A Src Homology 3-binding Sequence on the C Terminus of Sprouty2 Is Necessary for Inhibition of the Ras/ERK Pathway Downstream of Fibroblast Growth Factor Receptor Stimulation*

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Because the Sprouty (Spry) proteins were shown to be inhibitors of the mainstream Ras/ERK pathway, there has been considerable interest in ascertaining their mechanism of action especially since a possible role as tumor suppressors for these inhibitory proteins has been suggested. We compared the ability of the mammalian Spry isoforms to inhibit the Ras/ERK pathway in the context of fibroblast growth factor receptor (FGFR) signaling. Spry2 is considerably more inhibitory than Spry1 or Spry4, and this correlates with the binding to Grb2 via a C-terminal proline-rich sequence that is found exclusively on Spry2. This PXXPR motif binds directly to the N-terminal Src homology domain 3 of Grb2, and when added onto the C terminus of Spry4 the resultant chimera inhibits the Ras/ERK pathway. The ability to inhibit neurite outgrowth in PC-12 cells correlates with the propensity of Spry isoforms and engineered constructs to inhibit the phosphorylation of ERK1/2. The PXXPR motif is cryptic in unstimulated cells, and it is postulated that Spry2 undergoes a conformational change following FGF stimulation, enabling the subsequent interaction with Grb2. We present evidence that Spry2 can compete with the RasGEF (guanine nucleotide exchange factor) SOS1 for binding to Grb2, resulting in the inhibition of phosphorylation of ERK1/2.

The gene for Drosophila Sprouty (Spry) was first discovered in a screen aimed at identifying genes and subsequent protein products that participate in the branching of the trachea in the developing fly (1). Evidence indicated that the Drosophila Sprouty gene expression was induced by stimulation of the Ras/ERK pathway downstream of the fly-equivalent of the fibroblast growth factor receptor (FGFR).2 The expressed protein, in a classic feedback inhibition, interacted with components of the originating pathway to fulfill its function. The amino acid sequence of dSpry gives few clues as to how it may function in its designated capacity. The one outstanding sequence-related feature of dSpry is a Cys-rich “domain” in the C-terminal half of the protein that is reiterated in the four mammalian isoforms (2, 3). In Drosophila, a candidate approach was employed where various proteins involved in the early phase of the Ras/ERK pathway were investigated as to whether they interacted with dSpry. Two proteins, Drk (the Drosophila equivalent of Grb2) and Gap1 (a RasGAP protein), were demonstrated to bind to dSpry, although no functional mechanism has been proven (4).

All of the mammalian Spry proteins are shorter than dSpry, yet they all retain the novel Cys-rich domain. Careful sequence alignment and comparison indicate that there are additional smaller sequences that appear conserved within the various Spry proteins; there is a tyrosine residue (Tyr-55 on Spry2) that is also flanked by conserved residues (5), and in the mammalian Spry isoforms (but not dSpry) there are a number of Ser residues that are reiterated.

Several mechanisms of action for Spry inhibition of the Ras/ERK pathway have been proposed; in the case of vascular endothelial growth factor stimulation, the various Spry and SPRED (Sprouty-related EVH1 domain-containing protein) proteins bind to Raf via a conserved sequence in the C terminus and inhibit its function (6). Another mechanism, proposed for the inhibition of the FGF-mediated activation of the ERK pathway, suggests that the SH2 domain of Grb2 recognizes and binds to the phosphorylated Tyr-55 residue of Spry2 (7). Such an interaction would compete for the binding of Grb2 to Tyr-phosphorylated residues on FRS2/Shp2 and thus derail the recognized FGF-specific ERK-activating pathway.

The interaction between the Grb2 SH2 domain and phosphorylated Tyr-55 has not been shown to occur directly and would represent a novel departure for the well characterized binding sequence of the Grb2 SH2 domain. All mammalian Spry proteins are Tyr-phosphorylated (5) and thus potentially are all capable of binding to the Grb2 SH2; but it has been a consistent observation in our laboratory that not all Spry proteins are capable of inhibiting the FGF-activated Ras/ERK pathway equally (8).

The abbreviations used are: FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; EGFR, epidermal growth factor receptor; SH, Src homology domain; PBS, phosphate-buffered saline; GST, glutathione S-transferase; WT, wild type; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; WCL, whole cell lysate.

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2 The abbreviations used are: FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; EGFR, epidermal growth factor receptor; SH, Src homology domain; PBS, phosphate-buffered saline; GST, glutathione S-transferase; WT, wild type; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; WCL, whole cell lysate.
**Mechanism of Action of Sprouty2**

Phosphorylation of the conserved Tyr-55 of Spry2 is perhaps the most universally accepted biochemical observation with respect to Spry proteins (6, 9–12). This Tyr residue is contained within a recognition sequence for the atypical SH2 domain of the Cbl isoforms. Several groups have reported that over-expression of Spry2 can inhibit the endocytosis of EGFRs (resulting in extension of ERK activation). This is thought to occur because of competition between Tyr-phosphorylated Spry2 and the Cbl-binding Tyr residue on EGFRs, whereby Cbl interacts and initiates ubiquitination that leads to the eventual compartmentalization and disarming of active EGFRs (9, 10, 11). Spry is also reported to be inactivated and tagged for destruction by Cbl-mediated ubiquitination (10, 11).

Taking into consideration the inconclusive and conflicting evidence, we sought to unravel the mechanism of action of Spry proteins as it pertains to the inhibition of the Ras/ERK pathway. We chose to do this with respect to FGFR activation, because of the mounting evidence that Spry function is associated mainly with the FGFR pathway (13).

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Wild type full-length constructs of the different isoforms of Spry, FGFR1, and ERK2 have been described previously (8, 14, 15). FLAG-T282-(1–282), FLAG-T301-(1–301), FLAG-D283-(A283–301), S4-PXAP (chimera of full-length Spry4 and last 14 amino acid residues of Spry2) were 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).

**Antibodies**—Mouse and rabbit anti-FLAG, rabbit anti-HA, agarose-conjugated anti-FLAG M2 beads, rabbit anti-Sprouty2 (N-terminal), anti-Sprouty2 (C-terminal), and Cy3-conjugated mouse anti-β-tubulin were from Sigma. In addition, affinity-purified rabbit polyclonal antibodies against amino acids 66–80 of Spry2 and last 14 amino acid residues of Spry2 were 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).

**Immunoprecipitation and Western Blot Analyses**—Immunoprecipitation and Western blot analyses were carried out as described previously (8). Quantification of phospho-ERK assay and Spry expression level was performed with the GS-800 calibrated densitometer (Bio-Rad).

**Immunofluorescence Microscopy**—PC-12 differentiation experiments were performed as described previously (14, 15). For the neurite outgrowth assay, PC-12 cells were transfected with the various Spry constructs. 48 h post-transfection, the cells were serum-starved for 12 h followed by stimulation with bFGF (50 ng/ml) for 6 days or left in serum-free medium as control.

Cells were washed 2–3 times with PBS (rocked gently for 2–3 min between each wash) and fixed on coverslips using 4% paraformaldehyde for 20 min at room temperature. Cells were then washed once with PBS, twice with PBS/100 nM NH4Cl, and once with PBS (rocked gently for 2–3 min between each wash interval). Cells on the coverslips were then permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. Permeabilized cells were then blocked with blocking buffer (PBS, 2% bovine serum albumin, 5% fetal calf serum, sterile filtered) for 1 h. About 100 μl of primary antibody diluted in blocking buffer was added (mono-FLAG (1:500)) onto coverslips of each well. Parafilm was used to lay over the coverslips to ensure the solution was well spread throughout. Primary antibody incubation lasted about 1 h at room temperature. After primary antibody incubation, the parafilm was removed, and the coverslips were washed three times with PBS. About 100 μl of secondary antibody was added Alex Fluor® 647 goat anti-mouse IgG (H+L) (mCy5 (1:200 = 10 μg/ml)). Incubation was done in the dark for 1 h. Parafilm was laid as stated above and was followed by the washing step. Finally the samples were incubated with the monoclonal anti-β-tubulin Cy3 conjugate, mCy3 tubulin (1:100 = 10 μg/ml), for 1 h in the dark. Parafilm was laid as stated above and was followed by the washing step. After completion of the washing steps, coverslips (with the stained cells on them) were transferred over to glass slides to be mounted with mounting medium (the cell surface should be in contact with the mounting medium and the coverslips should not be moved). Glass slides were placed in the dark in an enclosed box/container for about 1 day before viewing on the microscope.

**Grb2 Domain Dot Blot Assay**—A biotin-conjugated 18-amino acid peptide coding for the C terminus of Spry2 (TV-CCKVPTVPFRPRNF EKPT) and a control peptide (TVCCVK ATVAPANFEKPT) carrying alanine substitutions at the canonical residues (underlined) were synthesized commercially (NeoMPS Inc.). GST fusion proteins of Grb2 full-length and N- and C-SH3 and SH2 domains were spotted onto a nitrocellulose membrane at two different concentrations (50 and 200 ng). The membrane was first incubated in blocking buffer (3% milk and 0.1% Tween 20 in PBS) and subsequently with the biotin-conjugated peptides at a final concentration of 1 μg/ml overnight. Visualization of binding was determined by a streptavidin-horseradish peroxidase-conjugated antibody and enhanced chemiluminescence.

**Far-Western Assay**—The Far-Western assay was performed according to Dans et al. (16). HA-tagged FRS2α and FLAG-tagged Spry2 were expressed in 293T cells and immunoprecip-
tated before electrophoresis in SDS-PAGE and transfer to polyvinylidene difluoride membrane. A GST protein fused to the SH2 domain of Grb2 was incubated with the blot at 1 μg/ml. Binding was determined using a rabbit anti-GST antibody (Santa Cruz Biotechnology).

RESULTS

Spry2 Is the Most Effective Spry Isoform in Inhibiting FGFR-activated ERK Phosphorylation—Since the original study in Drosophila, there have been various reports on the ability of mammalian Sprouty proteins to inhibit the Ras/ERK pathway. Most of these studies involved only one of the Spry isoforms in a specific context. The various Sprys contain several conserved and nonconserved sequences that may reflect different physiological functions. We have previously observed that there is a considerable difference in the ability of the various isoforms to inhibit the Ras/ERK pathway (8), and thus we asked whether sequence differences could provide a lead into the mechanism of action by which this inhibition occurs.

FIGURE 1. Spry2 is the strongest inhibitor of ERK phosphorylation compared with other Spry isoforms in chronic and acute activation of FGFR. A, 293T cells were transfected with plasmids encoding HA-ERK2, FGFR1, various FLAG-Spry isoforms and mutants, and empty vector constructs. Expression of these constructs was ascertained by immunoblot (IB) analysis with anti-FLAG or anti-FGFR1. Whole cell lysates (WCLs) were subjected to Western blotting analysis to detect phospho-ERK2 using anti-phospho-ERK1/2. The same blot was stripped and reprobed with anti-ERK2 to confirm equal loading. The equal loading of lysates was additionally demonstrated by Western blotting to detect β-actin. B, graphical representation of three separate experiments similar to that depicted in A. The phospho-ERK signal was normalized to the ERK2 level and plotted as a percentage of the positive control while taking Spry expression levels into account. C and E, 293T and PC-12 cells over-expressing various FLAG-Spry isoforms were serum-starved and stimulated with 20 ng/ml bFGF at 37 °C for various periods of time as indicated. The total protein content of the whole cell lysates was equalized using the Bio-Rad Protein Assay Reagent™. Levels of phosphorylated ERK, total ERK, and FLAG-Spry were analyzed by Western blotting with the indicated antibodies. D and F, quantification was determined as described in B. Closed and open bars represent bFGF treatment for 10 and 120 min, respectively.
We first set up a comparative study whereby the ability of the various Spry isoforms could be compared directly in the context of Ras/ERK inhibition. We used both acute stimulation with FGF and over-expressed FGFR1, which is auto-activating. The latter system was employed because we thought this best mimics the longer term signaling seen in development and cancer progression.

The representative Western blot shown in Fig. 1A demonstrates that wild type (WT) Spry2 is the strongest inhibitor of ERK phosphorylation under the parameters described. Neither WT Spry1 nor Spry4 has a pronounced inhibitory effect, as can be seen in the graphical presentation derived from three separate experiments and illustrated in Fig. 1B. The various point mutants were included in this experiment, as it had been shown previously that all Spry isoforms contain a conserved tyrosine residue, which may be a key to the protein function (9).

We investigated the ability of the various Spry isoforms to inhibit ERK phosphorylation in two cell lines. 293T and PC-12 cells were stimulated with bFGF for several periods of time. It can be observed in Fig. 1, C and E, that in both cell lines and at the times shown, Spry2 exhibited the strongest phospho-ERK1/2 inhibition. The degree of inhibition is shown graphically in Fig. 1, D and F, where the data were derived from three separate experiments; this shows a similar trend to that seen with FGFR1 over-expression.

Spry2 Inhibition of ERK Correlates with Grb2 but not c-Cbl Binding—Both Grb2 and c-Cbl proteins have been shown to bind to Spry proteins (4, 7, 9). Earlier work with dSpry indicated that Grb2 may play a role in its mechanism of action in the inhibition of the Ras/ERK pathway (4). Subsequent data indicated that Grb2 binds to phosphorylated Tyr-55 on Spry2 via its SH2 domain; this interaction derails the connection to the downstream ERK pathway by diverting Grb2 away from the FGFR/FRS2/Shp-2 complex (7). We therefore analyzed the same lysates used in Fig. 1A to investigate the binding of endogenous c-Cbl and Grb2 to the Spry isoforms and their mutated Y55F equivalents. From the representative result shown in Fig. 2A some interesting points can be made: while both Spry1 and Spry2 bind to c-Cbl, Spry4 does not bind even though it is significantly tyrosine

**FIGURE 2. Inhibition of ERK phosphorylation by Spry2 correlates with Grb2 but not c-Cbl binding and requires a PXXPXR motif in the C terminus.** A, The WCLs from the above experiment were subjected to immunoprecipitation (IP) with anti-FLAG. Bound proteins were separated by SDS-PAGE and then immunoblotted (IB) with anti-phospho-Tyr PY20, anti-Cbl, anti-Grb2, or anti-FLAG. B, 293T cells were starved and stimulated with 20 ng/ml bFGF for various periods of time at 37 °C as indicated. WCLs were subjected to immunoprecipitation with anti-Spry2 or protein A-agarose beads as control and immunoblotted with anti-Cbl, anti-Grb2, and anti-Spry2. C, sequence alignment of the C termini of the four mammalian Spry isoforms. The arrowheads indicate the canonical residues (PXXPXR) on Spry2 for binding to the Grb2 SH3 domain. D, diagrammatic representation of various constructs of Spry2 and a chimera of Spry4 made to define the importance of the C-terminal 14 amino acid residues of Spry2. E, WCLs derived from 293T cells over-expressing the described constructs were prepared and analyzed as detailed in the legend for Fig. 1A.
phosphorylated; and the Tyr point mutants of Spry1(Y53F) and 2(Y55F) are still significantly Tyr-phosphorylated but have no c-Cbl binding ability. It appears that Tyr-53 of Spry4 is essentially the only Tyr residue phosphorylated on that protein, as its removal leaves little to no residual phosphotyrosine signal. Perhaps the most significant feature of the data is that only WT Spry2 binds Grb2, with none of the other isoforms or derivatives showing significant binding. The basic conclusion drawn from these data is that although the inhibitory action of the Spry isoforms in the context of FGFR1-downstream signaling does not correlate with c-Cbl binding, it does correlate with the binding to Grb2.

Although Spry2 is weakly expressed in 293T cells, we wanted to provide evidence that Spry2 and Grb2 could interact at endogenous levels. Antibodies against Spry2, with normal rabbit IgG as a control (supplemental Fig. S1), were used to precipitate the protein as described. The resultant Western blots were probed with antibodies for Spry2, c-Cbl, and Grb2 (Fig. 2B). Binding of Spry2 to Grb2 increased with stimulation, whereas its binding to c-Cbl was maximal at 10 min post-stimulation and was subsequently reduced by 2 h.

The above data indicated that Spry2 contains a unique sequence that allows binding to Grb2 in a stimulation-dependent manner. There are several reasons to believe that this is not via the Grb2 SH2 domain binding to phospho-Tyr-55. First, there is no precedent in the literature for Grb2 SH2 binding to such a sequence, and there is overwhelming evidence that it prefers the pYXN sequence (17, 18). Second, it would be expected that the Grb2 SH2 would also bind Tyr-53 on Spry1 as c-Cbl does. These criteria raised the possibility that Grb2 interacted via one or both of its SH3 domains, binding to a proline signature on Spry2 that is cryptic in unstimulated cells. The optimal Grb2 SH3-binding sequence had previously been shown to be PXX-PXR (19). Such a sequence was located on the C terminus of Spry2 and was not present on the other Spry isoforms (Fig. 2C). We next set out to provide experimental evidence that this sequence is necessary for the inhibitory action of Spry2.

To further define the sequence necessary for inhibition, constructs were made as indicated in Fig. 2D. The C-terminal 14 amino acids are represented as the abbreviated form PXX-PXR. The T282 construct arose from previous work where the translocation domain was shown to be contained between residues 178 and 282 in the Spry2 C terminus (14). T301 had the last 14

**FIGURE 3.** A PXXPXR motif on the C-terminal of Spry2 binds directly to the N-terminal SH3 domain of Grb2. A, WCLs derived from 293T cells transfected with plasmids encoding HA-FRS2 or FLAG-Spry2 in the absence or presence of FGFR1 were subjected to precipitation with anti-HA or anti-FLAG as indicated. The immunoprecipitated proteins (IP) were analyzed using the Far-Western technique described under “Experimental Procedures.” Asterisk, indicates FRS2; arrowhead (†), indicates Spry2; IB, immunoblot. B, WCLs derived from 293T cells co-expressing HA-ERK2, FGFR1, and the indicated FLAG-Spry2 mutant proteins (circles beneath) were prepared and analyzed as indicated in the legend for Fig. 1. C, GST-conjugated full-length Grb2 as well as the various Grb2 domains were spotted onto a nitrocellulose membrane at two different concentrations (50 and 200 ng) as described under “Experimental Procedures.” The membrane was incubated with the Spry2 C-terminal 14-amino acid peptide or a control peptide (where the canonical Pro, Pro, and Arg residues were substituted with alanine) and further analyzed as described under “Experimental Procedures.”
residues deleted, whereas D283 was the T282 construct with the C-terminal 14 residues fused to it. In addition, a chimera was constructed between the weakly inhibitory Spry4 and the C-terminal PXXPXR motif from Spry2 to see if the derived protein (designated as S4-PXXP) could inhibit ERK phosphorylation.

The result, shown in Fig. 2E, indicates that both T282 and T301 have lost their ERK inhibitory action, which is recovered in the D283 mutant. This indicates that the determining factor is contained within the 14 amino acids. In addition, the chimeric S4-PXXP protein acquired the ability to inhibit ERK phosphorylation and to bind Grb2 (shown later in Fig. 5).

The PXXPXR Motif on the C Terminus of Spry2 Binds Directly to the N-terminal SH3 Domain of Grb2—As it is relevant to our proposal that an SH3 domain of Grb2 interacts with Spry2 upon stimulation and not the SH2 domain, we set up an experiment to see whether the SH2 domain of Grb2 could interact with Spry2 from stimulated cells, using FRS2 as the positive control. We employed a Far-Western technique in which lysates from stimulated and nonstimulated cells were precipitated with antibodies against HA (for FRS2) and FLAG (for Spry2). The resultant Western blots were incubated with GST-tagged Grb2 SH2 followed by immunoblotting with anti-GST to ascertain direct binding. The result (Fig. 3A) shows that although the FRS2 in stimulated cells binds the SH2 domain of Grb2 directly, there is no binding evident with Spry2.

To determine whether the PXXPXR motif in the Spry2 C terminus is necessary for Grb2 binding, a series of point mutations (indicated by circles under the sequence in Fig. 3B) were constructed, and their ability to bind Grb2 or c-Cbl and inhibit ERK was assessed. A double point mutant (designated YR) consisting of Y55F and R309A was also included in this experiment. The result of such an experiment is shown in Fig. 3B. WT Spry2 and the Spry2 P314A mutant are the only constructs tested that significantly inhibited ERK activity. In line with this finding, these two constructs are the only ones in the experiment that bound to Grb2 to any degree. Significantly, point mutations at Pro-304, Pro-307, and Arg-309 (closed circles) showed decreased binding to Grb2; and these are all located within the PXXPXR sequence, whereas the proline 314 mutation (open circle) that is outside the signature sequence showed no decrease in Grb2 binding. There is equal binding to c-Cbl with the exception of Spry2 Y55F, as each C-terminal mutant has an intact Tyr-55 and surrounding sequence, shown previously to be necessary for c-Cbl binding (9). As expected, the YR double point mutant did not bind to either Grb2 or c-Cbl and had no ERK inhibitory action.

We next set out see whether Spry2 binding to Grb2 was direct and what domain of Grb2 interacted with Spry2. A peptide binding experiment was set up whereby GST-conjugated Grb2 domains were spotted onto a membrane and challenged with the Spry2 C-terminal 14-amino acid peptide or a control sequence in which the consensus Pro and Arg residues had been deleted. Results are shown in Fig. 4. For example,FLAG-Spyr4-PXXP were grown on poly-L-lysine-coated coverslips and stimulated with bFGF as described under "Experimental Procedures." The Spry isoforms were tagged with Cy5, and the cells were counterstained with Cy3-conjugated anti-tubulin. Scale bar = 20 μm.
been mutated to alanine. The results, shown in Fig. 3C, indicate that the N-terminal SH3 domain of Grb2 is capable of binding directly to the \textit{bona fide} C-terminal sequence and not to the control peptide. Neither the Grb2 C-terminal SH3 domain nor the SH2 domain bound to either peptide sequence.

**Inhibition of FGF-stimulated Neurite Outgrowth Parallels Biochemical Data**—We next investigated the ability of the various Spry isoforms to inhibit neurite outgrowth in bFGF-stimulated PC-12 cells. The Spry isoforms were stained with Cy5, and the cells were counterstained with anti-tubulin conjugated with Cy3 to better visualize the neurites. From the images shown in Fig. 4 it is apparent that Spry2 completely inhibits bFGF-stimulated neurite outgrowth. Neurites are still present without FGFR1 and various FLAG-Spry constructs were lysed and immunoprecipitated (IP) with anti-FLAG and analyzed as indicated. The WCLs were subjected to Western analysis to detect the over-expressed proteins. IB, immunoblot.

**The C-terminal PXXPXR Sequence Is Cryptic in Unstimulated Cells**—To this point we had mostly compared the binding effects of various Spry isoforms and constructs on a FGFR-stimulated background. We next compared the potential of Grb2 to bind to the various mammalian Spry proteins and selected derivatives with and without FGFR1 activation (Fig. 5). For this study, in addition to the wild type Spry isoforms, we chose the Spry2 Y55F point mutant as well as Spry2 R309A, which represents one of the Grb2-SH3 binding points. In addition, various C-terminal truncations, T301 and D283 as well as the chimeric construct S4-PXXP (illustrated in Fig. 2D), were employed. Several conclusions can be drawn from this experiment in respect to Grb2 binding: WT Spry2 shows the strongest binding on activation of FGFR1; Spry1, Spry4, Spry2 Y55F, and Spry2 R309A all show less binding to Grb2 compared with WT Spry2; the D283 deletion has close to full capacity to bind to Grb2; and the T301 construct that does not contain the binding signature does not bind to Grb2, whereas the S4-PXXP construct does bind to Grb2.

**Mechanism of Action of Sprouty2**

It is also apparent that both endogenous and over-expressed Spry2 run as multiple bands on SDS-PAGE and that stimulation by bFGF or FGFR1 favors the faster migrating species. This is suggestive of covalent changes in the protein and is the subject of ongoing research in our laboratory.

Because the binding of SH3 domains to Pro-rich sequences does not theoretically need stimulation-dependent covalent modification of any interacting residue, a logical conclusion from the above data is that the PXXPXR-containing sequence is cryptic in unstimulated cells. Spry1 and Spry4 cannot interact, pre- or post-stimulation, as neither isoform has the SH3-binding signature. Somewhat unexpectedly, the Y55F point mutant, which is distal to the C terminus, is seen to have reduced Grb2 binding when the cells are stimulated, which correlates with its impaired ERK inhibitory ability.

**Activated Spry2 Competes with SOS1 for Binding to Grb2**—We have demonstrated that the inhibitory ability of Spry2 relates to the presence of a SH3-binding PXXPXR motif in the C-terminal end of the protein that is available for binding only upon stimulation. A predicted, a corollary of this model would be that Spry2 therefore binds the N-terminal SH3 of Grb2 and competes with the proline-rich domain of the Ras-GEF SOS for this binding. We therefore set up an experiment whereby 293T cells were transfected with FGFR1 and increasing amounts of WT Spry2, Spry2 YR mutant, and Spry4. Endogenous Grb2 was precipitated, the lysates were prepared for Western blotting, and the blots were probed for SOS1, Spry, and Grb2 (Fig. 6A). It can be seen that as the amount of Spry2 protein increases in the cells, the SOS1 precipitated by Grb2 decreases in a reciprocal manner. This does not occur with either the Spry2 YR mutant or Spry4, neither of which binds Grb2 nor inhibits ERK activation. The uncoupling of Grb2 from SOS1 by Spry2 was additionally confirmed through the immunoprecipitation of FRS2, where the precipitated amount of SOS1 decreases in the increasing presence of Spry2 while the precipitated Grb2 amount remains equal (Fig. 6B). These data further indicate that Spry2 does not compete with FRS2 for Grb2 binding.

**DISCUSSION**

A number of proteins have been demonstrated to bind to Spry isoforms, and understanding the corollary of the key interactions is likely to unravel the mode of action of this family of proteins. There is strong evidence for the association of two important signaling proteins, c-Cbl and Grb2, with Spry2. In this article we have demonstrated the following: in the context of FGF signaling, among the various Spry isoforms, Spry2 causes the most profound inhibition of ERK phosphorylation, an inhibitory function that correlates with binding to Grb2 but not c-Cbl; and Spry2 binds directly to the N-SH3 domain of
of diversion seems to be that Spry2 has a PXXPXR motif on its C terminus that binds to the Grb2 SH3 domain at the expense of SOS binding to the same site. The observation that this interaction occurs only upon stimulation is an unusual one for a typical constitutively binding domain. We have provided evidence that the SH3-binding motif on Spry2 is cryptic in unstimulated cells and is revealed upon activation of FGFR.

In terms of our current observation of the importance of the PXXPXR sequence on the C terminus of Spry2 and its interaction with the N-terminal SH3 of Grb2, the diminished ERK inhibition and Grb2 binding displayed by Spry2 Y55F, which is somewhat distal from the C terminus, is unexplained but likely involves interactions between protein(s) that associate with this region and modify or interact with residues elsewhere in the protein.

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