Biochemical and NMR characterization of the interactions of Vav2-SH2 domain with lipids and the EphA2 juxtamembrane region on membrane.

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Abstract

Vav2 is a ubiquitous guanine nucleotide exchange factor (GEF) for Rho family GTPases that is involved in regulating a wide range of biological processes. It interacts with several tyrosine-phosphorylated cell surface receptors, including the Eph family receptors, through its SH2 domain. The interaction of Vav2 with EphA2 is crucial for EphA2-mediated tumor angiogenesis. Here we show that Vav2-SH2 domain is a lipid-binding module that can recognize PI(4,5)P2 and PI(3,4,5)P3 lipids weakly but specifically. The specific lipid-binding site in Vav2-SH2 domain was identified by NMR chemical shift perturbation experiments using the head groups of PI(4,5)P2 and PI(3,4,5)P3, both of which bind to Vav2-SH2 with millimolar binding affinities. In addition, the interaction between Vav2-SH2 and the phosphorylated juxtamembrane region (JM) of EphA2 (Y594 phosphorylated) was investigated using NMR techniques. Furthermore, by using a nickel-lipid containing peptide-based nanodiscs system, we studied the binding of Vav2-SH2 to the phosphorylated JM region of EphA2 on lipid membrane and uncovered a role of membrane environment in modulating this protein-protein recognition.

Introduction

The Src-homology 2 (SH2) domains are prototypical protein interaction modules that specifically recognize phosphotyrosine (pY) containing motif [1]. They are found in diverse cell-signaling proteins and play crucial roles in cellular signal transduction. The human genome encodes 121 SH2 domains in 111 different proteins [1]. SH2 domains typically contain approximately 100 amino acid residues and share a common fold composed of a central antiparallel β-sheet flanked by two α-helices [2]. They specifically recognize pY and a few residues C-terminal to pY using a conserved positively charged pY-binding pocket and a more variable secondary binding site, respectively [3].
Interestingly, in addition to mediate pY-dependent protein-protein interactions, accumulating evidence suggests that SH2 domains also serve as lipid-binding modules. It was reported earlier that a small number of SH2 domains could bind lipids, including the SH2 domains of PI3-Kinase [4] and PLC-γ [5]. More recently, Park et al. systematically measured the binding abilities of 76 of 121 human SH2 domains for plasma membrane (PM)-mimetic vesicles by surface plasmon resonance (SPR) analysis and revealed that ~90% of the SH2 domains tested are capable of binding PM-lipids [6]. Despite recent progress, the lipid-binding properties and the recognition mechanisms for most SH2 domains are still unclear.

Vav2, a member of Vav family (Vav1, Vav2 and Vav3), functions as a guanine nucleotide exchange factor (GEF) for the Rho family GTPases [7]. It is broadly expressed in human tissues and plays essential roles in various biological processes, including phagocytosis, angiogenesis, cell spreading and migration, and neuronal development [7-12]. Vav2 structurally consists of a calponin homology (CH) domain, an Acidic (Ac) region, a catalytic Dbl homology (DH), a pleckstrin homology (PH) domain, a zinc finger (ZF) domain and a Src homology 2 (SH2) domain flanked by two Src homology 3 (SH3) domains. The presence of an SH2 domain is a unique structural feature of Vav members different from any other Ras superfamily GEF. Through its SH2 domain, Vav2 is able to interact with several tyrosine-phosphorylated transmembrane receptors or coreceptors, including Eph family receptors [10, 12, 13], platelet-derived growth factor receptor (PDGFR)[14], epidermal growth factor receptor (EGFR) [15] and CD19 [16]. Through these SH2-mediated interactions, Vav2 is translocated to the plasma membrane and then mediates different extracellular signals to intracellular responses. EphA2 is a member of the Eph family receptors. The interaction of Vav2 with EphA2 is crucial for EphA2-mediated tumor angiogenesis [13]. Two tyrosine phosphorylation sites in the JM region of EphA2, Y588 and Y594, have been shown to be involved in Vav2-SH2 binding. However, until now, the detailed molecular mechanism of this binding has not been determined. In addition to mediate protein-protein interactions, Vav2-SH2 domain has been recently shown to be also capable of binding PM-mimetic vesicles [6]. However, the lipid recognition specificity of Vav2-SH2 domain and the underlying molecular mechanisms are unclear. Moreover, it is also unknown whether the lipid membrane environment has any effect on the interaction of Vav2-SH2 with its membrane-anchored partners.

To study membrane-associated events in vitro, a suitable membrane mimetic is needed. Detergent micelles and liposomes are commonly used as membrane mimetics. However, micelles lack a flat lipid bilayer. In addition, the detergents often have deteriorating effects on the structure and activity of a protein. Liposomes contain a lipid bilayer, but they are too large for solution NMR studies. Nanodisc is a soluble nanoscale membrane mimic. It is composed of a planar phospholipid bilayer surrounded by two copies of amphipathic membrane scaffold proteins (MSPs) [17, 18].
to its high solubility in water, variability in the lipid composition, and lipid bilayer characteristics, nanodisc has been widely used for structural and functional analyses of membrane proteins in solution [19, 20]. It also emerges as an ideal model membrane system for investigating various membrane recognition events [21]. In addition to the MSP-based nanodisc, an alternative strategy, the peptide-based nanodisc has been developed recently [22-24]. In the peptide-based nanodisc, short amphipathic peptides are used in place of large MSPs and the nanodisc sizes can be conveniently controlled by varying the lipid/peptide ratio [22]. Unlike the MSP-based nanodisc, recent studies have shown that peptide-based nanodisc has the ability to allow lipid exchange between nanodiscs via collisions, which would be useful to probe lipid-protein interaction and to study the effect of lipid on the structure and function of membrane proteins [25-27].

Here, we have investigated the lipid binding properties of Vav2-SH2 domain by biochemical and NMR assays. We show that Vav2-SH2 can recognize PI(4,5)P2 and PI(3,4,5)P3 lipids weakly but specifically. In addition, the interaction between Vav2-SH2 and the phosphorylated juxtamembrane region (JM) of EphA2 was investigated using NMR techniques. Furthermore, by using a nickel-lipid containing peptide-based nanodisc system, we studied the binding of Vav2-SH2 to the phosphorylated JM region of EphA2 on lipid membrane and uncovered a role of membrane environment in modulating this protein-protein recognition.

Materials and methods

Materials

Lipids 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1, 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1,2-dihexanoyl-sn-glycero-3-phospho-L-serine (diC6-PS), Cardiolipin (CL), phosphatidylinositol (PI), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) and 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt) (DGSNTA(Ni)) were purchased from Avanti Polar Lipids. diC4-PI(4,5)P2 and diC4-PI(3,4,5)P3 were purchased from Echelon, USA. 15N-NH4Cl and D2O were purchased from Cambridge Isotope Laboratories, Inc.

Protein expression and purification

The DNA fragment encoding human Vav2-SH2 domain (residues 659 to 771) was amplified by PCR from the pCMV5-Vav2 plasmid as described previously [28], and cloned into the pET28a vector containing a 6xHis-tag and a thrombin protease cleavage site at the N terminus. The resulting construct was transformed into E. coli BL21 (DE3) (Novagen) for protein expression. The cells were cultured in LB medium at 37 °C until OD600 reached 0.8, and then were with 0.5 mM IPTG at
25 °C for 8 h. For the production of uniformly $^{15}$N-labeled sample, cells were grown in M9 minimal medium using $^{15}$N-NH$_4$Cl (0.5 g/L) as the sole nitrogen source. Cells were harvested by centrifugation and lysed by sonication in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8.0). The recombinant protein was purified by a chelated-nickel column (Qiagen), followed by tag removal by thrombin protease at 4 °C and size exclusion chromatography (SEC) on a Superdex 75 16/60 column.

The N-terminal 6×His-tagged phosphotyrosine peptide EphA2-JM-pY594 corresponding to the JM region of EphA2 (residues 559-607 with Y594 phosphorylated) (HHHHHHHRKQRARQSPEDVYFSKSEQUKLKTYVDHTPYEDPNQAVLKFTE) and the 22-residue peptide (PVLDLFRELLNELLEALKQKL) for nanodiscs preparation were synthesized by GL Biochem Ltd. (Shanghai).

**Liposome preparation and liposome pull-down assay**

For liposome preparation, the phospholipids were dissolved in chloroform and then mixed in the indicated proportions in a glass tube. Chloroform was evaporated under a stream of nitrogen gas for 20 min. After further drying with a vacuum pump overnight, lipid films were hydrated at room temperature with constant mixing in buffer A (20 mM Tris-HCl, 130 mM NaCl, pH 7.5). Following hydration, lipid vesicles were subjected to freeze–thaw cycles in liquid N$_2$ and a room temperature water bath, and then sized by an extruder (Avanti) using 100-nm polycarbonate filters. For liposome-binding assays, 20 μg of Vav2-SH2 protein in buffer A was incubated with different liposomes (640 μg as indicated in Figure 1) at 4 °C for 30 min, with a total volume of 100 μL. The mixtures were then centrifuged at 50,000 rpm in a Beckman Optima MAX-XP ultracentrifuge at 4 °C for 30 min. The resulting supernatant was removed to determine the free proteins, and the pellets were washed twice with buffer A and then dissolved in 100 μL of SDS/PAGE loading buffer. The supernatant and pellet fractions were subjected to SDS/PAGE and visualized by Coomassie Blue staining.

**Nanodiscs preparation**

The 22-residue peptide based nanodiscs were prepared as described previously [22]. Briefly, the 22-residue peptides were dissolved in buffer B (40mM potassium phosphate, pH 7.8) to make a stock solution at 10 mg/mL. Lipids of DMPC, P(4,5)P2, P(3,4,5)P3 and DGS-NTA(Ni) powders were dissolved in buffer C (10 mM potassium phosphate, pH 7.4) to make a stock solution at 20 mg/mL, respectively. To prepare nanodiscs, lipid stock solutions were mixed together at molar ratios of 98 : 2 (DMPC : DGS-NTA(Ni)) and 93 : 5 : 2 (DMPC : P(4,5)P2/P(3,4,5)P3 : DGS-NTA(Ni)) for Ni-NTA-nanodiscs and P(4,5)P2 or P(3,4,5)P3-containing Ni-NTA-nanodiscs, respectively. Peptide and lipids were then mixed at 1:1 w/w ratio and incubated at 50 °C for 10 min. Then the mixture was allowed to cool down at room temperature for 10 min. This procedure was repeated 3 to 5 times.
until the mixture became clear, followed by several cycles of freeze and thaw between -80 °C and room temperature to make the nanodiscs more homogeneous. The resulting solution containing nanodiscs was then purified by SEC on a Superdex 200 10/300 GL column.

**Dynamic light scattering (DLS)**

DLS were performed using a Malvern Zetasizer nano ZS (Malvern, UK) instrument. The temperature was set to 25°C and refractive index was assumed to be equal to that of Tris/NaCl. Samples were analyzed in triplicate. The size and size distributions of nanodiscs were calculated through analyses of autocorrelation functions using Zetasizer software.

**Transmission electron microscopy (TEM)**

Purified 22-residue peptide based nanodiscs were adsorbed onto copper grids and negative-stained with phosphorotungstic acid. Images were taken on an FEI Tecnai Spirit Transmission Electron Microscope (120 kV).

**Nuclear Magnetic Resonance (NMR) experiments**

All NMR experiments were carried out at 293 K on a Bruker Avance 600 MHz NMR spectrometer equipped with a CryoProbe. The NMR data were processed with NMRPipe [29] and analyzed using Sparky program [30]. For titration experiments, uniformly 15N-labeled Vav2-SH2 protein was prepared in buffer D (20 mM Tris-HCl, 100 mM NaCl, 5 mM DTT, pH 7.2) at a concentration of 40 or 50 μM with 10% D2O. Stock solutions of the water-soluble lipid analogs (diC4-Pl(4,5)P2, diC4-Pl(3,4,5)P3 and diC6-PS) and the EphA2-JM-pY594 peptide were prepared in the same buffer, and the pH was adjusted to 7.2. A series of 1H-15N HSQC spectra were recorded as the titrant gradually titrated into the protein solution. The combined chemical shift perturbations (CSPs) were calculated using the following equation: \( \Delta \delta = [(\Delta \delta_{1H})^2 + (0.17 \Delta \delta_{15N})^2]^{1/2} \), where \( \Delta \delta_{1H} \) and \( \Delta \delta_{15N} \) are chemical shift perturbations in the 1H and 15N dimensions, respectively. The dissociation constant \( K_D \) is deduced by fitting the combined chemical shift change to the equation for fast chemical exchange [31]. To obtain Vav2-SH2/EphA2-JM-pY594 backbone assignments, 0.5 mM 15N, 13C-labeled SH2 protein mixed with 1.5 mM unlabeled peptide was dissolved in buffer D. The following spectra were recorded: 2D 1H-15N HSQC, 3D-HNCO, HN(CA)CO, HNCA, HN(CO)CA, CBCANH.

**Isothermal titration calorimetry**

Isothermal titration calorimetry (ITC) experiments were performed on a MicroCal PEAQ-ITC instrument (Malvern) at 298K. The Vav2-SH2 protein (320 μM) in buffer E (20 mM Tris-HCl, 100 mM NaCl, pH 7.4) was loaded into the syringe. The free EphA2-JM-pY594 peptide (12 μM) and the peptide tethered on different nanodiscs, as indicated in Figure 5, were prepared with the same buffer and loaded into the sample cell, respectively. The titration protocol consisted of a single initial
injection of 0.4 µL, followed by 19 injections of 2 µL the Vav2-SH2 protein into the sample cell. The heat of dilution was determined by titration of the protein into buffer E under the same condition and subtracted from the corresponding raw experimental data. Thermodynamic data were analyzed with a single-site binding model using MicroCal PEAQ-ITC Analysis Software provided by the manufacturer.

Results and Discussion

The lipid-binding preference of Vav2-SH2 domain

Previous SPR analysis shows that Vav2-SH2 domain can bind to PM-mimetic vesicles [6]. To further investigate the lipid binding specificity of Vav2-SH2 domain, liposome pull-down assays were performed. According to the lipid composition of the PM, seven distinct liposomes were prepared, including 100% POPE, 100% POPC, 70% POPC + 30% PS, 70% POPC + 30% CL, 90% POPC + 10% PI, 90% POPC + 10% PI(4,5)P2 and 90% POPC + 10% PI(3,4,5)P3. The Vav2-SH2 protein was recombinantly expressed and purified to electrophoretic homogeneity. In the liposome-binding assay (Figure 1A), the purified Vav2-SH2 was pre-incubated with liposomes. After ultracentrifugation, the supernatant and pellets were analysed by SDS-PAGE. As shown in Figure 1B, only in the presence of PI(4,5)P2- or PI(3,4,5)P3-containing liposomes, a small amount of the Vav2-SH2 protein could be detected in the pellet fraction, indicating that Vav2-SH2 can bind directly, albeit weakly to these two liposomes. The purified GST, which was used as a negative control did not exhibit any binding to PI(4,5)P2- or PI(3,4,5)P3-containing liposomes. No detectable interaction of Vav2-SH2 with other types of liposome was observed. These results suggest that Vav2-SH2 has a binding preference for PI(4,5)P2 and PI(3,4,5)P3.

NMR analysis of the specific binding of Vav2-SH2 to diC4-PI(4,5)P2 and diC4-PI(3,4,5)P3.

Recent studies indicate that the molecular location and morphology of lipid-binding sites in SH2 domains are highly variable [6, 32, 33]. To investigate the specific lipid recognition mechanism of Vav2-SH2, we took advantage of the previously published Vav2-SH2 solution structure and backbone amide assignments and performed NMR titration experiments using diC4-PI(4,5)P2 and diC4-PI(3,4,5)P3, the water-soluble analogs of PI(4,5)P2 and PI(3,4,5)P3, respectively. As a control, a short-chain phosphatidylserine (diC6-PS) was also included. 2D ¹H-¹⁵N HSQC spectra of Vav2-SH2 at a series of protein to lipid ratios were collected, respectively. The titration with diC6-PS did not lead to any significant chemical shift perturbations (CSPs) of the Vav2-SH2 amide protons (Figure S1), indicating no detectable binding of diC6-PS to the protein. In contrast, both diC4-PI(4,5)P2 and diC4-PI(3,4,5)P3 induced distinct chemical shift perturbations for specific residues in the ¹H-¹⁵N HSQC spectra of Vav2-SH2 (Figure 2A and Figure S2), indicating that the protein recognizes diC4-PI(4,5)P2 and diC4-PI(3,4,5)P3 specifically. The NMR results are consistent with
that from the liposome-binding assays. In the case of diC4-PI(4,5)P2 binding, the significantly
perturbed residues (with CSPs above mean value plus one standard deviation) are Q682, A702,
A704, R706, F707, I720, K721, V722, V723 (Figure 2B-D). Among them, residues Q682, A702,
I720, K721 and V722 displayed fast exchange on the NMR timescale, while residues A704, R706,
F707 and V723 also exhibited decreased intensities upon titration, suggesting fast to intermediate
chemical exchange (Table S1). Besides, the less perturbed residues Q681 and A708 also revealed
intensity reductions. Structural mapping showed that all these residues, except Q681 and Q682,
formed a contiguous surface on Vav2-SH2, indicating of the specific binding site for diC4-PI(4,5)P2.
Titration of diC4-PI(3,4,5)P3 into Vav2-SH2 induced a similar pattern of chemical shift perturbation
and intensities reduction (Figure S2 and Table S1), suggesting both the lipids bind to the same region
of the protein. Besides, the revealed lipid-binding site in Vav2-SH2 was adjacent to its highly
cationic pY-binding pocket (Figure 2D and Figure S3). However, the invariable R698 residue
locating in the pY-pocket was less perturbed upon diC4-PI(4,5)P2 or diC4-PI(3,4,5)P3 binding,
suggesting that the pY-pocket may not be involved in lipid-binding. Interestingly, the lipid-binding
site in Vav2-SH2 is composed of cationic residues as well as hydrophobic and aromatic residues,
which is reminiscent of lipid-binding sites of membrane-binding proteins [34]. It should be noted
that the affinities of Vav2-SH2 to diC4-PI(4,5)P2 or diC4-PI(3,4,5)P3 are very weak, with the
dissociation constants (Kd) both estimated through a global fitting the CSPs as ~ 10 mM (Figure
2E and F). Recent studies show that the lipid binding affinities of SH2 domains are highly variable
[6]. Like Vav2-SH2, several SH2 domains have been reported to recognize lipid head groups with
millimolar affinities, including ABL-SH2 [35] and p85-SH2 [36].

To date, the lipid recognitions of several SH2 domains have been characterized. Despite the
low sequence similarity (33.3% sequence identity) (Figure 3A), the lipid-binding site of Vav2-SH2
is quite similar with that of Lck-SH2 [32]. As shown in Figure 3B left, the cationic residue K182 ,
the aromatic residue F163 as well as the hydrophobic residues A160 and I183 in the lipid-binding
site of Lck-SH2 have been shown to be essential for its binding to PI(4,5)P2. Vav2-SH2 has all these
critical residues in the corresponding sites (K721, A702, F707 and V722), which are significantly
perturbed by diC4-PI(4,5)P2 and diC4-PI(3,4,5)P3 according to the NMR titration data (Figure 3B
right). Differently, Lck-SH2 also contains another key cationic residue R184 for its lipid binding
and this residue is replaced by a hydrophobic residue V723 in Vav2-SH2, which may explain the
weaker lipid-binding of Vav2-SH2.

NMR analysis of the interaction between Vav2-SH2 and the Y594-phosphorylated EphA2 in
solution and on membrane
Vav2 interacts with phosphorylated EphA2 receptor through its SH2 domain. Y594, one of the
tyrosine phosphorylation sites in the JM region of EphA2, is within a YEDP motif, which matches
the consensus motif of YEXP preferred by Vav2-SH2. To test the interaction of Vav2-SH2 with Y594-phosphorylated EphA2, a 6xhis-tagged phosphorytrosine peptide (named EphA2-JM-pY594) corresponding to the JM region of EphA2 (residues 559-607 with Y594 phosphorylated) was synthesized. NMR titration experiment was then carried out. 1H-15N HSQC spectra were recorded for 15N-labeled Vav2-SH2 domain before and after addition of EphA2-JM-pY594 peptide to different molar ratios of protein/peptide. As shown in Figure S4A, the peptide induced large changes in the 1H-15N HSQC spectrum of the Vav2-SH2, indicating a direct binding. Assignment of the signals in the bound form was made by performing a series of triple resonance NMR experiments using 15N, 13C-labeled Vav2-SH2 in the presence of unlabeled peptide with a molar ratio of 3:1 peptide:protein (Figure S4B). The peptide binding caused a global decrease in signal intensities. The relative resonance peak heights measured from Vav2-SH2 in the presence of peptide (3:1), represented as a percentage of the corresponding resonance peak heights from free Vav2-SH2 are plotted as a function of Vav2-SH2 residue number (Figure S4C). The average relative peak height is 42.8%. In addition to intensity reductions, CSPs for many residues were observed (Figure S4A and S4D). Some residues showed small CSPs, which is traceable, suggesting fast to intermediate exchange on the chemical shift NMR time scale. Other residues exhibited large and untraceable CSPs, indicating slow to intermediate exchange. All these residues that displayed slow to intermediate exchange were mapped on the structure of Vav2-SH2 (Figure 4A, B). The majority of these residues are located in and around the pY-binding pocket of Vav2-SH2, which is formed by the central β-sheet, the BC loop and N-terminus of αA, including αA (E679-D684, L686-L687), βB (R698 and E699), βC (A708-I709 and I711), βD (K718-V722), BC loop (R700, A702, A704-F707) and βD’ (V723). Among them, the pY-binding pocket residues R680, R698 and R700 interact electrostatically with the oxygen atoms of pY, as seen in the complex structures of Vav2-SH2 with its pY ligands [28, 37]. Other residues that displayed slow exchange are located in BG loop (S755-Q758), EF loop (I731, E733, A734, F737) and βE (I729 and H730). These may be involved in the contact with the residues adjacent to pY594 in the EphA2 peptide. As seen in the SH2-pY complexes, the pY+3 pocket is formed by loops EF and BG that is used to recognize the pY+3 residues in their pY-ligands and to determine the specificity of an SH2 domain. Overall, it could be clearly seen that the region with significant CSPs contained a central pY-pocket along with a larger binding interface that could accommodate the extended peptide (Figure 4B). The results indicates that Vav2-SH2 binds to the phosphorylated JM region of EphA2 in a canonical SH2-ligand recognition manner.

Under physiological condition, the recognition of the phosphorylated EphA2 JM region by Vav2-SH2 takes place on the plasma membrane interface. Therefore, we further studied the influences of membrane environment on this interaction. To better mimic the physiological state of the peptide EphA2-JM-pY594, we adopted a strategy of tethering this histidine-tagged peptide to
the membrane mimic containing nickel chelating lipid. Herein, the peptide-based nanodiscs were
chosen as the membrane mimetics, which were assembled using a 22-residue peptide derived from
apolipoprotein A-I (Apo A-I) and neutral lipid DMPC [22]. The assembled nanodiscs (lipid:peptide
ratio of 1:1 w/w for all preparations) were purified by size exclusion chromatography (SEC). The
single peak of nanodiscs was eluted at 13.3 ml (Figure S5A). Transmission electron microscopy
(TEM) images showed the formation of disc shaped, monodispersed particles (Figure S5B). The
diameter of peptide nanodiscs was determined as about 8.7 nm by dynamic light scattering (DLS)
analysis (Figure S5C). To prepare the nickel-chelating nanodiscs, which is hereafter referred to as
Ni-NTA-nanodiscs, 2% DGS-NTA(Ni) was incorporated into the peptide nanodiscs. The efficient
tethering of the histidine-tagged EphA2-JM-pY594 peptide to Ni-NTA-nanodiscs was confirmed
by ITC assay. As shown in Figure S6, the peptide forms stable complex with Ni-NTA-nanodiscs,
with a binding $K_D$ of about 1.4 μM. Again, the interaction between Vav2-SH2 and membrane-
tethered EphA2-JM-pY594 were characterized by NMR. The 2D $^1$H-$^15$N HSQC spectrum of Vav2-
SH2 in the presence of membrane-tethered EphA2-JM-pY594 (SH2/peptide 1:3) was recorded and
compared with that mixed with the free peptide at the same SH2/peptide molar ratio. As shown in
Figure 4C, the membrane-tethered peptide and free peptide induced nearly identical CSPs in the
Vav2-SH2 spectrum, indicating that the protein binds to the free and membrane-tethered peptide
with the same binding site. The spectrum of Vav2-SH2 binding to membrane-tethered peptide
exhibited an overall reduction in signal intensities compared with that binding to the free peptide
(Figure S7A, B). An average relative peak height of 62.9% was observed. The global reduction in
signal intensities was likely caused by the large molecular weight of membrane-tethered peptide.
Interestingly, significant intensity reduction for several residues was observed (Figure S7C),
including A670, H690, K718, D726, H730, D738, L740, E742, H750, S751, E754 and K764. We
guess this may due to the effect of membrane environment.

Lipid membrane environment increases the binding affinity of Vav2-SH2 to Y594-
phosphorylated EphA2

To further quantitatively analyze the binding of Vav2-SH2 to the peptide EphA2-JM-pY594 with
or without membrane tethering, ITC experiments were then carried out. The results are shown in
Figure 5 and Table S1. In all cases, Vav2-SH2 binds to the peptide with a binding stoichiometry of
1:1 and the binding is strongly enthalpically driven. The binding $K_D$ of Vav2-SH2 with the free
peptide was determined to ~3.6 μM (Figure 5A). Interestingly, the $K_D$ of Vav2-SH2 to the
membrane-tethered peptide was measured as ~1.8 μM (Figure 5B), which is about 2-fold lower than
that to the free peptide. As a control, we also measured the binding $K_D$ of Vav2-SH2 to the free
peptide in the presence of Ni-NTA-free nanodiscs (100% DMPC). The presence of Ni-NTA-free
nanodiscs did not show any significant effect on the affinity of Vav2-SH2 to the free peptide (Figure
Taken together, these results clearly demonstrated that membrane-tethering enhances the affinity of the Vav2-SH2 domain to the EphA2-JM-pY594 peptide, revealing a critical regulatory role of membrane in this protein-protein interaction. A likely explanation is that the membrane-tethered EphA2-JM-pY594 peptide may adopt a conformation that binds stronger to Vav2-SH2 than its water-soluble conformation. SH2 domains are known to bind pY-containing peptides with variable affinity and a significant degree of promiscuity [38]. However, exquisite protein interaction specificity is required for high-fidelity pY signaling in vivo. This membrane enhanced protein-protein interaction maybe of great regulatory significance for cell signal transduction.

Finally, given that Vav2-SH2 can bind weakly to PI(4,5)P2 or PI(3,4,5)P3, we next probe whether the specific lipid binding of Vav2-SH2 has any effect on its pY-binding using this Ni-NTA-nanodiscs system. PI(4,5)P2 or PI(3,4,5)P3 was then introduced into the Ni-NTA-nanodiscs with a mole ratio of 5%. The $K_D$ values of Vav2-SH2 binding to the peptide tethered to the PI(4,5)P2 or PI(3,4,5)P3 containing Ni-NTA-nanodiscs were similar to that of peptide tethered to Ni-NTA-nanodiscs (Figure 5D, E), indicating that PI(4,5)P2 or PI(3,4,5)P3 did not affect the binding affinity of Vav2-SH2 to the membrane-tethered pY-peptide. In other words, the SH2-pY recognition is independent of PI(4,5)P2 or PI(3,4,5)P3 lipid binding. This may due to the fact that the lipid-binding pocket of Vav2-SH2 is separated from the pY binding site (Figure 2D and Figure 5F). Similar phenomenon has been observed in several other SH2 domains such as Lck-SH2 [32] and ZAP70-SH2 [6]. Lck-SH2 and ZAP70-SH2 exhibited identical pY peptide binding with and without PM-mimetic vesicles and unaltered vesicle binding in the presence of pY peptides, and molecular modeling suggested that they can bind the pY motif and lipids simultaneously and independently. The weak PI(4,5)P2 or PI(3,4,5)P3 binding ability of Vav2 may contribute to its membrane recruitment or other related events in vivo. Recent molecular dynamic simulations revealed that the proximal JM domain as well as the kinase domain of EphA2 interacts with PI(4,5)P2 and both domains induce formation of nanoclusters of PI(4,5)P2 in the membrane [39, 40]. These findings together with our observations that Vav2-SH2 domain is capable of binding phosphorylated EphA2 JM and PI(4,5)P2 lipid, imply a complex functional cross-talk among EphA2, Vav2 and PI(4,5)P2 in vivo, which should be further investigated.
Conclusions

In present work, we have characterized the binding of Vav2-SH2 domain with lipids and the Y594 phosphorylated EphA2 juxtamembrane region by biochemical and NMR methods. Our data shows that Vav2-SH2 can bind to PI(4,5)P2 and PI(3,4,5)P3 lipids weakly but specifically. NMR titrations demonstrated that Vav2-SH2 recognized the head groups of these two lipids with millimolar binding affinities through a lipid binding site separated from its pY-pocket. In addition, NMR titrations also revealed the structural basis of Vav2-SH2 for binding to Y594-phosphorylated EphA2. In order to further explore the effect of membranes on this interaction, we developed a strategy in which the histidine-tagged Y594-phosphorylated EphA2 peptide was attached to peptide-based nanodiscs using nickel chelating lipids. Our ITC analysis demonstrated that membranes increase the binding affinity of Vav2 to EphA2 by ~2 fold. Taken together, this study provides a way to study the protein-protein interaction on complex membrane environment and also a point of view for future work in the significance of membrane environment in membrane-spanning protein-protein interactions.

Author Contribution

Liang Ge and Bo Wu designed the experiments; Liang Ge, Bo Wu, Youjia Zhang and Jiarong Wang conducted the experiments and analyzed the data; Hongxin Zhao contributed to the experimental design; Bo Wu and Junfeng Wang supervised the project and wrote the paper.

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FIGURE LEGENDS

Figure 1. Phospholipid-binding preference of Vav2-SH2 analysed using liposome-binding assay. (A) Schematic diagram of the liposome-binding assay. (B) Liposome-binding assays of Vav2-SH2. Vav2-SH2 (20 μg) was mixed with different liposomes (640 μg), as indicated. The GST protein was used as negative control. After centrifugation, the pellet (P) and supernatant (S) were analysed by SDS/PAGE and Coomassie Blue-staining.

Figure 2. The binding interface of Vav2-SH2 for diC4-PI(4,5)P2 and its binding affinities to diC4-PI(4,5)P2 and diC4-PI(3,4,5)P3 revealed by NMR titration. (A) Overlay of 1H,15N HSQC spectra of Vav2-SH2 in the absence (black) and in the increasing amounts of diC4 PI(4,5)P2. The spectral resolutions in 1H and 15N dimensions are 3.521 Hz (0.0059 ppm)/point and 6.177 Hz (0.1016 ppm)/point, respectively. The molar ratios of the protein to diC4-PI(4,5)P2 are shown in the inset: 1:0 (black), 1:3.5 (royal blue), 1:7 (dark green), 1:14 (purple), 1:42 (turquoise), 1:63 (orange) and 1:84 (red). Residues Q681, Q682, A702, A704 and I720-V722 are zoomed in respectively. 1D spectral slices of Q681 and Q682, A702, A704 and I720-V722 are zoomed in respectively. 1D spectral slices of Q681 and A704 are included in subfigure. (B) The chemical shift perturbations (CSPs) of each residue during NMR titrations are calculated and shown with the secondary elements on top. The mean value and the mean value plus one standard deviation are indicated by dash and solid lines, respectively. (C and D) The
cartoon (C) and surface (D) representations of the Vav2-SH2 structure (PDB code: 2LNX) with the significantly perturbed residues upon binding to diC4-PL(4,5)P2 are colored and labeled. Residues that showed only significant CSPs are colored red. Residues that showed both significant CSPs and intensity reduction are colored in orange. Residues that showed significant intensity reduction but moderate CSPs are colored green cyan. (E and F) Fittings of chemical shift perturbations of diC4-PL(4,5)P2 (E) and diC4-PL(3,4,5)P3 (F) to the fast-exchange equation. The dissociation constant $K_D$ is shown in subfigure.

Figure 3. **Comparison of the lipid-binding sites in Vav2-SH2 and Lck-SH2.** (A) Sequence alignment of Vav2-SH2 and Lck-SH2. Alignment was performed using Clustal X and illustrated with ESPript 3. Strictly conserved (white letters filled with red color) and conservatively substituted (red letters with blue box) residues are denoted. The secondary structure element for Vav2-SH2 is labeled on the top. The conserved residues responsible for lipid-binding are marked by green dots and the different residue (V723 in Vav2-SH2 and R184 in Lck-SH2) is marked by a blue up-arrow. (B) The cartoon presentation of the structures of Lck-SH2 (left, PDB code: 1LCK) and Vav2-SH2 (right, PDB code: 2LNX). The lipid-binding site residues are depicted as sticks and labeled.

Figure 4. **Binding of Vav2-SH2 to Y594-phosphorylated JM of EphA2.** (A and B) The cartoon (A) and surface (B) representations of Vav2-SH2 structure with the significantly perturbed residues upon binding to the peptide EphA2-JM-pY594 are colored and labeled. (C) Overlay of the $^1$H-$^{15}$N HSQC spectra of 45 μM Vav2-SH2 in the absence (black) and presence, as indicated, of the peptide EphA2-JM-pY594 in buffer (green) or the peptide tethered on nanodiscs (red) at a protein/peptide molar ratio of 3:1. The spectral resolutions in $^1$H and $^{15}$N dimensions are 4.110 Hz/point and 8.077 Hz/point, respectively. Selected peaks that exhibit large chemical shift changes are labeled.

Figure 5. **The effect of membrane environment on the binding of Vav2-SH2 to the EphA2-JM-pY594 peptide.** (A-E) ITC measurements of the binding affinities of Vav2-SH2 to the EphA2-JM-pY594 peptide under different conditions, as indicated. The peptide was in buffer without nanodiscs (A), tethered on the Ni-NTA-nanodiscs (B), in the presence of Ni-NTA-free nanodiscs (C), tethered on PL(4,5)P2-containing Ni-NTA-nanodisc (D) and tethered on PL(3,4,5)P3-containing Ni-NTA-nanodiscs (E). (F) A schematic presentation of Vav2-SH2 binding to pY and lipid PL(4,5)P2 or PL(3,4,5)P3 independently.
