Sprouty2 Interacts with Protein Kinase Cδ and Disrupts Phosphorylation of Protein Kinase D1

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The Sprouty (Spry) proteins act as inhibitors of the Ras/ERK pathway downstream of receptor tyrosine kinases. In this study, we report a novel interaction between protein kinase Cδ (PKCδ) and Spry2. Endogenous PKCδ and Spry2 interact in cells upon basic fibroblast growth factor stimulation, indicating a physiological relevance for the interaction. This interaction appeared to require the full-length Spry2 protein and was conformation-dependent. Conformational constraints were released upon FGFR1 activation, allowing the interaction to occur. Although this interaction did not affect the phosphorylation of PKCδ by another kinase, it reduced the phosphorylation of a PKCδ substrate, protein kinase D1 (PKD1). Spry2 was found to interact more strongly with PKCδ with increasing amounts of PKD1, which indicated that instead of competing with PKD1 for binding with PKCδ, it was more likely to form a trimeric complex with both PKCδ and PKD1. Formation of the complex was found to be dependent on an existing PKCδ-PKD1 interaction. By disrupting the interaction between PKCδ and PKD1, Spry2 was unable to associate with PKCδ to form the trimeric complex. As a consequence of this trimeric complex, the existing interaction between PKCδ and PKD1 was increased, and the transfer of phosphate groups from PKCδ to PKD1 was at least partly blocked by Spry2. The action of Spry2 on PKCδ resulted in the inhibition of both ERK phosphorylation and invasion of PC-3 cells via PKCδ signaling. By disrupting the capacity of PKCδ to phosphorylate its cognate substrates, Spry2 may serve to modulate PKCδ signaling downstream of receptor tyrosine kinases and to regulate the physiological outcome.

Sprouty (Spry) was first discovered in Drosophila as a negative feedback inhibitor of receptor tyrosine kinase (RTK) signaling in a screen aimed at identifying genes involved in tracheal branching (1). Further work identified four murine homologues (Spry1–4), of which Sprouty2 (Spry2) bears the highest homology to Drosophila Spry (2). Although lacking any previously known domains, all four homologues retain a conserved cysteine-rich region (2), and like dSpry, murine Spry2 also negatively modulates lung branching morphogenesis (3). Subsequent studies revealed that Spry expression was induced by the Ras/ERK signaling pathway and acts as a negative feedback inhibitor of that same pathway (4). Various studies have alluded to points of action of Spry2 on the Ras/ERK pathway, including upstream of Ras, by disrupting the Grb2-Sos interaction (5, 6) or at the level of Raf (7). Therefore, it appears that there may be multiple points at which Spry2 inhibits Ras/ERK signaling.

The Ras/ERK pathway is a convergence point of the many signaling pathways that originate from activated RTKs (8). In addition to the key proteins involved in propagating the signal, this central pathway is further regulated by a number of positive and negative modulators that affect the pathway at all levels (9). At least three of the core components, RTKs together with Ras and Raf, have been shown to have many mutations in cancers (10). This knowledge has stimulated interest in understanding and identifying the positive and negative regulators of the pathway and how they function. Spry2, which is transcriptionally controlled by and inhibits this central pathway, is therefore well placed to act as a key regulator.

Unraveling the mechanism by which Spry2 inhibits the Ras/ERK pathway may be assisted by studying its interaction partners. To date, a number of interacting partners of Spry have been identified, including c-Cbl, Grb2, Tesk1, Raf1, PP2A, and DYSK1A (11–15); several of these interactions contribute to the function of Spry2 as an inhibitor of the Ras/ERK pathway. In addition to binding to other proteins, studies have shown that Spry2 can affect the signaling effect of certain proteins, such as protein kinase Cδ (PKCδ). Physiologically, Spry2 has been shown to block PKCδ signaling in Xenopus cells (16). In this context, Spry2 was reported to block morphogenesis and cell polarity, although the exact mechanism was not described.

PKCδ is a member of the PKC family of isozymes, which are classified into conventional, novel, and atypical subfamilies. Current models of PKC activation are based on the conventional PKC proteins, which are activated by diacylglycerol and calcium. A series of “priming” phosphorylations on conserved residues must also occur before the kinases can be fully active (17, 18). In contrast, PKCδ, as a novel member of the family, only requires diacylglycerol to be activated, but similar phosphorylations on PKCδ also take place (19). Like most members of the family, PKCδ consists of an N-terminal regulatory domain, which contains a pseudosubstrate domain, and a C-terminal catalytic domain, which includes the activation loop, the auto-phosphorylation site, and the ATP-binding site.
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(19). Various signals, including but not restricted to G-protein-coupled receptors, RTKs, and oxidative stress, can activate PKCδ (20–24). Among the different RTK activation mechanisms, PKCδ has been reported to be activated by FGF, vascular endothelial growth factor, and platelet-derived growth factor (16, 22–24), leading to the activation of ERK1/2 (23, 24). Recent reports have indicated that the activation of ERK1/2 via PKCδ signaling takes place through protein kinase D1 (PKD1), formerly known as PKCμ (25–27). This represents a relatively poorly studied pathway through which ERK1/2 is activated, especially with respect to the control mechanisms that regulate it.

Current evidence places PKCδ as an important regulator of various physiological functions and disease states, including development (28), cardiac development (29), cancer (30, 31), and apoptosis (32, 33). Because different factors can stimulate the PKCδ pathway, it is important that regulatory mechanisms are in place to control the gain, amplitude, and length of the signal initiated by PKCδ. The factors upstream of PKCδ that influence its activity have been relatively well documented (19, 34, 35). On the other hand, the mechanisms that specifically regulate the duration of PKCδ signaling remain inconclusive. Recent reports have indicated that signaling by PKCs can be controlled by ubiquitination following interaction with RINCK (RING finger protein that interacts with C kinase), a ubiquitin-protein isopeptide ligase (E3 ubiquitin ligase) (36), or dephosphorylation by a phosphatase, pleckstrin homology domain leucine-rich repeat protein phosphatase (37).

Despite the involvement of PKCδ in the ERK pathway, it is currently unclear at which point in the pathway it acts, although there are suggestions that it is at the level of Raf (38). Notably, this is also one point where Spry2 has been suggested to interfere with ERK1/2 signaling (7). Although Spry2 was shown to inhibit PKCδ signaling in Xenopus, this was not reported to be linked to ERK. Furthermore, Spry2 was also observed to associate with a number of other interacting partners, which serve to regulate the Ras/ERK pathway. Given these separate observations, we sought to investigate whether Sprouty2 played a role in regulating PKCδ signaling feeding into the ERK pathway, and whether this effect was exerted through an interaction with PKCδ.

In this study, we present evidence that PKCδ is an interacting partner of Spry2 and that this interaction serves to block the phosphorylation of the PKCδ substrate. Furthermore, we also show that Spry2, by interacting with PKCδ, is able to block another mode of activating the Ras/ERK pathway, and consequently exerts a negative impact on the pathway in a hitherto unidentified manner.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Mouse PKCδ was a kind gift from Peter J. Parker (London Research Institute, Cancer Research UK, London) and was subsequently subcloned into pXJ40-myc and pXJ40-HA mammalian expression vectors. The kinase-dead K376R mutant was generated by site-directed mutagenesis using the proofreading *Pfu* DNA polymerase (Promega, Madison, WI). Human PKD1 was purchased from Addgene (deposited by Alex Toker, plasmid 10808) and subsequently subcloned into pXJ40-HA vector. FGFR1, FLAG-tagged Spry1, Spry2, and Spry4 have been described previously (5, 7). Point mutants and truncations of Spry2 were generated using *Pfu* DNA polymerase.

**Antibodies and Reagents**—Mouse anti-FLAG M2, rabbit anti-FLAG, agarose-conjugated anti-FLAG M2 beads, rabbit anti-HA, rabbit anti-Spry2 (N-terminal), and bFGF were from Sigma. Rabbit and goat anti–PKCδ and rabbit antibodies against PKD1 (PKCμ), FGFR1, GST, PKCα, PKCε, PKCζ, and Myc were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse and rat anti-HA were from Roche Applied Science. Mouse antibodies against pan-ERK and PKCδ were from BD Transduction Laboratories. Mouse anti-phospho-ERK1/2, rabbit anti-phospho-PKCδ Thr(P)505, and rabbit anti-phospho-PKD1 Ser(P)744/Ser(P)748 were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-β-actin was from Abcam (Cambridge, UK). Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 546 goat anti-rabbit IgG, Alexa Fluor 546 donkey anti-rabbit IgG and Alexa Fluor 647 donkey anti-goat IgG were purchased from Molecular Probes Inc. (Eugene, OR).

**Cell Lines and Transfection**—All cell lines used were purchased from ATCC (Manassas, VA). HEK293T and PC-3 were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 2 mM L-glutamine. COS1 cells were cultured in Dulbecco’s modified Eagle’s medium with 1000 mg/liter glucose and supplemented with 10% fetal bovine serum and 2 mM L-glutamine. All transfections were carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions.

**Immunoprecipitation and Immunoblotting**—Immunoprecipitation (IP) and immunoblotting were carried out essentially as described previously (7) with the following modifications. Cells were harvested 24 h post-transfection and harvested in RIPA buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.5, 0.25 mM EDTA, 0.2% NaF, 1% sodium deoxycholate), which were then used for immunoprecipitation and subsequent immunoblotting, as described previously (7). Quantification of immunoblots was performed with the GS-800 calibrated densitometer (Bio-Rad). All Western blot data shown are representatives of at least three separate individual experiments, unless otherwise stated.

**Endogenous Interaction**—PC-3 cells were serum-starved for 3 h and subsequently stimulated for 2 h with bFGF (50 ng/ml). Cells were harvested using RIPA buffer, and the cell lysates were subjected to immunoprecipitation using rabbit PKCδ antibody, and subsequent immunoblotting was carried out using the immunoprecipitates. Reverse endogenous immunoprecipitation was carried out by using rabbit Spry2 antibody, and the immunoprecipitates were subsequently used for immunoblotting.

**Far-Western Analysis**—Mammalian proteins were immunoprecipitated and transferred onto polyvinylidene difluoride membranes as described previously (5). Briefly, membranes were blocked in 5% skim milk overnight and subsequently overlaid with GST-tagged Spry2 protein produced in Escherichia coli DH5α. Immunoblotting was then carried out using GST antibodies as described above.
**RESULTS**

**Sprouty2 Interacts with PKCδ**—It has been reported previously that Sprouty2 has a negative regulatory effect on PKCδ signaling in *Xenopus* (16). Several studies have indicated that PKCδ can target various substrates that feed into the Ras/ERK pathway, particularly centered on the complex phosphorylation/dephosphorylation events required to activate/inhibit the Raf isoforms. To study this effect in a mammalian context, we first sought evidence of an interaction between Sprouty isoforms and PKCδ. The different Sprouty isoforms, Spry1, -2, and -4, as well as the Y55F mutant of Spry2 were all tested for their interaction with endogenous PKCδ using immunoprecipitation. Of the isoforms tested, only Spry2 was present in a complex with PKCδ upon FGF stimulation (Fig. 1A). Neither Spry1 nor Spry4 had any discernible interaction with PKCδ.

The Tyr55 residue of Spry2 is seen as important, because mutagenesis experiments. Similar to our findings with FGF1 stimulation, bFGF stimulation also induced binding between Spry2 and PKCδ (Fig. 1B). We next wanted to determine whether PKCδ interacts with Spry2 endogenously. PKCδ was immunoprecipitated from PC-3 cell lysates and assayed for the presence of Spry2 in the immunoprecipitates. Spry2 co-immunoprecipitates with PKCδ upon FGF stimulation, indicating that these

**Immunofluorescence Microscopy**—COS1 cells were treated for immunofluorescence microscopy essentially as described previously (13), with a few modifications. Permeabilized cells were blocked for 1 h with phosphate-buffered saline supplemented with 2% bovine serum albumin and 5% fetal bovine serum, and incubated with primary antibodies overnight at 4 °C. After washing, cells were incubated with secondary antibodies for 1 h. Images were visualized and captured using a Zeiss LSM 510 META (Carl Zeiss Microimaging, Germany).

**Small Hairpin RNA Knockdown**—Small hairpin RNA (shRNA) constructs against human PKCδ were purchased from OriGene Technologies, Inc. (Rockville, MD) (catalogue number TR320468; sequence AGGGCAATGTAACGCTGCCATC-CACAAGAA) and from Sigma (catalogue number NM_006254; clone no. TRC0000010202; shRNA sequence CCGGGCAAGACAACAGTGAGCCTACTGAGTACGGCTCCACTGT-TGTCTTGCTTTTT). The plasmids containing the shRNA constructs were transfected into cells with about 30% confluency, using the transfection methods described above. Transfected cells were harvested 48 h post-transfection.

**PC-3 Cell Invasion Assay**—The cell invasion assay was carried out on PC-3 cells using the QCM 24-well cell invasion assay system from Chemicon (Billerica, MA) according to the manufacturer’s instructions. Briefly, PC-3 cells were serum-starved 24 h post-transfection before being harvested and seeded in Boyden chambers for the assay. PC-3 cells were allowed to invade for another 24 h before the number of cells that had invaded was quantified using the Spectra Max Gemini EM fluorescence plate reader (Molecular Devices, Sunnyvale, CA).

**FIGURE 1. Sprouty2 interacts with PKCδ**. A, lysates of 293T cells transfected with Spry2, Spry2Y55F, Spry1, or Spry4, with or without FGF1, were immunoprecipitated (IP) using agarose-conjugated anti-FLAG. The immunoprecipitates were separated on SDS-PAGE and immunoblotted (WB) with the antibodies indicated on the left. Expression of the respective proteins was verified by immunoblotting WCL with the indicated antibodies. B, transfections were carried out in PC-3 cells as described in A, but stimulation was carried out using bFGF (50 ng/ml) for 2 h after 3 h of serum starvation, instead of transfected FGF1. Lysates were subjected to IP using FLAG antibody, and immunoprecipitates and WCL were subjected to immunoblotting using the antibodies stated on the left. C, PC-3 cells were either stimulated with bFGF or left untreated as in B, and the lysates were immunoprecipitated with PKCδ antibody or normal rabbit IgG as control. The position of the Spry2 band is indicated with an asterisk. D, PC-3 cells were either stimulated with bFGF or left untreated as in C, and the lysates were immunoprecipitated with Spry2 antibody or normal rabbit IgG as control. The position of the PKCδ is indicated with an asterisk.
two proteins form a complex endogenously (Fig. 1C), and reflects a physiological relevance for the interaction between PKCδ and Spry2. To further verify the endogenous interaction, the reverse immunoprecipitation was performed, where Spry2 was immunoprecipitated, and the immunoprecipitates were assayed for the presence of PKCδ. Similar to the previous experiment, endogenous PKCδ co-immunoprecipitated with endogenous Spry2 (Fig. 1D). Direct binding between the two proteins also occurs, which is demonstrated later in the study.

Sprouty2-PKCδ Interaction Occurs at Multiple Sites and Is Conformation-dependent—Our next aim was to determine the region of interaction between PKCδ and Spry2. To this end, N- and C-terminal truncated constructs of Spry2 and PKCδ were made (Fig. 2A). The hSpry2N1–179 and C179–315 constructs essentially divide Spry2 into its less conserved N terminus and its conserved Cys-rich C terminus. Two other sets of Spry2 N- and C-terminal truncations were made. PKCδ truncations were made to separate the N-terminal regulatory domain and C-terminal catalytic domain. These constructs were transfected into HEK293T cells, subjected to immunoprecipitation experiments, and assayed for the presence of the interaction partner.

Besides several short motifs, the N-terminal domain of the different Spry isoforms is not conserved. However, there is a high degree of conservation in the Cys-rich C-terminal domain between the Sprouty isoforms. Because PKCδ interacted only with Spry2, it was postulated that PKCδ would interact with the more nonconserved N-terminal region of Spry2. However, we observed that PKCδ only interacted with the full-length Spry2 protein upon FGFR1 activation (Fig. 2B). To rule out the possibility that the binding site for PKCδ was cleaved upon truncation of the Spry2 protein, two other sets of truncation constructs with the truncation site moved progressively toward the N terminus were employed. Again, only the full-length Spry2 protein was able to interact with the full-length endogenous PKCδ protein (supplemental Fig. 1). Because the full-length Spry2 protein is required for its interaction with PKCδ, it was hypothesized that the interaction occurred on at least two sites of Sprouty2.

To determine which region of PKCδ interacted with Spry2, the N-terminal regulatory domain and C-terminal catalytic domain of PKCδ were tested for interaction with full-length Spry2. As before, the two full-length proteins interacted with FGFR1 activation (Fig. 2C). Unexpectedly, we observed that both the N- and C-terminal domains of PKCδ interacted with Spry2 in the absence of FGFR1 activation (Fig. 2C), which was strengthened in the presence of FGFR1 activation. These results were then validated by co-expressing the N- and C-terminal domains of PKCδ with the regulatory and catalytic domains of PKCδ (supplemental Figs. 2 and 3). Together, our findings support the assumption that there is more than one point of interaction between the two proteins, and imply that conformational constraints regulate the interaction of these two proteins. These constraints were released by truncating PKCδ, allowing the interaction to occur even in the absence of stimulation. Upon FGFR1 activation, the interaction strengthens, suggesting that post-translational modifications, such as phosphorylation, are also required for maximal interaction.

To validate the biochemical data, we examined the localization of Spry2 and PKCδ in COS1 cells under both control and stimulated conditions. In the absence of FGF stimulation, the proteins show distinct distribution patterns (Fig. 2D and supplemental Fig. 4) with Spry2 localized on the microtubules (40) and PKCδ in the cytoplasm. Upon FGF stimulation, the proteins both localize at the cell membrane, suggesting that the two proteins co-localize upon FGF stimulation and supporting the possibility of a physical interaction.

Sprouty2 Prevents Phosphorylation of a PKCδ Substrate—Like many other kinases, PKCδ requires post-translational modifications upon stimulation for it to be catalytically active. PKCδ has been reported to be phosphorylated within the activation loop on Thr505 by an upstream kinase. This phosphorylation is essential for the full PKCδ activity (41). Because Spry2 has been reported to inhibit PKCδ signaling, we investigated the possibility that Spry2 inhibited the activity of PKCδ through this residue, in the presence of FGFR1. In this experiment, the phosphorylation of PKCδ on Thr505 was examined in the presence and absence of Spry2. Although the results in Fig. 3A indicate that PKCδ is phosphorylated on Thr505 upon FGFR1 stimulation, this phosphorylation did not decrease in the presence of Spry2.

As Spry2 did not affect PKCδ phosphorylation on Thr505, we wanted to determine whether Spry2 affected the phosphorylation of downstream substrates by its association with PKCδ. PKD1 (formerly known as PKCμ) is a substrate of a number of members of the PKC family, including PKCδ (21, 42–44). Activation of PKD1 occurs upon phosphorylation of two residues within the activation loop as follows: Ser738 and Ser742 (Ser744 and Ser746 in mouse) (43). Using the wild type and kinase-dead PKCδ, we first wanted to ascertain the effect of PKCδ on the phosphorylation of PKD1 on Ser738/742 before challenging the activity of PKCδ with the addition of Spry2. In the absence of stimulation, PKD1 is not phosphorylated in the presence of endogenous PKCδ (Fig. 3B). When activated with FGFR1, however, PKD1 is phosphorylated. On the other hand, overexpression of PKCδ causes auto-activation of the kinase, resulting in phosphorylation of PKD1 on Ser738/742 even in the absence of FGFR1 stimulation. This phosphorylation can be suppressed by the presence of kinase-dead PKCδ alone, which persists even in the presence of FGFR1 stimulation, supporting previously reported data that PKD1 is a substrate of PKCδ.

The next step was to determine whether Spry2 affected the phosphorylation of PKD1 through its association with PKCδ. PKD1 showed basal levels of phosphorylation with endogenous PKCδ (Fig. 3C, lane 1–3) and, like before, overexpressed PKCδ increased phosphorylation on Thr505 (Fig. 3C, lanes 4–6), as did activation with FGFR1 (lane 7). However, in the presence of Spry2 and FGFR1, the phosphorylation of PKD1 was markedly reduced (Fig. 3C, lane 8 compared with lane 7), almost to the same level as seen without FGFR1 stimulation (lane 2). The Y55F mutant of Spry2, which does not interact with PKCδ, had no effect on PKD1 phosphorylation (Fig. 3C, lane 9). Interestingly, the reduction of PKD1 phosphorylation by Spry2 was rescued by overexpressed wild type PKCδ (Fig. 3C, lane 11). This would imply that Spry2, by interacting with PKCδ, is able to inhibit the
kinase activity of PKC\(\beta\). Furthermore, by increasing the levels of PKC\(\beta\) in the cell, the inhibitory effect of Spry2 on the kinase activity of PKC\(\beta\) is countered so that PKD1 phosphorylation is rescued. In Fig. 3C, the bar chart representation of PKD1 phosphorylation is shown below the Western blot data.

**Spry2 Interacts with Both PKC\(\beta\) and PKD1**—Our results so far indicate that Spry2 is able to inhibit the phosphorylation of a PKC\(\beta\) substrate by intervening somehow in the kinase-substrate interaction and abrogating the downstream signaling from PKC\(\beta\), as seen with the reduction in phosphorylation of

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**FIGURE 2.** Sprouty2-PKC\(\beta\) interaction is conformation-dependent and takes place on more than one site of each protein. A. schematic diagram showing the different domains and truncation constructs of Sprouty2 and PKC\(\beta\); Tyr\(^{55}\) (Tyr\(^{55}\) residue of the conserved Tyr\(^{55}\) motif of PKC\(\beta\)) of Sprouty2), C2 (C2 domain of PKC\(\beta\)), and Lys\(^{376}\) (ATP-binding site Lys\(^{376}\) of PKC\(\beta\)). B, 293T cells were transfected with full-length, N-terminal (Spry2N1–179) or C-terminal (Spry2C179–315) Spry2, and FGFR1. Cell lysates were immunoprecipitated (IP) using anti-FLAG-conjugated beads and then used for immunoblotting (WB) with the antibodies indicated on the left. WCL samples were also immunoblotted to indicate expression of the proteins. C, full-length, N-terminal (N1–345), and C-terminal (C346–674) Myc-tagged PKC\(\beta\) constructs were transfected into 293T cells. Lysates were IP with Myc antibody; immunoprecipitates and WCL were immunoblotted with the antibodies indicated on the left. D, COS1 cells were transfected with FLAG-tagged Spry2. Transfected cells were serum-starved overnight and either left untreated (panel c), or stimulated with 100 ng/ml bFGF (panel d) for 1 h. Cells were fixed and stained with mouse anti-FLAG and rabbit anti-PKC\(\beta\), followed by anti-mouse IgG (green) and anti-rabbit IgG (red) staining. Panels a and b show untransfected COS1 cells stained for PKC\(\beta\) as a control. The correlation coefficient for Spry2 and PKC\(\beta\) co-localization, as determined by regression analysis, is 0.967.
Sprouty2 blocks PKCδ from phosphorylating its substrate PKD1. A, 293T cells were transfected with Spry2-FLAG and FGFR1. Cell lysates were immunoprecipitated (IP) using PKCδ antibody immunoblotted (WB) using the antibodies indicated on the left. WCL were also immunoblotted to verify the expression of the proteins. B, wild type or KD PKCδ were co-expressed with FGFR1 in 293T cells. Endogenous PKD1 was IP from cell lysates using PKD1 antibody, and both the immunoprecipitates and WCL were subjected to immunoblotting with the antibodies stated on the left. C, wild type or Y55F mutant of Spry2 was co-expressed in 293T cells with either endogenous PKCδ or Myc-tagged wild type PKCδ and FGFR1. Cell lysates were IP using PKD1 antibody, and the immunoprecipitates were immunoblotted with the antibodies shown on the left. WCL were also immunoblotted to confirm the presence of equal levels of proteins. The immunoblot data were scanned using a densitometer and represented in the bar chart. The bar chart shows the level of phosphorylated PKD1 relative to total IP PKD1 (mean ± S.E., p < 0.05, n = 5).
PKD1. At this point, we advanced two scenarios by which Spry2 may accomplish this. The first involves the sequestration of PKC\(\delta\), or the competition between PKD1 and Spry2 for the interaction with PKC\(\delta\), such that the physical interaction between PKC\(\delta\) and PKD1 is prevented, leading to a reduction in PKD1 phosphorylation. A second possible mechanism is that Spry2 blocks the active site on PKC\(\delta\) so that, although PKC\(\delta\) may still bind to PKD1, it will be unable to phosphorylate the key residues (Ser\(^{738}\) and Ser\(^{742}\)). In this second scenario, the interaction between PKC\(\delta\) and PKD1 need not be disrupted.

To distinguish between the two alternative mechanisms, increasing amounts of PKD1 were expressed in 293T cells to determine whether PKD1 would influence the relative association between PKC\(\delta\) and Spry2. PKC\(\delta\) was immunoprecipitated from cell lysates, and the relative amounts of Spry2 that co-immunoprecipitated were examined. We observed that, under stimulated conditions, the amount of Spry2 co-immunoprecipitating with PKC\(\delta\) increased in the presence of increasing amounts of PKD1, suggesting a strengthening of the Spry2–PKC\(\delta\) interaction (Fig. 4B). This also indicates that Spry2 does not sequester PKC\(\delta\) nor does it compete with PKD1 for the interaction with PKC\(\delta\). The experiment was repeated by immunoprecipitating Spry2 instead of PKC\(\delta\), and similar results were obtained (Fig. 4C). Together, the data from these experiments suggest that sequestration is not the mechanism by which Spry2 inhibits PKC\(\delta\) signaling, and it leads us to the alternative theory that Spry2 can block the active site of PKC\(\delta\), so that it is unable to phosphorylate PKD1. Because PKD1 seemingly increased the interaction between Spry2 and PKC\(\delta\), it is possible that the three proteins act together in a trimeric complex.

For PKD1 to interact with both Spry2 and PKC\(\delta\) simultaneously, these three proteins would need to have the same cellular localization after stimulation. Prior to stimulation, both endogenous PKC\(\delta\) and PKD1 were in the cytoplasm, whereas Spry2 was mainly localized to the microtubules of COS1 cells (40). Upon FGF stimulation, all three proteins co-localized at the cell membrane (Fig. 4D and supplemental Fig. 5), supporting the possibility of a trimeric complex.

**Spry2–PKC\(\delta\) Interaction Depends on PKC\(\delta\)-PKD1 Binding—**Our next aim was to determine whether a trimeric complex exists between Spry2, PKC\(\delta\), and PKD1, and if so, it follows that Spry2 should be able to interact directly with both PKC\(\delta\) and PKD1. To validate this, we performed a far-Western blot using GST-tagged Spry2 proteins. PKC\(\delta\) and PKD1 were immunoprecipitated from 293T cell lysates using HA antibodies, with Cdc2 employed as a negative control. Both PKC\(\delta\) and PKD1 were found to bind directly to Spry2, whereas binding was not observed with Cdc2 (Fig. 5A). This direct binding between PKC\(\delta\) and PKD1 with Spry2 supports the hypothesis that these proteins are able to bind in a trimeric complex.

Next, we wanted to determine whether Spry2 blocked the active (ATP-binding) site of PKC\(\delta\) to prevent phosphorylation of its substrate. To this end, a kinase-dead mutant of PKC\(\delta\) was made (PKC\(\delta\)-KD), where the ATP-binding site Lys\(^{376}\) was mutated to arginine (K376R) (Fig. 5B). PKC\(\delta\)-KD was then used to assay for interactions with Spry2, using wild type PKC\(\delta\) as a basis for comparison. As shown in Fig. 5C, Spry2 co-immunoprecipitated with endogenous and overexpressed PKC\(\delta\) in the presence of FGFRI1 but not with PKC\(\delta\)-KD (top panel, lanes 10–12), suggesting that the ATP-binding site (Lys\(^{376}\)) is important for the Spry2–PKC\(\delta\) interaction. Interestingly, there is little discernible interaction between Spry2 and endogenous PKC\(\delta\) in the presence of PKC\(\delta\)-KD (Fig. 5C, lane 12). The following argument is presented to explain these observations. Although PKC\(\delta\)-KD is unable to bind to Spry2, it can still bind to its substrate PKD1, but without its activating effects. Because PKC\(\delta\)-KD is present in cells at a higher level than the endogenous PKC\(\delta\), most of the cellular PKD1 would have been sequestered by PKC\(\delta\)-KD, leaving little PKD1 available to interact with the endogenous PKC\(\delta\). As shown earlier, PKD1 increases the interaction between PKC\(\delta\) and Spry2. With less PKD1 available for interaction with endogenous PKC\(\delta\), binding of Spry2 to PKC\(\delta\) is likewise reduced, resulting in little detectable interaction between endogenous PKC\(\delta\) and Spry2 in the presence of PKC\(\delta\)-KD.

To validate this argument, another PKC\(\delta\) mutant without the C2 domain was constructed (PKC\(\delta\)-ΔC2) (Fig. 5B). It has been reported previously that the C2 domain of PKC\(\delta\) is able to recognize and bind to the motif Y/F/S/A/V/I/Y(Q/R)(X/Y/F)(X) (45), which is present in PKD1 (FYGMYD) (44). If the C2 domain and this motif are indeed important for the interaction between PKC\(\delta\) and PKD1, then there should be no interaction between PKC\(\delta\) and PKC\(\delta\)-ΔC2. More importantly, there should also be a reduction in interaction between Spry2 with either PKD1 or PKC\(\delta\) for the argument to be substantiated.

Using the two PKC\(\delta\) mutants, PKD1 immunoprecipitates were assayed for the presence of total PKC\(\delta\) and Spry2. In line with our earlier results, Spry2 failed to co-immunoprecipitate with PKD1 in the presence of PKC\(\delta\)-KD and FGFRI1 (Fig. 5D, top panel, lane 9), compared with endogenous and wild type PKC\(\delta\) (top panel, lanes 7 and 8). It should be noted that PKC\(\delta\)-KD still bound to PKD1 (Fig. 5D, 2nd panel, lane 9). At the same time, PKC\(\delta\)-ΔC2 showed significantly reduced binding to PKD1, indicating that the C2 domain of PKC\(\delta\) is important for the PKC\(\delta\)-PKD1 interaction (Fig. 5D, 2nd panel, lane 10). Consistent with this observation is the reduced interaction between Spry2 and PKD1 (Fig. 5D, top panel, lane 10), where the interaction is about 50% of the interaction that occurs in the presence of endogenous PKC\(\delta\) (top panel, lane 7).

Taken together, the results show that the ATP-binding site is important for Spry2–PKC\(\delta\) interaction. Spry2 appears to inhibit phosphorylation of PKD1 by blocking this site on PKC\(\delta\). At the same time, PKC\(\delta\)-PKD1 interaction is important for Spry2 to interact with PKC\(\delta\). The absence of an interaction between PKC\(\delta\) and PKD1 negatively affects the interaction between Spry2 and PKC\(\delta\). This supports our earlier argument for the formation of a trimeric complex between Spry2, PKC\(\delta\), and PKD1, where its formation is dependent on a PKC\(\delta\)-PKD1 interaction, and where Spry2 blocks the ATP-binding site of PKC\(\delta\). The resulting reduction in phosphorylation of PKD1 seems to hinge on Spry2 being a key player within this complex. Because the interaction of Spry2 is dependent on the presence and interaction of PKC\(\delta\) and PKD1, it would seem probable that the role of Spry2 is to increase the existing interaction between the two kinases. At the same time, by interacting with the ATP-binding site of PKC\(\delta\), Spry2 effectively blocks the transfer of a

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FIGURE 4. Spry2 interacts with PKCδ and PKD1. A, 293T cells were transfected with increasing amounts of PKD1, with or without FGFR1 activation. Cell lysates were immunoprecipitated (IP) using PKCδ antibody and subsequently immunoblotted (WB) using the antibodies shown on the left. WCL were also immunoblotted to verify the presence of equal levels of proteins. The relative amounts of immunoprecipitated Spry2 are indicated in the bar chart (mean ± S.E., p < 0.05, n = 3). B, experiment was carried out as described in A, except that IP was carried out using FLAG antibody instead of PKCδ antibody. Immunoblots were done with the antibodies indicated on the left for both the immunoprecipitates and WCL. The relative amounts of immunoprecipitated PKCδ are shown in the bar chart (mean ± S.E., p < 0.05, n = 3). C, COS1 cells were transfected with FLAG-tagged Spry2 and serum-starved overnight. The cells were then either left untreated (panel e) or stimulated with bFGF (100 ng/ml) (panel f) for 1 h. Cells were fixed and stained with mouse anti-FLAG, rabbit anti-PKD1, and goat anti-PKCδ, followed by anti-mouse IgG (green), anti-rabbit IgG (red), and anti-goat IgG (beige) staining. Panels a–d show COS1 cells stained for PKD1 and PKCδ in the absence of Spry2, as controls. The correlation coefficients for Spry2 and PKCδ co-localization and Spry2 and PKD1 co-localization as determined by regression analyses, are 0.967 and 0.919, respectively.
FIGURE 5. Spry2 forms a trimeric complex with both PKCδ and PKD1. A, 293T cells were transfected with HA-tagged PKCδ, PKD1, or Cdc2 (negative control) and FGFR1. The cell lysates were then immunoprecipitated (IP) using HA antibody, separated on SDS-PAGE, and transferred onto polyvinylidene difluoride membrane. These membranes were overlaid with either Spry2-GST proteins or GST proteins generated in E. coli DH5α and immunoblotted (WB) with GST antibody; a, HA-PKD1; b, HA-PKCδ; and c, HA-Cdc2. For each set of overlay, control blots were directly immunoblotted with anti-HA to verify successful immunoprecipitation. B, schematic diagram showing the different mutation constructs of PKCδ as follows: wild type PKCδ (WT-PKCδ), PKCδ-KD (K376R, lysine at 376 is substituted with arginine), and PKCδ-ΔC2 (C2 domain of PKCδ has been deleted). C, Myc-tagged WT-PKCδ or kinase-dead PKCδ (PKCδ-KD) were expressed in 293T cells with FGFR1. Total PKCδ was IP using PKCδ antibody, and the immunoprecipitates were immunoblotted according to the antibodies shown on the left. WCL were also immunoblotted to confirm the presence of equal amounts of proteins between the samples. D, 293T cells were transfected with Myc-tagged PKCδ, PKCδ-KD, or PKCδ-ΔC2, and FGFR1. Endogenous PKD1 was IP with anti-PKD1, and the immunoblotting was carried out on both immunoprecipitates and WCL using the antibodies indicated on the left. The position of the PKCδ-ΔC2 band is indicated with an asterisk.
Spry2 Inhibits Phosphorylation of a PKCδ Substrate

A

| PKCδ shRNA | -FGF | +FGF |
|------------|------|------|
| neg ctrl   | 1    | 2    |
| ctrl       | 4    | 5    |
| neg ctrl   | 7    | 8    |

WB: anti-pERK
WB: anti-PKCδ
WB: anti-pan ERK
WB: anti-PKCα
WB: anti-PKCε
WB: anti-PKCζ
WB: anti-β-actin

B

PKCδ (myc) | - | PKCδ | KD
FGF | - | + | +

WB: anti-pERK
WB: anti-pan ERK
WB: anti-PKCδ

C

Spry2 (FLAG) | - | +
PKCδ (myc) | - | +

WB: anti-pERK
WB: anti-pan ERK
WB: anti-FLAG
WB: anti-PKCδ

Endogenous PKCδ
Overexpressed PKCδ

Phospho-ERK level: proportion of vector

Vector FGF
FGF
shRNA 1 FGF
shRNA 2 FGF

Vector FGF
PKCδ FGF
PKCδ-KD FGF

Phospho-ERK level: proportion of vector

Vector FGF
Spry2 FGF
Y55F FGF
Vector FGF
Spry2 FGF
Y55F FGF
y-phosphate group to PKD1. Therefore, the net result of this trimeric complex is to create a dominant negative or kinase-dead PKCδ from the wild type PKCδ.

Spry2 Inhibits ERK1/2 Phosphorylation by PKCδ—There have been several reports to date implicating PKCδ in ERK1/2 activity (23, 24). However, the regulatory mechanisms regarding this particular pathway have not been well studied. First of all, to verify that PKCδ contributes to the activation of ERK1/2, endogenous PKCδ was knocked down using shRNA (Fig. 6A). PKCδ-specific shRNA significantly knocked down the level of endogenous PKCδ, as compared with a vector control and nonspecific shRNA control (Fig. 6A, 2nd panel). In shRNA-transfected cells stimulated with FGF, phospho-ERK1/2 levels decrease markedly, but not completely, compared with cells transfected with either the vector or nonspecific shRNA (Fig. 6A, top panel, lanes 5–8). The decrease in phospho-ERK1/2 levels was also due solely to the decrease in PKCδ expression, because the shRNA constructs used did not affect the levels of three other PKCs tested, PKCa, -ε, and -ζ (Fig. 6A, 4th to 6th panels).

To verify that the kinase activity of PKCδ, and not just its presence, is required for activation of ERK1/2, the phosphorylation of ERK1/2 was studied in the presence of kinase-dead PKCδ (PKCδ-KD). Upon FGF stimulation, ERK1/2 showed a marked decrease in phosphorylation in the presence of PKCδ-KD (Fig. 6B, lane 6) when compared with endogenous PKCδ or overexpressed wild type PKCδ (Fig. 6B, lanes 2 and 4), indicating that the kinase activity of PKCδ, while not being the sole determinant of ERK1/2 phosphorylation, plays an important role in affecting ERK1/2 activation. It is noted that although overexpressed PKCδ alone is able to phosphorylate PKD1, there is no effect on ERK1/2 phosphorylation (Fig. 6B, lane 4). This is further analyzed under “Discussion.”

Given that Spry2 has been shown to inhibit the kinase activity of PKCδ, we decided to determine whether Spry2 affects ERK1/2 phosphorylation via PKCδ signaling. There are several reports that show that Spry2 inhibits ERK1/2 activation by blocking the Ras/Raf/MEK/ERK pathway (5–7). However, the effect of Spry2 on ERK1/2 signaling downstream of PKCδ signaling is currently unknown. Consistent with earlier reports, Spry2 inhibits ERK1/2 phosphorylation upon FGF stimulation (Fig. 6C, lane 8), and Spry2Y55F, which does not interact with PKCδ nor inhibit PKD1 phosphorylation, also does not inhibit ERK1/2 phosphorylation (Fig. 6C, lane 9). However, when PKCδ expression is increased in cells to levels such that there is insufficient Spry2 available to block its ATP-binding site, the inhibition of ERK1/2 phosphorylation by Spry2 is reversed (Fig. 6C, lane 11). This indicates that Spry2 is able to inhibit ERK1/2 activation by negatively affecting the ability of PKCδ to phosphorylate its substrate.

FIGURE 6. Spry2 blocks PKCδ signaling to ERK. A, 293T cells were transfected with vector control, nonspecific shRNA, or PKCδ-specific shRNA constructs; 1, shRNA construct from OriGene; 2, shRNA construct from Sigma, and stimulated with bFGF (20 ng/ml) for 2 h or left untreated. Lysates were collected and separated on SDS-PAGE before immunoblotting (WB) with the antibodies indicated on the left. The relative levels of phospho-ERK as a proportion of the vector control are shown in the bar chart (mean ± S.E., p < 0.05, n = 4). neg ctrl, negative control. B, wild type PKCδ or PKCδ-KD were expressed in 293T cells. The cells were stimulated with bFGF or left untreated after 4 h of serum starvation. Immunoblotting was performed on WCL using the antibodies shown on the left. The relative amounts of phospho-ERK as a proportion of the vector control are shown in the bar chart (mean ± S.E., p < 0.05, n = 4). C, Spry2 or the Y55F mutant was co-expressed with either Myc-tagged PKCδ or a vector control and stimulated with bFGF or left untreated after 4 h of serum starvation. Lysates were subjected to SDS-PAGE and immunoblotting using the antibodies indicated on the left. The levels of phospho-ERK as compared with the vector control are shown in the bar chart (mean ± S.E., p < 0.05, n = 5).

Spry2 Inhibits Cell Invasion of PC-3 Cells—PKCδ has been reported to be involved in several physiological functions in development and disease (28–31). In prostate cancer cells, PKCδ has been shown to be important for cell invasion (46). To gain insight into the physiological relevance of this interaction, we investigated whether Spry2 could affect the invasiveness of a prostate cancer cell line, PC-3. We first ascertained that PKCδ is indeed involved in PC-3 cell invasion using a Boyden chamber cell invasion assay. PC-3 cells transfected with PKCδ-KD or PKCδ-specific shRNA showed about 50% decrease in “invasiveness” (Fig. 7). PC-3 cells that express Spry2 show a similar level of reduction in cell invasion. This reduction is rescued with the co-expression of Spry2 and PKCδ, supporting the biochemical results. On the other hand, Spry2Y55F did not exert a negative effect on cell invasion when compared with vector-transfected cells (data not shown). This result was expected as our results have so far indicated that, by not interacting with PKCδ, Spry2Y55F cannot exert an effect on PKCδ. Taken together, these results show the physiological importance of the biochemical interaction between PKCδ and Spry2.

DISCUSSION

The recent identification of different interacting partners of Spry2 has allowed us to better understand its complex mechanisms of control and modes of action. Here we identified that PKCδ is an interacting partner of Spry2. PKCδ is able to bind to endogenous Spry2 upon FGF stimulation, implicating a physiological role for this interaction. As several other known interacting partners of Spry2 play a role in the function of Spry2 inhibiting ERK1/2 phosphorylation, we postulated that this interaction may have a similar outcome. Although Spry2 was
Spry2 Inhibits Phosphorylation of a PKC\(\theta\) Substrate

able to interact with PKC\(\theta\), it was observed that the Y55F point mutant did not. Phosphorylation on the Tyr\(^{55}\) residue leads to a conformational change in Spry2, as reported previously by our group (5), and because PKC\(\theta\) does not appear to recognize the specific motif surrounding the Tyr\(^{55}\) residue, it is likely that phosphorylation of the Tyr\(^{55}\) residue is required to induce a conformational change in Spry2 to facilitate its binding to PKC\(\theta\). Therefore, it is likely that the association of PKC\(\theta\) with Spry2 is a corollary of a conformation change and is not associated with direct binding, as is the case with the binding of Cbl via its TKB domain to a canonical recognition site centered around Tyr\(^{55}\) (47).

Unlike its interaction with other proteins, Spry2 does not interact with PKC\(\theta\) on just one specific region (12, 47). Instead, our results suggest that multiple sequences within the full-length Spry2 protein are required for interaction with PKC\(\theta\). This is further supported by the finding that Spry2 forms a trimeric complex with PKC\(\theta\) and PKD1 via distinct regions of the protein.

The Spry2-PKC\(\theta\) interaction occurs at the plasma membrane in mammalian cells following stimulation with FGF. Our findings contrast with those seen in Xenopus cells, where Spry2 was reported to inhibit the activation of PKC\(\theta\) (16). This was observed by the lack of translocation of PKC\(\theta\) to the cell membrane in the presence of Spry2 in those cells. We expanded on our findings by showing that the phosphorylation of the Thr\(^{505}\) residue of PKC\(\theta\) was not affected by the presence of Spry2, implying that the cognate kinase for PKC\(\theta\) was not blocked from phosphorylating its substrate. Although others have reported a glutamate residue at position 500 renders the Thr\(^{505}\) residue within the activation loop of PKC\(\theta\) redundant, maximal activity and specificity still require the phosphorylation of Thr\(^{505}\) (48). Therefore, from these two observations, Spry2 does not appear to interfere with the phosphorylation and enzymology of PKC\(\theta\). Although the binding between PKC\(\theta\) and Spry2 did not inhibit the catalytic activity of PKC\(\theta\) itself, the formation of a trimeric complex between Spry2, PKC\(\theta\), and PKD1 caused a reduction in the phosphorylation of PKD1 on Ser\(^{738}/\)Ser\(^{742}\). Furthermore, the C2 domain of PKC\(\theta\) was shown to be important in mediating the formation of this trimeric complex, as a loss of binding between PKC\(\theta\) and PKD1 in the absence of the C2 domain of PKC\(\theta\) also diminished the interaction between PKC\(\theta\) and Spry2.

As a kinase, in the presence of a stimulus, PKC\(\theta\) binds ATP on its ATP-binding site, which is centered on Lys\(^{376}\). Upon interaction with its substrate PKD1 (via the C2 domain on PKC\(\theta\) and the FYGMYD motif on PKD1), PKC\(\theta\) is able to phosphorylate PKD1 on Ser\(^{738}/\)Ser\(^{742}\) and trigger downstream signaling (Fig. 8). When Spry2 is present, however, it interacts with both PKC\(\theta\) on Lys\(^{376}\), so as to block the transfer of its phosphate groups to PKD1, and with PKD1 so that the interaction between PKC\(\theta\) and PKD1 is tightened (Fig. 8). This explains the necessity of a full-length Spry2 protein. In essence, Spry2 creates the situation where a wild type, kinetically competent PKC\(\theta\) is rendered kinase-inactive because of its inability to phosphorylate its substrate.

Spry2 has been reported to interact with at least two other kinases, namely Tesk1 and DYRK1A (13, 14). Although the interaction with these two kinases abrogated the capacity of Spry2 to inhibit ERK1/2 activation, the reverse was true with the Spry2-PKC\(\theta\) interaction, with Spry2 curtailing the contribution of PKC\(\theta\) to ERK1/2 phosphorylation. The ability of Spry2 to associate with different kinases suggests that Spry2, in addition to being a negative feedback inhibitor of RTK signaling, may also serve as a conduit through which the effect of kinases can be regulated.

To demonstrate the physiological impact of Spry2 on PKC\(\theta\), a Boyden chamber cell invasion assay was performed. Previous studies have shown that invasion of prostate cancer cells (PC-3) is dependent on the activity of different PKCs, among them PKC\(\theta\) (46, 49). PC-3 cells expressing Spry2 were shown to be less invasive, comparable with those cells where endogenous

![Figure 8: Spry2 interacts with PKC\(\theta\) and PKD1 to block phosphorylation of PKD1 by PKC\(\theta\). A summary schematic diagram shows the possible mechanism of interaction between Spry2, PKC\(\theta\), and PKD1, and the domains involved are as follows: C2 (C2 domain on PKC\(\theta\)), FYGMYD (the C2 recognition motif on PKD1), Lys\(^{376}\) (ATP-binding site on PKC\(\theta\)), Thr\(^{505}\) (phosphorylation site within the activation loop of PKC\(\theta\)), and Ser\(^{738}\) and Ser\(^{742}\) (phosphorylation sites within the activation loop of PKD1).](image-url)
PKC8 was knocked down or where the activity of endogenous PKC8 was blocked by a kinase-dead, dominant negative PKC8. This effect of Spry2 could be reversed when PKC8 levels were raised so that Spry2 could no longer block the activity of PKC8, suggesting that Spry2, by inhibiting the transfer of phosphate groups from PKC8 to its substrate, is able to block PKC8 signaling. As a result, cell invasion, which is dependent on PKC8 signaling, is partially abated.

There have been several reports describing Spry2 as an inhibitor of ERK1/2 signaling, and it is likely that this inhibition occurs at several points in the pathway. PKC8 activation of ERK1/2, on the other hand, has been reported to occur via PKD1 and its downstream effectors, possibly resulting in the activation of Raf (38, 50). Although many pathways feed into the ERK signaling pathway, Spry2 has, to date, been generally considered to be a negative feedback inhibitor of the classical Grb2/SOS/Ras/Raf/MEK/ERK pathway. The recent increase in literature related to Spry proteins has served to underscore the many possible pathways upon which Spry proteins may act (51–53). Our findings here demonstrate an alternative mode of inhibition of ERK1/2 signaling for Spry2. We showed in Fig. 3B that overexpressed PKC8 alone phosphorylated PKD1 without FGFR1 activation. Overexpression of PKC8, however, did not result in ERK1/2 phosphorylation, as shown in Fig. 6B. This, combined with results shown in Fig. 6C, indicates that although PKC8 is able to activate ERK1/2 via PKD1, this activation is likely to involve a parallel signal or signals. Evidence for this was reported by Wang et al. (50), who demonstrated that RIN1 was a downstream substrate of PKD1. RIN1, when phosphorylated, is released from activated Ras, thus allowing Ras to activate Raf1, and subsequently ERK1/2. Unphosphorylated RIN1 is unable to be released from active Ras, thereby effectively competing with Raf1. In this scenario, ERK1/2 activation would therefore be dependent on two separate inputs as follows: the activation of Ras by stimulants such as growth factors, and the phosphorylation of PKD1 by PKC8, resulting in a phosphorylated RIN1. Absence of either of these two inputs would result in nonactivation of ERK1/2. Given that PKC8 has been implicated in a variety of physiological functions downstream of RTK signaling (16, 28, 29, 54–55), it is plausible to ask if Spry2 serves as an inhibitor of PKC8 signaling in any of these functions. It is worth noting that most of these reported functions require the kinase activity of PKC8, and Spry2, in this study, has been shown to be able to inhibit the phosphorylation of the PKC8 substrate. Further studies with regard to these functions are required to determine in more PKC8 substrates to determine whether the mechanism described in this study applies to other substrates. If so, this could provide a link between Spry2 and the physiological processes regulated by PKC8.

Physiologically, there is significant overlap in the functions that the two proteins regulate. Both PKC8 and Spry2 have been reported to be involved in embryo development, for example during mesoderm convergent extension and formation in *Xenopus* (16, 28). It was observed that Spry2 was able to inhibit PKC8 signaling (16). It was also reported that Dishevelled (Dsh), which was required for convergent extension movements in *Xenopus*, required the catalytic activity of PKC8 (28). It would therefore be interesting to observe if Spry2 could regulate Dsh signaling in embryo development through its effect on PKC8. Both PKC8 and Spry2 have also been implicated in angiogenesis (54–56). In this case, PKC8 was found to phosphorylate Syndecan-4 on Ser183 (54). Once again, the kinase activity of PKC8 was required. Should Syndecan-4 prove to be a direct substrate of PKC8, the effect of Spry2 on the phosphorylation status of Syndecan-4 and subsequent angiogenesis could also be more closely examined. In disease states, PKC8 has also been reported to be active in breast cancer (30, 31), a cancer that has been reported to show down-regulation of Spry proteins (57). Although the specific mechanisms leading to the disease are not fully understood, it was suggested in one instance that phosphorylation of glycogen synthase kinase-3 by PKC8 was involved (31). The full spectrum of the PKC8 substrates is currently unknown, and the effect of Spry2 on the catalytic function of PKC8 could prove to have an impact on several physiological functions involving PKC8. Given the significant degree of overlap in functions between the two proteins, a greater physiological link may exist between Spry2 and PKC8 than currently known.

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