Phosphorylation-mediated 14-3-3 Protein Binding Regulates the Function of the Rho-specific Guanine Nucleotide Exchange Factor (RhoGEF) Syx*5

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Background: The junctional localization and RhoGEF activity of Syx mediate cell junction integrity.

Results: PKD-mediated phosphorylation and 14-3-3 binding prevent the junctional localization and suppress the GEF activity of Syx.

Conclusion: 14-3-3 proteins modulate the function of Syx in the context of cell-cell adhesion.

Significance: Understanding mechanisms that regulate junction integrity is crucial in vascular and tumor biology.

Syx is a Rho-specific guanine nucleotide exchange factor (GEF) that localizes at cell-cell junctions and promotes junction stability by activating RhoA and the downstream effector Diaphanous homolog 1 (Dia1). Previously, we identified several molecules, including 14-3-3 proteins, as Syx-interacting partners. In the present study, we show that 14-3-3 isoforms interact with Syx at both its N- and C-terminal regions in a phosphorylation-dependent manner. We identify the protein kinase D-mediated phosphorylation of serine 92 on Syx, and additional phosphorylation at serine 938, as critical sites for 14-3-3 association. Our data indicate that the binding of 14-3-3 proteins inhibits the GEF activity of Syx. Furthermore, we show that phosphorylation-deficient, 14-3-3-uncoupled Syx exhibits increased junctional targeting and increased GEF activity, resulting in the strengthening of the circumferential junctional actin ring in Madin-Darby canine kidney cells. These findings reveal a novel means of regulating junctional Syx localization and function by phosphorylation-induced 14-3-3 binding and further support the importance of Syx function in maintaining stable cell-cell contacts.

The Rho GTPases function as molecular switches that cycle between a GTP-bound active state and a GDP-bound inactive state and are involved in a range of signaling pathways that control the actin cytoskeleton and fundamental cellular processes, like cell adhesion and cell migration (1). Guanine nucleotide exchange factors (GEFs)2 catalyze the exchange of GDP for GTP. Coordinated signaling by RhoA, Rac1, and Cdc42 GTPases regulates cell junction formation and cell polarization by modulating junctional components and the actin cytoskeleton (2). Precise spatiotemporal regulation of Rho GTPases by GEFs is thought to regulate the cross-talk between adhesion and polarity complexes (3, 4). Despite extensive characterizations of the Rho GTPases, their activation by junction-associated GEFs, and especially the mechanisms by which these GEFs are regulated, remain poorly understood.

We recently identified Syx, as a junctional RhoGEF that associates with multiple members of the Crumbs polarity complex, promotes junction stability by signaling through RhoA and Diaphanous homolog 1 (Dia1), and mediates the opposing effects of VEGF and angiotatin 1 on endothelial junction integrity (5). Depletion of Syx results in endothelial cell junction defects in vitro and in vivo. Syx belongs to the Dbl family of GEFs (6). Besides its Dbl/pleckstrin homology domains, Syx contains a PDZ binding motif (PBM) that is required for its recruitment to the junctions (5). Syx is proposed to play a role in endothelial cell migration and is important for angiogenesis and vascular barrier function in vivo (5, 7, 8). Both Syx activity and localization to junctions are critical for these effects, suggesting that misregulation of Syx function results in vascular defects. The mechanisms that regulate Syx localization and function are largely unclear.

In addition to its interaction with the myosin VI adaptor protein synectin (9), the scaffold protein multiple PDZ domain protein 1 (Mupp1) (7, 10), the protein associated with Lin7 (PALS1), and Lin7, we identified several 14-3-3 isoforms as novel Syx-binding partners (5). 14-3-3 family members associate with a diverse number of proteins, including many with oncogenic or tumor suppressor properties (11, 12). Homo- or heterodimers of 14-3-3 proteins bind to select phosphoserine/threonine residues, induce conformational change, and alter the localization, stability, and/or function of the bound protein (13). The localization and dimerization of 14-3-3 proteins are in turn regulated by post-translational modifications such as phosphorylation and acetylation (13). 14-3-3ζ and 14-3-3σ have been suggested to play a role in cell polarization by associating with Par3 (14, 15). However, the role of 14-3-3 proteins on junction stability remains unknown.

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2 The abbreviations used are: GEF, guanine nucleotide exchange factor; Dia1, Diaphanous homolog 1; DMSO, dimethyl sulfoxide; MDCK, Madin-Darby canine kidney; PBM, PDZ binding motif; PMA, phorbol 12-myristate 13-acetate; RBD, Rho binding domain.

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In this study, we explored the functional significance of the interaction between Syx and 14-3-3 proteins. Our data suggest that PKD phosphorylation regulates 14-3-3 binding to Syx. More importantly, a phospho-deficient, 14-3-3-uncoupled Syx mutant S92A/S938A displays elevated GEF activity and enhanced localization to areas of cell-cell contact. Altogether, these findings provide a mechanistic insight into how 14-3-3 proteins can modulate junction stability by altering the localization and GEF activity of Syx.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HeLa and MDCK cells were cultured in DMEM (Cellgro) with 10% fetal bovine serum (Invitrogen). HeLa and MDCK cells were transfected with TransIT-HeLaMonster (Mirus) and Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions, respectively.

**Antibodies and Reagents**—The following antibodies were used: mouse anti-Syx (KIAA0770, 5A9; Abnova); mouse and rabbit anti-HA (Cell Signaling); mouse anti-GFP/YFP 3E6, mouse anti-ZO1, monoclonal rabbit anti-GFP/YFP (Invitrogen); rabbit pan anti-14-3-3 (K-19), mouse anti-RhoA (26C4) (Santa Cruz Biotechnology); rabbit anti-GST, rabbit anti-actin (Sigma). Phalloidin 594 (Molecular Probes) was used to stain for actin filaments in immunofluorescence experiments.

Phorbol 12-myristate 13-acetate (PMA; Sigma) was dissolved in DMSO to a stock concentration of 100 μM. Protease and phosphatase inhibitor mixtures (Pierce) were used in all buffers (refer to immunoprecipitation section) for the generation of cell lysates.

**DNA Constructs and Recombinant Protein**—Full-length YFP-tagged murine Syx and HA-tagged PKD WT, kinase-active, and kinase-dead have been described previously (9, 16). Murine Syx truncation mutants were PCR-amplified from pEYFP-mSyx and then subcloned into pEYFP-C1 using HindIII and BamHI restriction sites. Point mutations were introduced in the respective Syx constructs (YFP-Syx, YFP-Syx(1–630), and YFP-Syx(791–1073)) to encode alanine substitutions at Ser92, Ser167, Ser294, Ser806, Ser936, Ser938, and Ser964 using the QuikChange Multisite-directed Mutagenesis kit (Stratagene). GST-tagged 14-3-3 epsilon (ε), HA-tagged 14-3-3 beta (β), epsilon (ε), gamma (γ), sigma (σ), and zeta (ζ) were purchased from Addgene. All DNA constructs generated were verified by DNA sequencing. pSuper-PKD1-RNAi and pSuper-PKD2-RNAi vectors were used as described previously to knock down PKD1 and PKD2 (17).

Recombinant GST-14-3-3ε was produced in Escherichia coli BL21 DE3 (Invitrogen). Briefly, overnight culture of BL21 cells transformed with pGEX-4T1-14-3-3ε was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (Sigma) at room temperature for 3 h and harvested by centrifugation; harvested bacterial pellet was lysed with extraction buffer (0.5% Nonidet P-40 in 1× PBS, pH 7.4, plus protease inhibitor mixture), sonicated, and clarified by centrifugation. The supernatant was incubated with glutathione-agarose beads (Sigma) at 4 °C for 1 h. The beads were then washed five times with extraction buffer, and bound proteins were eluted with elution buffer (50 mM Tris, 100 mM NaCl, 1 mM DTT, 20 mM glutathione, pH 8.4). The concentration and purity of the eluted protein were evaluated by SDS-PAGE and Coomassie Blue staining (Pierce).

**Immunofluorescence, Immunoprecipitation, and Immunoblotting**—MDCK cells were seeded on coverslips in 35-mm 6-well tissue culture dishes and transfected with Lipofectamine 2000; cells were fixed with methanol (10 min, ~20 °C) or 3% paraformaldehyde (30 min, followed by 5-min permeabilization with 0.2% Triton X-100 containing 1× PBS) the following day as reported previously (18) and probed with primary antibodies followed by incubation with Alexa Fluor secondary antibodies (Invitrogen). Images were acquired with a Zeiss LSM 510 META confocal laser-scanning microscope.

For immunoprecipitation, protein G beads (Invitrogen) were conjugated with either mouse monoclonal anti-Syx (KIAA0770; Abnova) or mouse anti-GFP 3E6 (Invitrogen) overnight at 4 °C in 1× PBS. HeLa cells transfected with the respective constructs were lysed with Triton X-100 lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.4). Cell lysates were cleared by centrifugation, and the supernatant was incubated with the antibody-coated protein G beads at 4 °C for 1 h; beads were subsequently washed four times with Triton X-100 lysis buffer, and bound proteins were eluted by boiling in loading buffer.

Total and immunoprecipitated protein samples were analyzed using SDS-PAGE, transferred to nitrocellulose, and probed with the respective primary antibodies. Peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) were detected using ECL (GE Healthcare).

**Active Rho and RhoGEF Pulldown Assay**—The level of activated Rho GTP was determined in HeLa cells using Rhotekin pulldown assays as described previously (19). Briefly, HeLa cells were transfected with the corresponding constructs and lysed with Rho activity lysis buffer (20 mM HEPES, 100 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 0.2% deoxycholic acid, 100 mM MgCl2, pH 7.5); cell lysates were collected and centrifuged, and the cleared supernatants were incubated with reconstituted GST-fused Rhotekin-Rho binding domain (RBD) protein beads (Cytoskeleton) at 4 °C for 1 h. Beads were washed four times with the lysis buffer, and bound proteins were eluted by boiling in loading buffer. Active RhoGEF pulldown assay was performed using Triton X-100 lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.4) and RhoA G17A-conjugated glutathione beads (Cell Biolabs); the procedure was identical as described above.

**RESULTS**

**Association of 14-3-3 with Syx Requires Ser92 and Ser938**—Previously, we identified 14-3-3 proteins as novel Syx-binding partners in our proteomics analysis (5). To verify the interaction, we initially used a pan 14-3-3 antibody to show the presence of endogenous 14-3-3 proteins in Syx immunoprecipitates from HeLa cells (Fig. 1A). We further demonstrated that ectopically expressed Syx associates with multiple 14-3-3 isoforms by co-immunoprecipitating HA-tagged 14-3-3β, ε, γ, σ, or ζ with YFP-Syx in HeLa cells (Fig. 1B). To provide biochemical evidence, we performed pulldown experiments where extracts of HeLa cells transiently expressing YFP or YFP-Syx were incubated with GST or purified recombinant GST-tagged 14-3-3ε
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A) Syx
    - Control
    - e-Syx
    - Total + yate

B) YFP
    - YFP.GST
    - YFP/IP
    - HA
    - HA + 14-3-3
    - Total

C) YFP
    - +
    - YFP.Syx
    - GST.14-3-3c

D) YFP
    - YFP.Syx
    - Syx 1-630
    - Syx 1-300
    - Syx 291-630
    - Syx 291-800
    - Syx 621-800
    - Syx 291-1073
    - Syx 621-1073
    - Syx 791-1073
    - Syx 1-800
    - Syx 291-1071
    - Syx 621-1071
    - Syx 791-1071

E) YFP
    - Pan 14-3-3
    - YFP
    - YFP.IP
    - YFP total

F) YFP
    - Pan 14-3-3
    - YFP
    - YFP IP
    - Total

G) YFP
    - 14-3-3 binding
    - Syx 1-630
    - Syx 1-300
    - Syx 291-630
    - Syx 291-800
    - Syx 621-800
    - Syx 291-1073
    - Syx 621-1073
    - Syx 791-1073
    - Syx 1-800
    - Syx 291-1071
    - Syx 621-1071
    - Syx 791-1071

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Regulation of RhoGEF Syx Function by 14-3-3 Binding

Phospho-peptide mass spectrometry analysis revealed motifs that do not conform to the established modes of interaction of Syx. Interestingly, unconventional motifs close to its C terminus (amino acids 791–1071) (Fig. 1D) strongly suggest that Syx contains two 14-3-3 binding sites: one toward its N terminus (amino acids 1–630) and the other close to its C terminus (amino acids 791–1071) (Fig. 1G).

Two 14-3-3 phospho-binding consensus motifs have been identified so far: RSXpSAP (mode I) and RXY/FpSXp (mode II) (22). However, neither consensus motif is found in the amino acid sequence of Syx. Interestingly, unconventional motifs that do not conform to the established modes of interaction have also been shown to mediate 14-3-3 binding (13). Phospho-peptide mass spectrometry analysis revealed numerous phosphorylated serine residues on Syx. To pinpoint the single residue responsible for 14-3-3 binding at each terminus of murine Syx, we generated single serine to alanine mutants using truncated Syx constructs (Syx(1–630) and Syx(791–1073)), to exclude 14-3-3-binding residue may exist for a given 14-3-3-interacting protein. We therefore hypothesized that 14-3-3 dimers bind to two distinct phosphorylated residues of Syx.

To narrow down the region that 14-3-3 interacts with Syx, we generated and tested a panel of murine Syx truncation mutants (Fig. 1D). 14-3-3 proteins co-immunoprecipitated with several Syx fragments (Fig. 1E). Notably, 14-3-3 proteins associated with both Syx(1–630) and Syx(791–1073), two nonoverlapping Syx fragments, suggesting that there are two distinct 14-3-3 binding sites on Syx. Because the PBM of Syx associates with multiple binding partners, it is possible that these proteins may themselves interact with 14-3-3 proteins. Therefore, we further tested three additional Syx truncation mutants that lack the PBM and found all three associate with 14-3-3 proteins (Fig. 1F). Thus, our co-immunoprecipitation analysis strongly suggests that Syx contains two 14-3-3 binding sites: one toward its N terminus (amino acids 1–630) and the other close to its C terminus (amino acids 791–1071) (Fig. 1G).

PKD-mediated Phosphorylation of Syx at Ser92 Induces 14-3-3 Binding—Phospho-peptide mass spectrometry analysis revealed the phosphorylation of Ser92, which conforms to a putative PKD phosphorylation motif (LXRXXS*) (Fig. 3A). To examine whether Ser92 is phosphorylated by PKD we utilized pMotif, an antibody that recognizes PKD phosphorylated motifs (23). Using pMotif, we showed previously that the major site of VEGF-induced, PKD-mediated phosphorylation on Syx is Ser906 (5). Consistent with this, the phosphorylation of Syx(791–1073) was readily detectable by the pMotif antibody (Fig. 3B, right). As expected, the PKD-mediated phosphorylation of Syx(791–1073) was due to the presence of Ser906 (Fig. 3C, right). Interestingly, basal level phosphorylation was observed on immunoprecipitated Syx(1–630) (when exposure of the immunoblot to the x-ray film was prolonged), and this

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Ser92 and Ser938 mediate 14-3-3 binding to Syx.

A, Ser92 is a putative 14-3-3-binding residue. HeLa cells were co-transfected with plasmids encoding HA-14-3-3β and YFP, YFP-Syx, YFP-Syx(1–630), or one of three YFP-Syx(1–630) mutants in which a single serine was mutated to alanine. Cells were subjected to immunoprecipitation, and protein samples were analyzed by Western blotting. Only the replacement of Ser92 with alanine abolished the interaction between Syx(1–630) and 14-3-3β/H9252.

B, Ser938 is a putative 14-3-3-binding residue. As in A, HeLa cells were co-transfected with plasmids encoding HA-14-3-3β and YFP, YFP-Syx, YFP-Syx(791–1073), or one of four YFP-Syx(791–1073) in which a single serine was mutated to alanine. Only the replacement of Ser938 with alanine abolished the interaction between Syx(791–1073) and 14-3-3β. Note that Ser806 was previously shown as a PKD-mediated phosphorylation target; mutating Ser806 to alanine, however, has no effect on the binding of 14-3-3β/H9252.

C and D, Ser92 and Ser938 are essential residues for 14-3-3 interaction. HeLa cells were co-transfected with the indicated constructs and subjected to immunoprecipitation. Protein samples were analyzed by Western blot analysis. Mutating Ser92 and Ser938 on Syx either abolished or diminished its interaction with 14-3-3 isoforms. Note that mutation of Ser938 alone in C has no clear effect on 14-3-3 binding, but resulted in a decrease or absence of 14-3-3 co-immunoprecipitates when combined with S92A mutation.

E, endogenous 14-3-3 proteins fail to co-immunoprecipitate with Syx S92A/S938A. HeLa cells were transfected with the indicated constructs and subjected to immunoprecipitation. Protein samples were analyzed by Western blotting. Mutating Ser92 and Ser938 on Syx significantly diminished its interaction with endogenous 14-3-3 proteins.
phosphorylation was increased upon PMA stimulation, a condition that induces PKD activation (Fig. 3B, left). Notably, mutating Ser92 to alanine abolished the PMA-induced increase of phosphorylation on Syx(1–630), further suggesting that Ser92 is a PKD phosphorylation target. Furthermore, binding of HA-14-3-3β positively correlated with increased phosphorylation of Syx(1–630) at Ser92 (Fig. 3C, B and C, left). In contrast, no change in HA-14-3-3β binding was detected for Syx(791–1073) or Syx(791–1073) S806A upon PMA treatment (Fig. 3C, right), indicating that PKD-mediated phosphorylation of Ser806 does not affect 14-3-3 binding.

To confirm this observation in full-length Syx, we repeated the same immunoprecipitation experiments using YFP-Syx S806A. As in B, transfected cells were subjected to immunoprecipitation, and protein samples were analyzed by Western blotting. Therefore, the data suggest that PKD is an important mod-
FIGURE 4. PKD1 induces binding of 14-3-3 to Syx. A, interaction between Syx and 14-3-3 increases in the presence of PKD1. HeLa cells were co-transfected with the indicated constructs and subjected to immunoprecipitation. Expression of wild type or kinase-active (KA) PKD1, but not kinase-dead (KD) PKD1, increases the binding of endogenous 14-3-3 proteins to YFP-Syx. B and C, PMA induces 14-3-3 binding to Syx. HeLa cells were transfected with YFP-Syx and subjected to DMSO (control) or PMA treatment (10 versus 100 nM PMA in B, 1, 5, or 10 min of 100 nM PMA in C prior to lysing and immunoprecipitation. Increased 14-3-3 binding to Syx was observed in a concentration- and time-dependent fashion. D, shRNA mediated down-regulation of PKD1 and PKD2. Western blot analysis shows the protein levels of PKD1/2 in nontarget versus PKD1 and PKD2 shRNA-expressing HeLa cells. E, association of YFP-Syx with 14-3-3 isoforms is enhanced by PKD1 activation. PKD1/2-depleted HeLa cells were transfected with YFP-Syx, pcDNA (empty vector (EV)) or PKD1, and HA-14-3-3α or -14-3-3ζ and subjected to DMSO (control) or PMA (100 nM) treatment prior to immunoprecipitation. Total and immunoprecipitated protein samples were analyzed by Western blotting. F, Down-regulation of endogenous PKD1/2 suppresses Ser92 phosphorylation and 14-3-3 binding. PKD1/2-depleted HeLa cells were transfected with YFP-Syx S806A and subjected to immunoprecipitation. A decrease in pMotif signal and 14-3-3 binding was observed in YFP-Syx S806A upon PKD1/2 depletion. G, Ser92 phosphorylation and 14-3-3 binding are increased by PMA stimulation and PKD1 expression. PKD1/2-depleted HeLa cells were transfected with YFP-Syx S806A and subjected to DMSO (control) or PMA treatment (100 nM, 10 min) prior to lysing and immunoprecipitation. Stepwise increase of pMotif staining and co-immunoprecipitated 14-3-3 was observed with PMA stimulation or PKD1 expression, or both. H, increased Ser92 phosphorylation and 14-3-3 binding is dependent on PKD1 expression. PKD1/2-depleted HeLa cells were co-transfected with the indicated constructs and subjected to immunoprecipitation. Expression of wild type or kinase-active PKD1, but not kinase-dead PKD1, increased the pMotif staining of YFP-Syx S806A and the binding of endogenous 14-3-3 proteins to YFP-Syx S806A.
To test further this hypothesis, we co-expressed YFP-Syx with PKD1 variants and observed a higher amount of co-immunoprecipitated endogenous 14-3-3 proteins in HeLa cells that expressed either wild type or kinase-active (but not kinase-dead) PKD1 (Fig. 4A). Increased endogenous 14-3-3 binding to YFP-Syx also correlated strongly with PMA-induced PKD activation in a concentration- and time-dependent manner (Fig. 4, B and C). We validated these observations by expressing YFP-Syx, PKD1, and HA-14-3-3ε or ζ in PKD1/2-depleted HeLa cells (Fig. 4D) and performing co-immunoprecipitation analyses. Binding of 14-3-3ε and ζ to Syx positively correlated with the expression of PKD1 (Fig. 4E). To determine whether the PKD-mediated phosphorylation of Ser92 is physiological and correlates with binding of endogenous 14-3-3 proteins, we expressed and immunoprecipitated YFP-Syx S806A from PKD1/2-depleted cells. Down-regulation of endogenous PKD1/2 decreased both Ser92 phosphorylation and 14-3-3 binding (Fig. 4F). Furthermore, increased Ser92 phosphorylation and 14-3-3 binding positively correlated with the expression of wild type PKD1 and PMA stimulation (Fig. 4G) and the expression of wild type and kinase-active PKD1 (Fig. 4H) in PKD1/2-depleted cells. Combined, the data indicate that the interaction between Syx and 14-3-3 proteins is regulated by PKD-mediated phosphorylation at Ser92.

FIGURE 5. Binding of 14-3-3 proteins inhibits the nucleotide exchange activity of Syx. A, 14-3-3-uncoupled Syx has high GEF activity. HeLa cells were transfected with plasmids encoding YFP, YFP-Syx, or YFP-Syx S92A/S938A. Cells were lysed 24 h after transfection, and the supernatants were incubated with GST-fused Rhotekin-RBD beads to bind active RhoA. Total and pulled-down active RhoA were determined by SDS-PAGE and immunoblotting. B, Syx S92A/S938A is highly active. As in A, cells were transfected with the indicated constructs and lysed 24 h after transfection. The supernatants were incubated with RhoA G17A-conjugated glutathione beads to pull down active RhoGEFs. Total and pulled-down active Syx were analyzed by SDS-PAGE and immunoblotting. C, expression of 14-3-3ε and ζ inhibits Syx-induced RhoA activation. HeLa cells were co-transfected with YFP-Syx and an increasing amount (0, 1, or 2 μg, respectively) of HA-14-3-3ε or ζ. As in A, active RhoA pulldown assay was performed, and protein samples were analyzed by Western blotting. D, Syx S92A/S938A-induced RhoA activation is unaffected by the expression of 14-3-3ε or ζ. As in C, active RhoA was pulled down from lysates of HeLa cells co-expressing YFP-Syx S92A/S938A and an increasing amount (0, 1, or 2 μg, respectively) of HA-14-3-3ε or ζ. Total and active RhoA were determined by SDS-PAGE and immunoblotting.
Binding of 14-3-3 Proteins Modulates the Function of Syx—
To determine the physiological significance of 14-3-3 binding,
we sought to evaluate the guanine nucleotide exchange activity
of 14-3-3-uncoupled Syx. Because Syx was classified as a RhoA-
specific GEF (6), experiments utilizing GST-tagged Rhotekin-
RBD protein beads were performed to pull down GTP-bound
RhoA from lysates of HeLa cells expressing YFP, YFP-Syx, or
YFP-Syx S92A/S938A. Expression of YFP-Syx increased the
global RhoA activation in cells compared with YFP alone;
meanwhile, Syx S92A/S938A-expressing cells exhibited signif-
icantly higher RhoA activity than Syx-expressing cells (Fig. 5
A).
To demonstrate that the observed increase in RhoA activation
was a direct result of increased GEF activity of Syx S92A/S938A,
we performed active RhoGEF pulldown assays. By coupling
recombinant RhoA G17A to glutathione beads, the nucleotide-
free RhoA mutant acts as a trap and binds activated RhoGEFs
with high affinity (24). In agreement with our active RhoA
assay, a substantially higher amount of Syx S92A/S938A was
brought down in the active RhoGEF pulldown assay (Fig. 5
B).
The observation that Syx S92A/S938A exhibits elevated
nucleotide exchange activity suggested that 14-3-3 binding
suppresses the GEF activity of Syx. To test this hypothesis, we
performed active RhoA pulldown assays using lysates from
HeLa cells that co-expressed YFP-Syx and HA-tagged 14-3-3
/H9280
or
/H9256
. A high level of RhoA activation
was observed when cells were transfected with Syx. The Syx-induced RhoA activation,
however, was counteracted by the increased expression of
14-3-3-uncoupled Syx S92A/S938A (Fig. 5C). In contrast, this suppression of RhoA
activation did not occur when 14-3-3-uncoupled Syx S92A/
S938A was co-expressed with 14-3-3 proteins (Fig. 5D), indi-
Regulation of RhoGEF Syx Function by 14-3-3 Binding

The ubiquitously expressed 14-3-3 isoforms bind to a large number of proteins and mediate a remarkable range of cellular activities. However, regulation of cell-cell adhesion by 14-3-3 proteins is largely unknown. Here, our data strongly suggest that Syx-regulated junction stability is in part modulated by PKD-mediated 14-3-3 binding.

There are two aspects that define the functional relationship between Syx and 14-3-3 proteins: the effects of 14-3-3 binding on the localization and the guanine nucleotide exchange activity of Syx. The 14-3-3-uncoupled mutant Syx S92A/S938A exhibits strong junctional localization, suggesting that the binding of 14-3-3 proteins has a negative impact on Syx localization and therefore its function to maintain junction stability. Our previous study identified Syx as a target of VEGF-induced, PKD1-mediated phosphorylation at Ser806, an event that induced the mislocalization of Syx away from cell junctions. Mutating Ser806 to alanine did not interfere with the ability of Syx to bind 14-3-3 proteins, but it did induce Mupp1 association and promoted the junctional localization of Syx (5). It also allowed us to detect the unexpected phosphorylation of Ser92 by PKD1. From a structural perspective, phosphorylation of Ser806 may induce conformational changes in Syx that promote not the association per se, but the dimerization of 14-3-3 proteins associated with the N- and C-terminal regions of Syx and the subsequent dissociation from cell junctions (Fig. 7). Importantly, prior data suggest that the N-terminal domain (Syx1–300) can suppress Syx exchange activity (5). Indeed, several RhoGEFs are subjected to inhibitory intramolecular interactions between their N-terminal domains and their catalytic DbI homology domains (25). Assuming a similar mode of action, 14-3-3 binding at the N and C termini and subsequent dimerization could promote an inactive Syx conformation, thus explaining the negative effect of 14-3-3 binding on Syx GEF activity (Fig. 7). An intramolecular interaction between Syx(1–300) with the DbI homology domain may also explain the observed lack of 14-3-3 binding to Syx(1–300) (Fig. 1E), despite the presence of Ser92 within this fragment. We postulate that the intramolecular interaction results in three-dimensional folding changes that allow Ser92 phosphorylation and association with 14-3-3 proteins. Whatever the mechanism, it is clear that PKD is a key regulator of junctional Syx function. Adhesion is a dynamic process, and as such, the function of Syx is highly regulated by a multitude of events, including its autoinhibitory...

FIGURE 7. Proposed mechanism of PKD-mediated 14-3-3 binding to Syx. A, membrane-localized Syx activates RhoA locally. B, PKD-mediated phosphorylation of Ser92 upon proper folding of Syx N terminus induces binding of 14-3-3 protein. C, phosphorylation of Ser806 promotes 14-3-3 binding at the C terminus. Additional phosphorylation of Ser938 (data not shown) by PKD induces conformational changes to the C terminus and brings the two 14-3-3 proteins into close proximity. As a consequence, dimerization of terminally bound 14-3-3 proteins locks Syx in an inactive state (suppresses GEF activity), and Syx is displaced from areas of cell-cell contact. PH, pleckstrin homology domain; DH, Dbl homology domain.
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N terminus, the phosphorylation of Ser^{92}, Ser^{106}, and Ser^{938} (of which the upstream kinase is currently unknown), and the association of 14-3-3 proteins.

RhoA is thought to regulate signaling events that contribute to cell junction formation and preservation (4, 26). Dia1, a RhoA effector that is proposed to regulate cell junction stability (27, 28), belongs to the formin family and can remodel the actin cytoskeleton (29). We previously identified Dia1 as the key downstream effector of Syx in regulating junction integrity (5). Here, we observed increased RhoA activation and intensified actin staining at areas of cell-cell contact in cells expressing Syx S92A/S938A. We postulate that the increased junctional targeting and elevated GEF activity of Syx S92A/S938A results in the subsequent Rho-mediated activation of Dia1, the expansion of the circumferential actin bundle, and the stabilization of cell-cell contacts.

In conclusion, our findings indicate a role for 14-3-3 proteins in the regulation of cell-cell junctions by modulating the function of Syx. Based on studies conducted on Syx thus far, it is likely that Syx is regulated by the integration of signals from multiple upstream pathways. Uncovering additional mechanisms will provide further insights as to how Syx functions. Interestingly, our data suggest that the junction disruptive effects of tumor promoting phorbol esters may be mediated by Syx dysfunction. Finally, despite its function in junction integrity, the role of Syx in human cancer is currently unknown.

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