 domain might adopt a two-stranded β-sheet. To confirm this, we conducted a detailed analysis of the NOE constraints over this region and identified characteristic NOEs to define an antiparallel sheet consisting of two β-strands: one over residues Phe83-Thr84-Ser85 and another over residues Glu89-Lys90-Leu91 (Fig. 3). Another interesting observation is that the secondary helix appeared to be shorter than those usually found in other SH2 domains.

Three-dimensional Structure—The NMR structure of the Nck2 SH2 domain was calculated from the experimental NMR constraints by the combined use of the CYANA and CNS programs. Table I summarizes the constraints used and structural statistics for the 10 lowest energy NMR structures accepted by CNS protocol, with distance violations less than 0.2 Å and dihedral angle violations less than 5°. Fig. 4 shows the stereoview of the superimposition of the 10 selected structures in the line (Fig. 4a) and ribbon modes (Fig. 4b). Globally, the Nck2 SH2 domain adopts a conserved tertiary architecture common to all SH2 domains. More specifically, the Nck2 SH2 domain has a core β-sheet composed of three long β-strands over residues 26–30, 37–43, and 48–56 followed by a short antiparallel β-sheet over residues 59–62 and 65–68. This central β-sheet was sandwiched by two helices: the N-terminal one over residues 12–17 and the C-terminal one over residues 73–76. It is very striking to note that the C-terminal region does adopt a short antiparallel β-sheet consisting of two strands over residues 83–85 and 89–91, respectively. Usually this C-terminal region with a very low sequence homology among different SH2 domain groups was considered not to be the core part of the common SH2 fold (15) and was demonstrated to adopt very
Table I
NMR constraints and structural statistics for the 10 lowest-energy structures of the human Nck2 SH2 domain

| Restraints for structure calculation | Total NOE restraints | Intraresidue | Sequential | Medium range | Long range | Hydrogen bond restraints | Dihedral angle restraints | Total NOE restraints |
|------------------------------------|---------------------|--------------|------------|--------------|------------|-------------------------|-------------------------|---------------------|
|                                    | 1348                | 663          | 285        | 121          | 279        | 36                      | 114                     | 1348                |

Final energies (kcal mol⁻¹)

- E (total)   409.4 ± 25.8
- E (bond)    16.4 ± 1.4
- E (angle)   111.4 ± 7.7
- E (improper) 18.1 ± 2.4
- E (van der Waals) 152.3 ± 10.5
- E (NOE)     103.8 ± 13.6

Root mean square deviations from idealized geometry

- Bond (Å)  0.003 ± 0.0002
- Angle (degree)  0.513 ± 0.0174
- Improper (degree)  0.380 ± 0.0244
- NOE (Å)  0.037 ± 0.0025

Ramachandran statistics (%)

- Most favored 67.2 ± 3.0%
- Additionally allowed 24.0 ± 3.7%
- Generously allowed 5.9 ± 2.7%
- Disallowed 3.0 ± 1.2%

Root mean square deviation from the lowest-energy structure (Å)

- All atoms (secondary structure region) 1.56
- Heavy atoms (secondary structure region) 1.37
- Backbone atoms (secondary structure region) 0.72

Solution Structure of the Human Nck2 SH2 Domain

Diverse secondary structures: some formed one short β-strand packed to the core β-sheet while the other had no regular secondary structures. The short antiparallel β-sheet observed for the Nck2 SH2 domain was unambiguously defined by the characteristic NOEs shown in Fig. 3, as well as many other long-range side chain NOEs between the residues on the two β-strands. In addition, this short β-sheet was found to pack to the core β-sheet as evident by many long-range NOEs. It seems that a hydrophobic cluster was formed between this short β-sheet and the core β-sheet by hydrophobic residues Leu²7, Val²8, and Ala³6 on the short β-sheet side and several hydrophobic residues on the rest of the protein. For example, Leu³³ had extensive NOEs with Phe³¹, Ile²⁸, Val³¹, Leu³², Ile³², Phe³³, Val³³, and Ile³³. Ala³⁶ had NOEs with Leu³³ plus the N-terminal aromatic residues Trp³ and Tyr³. Therefore structurally the C-terminal short β-sheet served to cap the hydrophobic surface of the core β-sheet and its absence would make the large hydrophobic core exposed, thus resulting in complete insolubility as observed for the truncated Nck2 SH2 domain with the C-terminal 14 residues deleted.

The properties of the electrostatic potential surface play a crucial role in controlling affinity and specificity of protein-protein interactions. We thus examined the electrostatic potential surfaces of many SH2 domains and it appears that the Nck2 SH2 domain had a potential surface very different from others (figures not shown). Fig. 5 presents the ribbon structure and potential surface of the human Nck2 SH2 domain together with those of two representative SH2 domains, namely Abl (34) and Src (35) SH2 domains, with the ligand binding surfaces all facing outside. It can be seen clearly that N-half positively charged pockets for binding to phosphotyrosine are well conserved in all three SH2 domains but it is much larger in the human Nck2 SH2 domain. It is especially interesting to note that the Nck2 SH2 domain also has a large positively charged surface over the C-terminal half of the ligand-binding interface that was proposed to be critical for modulating binding specificity (21, 36-38), whereas Src and Abl SH2 domains have more neutral surfaces over the C-halves.

Binding Interactions with Three Phosphorylated EphrinB Fragments—The ephrinB2 cytoplasmic domain contains five tyrosine residues out of which Tyr³⁰⁴, Tyr³¹¹, and Tyr³¹⁶ are located over the well structured β-hairpin region from residues 301 to 322, whereas Tyr³³⁰ and Tyr³³¹ are over the less-structured β-turn region from residues 324 to 333 (19). It was previously reported that the binding region between the ephrinB cytoplasmic domain and the Nck2 SH2 domain was located over the β-hairpin 22-residue region containing the first three tyrosine residues (14). In this regard, the 22-residue ephrinB peptides with either of three tyrosine residues phosphorylated were previously investigated and the results led to the identification of two peptides, [Tyr³⁰⁴]ephrinB²³⁰¹-³²² and [Tyr³¹¹]ephrinB²³⁰¹-³²², capable of binding to the human Nck2 SH2 domain prepared by the thrombin cleavage of the glutathione S-transferase fusion protein (20). Here we have studied the binding interactions of the three peptides with the His-tagged SH2 domain and the same binding profile was obtained, indicating that the presence of the His tag in the SH2 domain had no detectable effect on its binding profile. In the current study, we further examined the SH2 domain binding ability of the two fragments spanning the C-terminal 10 residues of the human ephrinB2 with either Tyr³³⁰ or Tyr³³¹ phosphorylated (designated as [Tyr³³⁰]ephrinB²³²⁴-³³³ and [Tyr³³¹]ephrinB²³²⁴-³³³), respectively. Very strikingly, the results indicated that only peptide [Tyr³³⁰]ephrinB²³²⁴-³³³ was able to bind to the SH2 domain.

Therefore, we monitored the binding interactions between the 15N-labeled SH2 domain and the three phosphorylated peptides by use of one-dimensional proton and two-dimensional heteronuclear HSQC spectra. Fig. 6 presents the binding profiles of the human Nck2 SH2 domain with all the three phosphorylated fragments. From the one-dimensional spectrum (Fig. 6a), it can be seen that the binding interaction with [Tyr³³⁰]ephrinB²³²⁴-³³³ induced shifts, and in particular extensive broadenings of the NMR resonance peaks arising from the SH2 domain. A detailed analysis of the HSQC titration results in Fig. 6b led to the identification of 38 perturbed peaks of the SH2 domain that either disappeared or underwent shifts. Of 38 perturbed peaks, 21 peaks disappeared because of the NMR resonance broadening, indicating that binding resulted in a significant increase in the conformational exchange on the microsecond-millisecond time scale. On the other hand, the binding profile in Fig. 6c indicated that the binding interaction with [Tyr³¹¹]ephrinB²³²⁴-³³³ caused less line broadening for the SH2 domain if compared with that with [Tyr³³¹]ephrinB²³²⁴-³³³. However, more HSQC peaks (44 peaks) were perturbed, of which 15 peaks disappeared (Fig. 6d). Very surprisingly, as seen in Fig. 6e, although the binding interaction with [Tyr³³⁰]ephrinB²³²⁴-³³³ resulted in the least line broadening and HSQC peak disappearance (only 11 peaks), it induced the most extensive HSQC peak perturbation, with a total of 67 HSQC peaks perturbed (Fig. 6f).

Further analysis of the perturbed HSQC peaks of the human Nck2 SH2 domain induced by binding with the three peptides allowed us to identify a set of 30 commonly perturbed HSQC peaks corresponding to residues Trp³, Tyr⁵, Val⁸, Arg¹⁰ His¹¹, Ala¹³, Glu¹⁴, Gly¹⁴, Leu²⁷, Ile²⁸, Arg³⁰, Asp³⁰, Ser³¹, Glu³², Ser³³, Ser³⁴, Ser³⁶, Asp³⁷, Phe³⁸, Ser³⁹, Val⁴⁰, Leu⁴², Ala⁴⁴,
Gly\(^{46}\), His\(^{50}\), Lys\(^{52}\), Val\(^{53}\), Val\(^{56}\), Asp\(^{57}\), and Gln\(^{98}\). As a result, the binding-affected HSQC peaks for each peptide could be regarded as the 30 commonly perturbed peaks plus a set of uniquely affected peaks for each peptide, which was composed of 8 peaks for [Tyr(P)\(^{304}\)]ephrinB\(^{301–322}\), 13 peaks for [Tyr(P)\(^{316}\)]ephrinB\(^{301–322}\), and 36 peaks for [Tyr(P)\(^{330}\)]ephrinB\(^{324–333}\). When the perturbed residues were mapped back to the NMR structure of the human Nck2 SH2 domain, a very interesting picture was emerging (Fig. 7). It seems that except for the last residue, Gln\(^{98}\), the commonly perturbed residues were mostly located over the N-terminal half of the SH2 domain sequence and their side chains were also clustering together to constitute the N-terminal half of the protein surface. Moreover, it seems that for [Tyr(P)\(^{304}\)]ephrinB\(^{301–322}\) and [Tyr(P)\(^{316}\)]ephrinB\(^{301–322}\), the perturbed interfaces were mainly located over the N-half part of the Nck2 SH2 domain. However, by sharp contrast, for [Tyr(P)\(^{330}\)]ephrinB\(^{324–333}\), the perturbed interface was much larger and a large number of residues (\(\sim 80\%\) of the total SH2 domain residues) on both the N- and C-halves of the molecule were involved. It is thus reasonable to speculate that the binding interfaces between the Nck2 SH2 domain with [Tyr(P)\(^{304}\)]ephrinB\(^{301–322}\) and [Tyr(P)\(^{316}\)]ephrinB\(^{301–322}\) were smaller than that with [Tyr(P)\(^{330}\)]ephrinB\(^{324–333}\). The present results are also consistent with the established notion that the N-half part of SH2 domains contributed a cluster of conserved residues for binding to the phosphorylated tyrosine, whereas the C-half used relatively diverse sets of residues to achieve the binding specificity. Furthermore, based on the results above, it is very likely that the binding mechanism for [Tyr(P)\(^{304}\)]ephrinB\(^{301–322}\) and [Tyr(P)\(^{316}\)]ephrinB\(^{301–322}\) was different from that for [Tyr(P)\(^{330}\)]ephrinB\(^{324–333}\). However, the exact contact surfaces and mechanism between the human Nck2 SH2 domain and the three phosphorylated ephrinB2 peptides can only be defined when the high resolution complex structures are available.

**DISCUSSION**

The binding interaction between the phosphorylated ephrinB with the Nck2 SH2 domain represents a key event for the reverse signaling mediated by Eph receptor-ephrinB, which allows signal transduction from the transmembrane ephrinB to the downstream multiple pathways, thus leading to cytoskeleton assembly and remodeling (6, 14). Previously the three-dimensional structures of the ephrinB were determined for both extracellular (7, 39–40) and cytoplasmic (19) domains. Furthermore, it has been showed that the well packed \(\beta\)-hairpin adopted by the ephrinB cytoplasmic needed to be disrupted by tyrosine phosphorylation so as to bind to the Nck2 SH2 domain (20). To have the story completed for the ephrinB-Nck2 interaction, in the present study we have succeeded in obtain-
ing suitable protein samples and determining the solution structure of the human Nck2 SH2 domain by use of heteronuclear NMR spectroscopy. Although globally the human Nck2 SH2 domain adopted a conserved SH2 fold, it does have some unique structural properties. For instance, the C-terminal helix of the Nck2 SH2 domain is shorter and its potential surface seems very different from those of other SH2 domains (Figs. 2, 4, and 5). However, the most striking finding is that its C-terminal tail, which was considered not to be the core part of the SH2 fold, adopted a short antiparallel $\beta$-sheet packed against the core $\beta$-sheet. To the best of our knowledge, this structural element has never been observed for other SH2 domains before. In addition to the involvement in binding with phosphorylated ephrinBs, one critical function of this short $\beta$-sheet appears to prevent the exposure of the hydrophobic surface mainly formed by the core $\beta$-sheet. This conclusion is strongly supported by our experimental observation that the deletion of this region made the truncated molecule totally insoluble.

SH2 domains are a family of small modular domains playing a critical role in directing and assembling signaling networks in many cellular processes by recognizing phosphorylated tyrosine (Tyr(P)) in their binding partners (41). Strikingly, SH2 domains from different proteins show a wide range of binding specificity. As a result, the principles underlying the ligand binding specificity for the SH2 domains still remain poorly understood. For example, although the SH2 domains from Nck1 and Nck2 share an extremely high sequence homology (Fig. 1), only the SH2 domain of the Nck2 was identified to bind to phosphorylated ephrinB (14). Interestingly, it was also reported that even a single mutation on the SH2 domain would switch its binding specificity (42–44). Intriguingly, on the other hand, one SH2 domain was usually found to be capable of binding to a variety of peptide ligands without any obvious consensus (38). For example, although the Nck1 SH2 domain prefers to bind to the pYDEP consensus motif (45), it does bind to pYVPM of the PDGF receptor (46), as well as pYDTG of IRS-1 (45). In our present study, we observed the similar situation in which the human Nck2 SH2 domain not only showed binding ability to [Tyr(P)304]ephrinB2301–322 and [Tyr(P)316]ephrinB2301–322 (20), it was also able to bind to [Tyr(P)330]ephrinB2324–333 as identified here. The detailed NMR study indicated that the binding mechanisms for the three ephrinB fragments might be different. The binding interaction with [Tyr(P)304]ephrinB2301–322 and [Tyr(P)316]ephrinB2301–322 with the same amino acid sequence might be mostly involved in residues over the N-half of the SH2 domain and in the meanwhile provoked a significant increase in the backbone and side chain dynamics on the microsecond-millisecond time scale. In contrast, the binding interaction with [Tyr(P)330]ephrinB2324–333 may have much more residues over both halves of the SH2 domain involved but induced less profound conformational dynamics on the microsecond-millisecond time scale. Our current observation thus strongly highlights the complexity of the backbone and side chain dynamic changes of the SH2 domains induced by ligand binding, consistent with previous observa-
tions that for some cases the binding of the SH2 domains with phosphorylated ligands increased conformational dynamics, whereas for other cases, the binding decreased the dynamics (47–50). The binding complexity revealed by NMR is also consistent with the results obtained by isothermal titration calorimetry. The isothermal titration calorimetry study on the interactions between the human Nck2 SH2 domain and the three phosphorylated ephrinB fragments offered very complex curves that might indicate multiphase binding interactions and consequently made it impossible to fit the data to obtain reliable thermodynamic parameters such as dissociation constants and the stoichiometry.\(^2\)

In summary, the successful determination of the NMR struc-

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\(^2\) X. Ran and J. Song, unpublished data.
tecture of the human Nck2 SH2 domain revealed the structural and potential surface properties unique for the SH2 domains in the Nck group, which may bear important implications in future understanding of the Eph receptor-ephrin-B-mediated signaling mechanism. For example, with the obtained structural knowledge, in the future it would be possible to design ephrinB mutants that are able to constitutively bind to the Nck2 SH2 domain, so as to better understand the biological role of the ephrinB-Nck2 pathway. To achieve this, some mutations will be introduced to disrupt the well packed β-hairpin, whereas other such as the Tyr → Asp mutation could be used to mimic phosphorylated tyrosine residues. Furthermore, the detailed binding results also offer interesting insights into the specificity determinants for ligand interactions with the human Nck2 SH2 domain, which may assist in designing molecules of therapeutic interest in the future.

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