Direct Binding of PP2A to Sprouty2 and Phosphorylation Changes Are a Prerequisite for ERK Inhibition Downstream of Fibroblast Growth Factor Receptor Stimulation*

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In the context of fibroblast growth factor (FGF) signaling, Sprouty2 (Spry2) is the most profound inhibitor of the Ras/ERK pathway as compared with other Spry isoforms. An exclusive, necessary, but cryptic PXXPXR motif in the C terminus of Spry2 is revealed upon stimulation. The activation of Spry2 appears to be linked to sequences in the N-terminal half of the protein and correlated with a bandshifting seen on SDS-PAGE. The bandshifting is likely caused by changes in the phosphorylation status of key Ser and Thr residues following receptor stimulation. Dephosphorylation of at least two conserved Ser residues (Ser-112 and Ser-115) within a conserved Ser/Thr sequence is accomplished upon stimulation by a phosphatase that binds to Spry2 around residues 50–60. We show that human Spry2 co-immunoprecipitates with both the catalytic and the regulatory subunits of protein phosphatase 2A (PP2A-C and PP2A-A, respectively) in cells upon FGF receptor (FGFR) activation. PP2A-A binds directly to Spry2, but not to Spry2Δ50–60 (Δ50–60), and the activity of PP2A increases with both FGF treatment and FGFR1 overexpression. c-Cbl and PP2A-A compete for binding centered around Tyr-55 on Spry2. We show that there are at least two distinct pools of Spry2, one that binds PP2A and another that binds c-Cbl. c-Cbl binding likely targets Spry2 for ubiquitin-linked destruction, whereas the phosphatase binding and activity are necessary to dephosphorylate specific Ser/Thr residues. The resulting change in tertiary structure enables the Pro-rich motif to be revealed with subsequent binding of Grb2, a necessary step for Spry2 to act as a Ras/ERK pathway inhibitor in FGF signaling.

The Ras/ERK2 pathway is central to many physiological processes, and several of its key components, for example, mem-

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§ The abbreviations used are: ERK, extracellular signal-regulated kinase; Spry2, Sprouty2; FGF, fibroblast growth factor; FGFR, FGF receptor; bFGF, basic FGF; PP2A, protein phosphatase 2A; TKB, tyrosine kinase binding domain; aa, amino acids; HA, hemagglutinin; GST, glutathione S-transferase; WT, wild type; OA, okadaic acid; MS/MS, tandem mass spectrometry; WCL, whole cell lysates; KSR, kinase suppressor of Ras.
Mechanism of Action of Sprouty2

We had noted in a previous study that endogenous Spry2 partitions into two major bands when run on SDS-PAGE and that stimulation with FGF favored the faster migrating band (15). The partitioning of Spry isoforms had been reported earlier together with the demonstration that treatment of cell lysates with a phosphatase resulted in the “loss” of the slower migrating band when analyzed on SDS-PAGE with subsequent Western blotting (7). Taking this evidence into account, we postulated that a Ser/Thr phosphatase could be involved in the activation of Spry2 via selective dephosphorylation of Ser residues.

Four major classes of protein phosphatases have been described, including PP1, PP2A, PP2B (calcineurin), and PP2C. PP2A is widely expressed in mammalian cells and regulates signaling pathways by a mechanism of phosphorylation/dephosphorylation with a variety of substrates including core components of the Ras/ERK pathway such as RAF, KSR1, and ERK (18–20). The predominant form of PP2A in cells is a heterotrimeric holoenzyme. The core enzyme consists of the 36-kDa catalytic subunit (PP2A-C) and a 65-kDa regulatory subunit (PP2A-A or PR65). In addition, there are a number of regulatory subunits (B subunits) that bind to the core enzyme and confer substrate specificity to its dephosphorylating activity (17).

With the long term goal of determining the mechanism of action of Spry2 in inhibiting the Ras/ERK pathway, we sought to locate and characterize binding proteins and their sites of activity that contribute to the conformational changes necessary to activate the protein. We chose to do this with respect to FGFR activation primarily due to evidence that suggests that Spry function is predominantly associated with the FGF pathway (21). In this study, we identify a role for PP2A in the activation of Spry2.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Wild type full-length constructs of the different isoforms of Spry, FGFR1, ERK2, HA-Cbl TKB have been described previously (8, 23). The Spry2 Δ50–60 mutant was generated using standard polymerase chain reaction and molecular cloning techniques. Point mutants of Spry2 were generated by site-directed mutagenesis using the proofreading Pfu DNA polymerase from Promega (Madison, WI). The HA-tagged PP2A-C and EE-tagged PP2A-A were kindly provided by Assoc. Prof. E. Sonntag (University of Texas Southwestern Medical Center, Dallas, TX). GST-PP2A-A was purchased from Abnova (Abnova Corp., Taipei City, Taiwan).

Antibodies

Mouse and rabbit anti-FLAG, rabbit anti-HA, agarose-conjugated anti-FLAG M2 beads, rabbit anti-Sprouty2 (N-terminal), Cy3-conjugated mouse anti-β-tubulin were from Sigma-Aldrich. In addition, affinity-purified rabbit polyclonal antibodies against aa 66–80 of Spry2 were also raised (BioGenes, Berlin, Germany). Mouse anti-PP2A-A, rabbit anti-Cbl, anti-Grb2, and anti-FGFR1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-phosphoTyr (PY20), mouse anti-ERK2, panERK, c-Cbl, and Grb2 were from BD Transduction Laboratories, mouse anti-phospho ERK1/2, anti-PP2A-C, and anti-PP2A-A subunits were from Cell Signaling Technology (Beverly, MA). Rabbit anti-Spry was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Texas Red-conjugated AffiniPure rabbit anti-mouse IgG and fluorescein isothiocyanate-conjugated AffiniPure goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Alexa Fluor 647 goat anti-mouse IgG-Cy5 was purchased from Molecular Probes Inc. (Eugene, OR).

Inhibitor

Okadaic acid was purchased from Upstate Biotechnology and reconstituted in Me2SO.

**FIGURE 1.** The aa 50–60 sequence in Spry2 controls the presentation of the C-terminal PXFXKR sequence upon stimulation and the electrophoretic properties of the protein. A, schematic illustration of the various point mutants and the Spry2 Δ50–60 deletion mutant used in subsequent experiments. The enlarged letters represent the location of the point mutations. B, WCLs derived from 293T cells co-expressing HA-ERK2, FGFR1, and various FLAG-Spry constructs were immunoprecipitated (IP) with anti-FLAG (Spry2), and the precipitated proteins as well as the WCLs were analyzed as indicated. IB, immunoblotting. C, WCLs derived from 293T cells expressing WT Spry2 or Δ50–60, with or without the addition of FGFR1, were separated on SDS-PAGE and analyzed by Western blotting, as indicated.
Peptides
The human Spry2 biotinylated peptides (48–61 aa) RKKRRQRRRIRNTNEYTEGPTV(KBtnX) and the phosphopeptide RKKRRQRRRIRNTNEpYTEGPTV(KBtnX) were custom made by Sigma-Genosys.

Cell Culture, Transfection, and Inhibitor Treatments
All cell lines used in this study were purchased from ATCC (Manassas, VA). Human embryonic kidney 293T and PC12 cells were cultured, maintained, and treated as described previously (23). For acute stimulation, bFGF (Sigma-Aldrich) was used at various concentrations and different time points as described in the text. 293T cells transfected with Spry2 constructs were treated with various doses of okadaic acid for 18 h. Cell lysates were prepared and analyzed for immunoblotting as described previously (8).

Immunoprecipitation and Western Blot Analyses
Immunoprecipitation and Western blot analyses were carried out as described previously (Ref. 8 or Ref. 24) for the PP2A experiments. The sequential precipitation with anti-PP2A-A was completed four times on lysates from FGFR1-activated Spry2-transfected cells. Four subsequent immunoprecipitations were then performed on the PP2A-A-depleted lysates using anti-c-Cbl. The precipitated proteins were then resolved on SDS-PAGE and analyzed using immunoblotting protocols.

Immunofluorescence Microscopy
Immunofluorescence in PC12 differentiation experiments were performed as described previously (15, 22, 23). For the neurite outgrowth assay, PC12 cells were transfected with the various Spry constructs. 48 h after transfection, the cells were serum-starved for 24 h followed by stimulation with bFGF (50 ng/ml) for 6 days or left in serum-free medium as control.

Protein Phosphatase 2A Assay
Cell extracts from 293T cells activated with overexpressed FGFR1 or stimulated with bFGF (50 ng/ml) for 30 min and 2 h were prepared in Tris buffer containing 0.2% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, protease inhibitors, and 1 mM Na₃VO₄, pH 7.5. Free phosphate from samples was removed using a desalting column and tested for phosphate...
contamination using the malachite green assay (Upstate Biotechnologies) before proceeding with the assay. 100 μg of samples were subjected to the PP2A immunoprecipitation assay using the PP2A assay kit purchased from Upstate Biotechnologies according to the manufacturer’s recommendations.

Alkaline Phosphatase Treatment

Cell lysates of 293T cells transfected with FLAG-Spry2 were harvested in lysis buffer without sodium orthovanadate and subjected to incubation with calf intestinal phosphatase from New England Biolabs (Beverly, MA) for 2 h at 37 °C using bovine serum albumin as a control.

Mass Spectrometry

Proteolysis—Proteins were separated using SDS-PAGE, and protein bands were excised into small pieces (1 × 1 mm) and transferred to a polypropylene 96-well microtitre plate (Greiner). Gel pieces were soaked in 25 mm Tris-HCl, pH 8.5, containing 50% acetonitrile (EM Science, Gibbstown, NJ) for 24 h followed by a brief rinse with the same solution and drying in a Savant SpeedVac centrifugal concentrator (Holbrook, NY). Enzymatic digestion performed by the addition of 10 μl of 0.02 μg/μl trypsin (Promega) in 25 mm ammonium bicarbonate buffer, pH 8.5 (Sigma-Aldrich). Samples were incubated overnight at 37 °C with shaking. Peptides were extracted with 50% acetonitrile in 0.1% trifluoroacetic acid (Pierce) using a Crest sonicating water bath for 10 min (Crest, Trenton, NJ), dried down in a SpeedVac and resolubilized in a solution of 2% methanol (Fisher) and 1% formic acid (Sigma-Aldrich) and stored at −80 °C until further analysis.

Liquid Chromatography-MS/MS—Digested samples were separated using a nano-flow high-performance liquid chromatography system (LC Packings). Each sample of 7 μl was injected and concentrated onto a trap cartridge (μPrecolumn, 300 μm × 5 mm, C18 PepMap 100, LC Packings) in 0.1% formic acid in water at a flow rate of 25 μl/min. After 5 min of washing, the flow was switched in line to a resolving column (75–μm internal diameter Picotip emitter, New Objective, Boston, MA) packed in-house with 10 cm of C18 reversed-phase packing material (Column Engineering, Ontario, CA), and the flow rate decreased to 100 nL/min. A gradient was then developed from 0 to 60% acetonitrile in 0.1% formic acid over 60 min at the same flow rate. Using a liquid junction at the distal end of the column, a voltage of 2300 V was introduced to form a spray at the tip of the column. The spray was directed at the inlet orifice of a quadrupole-time-of-flight hybrid tandem mass spectrometer (QSTAR-XL, Applied Biosystems, Foster City, CA). The mass spectrometer was run in information-dependent acquisition mode to capture and fragment doubly and triply charged mass ions automatically. Selected mass ions with a minimum signal of 8 counts/s were isolated and fragmented with nitrogen gas. The collision energy used was proportional to the mass of the cations were set to include carboxyamidomethyl cysteine, oxidized methionine, and phosphorylation of serine, threonine, and tyrosine residues. A peptide mass tolerance of 200 ppm was allowed. For a protein to be positively identified, at least two peptides had to match the protein with a score equal to or above that shown to be statistically significant (p < 0.05).

Data Base Analysis—All data files obtained were searched against the mammalian subset of the UniProt non-redundant protein data base (European Bioinformatics Institute, Cambridge, UK) using the MS/MS Ion Search mode of the Mascot search engine (Matrix Science, London, UK). Variable modifications were set to include carboxyamidomethyl cysteine, oxidized methionine, and phosphorylation of serine, threonine, and tyrosine residues. A peptide mass tolerance of 200 ppm was set, and an MS/MS tolerance of 0.5 Da was set. All doubly and triply charged spectra were considered. Up to one missed cleavage was allowed. For a protein to be positively identified, at least two peptides had to match the protein with a score equal to or above that shown to be statistically significant (p < 0.05).
**In Vitro Peptide Binding Affinity Assay**

The *in vitro* binding was carried out using the PP2A-A-GST, Cbl-TKB-GST, or GST vector with the non-phospho (48–61 aa RKKRRQRRRIRNTNEYTEGPTV(KBtnX)) or phospho-hS2 biotinylated (RKKRRQRRRIRNTNEpYTEGPTV(KBtnX)) peptide. 55/H9262 GST protein was incubated with various concentrations of the peptides or buffer for control at 4 °C overnight with rotation. 15/H9262 l of streptavidin-Sepharose beads were added to each tube the following day for 2 h. The complex was then washed three times with the PP2A lysis buffer, and after the final wash, the beads were boiled in 15/H9262 l of 2× Laemmli buffer. The boiled samples were then subjected to SDS-PAGE and analyzed using immunoblotting protocols.

**RESULTS**

**A Distal Motif on Spry2 Likely Controls the Presentation of the C-terminal PXXPXR Sequence**—We have recently shown that a PXXPXR motif on the C terminus of Spry2 is necessary for the ERK inhibitory activity of the protein in the context of FGFR signaling (15). The proline-rich motif is cryptic in unstimulated cells and is revealed upon FGFR stimulation. The question we next wanted to answer was: what was the mechanism that exposed the binding sequence? From our recent work, we had shown that a mutation of Tyr-55 can affect the Grb2 binding to the distal C-terminal sequence. We thought it was possible that some or all of the amino acids that surround Tyr-55 could be involved in regulating the exposure of the C-terminal proline-rich motif. With this in mind, we constructed a mutant by deleting aa 50–60, termed Δ50–60 (Fig. 1A), as well as the indicated point mutants and combinations of these. We investigated the effects of these mutations on Grb2 binding, c-Cbl binding, and ERK inhibition. A representative result is depicted in Fig. 1B. The YR (Y55F,R309A double mutant), Δ50–60, and Δ50–60R (deletion of aa 50–60 and R309A point mutant) mutations show no ability to inhibit ERK phosphorylation, none of them bind c-Cbl as they all lack the phosphorylated Tyr-55 residue, and all show minimal or no binding to Grb2. As shown previously, the Y55F mutant has a reduced binding to Grb2 and a diminished ability to inhibit ERK phosphorylation. An interesting observation here is that the Δ50–60 mutant of Spry2 separates into two obvious protein bands (panels 3 and 7, lanes 9 and 10). We had noted in a number of previous experiments that Spry2 separates on SDS-PAGE as two distinct bands in unstimulated cells (15). To investigate the disposition of the two major Spry2 bands on SDS-PAGE, we set up an experiment where 293T cells were left unstimulated or stimulated with overexpressed FGFR1 in the presence of WT Spry2 or Δ50–60. The result of a representative experiment is shown in Fig. 1C. Notably, in the absence of stimulation (lanes 1 and 3), the two Spry2 bands contain essentially equal amounts of protein. Upon FGFR1 stimulation, the cells that contained WT Spry2 showed a significant increase in the faster migrating Spry2 band (lane 2). Conversely, in the lysates from cells with Δ50–60 transfected, there is an increase in the slower migrating band (lane 4).
These changes in band migration in SDS-PAGE provided us with a clue that certain covalent modifications are necessary for Spry2 to function as an ERK inhibitor. We therefore set up a series of experiments to investigate the cause of the “bandshifting.”

**Spry2 Is Dephosphorylated upon FGFR Activation by a Ser/Thr Phosphatase That Targets Conserved Residues**—Spry1 and 2 had previously been shown to be phosphorylated predominantly on Ser residues (7). To investigate whether the Spry2 bandshift was due to phosphorylation/dephosphorylation, lysates from unstimulated cells, expressing either full-length Spry2 (plus and minus FGFR1) or the Spry2 Δ50–60 deletion mutant, were treated with either alkaline phosphatase or an equivalent amount of bovine serum albumin as a control. It can be seen in Fig. 2A (lanes 2 and 8) that treatment with alkaline phosphatase causes the “disappearance” of the slower migrating band in these lanes, indicating that the retardation in SDS-PAGE was most likely due to phosphorylation of specific residues on Spry2.

The predominance of the faster migrating band upon FGFR1 activation in Figs. 1C and 2A indicates that a Spry2-targeting phosphatase is activated upon cell stimulation. Since the deletion of aa 50–60 abrogated the accumulation of the faster migrating band, it can be assumed that the activated Ser/Thr phosphatase binds directly or indirectly to Spry2 via this sequence or possibly that the deletion may have caused a conformational change that affects the recruitment of a phosphatase at an adjacent, or less likely, a distal site on Spry2.

The next question that arose was: where are the amino acids that are targeted by the phosphatase located? An alignment of the various mammalian Spry proteins indicated that there is an N-terminal, Ser/Thr-rich sequence that is conserved to a relatively high degree in the various mammalian Spry isoforms (Fig. 2B, upper panel). We had shown that Ser/Thr phosphorylation was potentially responsible for the multiple bands of Spry2 seen on SDS-PAGE, and one way of seeing which of these putative phospho-targets contributed to the bandshifting was to perform a series of “alanine scan” point mutations over this sequence and compare the outcome on SDS-PAGE and subsequent immunoblotting.

From the data shown in Fig. 2B (middle panels), it can be observed that a loss of the slower migrating band can be seen on Spry2 with point mutations of Ser-110, Ser-112, Ser-115, Ser-118, Ser-121, Thr-124, Ser-125, Ser-127, Ser-128, Ser-130, Ser-131, and E132A. No difference was noted with point mutations of Ser-118, Ser-121, Thr-124, Ser-125, Ser-127, Ser-128, Ser-130, Ser-131, and E132A. No difference was noted with point mutations of Ser-108, Thr-113, Ser-116, Ser-120, Thr-122, Thr-126, and Ser-129. These data are summarized in Fig. 2B (lower panel); the arrows indicate changes in the upper band, and underlined letters indicate no change.

The working hypothesis at this point is that some or all of these conserved Ser/Thr residues would be phosphorylated in unstimulated cells and would be dephosphorylated upon stimulation and there would be a subsequent structural change that exposes the proline-rich C-terminal tail. CK2 is a likely “priming” kinase that phosphorylates residues in resting cells, and its recognition site starts with an acidic Glu or Asp residue that is C-terminal to multiple serines. In Fig. 2B, we included Glu-132 in the point mutational analysis, and this results in a downshifting of Spry2 similar to some of the serine point mutations, possibly implicating CK2 as the kinase that opposes a phosphatase in the phosphorylation/dephosphorylation cycle of these conserved residues.

To identify the sites of phosphorylation on Spry2 before and after FGFR1 stimulation, we next employed immunoprecipitation followed by peptide analysis utilizing mass spectrometry. We separated the bands of Spry2 as designated in the Coomasie Blue-stained protein separation blot in Fig. 2C (upper panel). To simplify visualization of the data, in Fig. 2C (lower panel), the table shows the residues revealed to be phosphorylated in several determinations. There are two points to note. Two residues, Ser-112 and Ser-115, are shown to be phosphorylated in unstimulated cells and not to be phosphorylated in FGFR1-stimulated cells. We have shown in the previous experiment that Ser-112 and Ser-115 were two of the residues that, when mutated, caused the slower migrating band to “disappear.” However, we could not detect peptides covering the majority of the conserved Ser/Thr sequence we used for the point mutation analysis despite repeated attempts. This arose due to the technical disadvantage of mass spectrometry in that all of the protein sequence is not usually recovered. We believe, however, that Ser-112 and Ser-115 provide a proof of concept in that the mass spectrometry-derived data agree with the point mutational analysis in that the phosphorylation status of certain residues can alter the gel migratory characteristics of Spry2 and that this potential “switch” may alter the tertiary structure of the protein with the discussed consequences.

The other feature to note is that the FGFR1-stimulated Spry2 is phosphorylated on multiple sites, and these do not appear to contribute profoundly to bandshifting in preliminary experiments (data not shown). Any functional outcome of this phosphorylation is currently not determined.

**PP2A Is the Likely Phosphatase That Controls the Activation of Spry2**—We wanted to identify the Ser/Thr phosphatase that is essential for the activation of Spry2. PP2A is a ubiquitous Ser/Thr phosphatase that has been shown to control many components of the Ras/ERK pathway with RAF, KSR1, and ERK all having been identified as substrates (18–20). We therefore employed the PP2A/1 inhibitor okadaic acid (OA) in a similar

*Figure 5. PP2A and c-Cbl compete in binding to Spry2. A, an illustration of the concept whereby the overexpressed TKB domain from c-Cbl competes with the PP2A-A that binds around aa 50–60 on Spry2. B, 293T cells were transfected with FGFR1, WT Spry2, Δ50–60, and HA-TKB of c-Cbl, as indicated. Cell lysates were precipitated with anti-FLAG (Spry2), and the eluted proteins were separated by SDS-PAGE, analyzed by Western blotting techniques, and immunoblotted (IB) with the indicated antibodies. IP, immunoprecipitation. C, 293T cells were co-transfected with HA-ERK2, FGFR1, WT Spry2, or Spry2-Y55F with or without HA-TKB of c-Cbl. WCLs were analyzed by Western blotting techniques and immunoblotted with the indicated antibodies. The arrows indicate the two major Spry2 migration bands. D, the effect of TKB domain and Spry2 on bFGF-induced neurite outgrowth. PC12 cells grown on poly-1-lysine-coated coverslips were transfected with FLAG-Spry2 and the HA-TKB of c-Cbl and stimulated with bFGF as described under “Experimental Procedures.” The Spry2 protein was stained with Cy5, whereas the HA-TKB was stained with fluorescein isothiocyanate (FITC), and the cells were counterstained with Cy3-conjugated anti-tubulin. Scale bar = 20 μm.*
**Mechanism of Action of Sprouty2**

A profound dose-responsive change was observed with the increased appearance of the slower migrating form of Spry2 with increasing doses of OA (Fig. 3A). In the FGFR1-expressing cells, the changes are observed at higher doses than seen in the unstimulated control samples, which are likely due to activation of the phosphatase by FGFR1. Because the dose response observed in this experiment was in the dose range to which PP2A, but not PP1, is reported to be sensitive in cell assays, we deemed this as preliminary evidence that PP2A was the phosphatase we were seeking.

An experiment was next conducted to see whether PP2A subunits bind to WT Spry2 in comparison with Δ50–60. PP2A consists of 3 subunits: A, a scaffold; C, the catalytic entity; and B, a modulator/locator. 293T cells were transfected with a combination of WT Spry2 (S2), Δ50–60, and FGFR1 as indicated in Fig. 3B. The cells were lysed and immunoprecipitated with anti-FLAG (Spry2). From Fig. 3B, it can be seen that PP2A-A and PP2A-C complex with WT Spry2 but not with Δ50–60.

Spry2 becomes dephosphorylated upon stimulation, which means that the potential phosphatase has to be activated under the same conditions. To demonstrate that bFGF stimulation or FGFR1 expression causes an activation of PP2A in cells, phosphatase assays were performed on cell lysates as described under “Experimental Procedures.” Fig. 3C shows that FGFR1 induces an increase in PP2A activity as compared with cells with no FGFR1 overexpression or containing kinase-dead FGFR1. With bFGF treatment, there is only a marginal increase in activity seen after 30 min, but there is a significant increase after 2 h of treatment.

**Spry2 Binds Directly to PP2A-A and Interacts Endogenously—** We next performed a series of far-Western blotting experiments designed to assess whether the binding of either PP2A-A or PP2A-C to Spry2 was direct. Representative results are shown in Fig. 4, A–C. In Fig. 4, A and B, PP2A-A or PP2A-C was overexpressed in 293T cells plus or minus FGFR1. The PP2A subunits were precipitated, the lysates were separated on SDS-PAGE and Western blotted; the membranes were incubated with GST-Spry2, and any specific binding was revealed by anti-GST antibody linked to horseradish peroxidase. From Fig. 4A, it can be seen that PP2A-C does not bind directly to Spry2, whereas in Fig. 4B, PP2A-A was observed to bind directly to Spry2.

Fig. 4C depicts a far-Western blot where WT Spry2 and Δ50–60 plus or minus FGFR1 were precipitated from 293T cell lysates, and the resultant Western blots were probed with PP2A-A-GST as described under “Experimental Procedures.” From these results, it can be seen that the direct binding of PP2A-A to Spry2 increases upon FGFR1 stimulation, whereas there is only minimal binding to Δ50–60 with or without FGFR1 stimulation.

To ascertain whether endogenous PP2A-A and Spry2 were in a complex within cells, 293T cells were left unstimulated or stimulated with bFGF (20 ng/ml) for 2 h. Precipitations were performed with anti-Spry2, and Western blotting was carried out to analyze the precipitates for the partner protein. The results shown in Fig. 4D indicate that PP2A-A and Spry2 associate in cells.

**PP2A and c-Cbl Compete in Binding to Spry2—** The accumulated evidence indicated that the PP2A-A subunit binds around aa 50–60 on Spry2, and c-Cbl binding requires several amino acids within the same sequence (12). We postulated that the binding of c-Cbl and PP2A-A would be mutually exclusive with most likely different physiological outcomes. We therefore performed an experiment designed to prove part of the above hypothesis. A scheme of how this may work is outlined in Fig. 5A. We employed the TKB domain from c-Cbl that constitutes the atypical SH2 domain and adjacent 4H and EF hand domains to compete with PP2A-A. The effects of the TKB domain were compared with those seen with Δ50–60. Spry2, Δ50–60, and Spry2 together with the TKB domain of c-Cbl were transfected into 293T cells and stimulated with FGFR1. The lysates were precipitated with anti-FLAG (Spry2) and then processed for Western blotting and subsequent immunoblotting as indicated in Fig. 5B. Analysis of this result indicates that only Spry2 binds PP2A-A and C and Grb2 as compared with the Δ50–60, all of which are enhanced upon stimulation, and that binding is inhibited when Spry2 is transfected with the TKB domain of c-Cbl. This reiterates that aa 50–60 of Spry2 are essential for PP2A binding and that binding of c-Cbl, centered on phosphorylated Tyr-55, competes away the PP2A. This was also the first evidence that PP2A and c-Cbl bind to different pools of Spry2.

A similar experiment was next performed to see what the effect of eliminating PP2A binding had on the phospho-ERK inhibitory action of Spry2. It can be seen from Fig. 5C that overexpression of the TKB domain in FGFR1-stimulated cells (lanes 7–12) abrogates the ability of Spry2 to inhibit ERK phosphorylation (compare lanes 5 and 11). Most interestingly, in the presence of the TKB domain (lane 11), the majority of Spry2 is in the slower migrating band, which has a distinctly different distribution as compared with lane 5 (in Fig. 5C, the arrows indicate the major Spry bands).

To identify the physiological relevance of these observations, we next transfected combinations of the TKB domain and Spry2 into PC12 cells with or without bFGF stimulation. It can be seen in Fig. 5D that the presence of the TKB domain in the cells abrogates the inhibitory effect of Spry2 on neurite extension.

**PP2A and c-Cbl Bind to Different Pools of Spry2 and Initiate Different Physiological Outcomes—** Since PP2A-A and c-Cbl bind to overlapping sequences on Spry2, it can be assumed that their binding is competitive and mutually exclusive. Our results here suggest that PP2A activity is necessary for the preactivation of Spry2 to enable it to inhibit the Ras/ERK pathway. Currently, the only reported outcome of c-Cbl binding to Spry2 is the ubiquitination of the protein (26) and its facilitated removal from the cell. Such a model is presented graphically in Fig. 6A.

To investigate the likelihood of two pools of Spry2 we performed sequential precipitations on lysates from FGFR1-activated cells whereby PP2A-A was precipitated in multiple rounds until no more Spry2 was detected. The PP2A-A-depleted lysate was then incubated with anti-c-Cbl to precipitate the associated Spry2 (Fig. 6B). As predicted, there is a diminishing amount of Spry2 complexed with PP2A-A with each successive precipitation, and there is noticeably no c-Cbl present in these precipitated complexes. When c-Cbl is then precipitated, relatively high amounts of Spry2 are associated, but no PP2A-A is present in this complex. This provides evidence for the mutually exclusive binding of either PP2A or c-Cbl to Spry2.
To gain an insight into the relative binding affinities of PP2A-A and c-Cbl TKB to the conserved sequence encompassing Tyr-55 (phosphorylated and non-phosphorylated) on Spry2, a peptide binding experiment was performed, as described under “Experimental Procedures.” The result of a typical experiment is shown in Fig. 6C. c-Cbl TKB does not bind

**FIGURE 6.** PP2A and c-Cbl bind to different pools of Spry2 and initiate different physiological outcomes. A, a summary diagram of the competition between c-Cbl and PP2A leading to different physiological outcomes. B, sequential precipitation of lysates from 293T cells transfected with Spry2 and FGFR1. The lysates were subjected to four rounds of precipitation with anti-PP2A-A prior to four more rounds of precipitation with anti-c-Cbl. The precipitated protein was separated by SDS-PAGE before Western blotting, as indicated. IP, immunoprecipitation; IB, immunoblotting. C, in vitro peptide binding affinity assay. Panel i, Western blot analysis of the GST proteins bound to the non-phospho (48–61 aa RKKRRQRRIRNTNEYTEGPTV(KBtnX)) or phospho-hS2 biotinylated (RKKRRQRRIRNTNEpYTEGPTV(KBtnX)) peptide at various concentrations. Panel ii, a graphic representation of the phospho-peptide binding to PP2A-A-GST (blue) and Cbl-TKB-GST (pink) data. The intensity of the bands was determined by densitometer scanning, and values obtained from the binding were corrected with the representative input value and expressed as the percentage of maximum binding. The graph shows the relationship between the percentage of binding and the concentration of the peptides. D, 293T cells were transfected with Spry2 and left unstimulated or stimulated by overexpressing FGFR1. Cells were treated with increasing doses of OA overnight. The lysates were precipitated with anti-FLAG (Spry2), and the associated proteins were separated on SDS-PAGE and subjected to Western analysis as indicated.
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to the non-phosphorylated peptide, whereas PP2A-A binds with reasonable affinity. Conversely, the c-Cbl TKB binds approximately with 8-fold higher affinity to the phosphorylated peptide when compared with PP2A-A.

We next conducted an experiment to show that the activity of PP2A was related to the status of the bandshifting of Spry2 that, in turn, was related to the binding to Grb2. We again utilized OA in a dose-response regime, precipitated Spry2, and analyzed the amount of Grb2 that bound to Spry2 at different doses. The result in Fig. 6D clearly indicates a progressive increase in the amount of the slower migrating Spry2 band with increasing doses of OA (see lanes 2–7 and 9–14). In the case of FGFR1 stimulation, the proportion of Grb2 binding to Spry2 decreases with increasing concentrations of OA. Taken together, the experiments shown in Fig. 6 lend credibility to the proposed model, which represents a mechanism by which Spry2 becomes activated before it can exert its role as an inhibitor of the ERK pathway in FGF signaling.

DISCUSSION

In recent experiments (15), we have demonstrated that, in the context of FGF signaling, Spry2 causes the most profound inhibition of ERK phosphorylation as compared with other Spry isoforms, and this inhibitory function correlates with binding to Grb2 but not c-Cbl. Spry2 binds directly to the N-SH3 domain of Grb2 through a cryptic PXXPXR motif on its C-terminus (15). In this study, we have demonstrated that Spry2 is phosphorylated on conserved Ser/Thr residues in resting cells, and dephosphorylation of these residues upon FGF stimulation, most likely by PP2A, constitutes a necessary step in the activation of Spry2, leading to likely changes in protein tertiary structure to unveil the cryptic C-terminal sequence. PP2A-A binds around aa 50–60, competing with c-Cbl that binds to phosphorylated Tyr-55, which is within the same sequence.

Currently, it is believed that phosphorylation on Tyr-55 attracts c-Cbl, seemingly with the sole purpose of docking onto Spry and subsequently targeting it for destruction via ubiquitination and the endosome sorting pathway (13, 25). As c-Cbl competes for binding to an overlapping sequence with PP2A and the active phosphatase is necessary for the dephosphorylation and subsequent shape change and exposure of the PXXPXR motif, it can be seen that c-Cbl and PP2A could not bind concurrently to the same Spry molecule. In this scenario, there is a balance between protein activation and protein degradation.

It is outside the scope of this work to identify the kinase that phosphorylates the phosphatase target residues in unstimulated cells. The kinase has certain hallmarks of CK2 and/or perhaps GSK3. CK2 has over 300 substrates ascribed to it, and in many cases, it is active in unstimulated cells (26). It appears to have a role in preparing the cell for physiological processes by phosphorylating certain proteins, which fits the role it may play in phosphorylating Spry2. Recently, evidence was presented that Mnk1 targets certain Ser residues within the conserved sequence indicated in Fig. 2B, upper panel (27).

There is a precedent with other proteins involved in regulating the Ras/ERK pathway in that they are regulated in the initial stages by PP2A that dephosphorylates key residues, resulting in a change of localization or association of regulatory proteins. Both Raf and KSR are recruited to the membrane following PP2A-facilitated dephosphorylation of Ser-259 and Ser-392, respectively (19, 20). It would appear that the Spry proteins function as small scaffolds, and their own conformation and interaction with other proteins are controlled by carefully orchestrated phosphorylation and dephosphorylation. We present evidence for one possible mechanism by which Spry2 inhibits the FGFR-stimulated Ras/ERK pathway, but it is possible that the same protein can curtail the pathway by other mechanisms. What is apparent, however, is the careful control on Spry2: induced expression, intricate activation, and high affinity targeting by an E3 ligase (c-Cbl), suggesting that it has an important role in cell regulation and that its own deregulation may have profound consequences.

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