Tyrosine Phosphorylation of the Well Packed EphrinB Cytoplasmic β-Hairpin for Reverse Signaling

STRUCTURAL CONSEQUENCES AND BINDING PROPERTIES*[S]

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Tyrosine phosphorylation of the 22-residue cytoplasmic region of ephrinB induces its binding to the SH2 domain of Grb4, thus initiating reverse signaling pathways controlling cytoskeleton assembly and remodeling. Recently, the region corresponding to this 22-residue motif was demonstrated to adopt a well packed β-hairpin structure with a high conformational stability in the unphosphorylated cytoplasmic subdomain. However, because the binding to Grb4 is phosphorylation-dependent and the hairpin contains three conserved tyrosine residues that may be phosphorylated, the key events remain unknown as to how tyrosine phosphorylation affects the structure of this well packed β-hairpin and which phosphorylation site is relevant to SH2 domain binding. By characterizing the structural and binding properties of six 22-residue SH2 domain-binding motifs with different phosphorylated sites, the present study reveals that, as shown by circular dichroism and NMR, the unphosphorylated 22-residue motif adopts a well formed β-hairpin structure in isolation from the ephrinB cytoplasmic subdomain. However, this β-hairpin is radically abolished by tyrosine phosphorylation, regardless of the relative location and number of Tyr residues. Unexpectedly, the peptides with either Tyr304 or Tyr316 phosphorylated show high affinity binding to SH2 domain, whereas the peptide with Tyr311 phosphorylated has no detectable binding. This implies that ephrinB with Tyr311 phosphorylated might have a currently unidentified binding partner distinct from the Grb4 protein, because Tyr311 is known to be phosphorylated in vivo. Based on the results above, it is thus proposed that the disruption of the tight side-chain packing by tyrosine phosphorylation in the well structured region of a signaling protein may represent a general activation mechanism by which a cryptic binding site is disclosed for new protein-protein interactions.

The Eph receptors, with a total of 14 members, is the largest known family of receptor tyrosine kinases. They are implicated in learning and memory formation (8), and differential expression of ephrinB was also correlated with tumorigenesis (9).

The cytoplasmic tail of the ephrinB proteins plays a central role in mediating reverse signaling via protein-protein interactions with intracellular protein binding partners (10–11). In particular, interaction between the 22-residue cytoplasmic region of ephrinB and the Grb4 SH2 domain initiates downstream signaling pathways regulating cytoskeleton assembly and remodeling (7, 10). Recently, we determined the NMR structure of the ephrinB cytoplasmic subdomain, and showed that, in the absence of the tyrosine phosphorylation, this 22-residue motif adopted a well packed β-hairpin structure (Fig. 1) with a high conformational stability as indicated by 15N backbone dynamics data (12). However, because the interaction between this 22-residue region and the Grb4 SH2 domain is phosphorylation-dependent (7, 10), and the β-hairpin region contains three conserved Tyr residues Tyr304, Tyr311, and Tyr316 (Fig. 1), the role of tyrosine phosphorylation in this interaction still remains unknown. On the other hand, although selective and reversible phosphorylation plays a pivotal role in controlling biological activity of proteins in many biochemical processes, the mechanism still remains poorly understood especially in structural terms (13). In this regard, the 22-residue ephrinB β-hairpin represents a unique model for addressing the structural consequence of tyrosine phosphorylation because: 1) it adopts a well packed β-hairpin structure with extensive long range side-chain packing, which would facilitate the assessment of structural changes upon tyrosine phosphorylation and 2) it contains three conserved tyrosine residues, thus offering the possibility of studying the consequence of multiple tyrosine phosphorylations. By characterizing structural and binding properties of a set of six 22-residue circular dichroism; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser single-quantum coherence spectroscopy; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.
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Fig. 1. Structural model of the 22-residue β-hairpin ephrinB2301–322. Ribbon representation of the eight lowest energy structures of ephrinB2301–322 derived from the corresponding region of the NMR structure of ephrinB2301–333 (12), superimposed over residues 303–317, with the side chains of Tyr304, Tyr311, and Tyr316 displayed in stick mode and colored red.

EphrinB peptides, the current study focuses on addressing the structural consequences of tyrosine phosphorylation and on identification of phosphorylation sites relevant to the SH2 domain binding by use of circular dichroism (CD) and NMR spectroscopy. The set of peptides includes one unphosphorylated, three single-, one double-, and one triple-phosphorylated forms. Detailed CD and NMR characterization of the six 22-residue peptides demonstrates that the isolated SH2-domain binding motif adopts a well packed β-hairpin structure in the unphosphorylated state as described before (12), but this β-hairpin structure is dramatically abolished by tyrosine phosphorylations. Furthermore, NMR-based binding study indicates that the unphosphorylated site Tyr304 is actually relevant to the SH2 domain binding, whereas the in vivo phosphorylated site Tyr311 is irrelevant. These results provide the first detailed picture showing how cryptic binding sites buried in a well packed β-hairpin of a signaling protein is activated by disrupting the tight side-chain packing through tyrosine phosphorylation. These observations may bear important implications in deciphering the Eph-ephrinB-mediated reverse signaling mechanism and contribute to our general knowledge of how tyrosine phosphorylation modulates signal transduction by switching the conformation of a signaling protein.

EXPERIMENTAL PROCEDURES

Cloning and Expression of the Grb4 SH2 Domain—A DNA fragment encoding human Grb4 SH2 domain (residues 283–380) was generated from Grb4 DNA by PCR with the forward primer, 5′-GGG GAA TTC AGA GAG TGG TAC TAC GGG-3′ from Grb4 DNA by PCR with the reverse primer, 5′-GGG CTC GAG TCA CTG CAG GGC CCT GAC G-3′. The PCR product was cloned into the pGEX-5X-1 vector (Amersham Biosciences) using EcoRI/XhoI restriction sites, and the DNA sequence was verified by automated DNA sequencing. The expression construct for the SH2 domain was transformed into the Escherichia coli strain BL21 to express GST fusion protein. The cleavage of the fusion protein was performed by incubating the fusion proteins with bovine factor Xa for 2 days. The purified SH2 domain protein was obtained by rebinding the released GST protein to the glutathione-Sepharose. For heteronuclear NMR experiments the SH2 domain protein was prepared in 15N-labeled form using a similar expression protocol except for growing E. coli cells in minimal M9 media instead of the 2YT media, with an addition of [15N]NH4)2SO4 for 15N labeling.

Peptide Synthesis and Purification—Six 22-residue peptides with different sites and numbers of phosphorylations at Tyr304, Tyr311, and Tyr316 were synthesized using standard Fmoc (N-(9-fluorenylethoxycarbonyl) chemistry. The set of peptides includes one unphosphorylated native peptide with a sequence of CPHYEYKSGSDYHPVYIVQEMP corresponding to the residues 301–322 of ephrinB2 (designated as ephrinB2301–322), three single-phosphorylated peptides, [pY304]ephrinB2301–322, [pY311]ephrinB2301–322, and [pY316]ephrinB2301–322, with phosphorylation at Tyr304, Tyr311, and Tyr316, respectively; one double-phosphorylated peptide [pY304,pY311]ephrinB2301–322, and one triple-phosphorylated peptide [pY304,pY311,pY316]ephrinB2301–322. For further mapping of the specificity and affinity determination of the ephrinB and SH2-domain interaction, three short phosphorylated peptides derived from the ephrinB2 sequence, pY304KSVG, pY311GHPV, and pY316QVEM, were also chemically synthesized using the same protocol. The peptides were purified by reverse-phase high-performance liquid chromatography (C18 column, and their identities were verified by MALDI-TOF mass spectrometry and NMR resonance assignments.

Circular Dichroism Spectroscopy—CD experiments were performed on a Jasco J-715 spectropolarimeter, with samples containing 50 μM peptides in 20 mM sodium phosphate buffer at pH 6.8. Far-UV CD spectra were collected at 20 °C using 1-mm path length cuvettes with a 0.1-nm spectral resolution. Fifteen independent scans were averaged for each peptide.

NMR Experiments and Structure Model Generation—An NMR sample of the 15N-labeled SH2 domain with a concentration of ~0.15 mM was prepared by exchanging the protein into 50 mM sodium phosphate buffer (pH 6.8). Samples of the synthetic 22-residue peptides with concentrations of ~2.0 mM were prepared by dissolving the lyophilized peptides in 400 μl of either H2O or D2O aqueous buffer containing 50 mM sodium phosphate (pH 6 or pH 6.8). Deuterated diethrioireitol was added to a final concentration of 15 mM to prevent the formation of intermolecular disulfide bridge. The deuterium lock signal for the NMR spectrometers was provided by the addition of 50 μl of D2O. A mixture of protease inhibitors was also added to inhibit the activities of any residual proteases not removed by the purification procedure.

NMR experiments, including two-dimensional NOESY (14), TOCSY (15), and 1H-15N HSQC (16) were collected on a Bruker Avance-500 spectrometer. A mixing time of 250 ms was used for NOESY, and 65 ms was used for TOCSY experiments. Spectral processing and analysis were carried out using the XwinNMR (Bruker) and NMRview software programs. Sequence-specific assignments for the synthetic peptides were achieved through identification of spin systems in the TOCSY spectra combined with sequential NOE connectivities in the NOESY spectra (17, 18). The structural model of ephrin-B2301–322 was generated from the corresponding region of the published NMR structure (12) by using the Insight II software package.

RESULTS

Structural Effects of Tyrosine Phosphorylations Characterized by Circular Dichroism—To gain insight into the globular secondary structure changes caused by tyrosine phosphorylation of the peptides, far-UV circular dichroism (CD) spectra of all six 22-residue peptides were collected at pH 6.8 and 20 °C. As seen in Fig. 2, the far-UV CD spectrum of the unphosphorylated ephrinB2301–322 has two main bands at 198.1 nm (positive) and at 212.5 nm (negative). The presence of a positive band at about 198 nm and a negative one in the range of 210–215 nm usually indicates a well formed antiparallel β-sheet or β-hairpin (19). This observation provides solid evidence that the 22-residue SH2-domain binding motif still adopts a well formed β-hairpin structure on its own in isolation from the 33-residue functional subdomain (12). Strikingly, all three single-phosphorylated ephrinB301–322 gave CD spectra without positive bands and with the negative bands shifted to around 200 nm, which are typical of very flexible loops or even random coil conformations (19). Similar CD spectra were also observed for the double- and triple-phosphorylated ephrinB2301–322. Taken together, these results strongly indicate that, upon tyrosine phosphorylation, the well formed β-hairpin adopted by 22-residue ephrinB2301–322 was drastically abolished, regardless of the position and numbers of the phosphorylation sites.

Structural Effects of Tyrosine Phosphorylations Characterized by NMR Spectroscopy—To further assess the detailed
Structural changes induced by tyrosine phosphorylations, all six peptides were extensively characterized by two-dimensional NMR spectroscopy. The sequential assignments were successfully achieved for all six peptides by analyzing the combination of TOCSY and NOESY spectra in H2O according to well-established methods (17, 18), and the proton chemical shifts are presented in the supplementary materials (Supplemental Tables S1–S6). Fig. 3a presents the chemical shift differences between the isolated 22-residue unphosphorylated peptide ephrinB2301–322 and the corresponding region in ephrinB2301–333 (12). No significant difference was observed for the amide protons. With regard to the CαH resonance shifts, only three residues Cys301, Tyr311, and Pro322 showed obvious changes, indicating that the secondary structure of the unphosphorylated ephrinB2301–322 is almost identical to that of the corresponding region in ephrinB2301–333, which is completely consistent with the CD study.

Fig. 3b shows the differences of amide proton chemical shifts between ephrinB2301–322 and its five phosphorylated forms. Overall, differences were observed among the sequence with some changes larger than 0.1 ppm. More profound changes were observed for CαH chemical shifts (Fig. 3c). Interestingly, there were always three residues, Cys301, Pro302, and Tyr311 showing significant changes on a similar scale, regardless of the position of individual phosphorylated sites in the all five phosphorylated peptides (Fig. 3c). Previously, it was shown that, unlike serine and threonine phosphorylations, tyrosine phosphorylation had no direct effect on the backbone conformation of model peptides (20). Therefore, the current observation implies that tyrosine phosphorylations of ephrinB2301–322, regardless of their locations in the hairpin (Fig. 1), all caused similar global structural disruption, which consequently gave rise to the similar patterns of CαH chemical shift changes.

NOESY spectra in D2O were collected for all six peptides to further address detailed changes in side-chain packing upon tyrosine phosphorylation. Fig. 4 (a–f) show the same aromatic side-chain regions of NOESY spectra of the six peptides in D2O. As seen in Fig. 4a, the existence of a large number of NOEs indicates that ephrinB2301–322 is well packed even through it was isolated from the functional subdomain. A further comparison of NOE patterns from ephrinB2301–322 with those from the corresponding region in ephrinB2301–333 indicates that their characteristic NOEs are almost identical. Together with CD and chemical shift data shown in Figs. 2 and 3a, results of the NOE analysis here strongly indicate that the structure of the isolated ephrinB2301–322 is very similar to that of the corresponding region in ephrinB2301–333, namely it is a well packed β-hairpin structure stabilized by extensive packing interactions among Tyr, His, Pro and the other hydrophobic side chains (12).

In striking contrast, upon phosphorylation of Tyr304, extensive NOE disappearances occurred (Fig. 4b), indicating a severe disruption of the tight side-chain packing (21). More specifically, phosphorylation of Tyr304 resulted in a complete disappearance of NOEs related to the ε protons of Tyr304. Phosphorylation of Tyr304 also disrupted all NOE connectivities related to the δ protons of His303 as well as all NOEs between proton pairs from the two hairpin strands, such as those between the δ and ε protons of Tyr311, and γ protons of Lys206 or γ protons of Glu206 (Fig. 4, a and b). These clearly indicated that a global and severe disruption of the β-hairpin structure had occurred. Similarly, phosphorylation of Tyr311 caused not only local structural perturbation, as indicated by the disappearance of all NOEs related to the ε H protons of Tyr311, but also abolished all the long range side-chain packing, particularly those between proton pairs from two hairpin strands, similar to those observed for [pY304]ephrinB2301–322 (Fig. 4, b and c). These observations are similar to those for
ephrinB2301–322, ephrinB2301–322, and triple-phosphorylated ephrinB2301–322 peptides (Fig. 4, d–f). It is particularly worthwhile to note that, for the [pY304,pY311,pY316]ephrinB2301–322 peptide, all NOEs related to H protons of Tyr304, Tyr311, and Tyr316 have dramatically disappeared (Fig. 4f). The disappearance of NOEs related to H protons of the phosphorylated tyrosines suggests that the introduction of a phosphate group to the aromatic ring of the tyrosine residues may place a large steric barrier, or change the dynamics, or both. All of which could lead to a severe...
disruption of the side-chain packing centered around the ε protons of the phosphorylated tyrosine residues. This notion is strongly supported by the dramatic changes of CD spectra upon tyrosine phosphorylation (Fig. 2) and NMR observation that, for all peptides, both δ and ε protons of the phosphorylated tyrosines can be unambiguously assigned in TOCSY spectra but NOEs related to ε protons have totally disappeared (Supplemental Table S1–S6). Strikingly, it appears that the disruption of the tight side-chain packing related to any of three tyrosine side chains is sufficient to abolish the entire ε-hairpin structure. However, although tyrosine phosphorylation severely abolished all long range packing interactions, some sequential and medium range NOEs can still be identified clustering over the Ω-loop-like region (residues Ser308-Gly312) in all five phosphorylated peptides. This observation is very similar to residual structures in partially folded proteins, implying that this loop region might serve as an initiation site in protein folding (21–24).

**Binding Properties of Six 22-Residue Peptides and Three Short Peptidomimetics**—Their binding properties of all six 22-residue ephrinB peptides for the Grb4 SH2 domain were monitored by following 1H-15N HSQC spectra of the 15N-labeled SH2 domain titrated with different peptides. The unphosphorylated native peptide ephrinB2301–322 causes no HSQC peak shifts even with a 5-fold excess of the peptide. In contrast, titration of the 15N-labeled SH2 domain with the peptide [pY304]ephrinB2301–322 showed that, if the molar ratio between the peptide and the SH2 domain was less than 1, two sets of the HSQC peaks were observed for many residues of the SH2 domain. One set is without any peak shift and another set with extensive peak shifts, which must have arisen from the SH2 domain complexed with the peptide [pY304]ephrinB2301–322. When the molar ratio reached ~1:1, the HSQC peak set from the free SH2 domain all shifted to merge with the set from the complexed SH2 domain. Further addition of the peptide up to a 5-fold excess caused no further peak shift (Fig. 5a). This observation implies that the dissociation of the complex between the SH2 domain and [pY304]ephrinB2301–322 is within the regime of slow chemical exchanges. For slow chemical exchange, the $K_d$ value is usually less than 10 μM (25). However, as pointed out previously (25), exceptions do exist and it would be important to establish the binding constants independently by other biophysical methods. Surprisingly, [pY311]ephrinB2301–322 causes no detectable change of the HSQC spectra even with a 5-fold excess of the peptide (data not shown), indicating that [pY311]ephrinB2301–322 does not bind to the Grb4 SH2 domain.

For the peptide [pY316]ephrinB2301–322, at a molar ratio (peptide/SH2 domain) less than 1, two sets of the HSQC peaks were observed for many residues of the SH2 domain. One set is without any peak shift and another set with significant peak...
shifts resulting from the complexed SH2 domain. When the molar ratio was increased to ~1:1, the HSQC peak set from the free SH2 domain shifted to merge with the set from the complexed SH2 domain, similar to that observed in [pY304]ephrinB2–322 titration. Furthermore, the addition of the peptide up to a 5-fold excess caused no further peak shift (Fig. 5b). This observation indicates that the dissociation between the [pY316]ephrinB2–301–322 peptide and the SH2 domain is also within the regime of slow chemical exchanges. Interestingly, the HSQC spectrum of the SH2 domain complexed with [pY304]ephrinB2–301–322 is different from that of the SH2 domain complexed with [pY316]ephrinB2–301–322. This observation may result from different conformational changes induced by the binding of the two peptides to the same site on the SH2 domain, or alternatively from the binding of the two peptides to two completely different sites. Given the fact that a large portion of the HSQC peaks of the 15N-labeled SH2 domain underwent significant shifts upon binding to either [pY304]ephrinB2–301–322 or [pY316]ephrinB2–301–322, discrimination of the above two possibilities can not be easily achieved by the HSQC mapping method, and would certainly require high resolution three-dimensional structures of the peptide-SH2 domain complexes to be determined by x-ray crystallography or NMR spectroscopy. Furthermore, the HSQC spectrum of the SH2 domain complexed with the double-phosphorylated peptide [pY311]pY316]ephrinB2–301–322 was almost identical to that of the SH2 domain complexed with the single-phosphorylated peptide [pY316]ephrinB2–301–322 (Fig. 5c). This clearly suggests that additional phosphorylation of Tyr311 does not enhance binding. The HSQC spectrum of the SH2 domain complexed with triple-phosphorylated peptide [pY304]pY311]pY316]ephrinB2–301–322 was almost identical to that of the SH2 domain complexed with the single-phosphorylated peptide [pY304]ephrinB2–301–322 peptide (Fig. 5d), indicating that the SH2 domain may bind to the triple-phosphorylated peptide [pY304]pY311]pY316]ephrinB2–301–322 in a similar manner as with the single-phosphorylated peptide [pY304]ephrinB2–301–322.

To map the determinant of the binding affinity and specificity between the phosphorylated ephrinB and the Grb4 SH2 domain, the binding interactions between the SH2 domain and three short phosphorylated peptides pY304EKVSG, pY311GHPV, and pY316IVQEM were studied by HSQC titrations. The pY304EKVSG peptide started to induce the shifts of a subset of HSQC peaks of the 15N-labeled SH2 domain at a molar ratio of 4:1 (peptide/SH2 domain), and the shifting proceeded until it was saturated at a molar ratio of ~16:1 (spectra not shown). This observation suggests that the dissociation between the SH2 domain and pY304EKVSG is within fast chemical exchange regime with a much weaker binding affinity (25) compared with the interaction with [pY304]ephrinB2–301–322. Interestingly, the saturated HSQC spectrum of the 15N-labeled SH2 domain in the presence of a 16-fold excess of the pY304EKVSG peptide is similar to that of the 15N-labeled SH2 domain complexed with [pY304]ephrinB2–301–322, indicating that both the short pY304EKVSG and [pY304]ephrinB2–301–322 peptides bind to the SH2 domain in a similar manner. Again, the unambiguous interpretation of the HSQC titration results undoubtedly requires the availability of the high resolution structures of the complexes between the SH2 domain and the different phosphorylated peptides. For pY311GHPV and pY316IVQEM, no detectable HSQC peak shift was observed even with a 16-fold excess of the peptides (data not shown). These results imply that, for both the phosphorylation sites Tyr311 and Tyr316, additional residues not present in the shortened peptides pY304EKVSG and pY316IVQEM are required to achieve high affinity bindings with the SH2 domain.

The transmembrane ephrinB ligands behave as "receptor-like" signaling molecules, controlling morphogenetic processes such as axon guidance and angiogenesis. Previous functional studies have highlighted the critical role of the cytoplasmic domain in these processes (6, 7, 26–28). Identification of two intracellular binding partners of ephrinB, namely PDZ-RGB and Grb4 proteins, provided critical insights into the molecular interactions underlying the Eph-ephrinB-mediated reverse signaling network (7, 10–11). Very recently, it was proposed that by phosphorylation/dephosphorylation of the ephrinB cytoplasmic domain, the reverse signaling could be controlled and switched between two critical pathways: one PDZ-dependent pathway leading to G protein-coupled receptor signaling and another phosphorylation-dependent pathway that modulates cytoskeletal dynamics (29). Despite the central role of tyrosine phosphorylation in Eph-ephrinB-mediated reverse signaling, it remained unknown how tyrosine phosphorylation affects the structure of the 22-residue SH2 domain binding motif with a well packed β-hairpin and which phosphorylation site is relevant to the SH2 domain binding.

Reversible phosphorylation of proteins is perhaps the most universal regulatory mechanism in controlling the biological activity of proteins by post-translational modifications, which occurs for a variety of cellular processes ranging from regulation of catabolism/metabolism to control of growth and gene expression (13, 20, 30–34). It has been widely thought that phosphorylation controls protein activity mainly by inducing conformational changes, but the detailed mechanisms remain poorly understood. Therefore, understanding the structural consequence and binding properties of tyrosine phosphorylations on the well packed ephrinB cytoplasmic β-hairpin might contribute to our general knowledge how tyrosine phosphorylation controls cellular signaling through conformational switches.

In the first part of this study, the detailed CD and NMR characterization of a set of six 22-residue ephrinB peptides demonstrates that the unphosphorylated ephrinB2–301–322 adopts a well packed β-hairpin structure as described previously (12), but this structure is radically disrupted upon tyrosine phosphorylation, regardless of the exact position of the phosphorylated site. Because many tyrosine side chains in proteins participate in the formation of the tertiary packing by interacting with other local and/or long range hydrophobic side chains, the introduction of a phosphate group with a large side-chain volume and strong negative charges will inevitably disrupt the hydrophobic tertiary packing. It is therefore very likely that destabilization of protein structures through disrupting the side-chain packing may represent a general mechanism by which tyrosine phosphorylation controls the functions of proteins.

In the second part, the phosphorylation of the 22-residue motif is further correlated to the binding interaction with the Grb4 SH2 domain. The NMR study reveals that both the in vivo unphosphorylated site Tyr304 and the in vivo phosphorylated site Tyr311 can bind the Grb4 SH2 domain with high affinities. Unexpectedly, however, the peptide with Tyr311 phosphorylated showed no detectable binding ability to the SH2 domain. Whether the phosphorylated Tyr304 can serve as an in vivo docking site for the Grb4 SH2 domain needs to be further investigated. Nevertheless, the observation that the peptide with Tyr311 phosphorylation shows no binding activity to the Grb4 SH2 domain suggests that phosphorylated Tyr311 might have other currently unknown in vivo binding partners, because both Tyr311 or Tyr316 are conserved tyrosine residues within the 22-residue β-hairpin and was shown to be phosphorylated in vivo (7, 29, 30). A recent report indicated that the protein Dishevelled (Dsh) formed a complex with either the
unphosphorylated or phosphorylated ephrinB cytoplasmic domain (35), followed by a further recruitment of other proteins. To this end, it would be of great interest to explore whether the phosphorylation of Tyr$^{311}$ contributes to the Xdsh-based protein-protein interactions.

Based on current results, it appears that for the interaction between the ephrinB cytoplasmic region and the Grb4 SH2 domain, three conditions have to be satisfied simultaneously: 1) the well packed hairpin structure has to be disrupted by tyrosine phosphorylation to expose the docking region for the Grb4 SH2 domain; 2) the phosphate group is needed to provide the binding energy (36); and 3) the specific amino acid sequences surrounding the phosphorylated tyrosine residues have to be present to achieve high affinity binding. The last point is particularly underscored by the results of NMR binding study on the interactions between the SH2 domain and three short peptides, which clearly indicates that the high affinity binding between the SH2 domain and phosphorylated ephrinB$_{2}^{304–322}$ requires regions larger than the short sequences C-terminal to the phosphorylated tyrosines. It is also striking to note that phosphorylation of Tyr$^{311}$, which satisfies conditions 1 and 2, but not 3, result in no detectable binding to the Grb4 SH2 domain. The finding that the pY$^{304}$EKVSG peptide is able to bind the Grb4 SH2 domain may provide a promising starting point for the design of inhibitors disrupting the protein-protein interaction between ephrinB and the Grb4 protein (27).

In summary, the present NMR study provides the first detailed picture showing how the well packed β-hairpin structure of ephrinB cytoplasmic domain can be dramatically disrupted by single, double, and triple phosphorylations at three conserved tyrosine residues. The NMR binding study further identifies two out of three tyrosine residues within the 22-residue hairpin of ephrinB as phosphorylation sites relevant for high affinity binding to the Grb4 SH2 domain. In particular, the observation that the 22-residue peptide with Tyr$^{311}$ phosphorylated shows no binding to the Grb4 SH2 domain implies that ephrinB phosphorylated at Tyr$^{311}$ might have currently unidentified in vivo binding partners other than Grb4. The current results can be extrapolated to suggest that the disruption of the tight side-chain packing by tyrosine phosphorylation in the well structured region of a signaling protein may represent a general activation mechanism by which the cryptic binding site is disclosed for further protein-protein interactions.

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