Structural Basis of Membrane Targeting by the Phox Homology Domain of Cytokine-independent Survival Kinase (CISK-PX)*

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The cytokine-independent survival kinase (CISK) in the serum and glucocorticoid-regulated kinase family plays an important role in mediating cell growth and survival. N-terminal to its catalytic kinase domain, CISK contains a phox homology (PX) domain, a phosphoinositide-binding motif that directs the membrane localization of CISK and regulates CISK activity. We have determined the crystal structures of the mouse CISK-PX domain to unravel the structural basis of membrane targeting of CISK. In addition to the specific interactions conferred by the phosphoinositide-binding pocket, the structure suggests that a hydrophobic loop region and a hydrophilic β-turn contribute to the interactions with the membrane. Furthermore, biochemical studies reveal that CISK-PX dimersize in the presence of the linker between the PX domain and kinase domain, suggesting a multivalent mechanism in membrane localization of CISK.

Restricting the localization of proteins spatially and temporally is one of the strategies cells have developed to ensure their proper function and activity during various cellular processes. The membrane-localized phosphatidylinositol (PtdIns) phospholipids can be phosphorylated on different positions of their inositol rings to generate phosphoinositides (PIs). PIs have been universally utilized by membranes in restricting the proteins with PI-binding motifs to their desired sites (1–5). Among the important PI-binding modules, PH, FYVE, FERM, and ENTH domains play important roles in membrane localization of a variety of signaling proteins (6–8). The phox homology domain (PX domain), recently identified as a PI-binding motif, has been found in more than 100 eukaryotic proteins with diverse functions (9–11). For example, p40phox and p47phox are involved in neutrophil defense; Vam7p and sorting nexins (SNXs) participate in protein trafficking; and PI3K C2γ, CISK, PLD1, and RGS-PX1 are important signal transducing molecules (12). Membrane targeting through the interaction between the PX domain and specific PIs appears to be a critical step in regulating PX domain-containing proteins. Consistent with the low sequence similarity among the PX domains, different PX domains exhibit different PI binding specificity (13). For example, p40phox, Vam7p, SNX3, SNX7, and SNX16 bind PtdIns(3)P specifically with moderate to high affinity, whereas p47phox interacts with PtdIns(3,4)P2 and PI3K C2γ interacts with PtdIns(4,5)P2 (13–18).

The cytokine-independent survival kinase (CISK), the only member of the serum and glucocorticoid-regulated kinase family to contain an intact PX domain, functions downstream of phosphatidylinositol (PI) 3-kinase and plays an important role in cell growth and survival (19–21). CISK is composed of an N-terminal PX domain, a conserved linker region, a Ser/Thr kinase domain, and a C-terminal regulatory domain. CISK is structurally homologous to the protein kinase B (PKB/Akt) kinase family that plays essential roles in cell survival and insulin response with the major difference being the N-terminal PH domain in PKB/Akt instead of the PX domain in CISK (Fig. 1). Because of their similar domain organizations and regulatory phosphorylation sites, CISK and PKB/Akt are believed to respond to the PI 3-kinase signal in a similar manner (20, 22, 23). PIs with a 3-phosphate group produced upon PI 3-kinase activation interact with the PH domain of partially phosphorylated PKB/Akt, which may induce a conformational change in PKB/Akt that facilitates further phosphorylation and complete activation of PKB/Akt (24–26). Whether the interaction between the CISK-PX domain and PIs has a similar effect on CISK activity in response to PI 3-kinase signaling is still unclear. Most interesting, mutations in the PX domain that disrupt the localization of CISK also impair CISK activity in vivo (27).

PX domains from p40phox/p47phox, Vam7p, and sorting nexins can bind PtdIns(3)P (14–18). The CISK-PX domain, on the other hand, binds D3-phosphoinositides (28) but exhibits high affinity for PIs containing a 5-phosphate group (27). Here we report the crystal structure of the mouse CISK-PX domain. The structure displays special features of the PI-binding pocket of CISK-PX and reveals additional structural elements involved in the membrane attachment by CISK. Our biochemical studies suggest that the linker region located C-terminal to the CISK-PX domain is necessary for PX domain dimerization, which supports a multivalent mechanism in membrane localization of CISK.

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† The abbreviations used are: PtdIns, phosphatidylinositol; CISK, cytokine-independent survival kinase; PX, phox homology; PI, phosphatidylinositides; PKB, protein kinase B; SNX, sorting nexin; SeMet, selenomethionine; MES, 4-morpholineethanesulfonic acid; r.m.s.d., root mean square deviation; DLS, dynamic light scattering; SH3, Src homology domain 3; PPH, polyproline type II; PH, pleckstrin homology; EGS, ethylene glycol bis(succinimidylsuccinate).

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Crystal Structure of CISK-PX Domain

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Crystallization—Two CISK-PX fragments were used for the crystallographic studies. One contained the PX domain (short-PX, residues 7–126) and another contained both the PX domain and the linker between the PX domain and kinase domain (long-PX, residues 7–160) (Fig. 1). Proteins were expressed and purified as described previously (29). Briefly, glutathione-S-transferase-tagged native mouse CISK-PX fragments (long-PX and short-PX) were expressed in Escherichia coli BL21(DE3) cells in LB medium under isopropyl-1-thio-β-galactopyranoside induction. Selenomethionine-derivated long-PX domain (SeMet-long-PX) was expressed in the auxotrophic E. coli cell line B834 in minimum LB medium based on a protocol for leaky protein expression (30). Both native PX (long-PX and short-PX) and SeMet-long-PX were purified with the glutathione affinity column, the Q-Sepharose HP column, and the SP-Sepharose HP column (29). Purified native PX and SeMet-long-PX were finally concentrated to ~10–15 mg/ml, respectively, into stock solution (20 mM Tris, pH 7.5, 50 mM NaCl, 2 mM dithiothreitol).

Purified native long-PX and SeMet-long-PX were crystallized by using the hanging drop vapor diffusion method. The reservoir solution contains 0.1M sodium citrate, pH 5.5, 5.8, and 2.0M sodium malonate buffered to pH 5.5 (29). The short-PX fragment was crystallized as thin plates (0.01 × 0.01 × 0.2 mm) in hanging drops using 0.1 M MES, pH 6.5, 0.15 M (NH4)2SO4, and 25% PEG 5000 as the reservoir solution.

For each CISK-PX fragment, co-crystallization with up to 4 mM Di-C4PtdIns(3,4,5)P3 or Di-C4PtdIns(3,5)P2 (Echelon, Utah) were extensively screened to obtain PI-bound CISK-PX crystals. All the crystals were further soaked in various cryoprotection solutions containing up to 4 mM PIs. To stabilize the interaction between CISK-PX and PIs in the crystal, we also extensively screened crystal stabilization conditions with lower salt concentrations. However, removal of sulfate ions from short-PX crystals or decrease of sodium malonate concentration in long-PX crystals during the soaking process resulted in the loss of diffraction ability of those crystals.

Data Collection and Structure Determination—These two CISK-PX fragments were crystallized in different crystal forms. A 1.6-Å native data set and a 2.0-Å selenomethionine SAD data set were collected for long-PX fragment crystals in space group I4. A 2.2-Å native data set was collected for short-PX fragment crystals in space group P21212. Data sets were processed with the HKL program suite (31). SAD phases were calculated using SOLVE with the data truncated to 3.0 Å. Initial phases were improved with density modification and phase extension to 2.0 Å using RESOLVE. Xtalview and CNS were used for model building and refinement. The crystal structure with space group P21212 was solved by molecular replacement using the molecular model from space group I4. Refinement was conducted against 1.6- (space group I4) and 2.2-Å (space group P21212) native data sets, respectively (Table I). The stereochemical quality of structural models was monitored using Procheck (32).

Structure superposition and structure-based sequence alignment were performed with SwissPDB viewer (33). The following programs were used to generate the figures: GRASP (34), Molscript (35), and RASTER3D (36).

Dynamic Light Scattering—DLS was carried out using a DynaPro-99 molecular-sizing instrument equipped with a microsensor (Protein Solutions, Inc.). Two CISK-PX fragments (long-PX and short-PX) were used in the experiment. Protein samples (5 mg/ml stored in 20 mM Tris, pH 7.5, 50 mM NaCl, and 2 mM dithiothreitol) were loaded into a 16-μl chamber quartz cuvette after centrifugation filtration in the presence or absence of 2 mM Di-C4PtdIns(3,4,5)P3 or Di-C4PtdIns(3,5)P2 (Echelon Corp.). Data were analyzed by using Dynamics 5.0 software. Interpretation of the statistical parameters was summarized in Table II.

Cross-linking Reaction—Sufo-EGS was used as the cross-linking reagent (Pierce), which is water-soluble and reacts primarily with the ε-amino of lysine residues. Both long-PX and short-PX fragments were dialyzed into the reaction solution (20 mM Hepes, pH 7.5, 20 mM NaCl, 160 mM NaCl, 0.15 M (NH4)2SO4, and 25% PEG 5000) as the reservoir solution. These crystals were flash-frozen with liquid nitrogen from the crystallization drops for data collection.

| Table I |
| --- |
| Structure determination and refinement of CISK-PX |

| Data collection | Long-PX | Native 1* | Native 2* |
| --- | --- | --- | --- |
| Space group | P21212 | I4 | I4 | P21212 |
| Wavelength (Å) | 0.97993 | 0.97993 | 1.00000 | 1.60000 |
| Resolution (Å) | 2.0 | 2.0 | 2.2 | 2.2 |
| Unique reflections | 21,722 | 18,760 | 16,082 | 16,082 |
| Completeness (%) (last shell) | 98.7 (92.1) | 98.6 (97.7) | 99.4 (94.5) | 99.4 (94.5) |
| Rmerge (%) (last shell) | 21.7 (3.7) | 34.0 (6.5) | 27.5 (3.2) | 27.5 (3.2) |
| completeness (%) (expected) | 10.0 (23.3) | 4.0 (19.9) | 9.0 (52.5) | 9.0 (52.5) |
| Figure of merit | 6.7 | 5.4 | 11.1 | 11.1 |

**Data sets were collected at the following photon sources. SAD, APS 19ID beam line; Native 1, APS 19BM beam line; Native 2, ALS 5.0.1 beam line.**

* R-factor = \( \frac{1}{\sum|F_{o}-|F_{c}|^2} \)

| Refinement statistics | Long-PX | Native 1* | Native 2* |
| --- | --- | --- | --- |
| Rwork/Rfree (%) | 18.7/22.0 | 20.0/24.0 | 20.0/24.0 |
| Average B factor | 22.6 | 36.8 |
| Ramachandran plot (core, disallowed, %) | 93.2, 0.00 | 90.5, 0.00 |
| r.m.d. from ideality | 0.020 | 0.010 |
| Bond angle (°) | 1.9 | 1.5 |
| Dihedral angle (°) | 24.1 | 23.6 |
| Protein atoms | 962 | 1862 |
| H2O molecules | 149 | 137 |
| Heterogenous atoms | 0 | 20 |

* As defined in PROCHECK (32).

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and 2 mM dithiothreitol). Reactions using protein concentrations of 1 mg/ml were performed under the conditions suggested by the vendor for 1 h. 0.7 μl of sulfo-EGS (76 mM) was added to 20 μl of PX domain fragments (1 mg/ml). The reaction mixtures with a final sulfo-EGS concentration of 2.6 mM were incubated at 20 °C. Samples were taken after 30 and 60 min, respectively, and analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Overall Structure of CISK-PX Domain—Crystal structure of the long-PX fragment in space group I4 has one molecule per asymmetric unit. Final model of the long-PX contains residues 10–125 (Fig. 2A). The structure of the short-PX fragment in space group P2₁2₁2 has two molecules per asymmetric unit with residues 11–121 in molecule A and 13–125 in molecule B (Fig. 2B). The overall fold of the CISK-PX domain is composed of a β-sheet with three antiparallel β-strands (β₁, β₂, and β₃) and a helical subdomain consisting of four α-helices (α₁, α₂, α₃, and α₃') together with a type II polyproline (PPII) region and a 3₁₀ helix. A β-bulge at residues 19 and 20 in the middle of strand β₁ twists the β-sheet (Fig. 2A).

Di-C₄PtdIns(3,4,5)P₃ or Di-C₄PtdIns(3,5)P₂ was not visible in either crystal form, although multiple data sets were collected for crystals of each CISK-PX fragment from co-crystallization and soaking. Instead, CISK-PX in the P2₁2₁2 crystal form is bound with two sulfates, which are introduced by the crystallization solution (Fig. 2B). Sulfates A from molecule A or sulfate B from molecule B in the P2₁2₁2 crystal form is found sitting in the putative PI-binding pocket of CISK-PX domain at the same position as the 3-phosphate of PtdIns(3)P bound to p40⁰ᵖₓ (37) (Fig. 2B and C). Another sulfate (sulfate A from molecule A or sulfate B from molecule B) is on a solvent-accessible surface (Fig. 2B). The presence of sulfate ions (0.15 M (NH₄)₂SO₄) in short-PX crystals or high ionic strength (2.0 M sodium malonate) in the long-PX crystal growth condition may account for the absence of phosphoinositides in the positively charged PI-binding pocket in both crystal forms of CISK-PX.

Structure superposition of the CISK-PX domain from two
different space groups results in an overall r.m.s.d. ~0.78 Å based on 106 Ca positions of molecule A and r.m.s.d. ~0.82 Å based on 107 Ca positions of molecule B in P2\textsubscript{1}2\textsubscript{1}2 crystal form. However, there is a significant backbone shift around the β-turn connecting the first and second β-strand involving 11 residues with the largest Ca displacement around 5 Å (Fig. 2B).

Comparison of CISK-PX domain structure with the crystal structures of p40\textsuperscript{phox}-PX and p47\textsuperscript{phox}-PX reveals that the core secondary elements of PX domains are closely superimposable (37, 38). r.m.s.d. of CISK-PX (molecule B from space group P2\textsubscript{1}2\textsubscript{1}2) and p40\textsuperscript{phox}-PX is ~1.37 Å based on 97 Ca atoms. CISK-PX (molecule B from space group P2\textsubscript{1}2\textsubscript{1}2) and p47\textsuperscript{phox}-PX has an r.m.s.d. ~1.46 Å based on 86 Ca atoms (Fig. 2C). The major structural difference of those PX domains is in the PPII region. The α1/PPII loop in the CISK-PX domain forms a compact turn, whereas the same region in p40\textsuperscript{phox}-PX and p47\textsuperscript{phox}-PX is a wide turn with a 10-residue insertion (Fig. 2, C and D). The PPII/α2 loop at the C-terminal end of the PPII, which forms one wall of the PI-binding pocket, assumes several distinct backbone conformations in different PX domain structures (Fig. 2C), which provides important clues to the selectivity of the PI-binding pocket (see below).

Many PX domain-containing proteins have one or more SH3 domains, which supports intermolecular or intramolecular interactions with proline PXXP motifs in some cases (10).
the N-terminal end of constitute the walls of the pocket. Overall, the PI-binding Pocket, which forms only one left-handed PPII helical turn (Fig. XX). In the analogous region with the sequence LXX in the turret loop (residue 75–82) and the N terminus of β2 in the β1/β2 loop (Fig. 3, A and B). This more spacious opening, caused partially by an average 4–5 Å movement of backbone atoms in the PPII/α2 loop, could favor the binding of a phosphoinositide with a highly phosphorylated head group (Fig. 3C), such as PtdIns(3,4,5)P3 and PtdIns(3,5)P2 targeted by CISK-PX domain (27).

Structural superposition suggests that the sulfate in the PI-binding pocket of CISK-PX mimics the binding of the 3-phosphate group of PtdIns(3)P in p40phox-PX (Figs. 2C and 3C). It is tightly coordinated with NE and NH2 of Arg-50 in α1, which is equivalent to Arg-55 in p40phox-PX. This arginine, conserved in most of the PX domains, was proposed as the binding determinant of the 3-phosphate according to the current crystal structures (37, 40). It is not conserved in the PX domain of P3K C2-γ, which binds preferentially to PtdIns(4,5)P2. Several other interactions that stabilize the binding of the 3-phosphate are also conserved in the binding of this sulfate to CISK-PX. For example, the backbone nitrogens of Tyr-51 and Ala-52, corresponding to the Tyr-59 and Arg-60 in the p40phox-PX, form hydrogen bonds with the sulfate. In addition, side chains of Tyr-51 in CISK-PX and Tyr-59 in the p40phox-PX domain are close to each other and assume the same conformation, which could provide the carbohydrate/aromatic stacking interaction with one side of the inositol ring as shown in the p40phox-PX/PtdIns(3)P structure (37). Residues with aromatic side chains are preserved at this spot (Fig. 2D), which indicates a common binding site for the inositol ring in the PI-binding pocket. Moreover, an important basic residue, Lys-75 in the PPII/α2 region of CISK-PX is positioned similarly as Lys-92 of p40phox-PX, which plays a critical role in stabilizing the nonbridging oxygens of the 1-phosphoryl group. These structural similarities reinforce the idea that phosphoinositides most likely bind in the PI-binding pocket of CISK-PX and p40phox-PX domains at the same location with a similar orientation.

A PtdIns(3,4,5)P3 head group was manually modeled into the PI-binding pocket of CISK-PX based on the sulfate-bound CISK-PX crystal structure and its superposition to the PtdIns(3)P-bound p40phox-PX (Fig. 3D). Only minor adjustment to the orientation of the inositol ring was made to avoid the steric clash with Arg-90 under the circumstances that all other interactions stabilizing the 3-phosphate and 1-phosphate binding were preserved.

These restrictions indicate that 4-phosphate of the model may interact tightly with Arg-90 of CISK-PX, a residue that is also conserved in the PX domain family (Fig. 2D) and has a strong effect on the interaction of CISK-PX with PtdIns (3–5) based on mutational analyses (27). In this model, ND2 of the Asn-81 side chain in the PPII/α2 loop may also form hydrogen bonds with 4-phosphate and 5-phosphate. Furthermore, 5-phosphate is positioned in the pocket near the PPII/α2 loop. The more spacious pocket resulting from the movement of the PPII/α2 loop in CISK-PX compared with p40phox-PX provides the necessary space to accommodate the binding of the 5-phosphate group. In the same region of p40phox-PX, positions of backbone atoms and carbonyl oxygens will potentially collide with the 5-phosphate (Fig. 3D), as also shown by a previous modeling in the crystal structure of p40phox-PX/PtdIns(3)P (37).

The PPII/α2 loop region varies significantly in both sequence and structure across the PX domain family (37, 38, 40–42). Our analysis indicates that the structural divergence of this region is responsible for the selective recognition of PIs by different PX domains.
molecules A and B from the P2 1212 crystal form of CISK-PX. The dimeric form of the CISK-PX fragment is labeled with an arrowhead.

Structural Elements Involved in the Membrane Targeting of CISK-PX Domain—Crystal structures of CISK-PX suggest that multiple structural elements are likely involved in CISK membrane localization. Whereas the PI-binding pocket of CISK-PX domain delivers the specific targeting, the hydrophobic membrane insertion loop and tandem basic residue region of CISK-PX may facilitate the overall membrane localization via nonspecific interactions with different membrane components.

In addition to binding to PIs, CISK-PX may insert a hydrophobic part of the PPII/α2 loop (Ile-77 and Phe-78) into the membrane to assist with the orientation during membrane targeting and to provide additional interactions with the membrane lipid bilayer. Large chemical shifts have been observed in the analogous loop region when Vam7p-PX domain binds to the micelle (17). Most interesting, the backbone conformation of residues 76–79 in the PPII/α2 loop is very different between molecules A and B from the P2_2_2 crystal form of CISK-PX (Fig. 4A). In the structure of molecule B, the conformational plasticity of the PPII/α2 loop allows side chains of hydrophobic residues Ile-77 and Phe-78 to flip outwards from the PI-binding pocket and become solvent-exposed, which may mimic the membrane insertion state of CISK-PX (Fig. 4A). Similarly, hydrophobic residues are present in the corresponding locations of p40phox, p47phox, Vam7p, and Grd19p PX domain structures (37, 38, 40–42). It is intriguing that the binding of PtdIns(3)P to p40phox and Grd19p PX domains is accompanied by a flipping-out conformation of those hydrophobic residues (37, 40). Mutations of those hydrophobic residues to alamines led to lower degree of penetration in the phospholipid monolayer by p40phax and p47phax PX domains (43). Therefore side-chain flipping of the hydrophobic residues in the PPII/α2 loop seems favored by the binding of PIs and could be a common structural feature coupled to the membrane targeting of PX domains.

The orientation of the phosphoinositide in the binding pocket of CISK-PX domain modeled in Fig. 3D also positions tandem lysine residues in the β1/β2 loop (27KKK29) in contact with the membrane surface (Fig. 4A). The flexibility of the β1/β2 loop in CISK-PX domain could assist with adjusting the interaction distance between the lysine residues and phospholipid head groups on the membrane. Although analogous regions in other PX domains are not abundant in charged residues, similar structural features have been observed with other membrane targeting motifs. In the crystal structure of inositol 1,3-bisphosphate-bound EEA1-FYVE domain, tandem lysine residues in the β3/β4 loop of EEA1-FYVE domain are suggested to interact with the head group region of the lipid bilayer upon membrane targeting (44). In addition, two uncharged residues at the tip of a loop (“turret loop”) preceding the β1 of EEA1-FYVE were predicted to penetrate into the membrane lipid bilayer in agreement with the shifted and broadened resonances for these residues in the presence of micelles (Fig. 4B) (44–46). Likewise, hydrophobic residues located in the PPII/α2 loop of PX domain family may play a similar role.

Membrane targeting via both hydrophobic and electrostatic interactions is a strategy utilized by the human factor VIII C2

### Table II

Dynamic light scattering of CISK-PX fragments

| CISK fragment | Concentration | \( R_H \) | \( C_p \) | Polydispersity (\( C_p/R_H \)) | Mass | Base line | SOS error |
|-------------|---------------|---|---|----------------|-----|---------|---------|
| Long-PX, residues 7–160 | 5 | 2.718 | 0.3059 | 11.25 | 35.14 | 1.000 | 2.46 |
| Short-PX, residues 7–126 | 5 | 2.120 | 0.2653 | 12.50 | 16.68 | 1.000 | 2.55 |

**Fig. 5.** SDS-electrophoresis analysis of the cross-linking products of CISK-PX fragments. The dimeric form of the CISK-PX fragment is labeled with an arrowhead.

**Fig. 6.** Models for the membrane targeting by dimeric CISK-PX. IP3 stands for the inositol head group of PtdIns(3,4,5)P3. The membrane insertion loop refers to the hydrophobic residues in the PPII/α2 loop. The β1/β2 loop contains tandem lysine residues that could interact with the membrane surface. The linker region (orange), C-terminal to the CISK-PX domain (blue), might facilitate the divalent membrane targeting of CISK-PX via either linker-linker interaction (A) or cross-talking with another CISK-PX domain (B).
domain as well. A group of hydrophobic residues presented by a loop and $\beta$-turns and a belt of positively charged residues lying behind the hydrophobic surface suggested an interaction of the C2 domain with both polar and nonpolar components of the membrane bilayer (47).

Oligomeric State of CISK-PX Domain.—The conserved linker (residues 128–160) between the PX domain and the kinase domain of CISK exists in the I4 crystal form of CISK-PX as confirmed by SDS-gel electrophoresis of the dissolved crystals after an extensive wash (data not shown). The density for this linker region, however, cannot be observed in the electron density map, which indicates that the linker region is much more dynamic compared with the PX domain in the crystallized CISK fragment. Our DLS data on different fragments of CISK-PX have shown that the fragment containing only the PX domain (residues 7–126, 14.34 kDa) is monomeric (DLS molecular mass, 16.68 kDa), whereas the fragment containing the PX domain and the linker region (residues 7–160, 18.00 kDa) is in an oligomeric form with DLS molecular weight around a dimer (35.14 kDa). Both samples have been characterized as monodisperse as indicated by the polydispersity (%) (Table II). Addition of 2 mM Dl-C$_{4}$PtdIns(3,4,5)P$_{3}$ or Dl-C$_{4}$PtdIns(3,5)P$_{2}$ has no apparent effect on the oligomeric state of the CISK-PX fragment.

We proceeded to study further the oligomerization tendencies of two different CISK-PX fragments through cross-linking reactions. Under the same reaction conditions, the CISK-PX fragment containing the linker region, but the fragment without the linker region, could be cross-linked into a dimeric form (Fig. 5). The tendency for the longer fragment of CISK-PX to dimerize suggests that the linker region is essential for the formation and stabilization of a dimer.

In addition to the linker region, the PX domain itself might also contribute to part of the dimer interface. At least two models exist for the dimeric state of CISK-PX. In the first model, the linker facilitates the dimerization via linker-linker interaction; in the other model, the linker from one CISK molecule interacts with another CISK-PX domain (Fig. 6).

Membrane Targeting by Dimeric CISK—Crystal structures of CISK-PX fragments have defined an identical PX domain interface (1158 Å$^2$, ~16% of the overall CISK-PX domain surface) in both P$_2$$_2$$_1$$\alpha$ and I4 crystal form. Two CISK-PX domains constituting such an interface present their PI-binding pockets, membrane insertion loops, and tandem lysine residues roughly on the same surface as indicated in Fig. 6. However, due to the absence of the linker region in the electron density map, whether it is a physiologically relevant dimer interface needs to be further investigated.

Nevertheless, the high tendency for CISK-PX to dimerize in the presence of the linker region implies that dimerization may play an important role in CISK membrane localization and regulation. As shown in the model of Fig. 6, CISK-PX domain dimer could strengthen the interaction between CISK-PX and the membrane via a multivalent additive effect. Such an effect on the membrane-binding affinity was also observed with the EE1/FFY domain. The crystal structure of inositol 1,3,4,5-biphosphate bound EE1/FFY dimer, which involves a parallel coiled-coil linker region and a homodimer of FFY domain itself, establishing a multivalent additive mechanism in the endosome localization of EE1 (44, 48).

Our structure and biochemical results suggest that several structural features of the CISK-PX domain cooperatively contribute to the membrane targeting by CISK. The PI-binding pocket of CISK-PX recognizes a specific phosphoinositide head group through both conserved residues and variable architecture of the pocket. The hydrophobic residues in the PPII/a2 loop and tandem lysine residues in the $\beta$/b$\beta$ loop of CISK-PX domain provide nonspecific interactions with different membrane elements. Moreover, dimerization of the CISK-PX domain driven by the linker region may increase the membrane localization efficiency via an additive manner, a feature that has not been reported with other PX domains. PKB/Akt shares sequence and structure homology with CISK in its kinase domain and C-terminal domain (Fig. 1). It is interesting to note that the linker between its pH and kinase domains is required in the homo-oligomization of PKB/Akt mediated through the pH domain (49). The interaction is sufficient to induce PKB/Akt activation in the in vitro kinase assay (49). Therefore, our work suggests that CISK and PKB/Akt may share a very similar regulatory mechanism.
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