Tyrosine phosphorylation of Sprouty2 enhances its interaction with c-Cbl and is crucial for its function

Running title: Conserved Spry motif essential for function

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SUMMARY

Mammalian Sprouty (Spry) proteins are now established as receptor tyrosine kinase- (RTK) induced modulators of the Ras/mitogen-activated protein kinase (MAPK) pathway. Specifically, hSpry2 inhibits the fibroblast growth factor receptor (FGFR)-induced MAPK pathway but conversely prolongs activity of the same pathway following epidermal growth factor (EGF) stimulation, where activated epidermal growth factor receptors (EGFRs) are retained on the cell surface. In this study it is demonstrated that hSpry2 is tyrosine phosphorylated upon stimulation by either FGFR or EGF, and subsequently binds endogenous c-Cbl with high affinity. A conserved motif on hSpry2, together with phosphorylation on tyrosine 55, is required for its enhanced interaction with the SH2-like domain of c-Cbl. A hSpry2 mutant (Y55F) that did not exhibit an enhanced binding with c-Cbl, failed to retain EGFRs on the cell surface. Furthermore, individually mutating hSpry2 residues 52-59 to alanine, indicated a tight correlation between their affinity for c-Cbl binding and their inhibition of ERK2 activity in the FGFR pathway. We postulate that tyrosine phosphorylation ‘activates’ hSpry2 by enhancing its interaction with c-Cbl and that this interaction is critical for its physiological function in a signal-specific context.
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Spry</td>
<td>sprouty</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated protein kinase</td>
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<td>GST</td>
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INTRODUCTION

*Drosophila* Sprouty (dSpry) protein was first discovered in a genetic screen for novel genes implicated in tracheal branching (1), which is under the control of various growth factors including FGF. dSpry was further shown to inhibit EGFR signaling as *dSpry* knockout mutants displayed excess photoreceptors, cone cells and pigment cells, while overexpression of dSpry mimics a loss of EGFR signaling (2). In mammals, the four Spry isoforms (Spry1-4) have been implicated as inhibitors of FGF, nerve growth factor and vascular endothelial growth factor signaling (3, 4, 5). Different genetic studies have indicated that dSpry inhibits the Ras/MAPK pathway at various points including downstream of Ras activation around the level of Raf (6), which coincides with studies on the point of inhibition of human Spry2 (3).

Mammalian Spry2 was recently shown to prolong EGF-stimulated MAPK signaling by attenuating EGFR ubiquitination and endocytosis (7, 8). Through its direct binding to the Ring Finger domain of c-Cbl (9), which is an E3-Ubiquitin ligase, hSpry2 appears to compete off the docking of c-Cbl onto the tyrosine phosphorylated EGFR. Consequently, signals emanating from the EGFR are not attenuated but rather sustained. This study illustrated the dual function of Spry proteins in modulating the intensity and duration of MAPK signaling initiated by different RTKs.

Two interesting observations were made during the course of our previous study; (a) upon stimulation of RTKs the association between c-Cbl and hSpry2 was significantly increased (b) hSpry2 was highly tyrosine phosphorylated. The question was asked whether Spry2 and c-Cbl exhibit another level of interaction following activation of RTKs, whether this centered around tyrosine phosphorylation of Spry2, and how this may impact on the modulatory effect of Spry2 on the Ras/MAPK pathway.
EXPERIMENTAL PROCEDURES

Antibodies, reagents and DNA constructs

Monoclonal antibodies and agarose-conjugated beads against HA and FLAG epitope tags were obtained from Roche Molecular Biochemicals (Indianapolis, IN) and Sigma Aldrich (St Louis, MO) respectively. Monoclonal anti-phospho-ERK1/2 (p44/p42) was from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated anti-phosphotyrosine (PY20), monoclonal antibody against ERK2 and c-Cbl were purchased from Transduction Laboratories (Lexington, KY). Anti-FGFR1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Human c-Cbl cDNA was a kind gift from Dr W. Langdon (University of Western Australia). The full length c-Cbl cDNA and the various deletion mutants were constructed and subcloned into pXJ40HA vector as previously described (9). hSpry2 mutants were constructed using the site-directed mutagenesis kit from Clontech according to the manufacturer’s instructions. The mutant products were derived using wild type FLAG-hSpry2 as template and verified by DNA sequencing.

Immunoprecipitation, western-blotting, cell culture and confocal microscopy

Immunoprecipitation, western-blotting and cell culture were performed as described previously (3). M2 (anti-FLAG conjugated agarose) beads were used for immunoprecipitating FLAG-tagged proteins, while anti-HA conjugated agarose beads were used for HA-tagged proteins. Confocal microscopy was carried out by seeding COS-7 cells onto 60mm diameter tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. At 70% confluency, cells were transfected with 1-2 µg of FLAG-tagged plasmid DNA using FUGENE™ 6 reagent (Roche Molecular
Biochemicals; Indianapolis, IN) according to the manufacturer’s instructions. On the following day, the cells were trypsinized and plated into 6-well plates containing sterilized glass coverslips at a confluency of 30%. The cells were treated with or without EGF (50 ng/ml) for 30 min before fixing in 3% paraformaldehyde in PBSCM (phosphate-buffered saline containing 10 mM CaCl₂ and 10 mM MgCl₂) buffer for 30 min at room temperature, then permeabilized with 0.1% saponin (Sigma Aldrich) in PBSCM for 15 min at room temperature. Anti-FLAG monoclonal antibody (Sigma Aldrich) was used at 1 µg/100 µl in fluorescence dilution buffer (3% BSA in PBSCM). After washing 3X with PBSCM buffer, Texas Red dye-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.; West Groove, PA) was added to the cells at 1 µg/100 µl. The coverslips were mounted using Gel-Mount and viewed with an MRC-1024 (Biorad) laser scanning confocal microscope.

Far-western assay

FLAG-tagged hSpry constructs were transfected into HEK293T cells using FUGENE™ 6 reagent. 48 h after transfection, the cells were lysed and the FLAG-tagged proteins immunoprecipitated with M2 beads, followed by SDS-PAGE and transfer onto PVDF membrane. The membrane was then blocked with 3% milk in PBST (phosphate-buffered saline containing 0.1% Tween 20), and then incubated with 1 µg/ml GST-c-Cbl protein diluted in 3% milk in PBST and 1 mM DTT for 2 h. Excess GST-c-Cbl protein was washed off thoroughly with PBST and probed with anti-GST monoclonal antibody (Santa Cruz Biotechnology), followed by anti-mouse HRP-conjugated secondary antibody (Sigma Aldrich).
Receptor down-regulation assay

EGF receptor down-regulation assay was performed essentially as previously described (7).
RESULTS

Spry2 is tyrosine phosphorylated upon stimulation resulting in an enhanced interaction with endogenous c-Cbl

Preliminary experiments indicated that hSpry2 was tyrosine phosphorylated upon stimulation by various growth factors, particularly on the relatively non-conserved N-terminal half. Point mutations replacing all tyrosine residues with phenylalanine were constructed and the following experiment was performed. The effect of EGF stimulation on these hSpry2 point mutations was studied by overexpressing FLAG-tagged hSpry2 in COS-7 cells (where relatively high levels of endogenous EGF receptors are present) followed by stimulation with EGF (50 ng/ml) for 10 min and immunoblotting the M2 (anti-FLAG) precipitated Spry2 protein with anti-phospho-tyrosine (PY20 antibody). Our results indicate that compared with wild type hSpry2, mutating tyrosine 55 to phenylalanine (hSpry2Y55F) strongly reduces the phosphorylation upon EGF stimulation (Fig. 1A; all tyrosine residues were mutated but only some of them are shown). Furthermore, the hSpry2Y55F mutant failed to interact with a 120 kDa phospho-tyrosine protein that was previously identified as c-Cbl (9). The wild type hSpry2 and phenylalanine mutants did not show any basal level of tyrosine phosphorylation as indicated by the paucity of signals in the unstimulated lanes (Fig. 1A).

The effects of FGFR activation on the hSpry2 tyrosine point mutations in HEK293T cells were next explored through the overexpression of FGFR1, which is constitutively active and hence mimics the sustained FGF signaling in developmental models. Similar to EGF stimulation, FGFR1 expression causes hSpry2 to be tyrosine phosphorylated, as are the other point mutants with the exception of hSpry2Y55F (Fig. 1B). Endogenous c-Cbl was also demonstrated to be co-immunoprecipitated with hSpry2, as were the various tyrosine to phenylalanine mutants, again with the exception of hSpry2Y55F.
Substituting phenylalanine for tyrosine results in a change of charge at the target site. To investigate whether the charge change, rather than phosphorylation, was likely to be responsible for the loss of binding to c-Cbl we further mutated Y55 to various other amino acids: alanine (neutral), proline (neutral), histidine (positively-charged) and glutamic acid (negatively-charged). These hSpry2 mutants were tested for binding to c-Cbl, and they all failed to do so (result not shown), indicating that the tyrosine residue is indeed necessary for c-Cbl interaction. These mutants also failed to be tyrosine phosphorylated when stimulated with EGF or when co-expressed with FGFR1.

We next asked whether the tyrosine phosphorylation-induced interaction resulted in direct binding between c-Cbl and hSpry2. To this end we employed a far-western assay; a GST-c-Cbl fusion protein was generated and used to overlay a PVDF blot of SDS-PAGE-resolved hSpry2 and hSpry2Y55F proteins overexpressed in HEK293T cells (Fig. 1C). The membrane was washed thoroughly to remove unbound GST-c-Cbl protein and subsequently probed with GST antibody. An intense band corresponding to the size of hSpry2 was observed in the immunoprecipitate of hSpry2 and FGFR1 co-expressing cells (Fig. 1C, lane 2), while a weaker signal was observed in the immunoprecipitate from unstimulated, hSpry2-expressing cells (Fig. 1C, lane 1). This clearly indicates that c-Cbl protein binds directly to hSpry2, both constitutively and with a significantly higher affinity for the tyrosine phosphorylated hSpry2. The mutant hSpry2Y55F (Fig. 1C, lane 3) also shows weak constitutive interaction with GST-c-Cbl with a slightly lower level of binding in the presence of FGFR1 co-expression (Fig. 1C, lane 4). The immunoprecipitate from vector-expressing cells did not bind GST-c-Cbl (Fig. 1C, lanes 5 and 6), hence indicating that the binding of c-Cbl to hSpry2 and hSpry2Y55F is specific.

We had previously characterized the constitutive binding of hSpry2 and c-Cbl (9). During these studies we observed that there appeared to be an enhanced interaction between
these two proteins following RTK activation. We were interested in ascertaining the relative strength of binding between c-Cbl and hSpry2 pre- and post-stimulation of RTKs and to investigate the likely physiological outcome of this apparent dual mode of interaction. We consistently found that the interaction between c-Cbl and tyrosine phosphorylated hSpry2 was significantly stronger than their constitutive binding. Both could be observed if sufficient protein was used in binding experiments. For clarity we tended to use lower amounts of lysates to “isolate” the phospho-tyrosine-dependent binding. Therefore, constitutive interactions between c-Cbl and hSpry2 may not be always observed unless the experimental conditions are optimal.

Tyrosine55 embedded in a conserved motif on Spry2 is necessary for enhanced c-Cbl interaction

In view of the close homology of Spry2 residues 52 to 59 among all the Spry isoforms (S/T-N-E/D-Y-T/V-E/D-X-P) (Fig. 2A), an alanine scan of these residues was performed to investigate their individual effect on c-Cbl binding. In the unstimulated state, these alanine mutants did not show any basal level of tyrosine phosphorylation and c-Cbl was not detectable in the immunoprecipitates under the chosen experimental conditions (Fig. 2B). We then studied the ability of these mutants to interact with endogenous c-Cbl upon coexpression of FGFR1. Interestingly, singly mutating residues 53 and 59 to alanine also resulted in a loss of c-Cbl interaction, similar to the hSpry2Y55F mutant (Fig. 2C). Moreover, both 53A and 59A hSpry2 mutants were tyrosine phosphorylated; notably, asparagine 53 and proline 59 (both with respect to hSpry2) are fully conserved among all Spry isoforms.

The collective data indicates that hSpry2 phosphorylation (which evidence suggests occurs on tyrosine 55), can only enhance its binding with c-Cbl when the residues at –2 (asparagine) and +4 (proline) positions relative to the tyrosine site (Y55) are both intact.
The SH2-like domain of c-Cbl binds to tyrosine phosphorylated hSpry2

c-Cbl possesses a sequence, the atypical SH2 domain, that has been characterized to bind directly to a tyrosine phosphorylated motif. We next performed an experiment to verify this interaction employing constructs of c-Cbl that were used previously to characterize the constitutive interaction with hSpry2 (9). Fig. 3A shows the c-Cbl constructs with either C-terminal or N-terminal deletions that were employed for subsequent interaction studies. FGFR1 was co-expressed with the various c-Cbl deletion mutants in the absence or presence of FLAG-hSpry2 in HEK293T cells. M2 beads were used for immunoprecipitation, followed by SDS-PAGE and western-blotting. The control experiment (Fig. 3B left panel) indicates that the c-Cbl constructs do not interact non-specifically with the M2 beads. In the presence of FLAG-hSpry2, c-Cbl constructs FL, N_R or N_O were co-immunoprecipitated (right panel; Fig. 3B) but not constructs lacking the SH2-like domain (N_S or C_R). As FLAG-hSpry2 protein in the current experiment was stimulated by FGFR1 over-expression, and hence tyrosine phosphorylated, compelling evidence (comparing the binding capabilities of N_O and N_S) indicates the SH2-like domain of c-Cbl is responsible for binding to tyrosine phosphorylated hSpry2.

As mentioned earlier there is a differential degree of binding between c-Cbl and hSpry2 in the unstimulated and stimulated conditions (Fig. 1C). The c-Cbl constructs containing the Ring Finger motif were previously shown to bind GST-hSpry2 constitutively (9). To further illustrate the existence of this constitutive interaction in-vivo, we repeated the experiment shown in Fig. 3B without FGFR1 co-expression. A similar set of c-Cbl constructs to those employed in Fig. 3A were used, with the addition of the c-Cbl construct C_O (which does not have the Ring Finger motif on the C-terminal; shown in Fig. 3C) as an appropriate control for C_R. The result depicted in Fig. 3D demonstrates that c-Cbl constructs retaining the
Ring Finger motif (FL, N$_R$ and C$_R$) can be co-immunoprecipitated with hSpry2. A larger amount of cell lysate was employed in this experiment which reinforces the notion that the constitutive interaction between c-Cbl and hSpry2 is relatively weak when compared with the phosphorylated tyrosine/SH2 interaction.

**Spry2Y55F mutant fails to retain EGFR at the cell surface**

We had previously shown that hSpry2, via an interaction with c-Cbl, inhibited the internalization of EGFRs, which subsequently led to a sustained activation of the MAP kinase pathway (7). c-Cbl has been shown to bind phospho-tyrosine 1045 on EGFR (10), which is contained within a sequence that shares close homology to the conserved Spry motif shown in Fig. 2A. A possible mechanism for the hSpry2-mediated inhibition of EGFR endocytosis is that Y55-phosphorylated hSpry2 offers an alternative binding site for the SH2-like domain of c-Cbl, which would compete for the binding of c-Cbl to tyrosine phosphorylated EGFRs and therefore subsequently inhibit receptor ubiquitination. In essence it would be the SH2/Y55 phosphorylation motif interaction that would mediate the observed EGFR inhibited endocytosis rather than the constitutive and weaker interaction between hSpry2 and the c-Cbl Ring Finger domain. In this modified hypothesis hSpry2Y55F should be unable to inhibit c-Cbl-mediated EGFR endocytosis. To this end we compared the effects of wild type hSpry2 and hSpry2Y55F on the rate of EGFR internalization. COS-7 cells were again employed in this experiment due to the high level of endogenous EGFR. As depicted in Fig. 4A, while c-Cbl-transfected COS-7 cells enhanced the rate of internalization of activated EGFR, cells doubly-transfected with c-Cbl and hSpry2 showed an impeded endocytosis, consistent with the reported role of hSpry2 (7, 8). Conversely, c-Cbl/hSpry2Y55F transfectants failed to block receptor endocytosis and the surface EGFR levels were observed to decrease at basal rates. The equal expression level of the various constructs is shown in Fig. 4B.
A prominent role of c-Cbl in regulating RTK signaling is to function as an E3-Ubiquitin ligase, acting on substrates such as the EGFR (10). We had previously shown that hSpry2 can interfere with c-Cbl ubiquitination of EGFR due to the competition for the Ring Finger domain of c-Cbl with the E2-Ubiquitin ligase, UbcH7 (7). As our current data indicates that hSpry2Y55F protein does not exhibit an enhanced interaction with c-Cbl upon RTK stimulation, this construct is therefore useful to address whether the constitutive or tyrosine phosphorylation-dependent binding with c-Cbl is important to modulate EGFR ubiquitination. HA-Ubiquitin, together with c-Cbl, hSpry2 and hSpry2Y55F constructs were transfected into HEK293T cells and the resultant SDS-PAGE-separated lysates were western-blotted and probed for the presence of Ubiquitin-conjugated proteins in EGFR immunoprecipitates, as previously described (7). Wild type hSpry2 was observed to significantly block the conjugation of Ubiquitin onto EGFR, while the Y55F mutant exhibited a reduced albeit significant ubiquitination of EGFR (Fig. 4C). This indicates that tyrosine phosphorylation of hSpry2 is a necessary step in the reduction of the c-Cbl-directed ubiquitination of EGFR and the subsequent attenuation in the endocytosis of activated receptors. The partial inhibition of EGFR ubiquitination by the Y55F mutant may be due to the previously characterized weak constitutive interaction via the N-terminal region of Spry2 with the Ring Finger domain of c-Cbl (9).

The C-termini of Spry proteins contain a cysteine-rich domain that is responsible for their translocation into membrane ruffles upon stimulation (11). The failure of Spry2 to translocate into the cell periphery is known to result in a loss of Spry2 function, including inhibition of cell proliferation and migration (12). It is possible hSpry2Y55F, because of a resultant alteration in tertiary structure, was simply unable to translocate, which would explain its inability to function like its wild type counterpart. We ascertained by employing confocal microscopy, the ability of hSpry2Y55F to translocate into membrane ruffles. In
unstimulated COS-7 cells, we observed that both wild type hSpry2 (Fig. 4Da) and hSpry2Y55F (Fig. 4Dc) exhibited strong staining that corresponded to a microtubule location, as described previously (11). Addition of EGF (50 ng/ml) for 10 min induced the translocation of both hSpry2 (Fig. 4Db) and hSpry2Y55F (Fig. 4Dd) to the cell periphery, notably membrane ruffles. It is therefore unlikely that hSpry2Y55F loses its ability to retain EGFR on the cell surface due to faulty targeting.

We had previously established a paradigm for Spry isoforms that an intact Spry translocation domain (residues 178 to 282) was necessary for function (11). Since the point mutation of hSpry2Y55F does not impact on this function it has become apparent that the phosphorylation of hSpry2 (probably on the Y55 residue) and the presence of the asparagine(-2) and proline(+4) that flank Y55 are necessary for hSpry2 interaction with c-Cbl in the context of inhibiting EGFR endocytosis. This further indicates that both the highly conserved cysteine-rich domain as well as the equally highly conserved three residues centered on Y55 (hSpry2) are essential for Spry functions.

**Tyrosine phosphorylated hSpry2 does not effect the ubiquitination of FGFR or FRS2**

As hSpry2 appears to divert a ubiquitination process away from the EGFRs we were interested to see whether there is any hSpry2/c-Cbl redistribution of ubiquitination in the FGFR system. Recently, c-Cbl was shown to partially attenuate FGFR signaling by both ubiquitination of FGFR and its necessary docker protein FRS2α (13). As these two molecules are key components of the FGFR signaling pathway, regulation of their activity or alteration of their cellular levels would be expected to have a profound effect on the magnitude of the FGFR-mediated signal. We therefore employed a similar protocol to that described for ubiquitination of EGFR (Fig. 4C) and the results of a typical experiment are shown in Fig. 5A and 5B. The conjugation of Ubiquitin molecules onto FGFR1 was clearly not affected by
hSpry2 but partially by the Y55F mutant. In the case of FRS2α, the basal level of ubiquitination was also not elevated with coexpression of FGFR1 (Fig. 5B). Overexpression of c-Cbl also did not result in enhanced ubiquitination, suggesting that the predominant E3-ligase for FRS2α may not be c-Cbl. This could account for the inability of hSpry2, which binds avidly to c-Cbl upon FGFR1 co-expression, to alter FGFR1 and FRS2α ubiquitination levels as it does for EGFR. This possibility was also eluded to in a previous study (13).

Inhibition of FGFR-induced MAP kinase inhibition by hSpry2 depends on its stimulated interaction with c-Cbl

While Spry effects EGF-pathways by binding c-Cbl, post tyrosine phosphorylation, and effecting ubiquitination targets it appears the hSpry2/c-Cbl interaction in the context of the FGFR pathway may alternatively center around the ability of c-Cbl to act as a docking protein for other proteins and the likely redistribution of protein complexes following hSpry2 tyrosine phosphorylation. We therefore addressed whether tyrosine phosphorylation of hSpry2 per se or the binding of c-Cbl was essential to the ability of hSpry2 to inhibit the FGFR-stimulated Ras/MAP kinase pathway. To address this question we employed the various alanine mutants characterized in the early part of this study: hSpry2Y55A, does not bind c-Cbl nor becomes tyrosine phosphorylated as well as hSpry2N53A and hSpry2P59A both of which are tyrosine phosphorylated but do not bind c-Cbl.

The basal activation/repression of ERK by the alanine mutants was initially assessed by probing SDS-PAGE separated and western-blotted lysates with a phospho-ERK antibody that detects the phosphorylation of p44/p42(ERK1/2) on threonine 202 and tyrosine 204 residues (Fig. 6A). FGFR1 was then co-expressed into these cells to elevate ERK activity. Interestingly, hSpry2 mutants that failed to bind c-Cbl in a stimulation-dependent manner displayed a parallel loss of ERK inhibitory function (Fig. 6B, 6C). It therefore appears that
the inhibition of the FGFR-stimulated MAPK pathway by hSpry2 likely also involves stimulated binding to c-Cbl and not solely on tyrosine phosphorylation of hSpry2 protein.

An additional ubiquitination experiment demonstrated that when Ubiquitin-incompetent c-Cbl(C381A) point mutant was transfected into 293T cells, hSpry2 was still able to attenuate the FGFR-induced MAP kinase pathway (result not shown). This indicates that the Spry-induced downregulation, while seemingly needing the activated involvement of c-Cbl does not require the inherent Ubiquitin E3 transferase activity of this associating protein to fulfill its physiological role.
DISCUSSION

Genetic evidence from several model systems that are conducive to ‘cause and effect’ analysis have indicated that Spry proteins inhibit the well-characterized Ras/MAPK pathway. Current studies in mammalian systems and cell lines demonstrate that of the four mammalian members, Spry2 is most effective in modifying this crucial pathway. While m/hSpry2 inhibits the FGFR-activated Ras/MAPK pathway, similar to the dSpry prototype, recent evidence shows that with EGFR-stimulated pathways hSpry2 actually enhances the Ras/MAP kinase signal by attenuating receptor endocytosis (7, 8).

The main targets of our current experiments were: (a) to investigate possible tyrosine phosphorylation of Spry proteins (b) to identify protein(s) that bind to hSpry2 and assist in the downregulation of the Ras/MAPK in the context of FGF-stimulated signaling or attenuate EGFR down-regulation (c) to advance towards plausible mechanisms that explain both phenomena. In this regard we established that Y55 of hSpry is potentially tyrosine phosphorylated following activation of several RTKs (EGFR and FGFR) and that this and two flanking amino acid residues (N53 and P59) located on a postulated effector loop are necessary to interact with a putative effector protein to fulfill the modulatory effects of hSpry2 on the Ras/MAPK pathway. Based on previous observations pertaining to the increased interaction of c-Cbl and hSpry following FGFR stimulation, we asked whether c-Cbl is a likely candidate for the effector protein. Strong correlative evidence presented suggests that c-Cbl may also play a role in hSpry2 inhibition of the Ras/MAPK pathway as well as the attenuation of EGFR downregulation. Point mutations of the protein are most likely to disrupt only the most pertinent interactions. It is also noteworthy that point mutations at N53, Y55 and P59 on the hSpry2 protein individually disrupt post-FGFR-stimulated binding to c-Cbl, and each mutation correlates with an inability to inhibit the
Ras/MAPK pathway. Previously Sasaki and coworkers (14) have also shown Spry2Y55A to be a dominant negative mutant that failed to inhibit FGF-induced ERK activation. Our current observation provides a cogent explanation as to why Spry2Y55A should function as a dominant negative mutant.

From the assembled evidence two models can be proposed that may shed light on the mechanism of action for hSpry2 in respect to (a) the retention of EGFRs on the cell surface and (b) the inhibition of the FGFR-induced Ras/ MAPK inhibition.

A summary of the ‘normal’ activation/downregulation of the EGFR in respect to the MAPK pathway can be described as follows. Binding of EGF induces receptor dimerization, which subsequently causes trans-phosphorylation of various tyrosine residues on the intracellular domain of EGFR. Two of these are described for illustrative purposes; Y1068 is a canonical target for the SH2 domain of Grb2 (YXN) and when phosphorylated results in the linked-activation of MAP kinase (15). At the same time another residue Y1045, when phosphorylated is a well-characterized binding motif for the SH2 domain of c-Cbl (10). The intimate association with c-Cbl allows for tagging of the EGFR with polyubiquitin, which further down the endocytic pathway, becomes the recognition signal for protein destruction (10). The Ring Finger domain of c-Cbl in this context fulfills its role as an E3-Ubiquitin ligase, accepting Ubiquitin moieties from an E2-Ubiquitin conjugating enzyme, which has been characterized as UbcH7 in the EGFR context. There is thus a balance between activation and attenuation, involving the covalent modifications of phosphorylation and ubiquitination on the signal-instigating EGFR.

It has been shown that the hSpry2 gene is induced as a result of MAPK stimulation (16). In the activated context hSpry2 would target PtdIns(4,5)P2 in the plasma membrane (17). Both hSpry2 and EGFR had been shown to be enriched in membrane ruffles (11). hSpry2 in this location also becomes tyrosine phosphorylated, either directly or indirectly by
the RTK and the Y55 residue offers an alternative site for the SH2 domain of c-Cbl to bind to. It is predicted that ‘activated’ hSpry2 therefore diverts the Ubiquitin destruction tag away from the EGFR, which is subsequently retained on the surface of the cells, with the Ras/MAPK pathway in the ‘switched on’ state (7, 8). hSpry2 itself is also a likely substrate of c-Cbl (see footnotes).

The weaker constitutive binding between hSpry2 and c-Cbl may play a role in positioning the interacting partners in close proximity to each other prior to activation. There are a number of examples in the cellular context where low affinity binding precedes a more definitive and physiologically relevant high-affinity interaction.

The proposed model for the interaction of hSpry2 with the FGFR system is currently incomplete but demonstrates clear differences from the EGFR system. The current best understanding of the upstream activation of the Ras/MAPK pathway comes from a series of publications from the Schlessinger laboratory (18, 19). There are four phospho-tyrosines on FRS2α possessing a canonical Grb2-SH2 binding motif (18) and two downstream phospho-tyrosines possessing a canonical binding site for the SH2 domain of the Shp2 tyrosine phosphatase (19). According to current concepts the majority of the MAPK activation pathway comes from the Shp2 connection, which then binds Grb2 and subsequently involves downstream molecules similar to those of the EGFR pathway. It is proposed that where the SH2 domain of Grb2 binds directly to FRS2, the SH3 domain binds to c-Cbl via a SH3/proline-rich binding interaction (13). c-Cbl competes with SOS for the N-terminal SH3 domain of Grb2 and thus nullifies signaling to the Ras/MAPK pathway. This represents another example of the balance of ‘off’ and ‘on’ signals juxtaposed on the same signaling molecule.

When hSpry2 appears on the scene in the FGFR context what happens next is less clear. The targeting into a receptor proximal location will be similar to that with the EGFR
‘Activated’ hSpry2 binds to c-Cbl and then may inhibit the activation of the
Ras/MAPK pathway via any of a number of plausible mechanisms; (a) by sequestering c-Cbl
away from FRS2 there may be some destabilisation of the signaling balance eluded to above,
(b) c-Cbl, functioning as a docker protein (20), may sequester a key effector of the MAPK
pathway or bring a regulator into a favourable location to fulfill its function. The evidence
presented in this manuscript indicates that ubiquitination diversion is less likely to play a role
in the FGFR signaling modification brought about by the strategic positioning of ‘activated’
hSpry2, although FGFR, FRS2 and hSpry2 are all ubiquitinated. However, it appears that the
interaction of hSpry and c-Cbl, is still central to both proposed mechanisms.

Sequence alignment and analysis of all the Sprys from various species ranging from
Drosophila to man indicates that two domains are stringently conserved. The first is the Spry
translocation domain that includes the majority of the C-terminal cysteine rich domain and
includes a R252 residue (also 100% conserved) that has been shown to be essential for
binding the PtdIns(4,5)P2 (17). The second is the NXY*XXXP motif (where Y* represents a
potential phospho-tyrosine site) that is essential for Spry2 functionality. Currently the only
known interacting protein with this sequence is c-Cbl. Various signaling proteins have been
implicated as being involved in the function of Spry isoforms (21, 22). A direct effect on
FRS2, for instance would provide a feasible target with respect to FGFR signaling as it is
strategically placed in originating and modifying the disposition of all downstream signaling.
FRS2 however is not found in Drosophila and if a universal mechanism of hSpry2 function
exists the ubiquitoustous Cbl family may be more plausible as partners at the center of Spry
function.
REFERENCES


FOOTNOTES

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NOTE ADDED IN PROOF

While this manuscript was under review, Hanafusa et. al. (2002) [Nat. Cell. Biol. 4, 850-858] reported the FGF-stimulated phosphorylation of Spry2 on the same tyrosine residue as described in this paper. At the same time, Rubin et. al. (2003) [Curr. Biol. 13, 297-307] and Hall et. al. (2003) Curr. Biol. 13, 308-314 also reported that Spry2 is a target for c-Cbl mediated-ubiquitination and degradation.
FIGURE LEGENDS

FIGURE 1

**hSpry2 is tyrosine phosphorylated upon stimulation by RTKs which subsequently enhances its interaction with endogenous c-Cbl**

A, *hSpry2 is phosphorylated and associates with a 120 kDa protein (p120) upon EGF stimulation.* Wild type FLAG-tagged hSpry2 (WT) and the various phenylalanine mutants were transfected into COS-7 cells. 48 h post transfection, the cells were stimulated with EGF (50 ng/ml) for 10 min (+) or left unstimulated (-). M2 (anti-FLAG agarose-conjugated) beads were added to the lysates to immunoprecipitate (IP) the FLAG-tagged proteins. The precipitated proteins were resolved on SDS-PAGE and immunoblotted (IB) for phosphotyrosine (PY20) and FLAG-tagged Spry proteins. B, *FGFR1 induces tyrosine phosphorylation of hSpry2 and association with endogenous c-Cbl.* HEK293T cells were transfected with the various FLAG-hSpry2 mutants in the absence or presence of FGFR1. The lysates were treated as before and the resultant blots were probed with the indicated antibodies. C, *c-Cbl binds directly to tyrosine phosphorylated hSpry2 with greater affinity than the non-phosphorylated form.* FLAG-hSpry2, FLAG-hSpry2Y55F and FLAG pXJ-40 vector (control) were transfected into HEK293T cells in the absence or presence of FGFR1. The far-western assay was performed as described in Experimental Procedures.
FIGURE 2

A conserved motif on hSpry2 together with tyrosine phosphorylation are responsible for binding to c-Cbl

A, amino acid sequence alignment of the various Spry isoforms indicates a conserved motif in the region around tyrosine 55. The asterisks indicate conserved residues among all the isoforms. B, residues 52 to 59 of FLAG-hSpry2 were singly mutated to alanine (52A to 59A) which show no basal tyrosine phosphorylation. The mutants were expressed in HEK293T cells and immunoprecipitated with M2 beads. hSpry2 wild type (WT) was used as a control. PY20 and c-Cbl antibodies were used to probe for the presence of phospho-tyrosine and c-Cbl proteins respectively. C, FGFR1 induces tyrosine phosphorylation of all alanine mutants except 55A. FGFR1 was coexpressed with all the alanine mutants (52A to 59A) of FLAG-Spry2. Tyrosine phosphorylation of the alanine mutants and presence of endogenous c-Cbl in the anti-FLAG immunoprecipitates were determined as before. Similar expression levels of FGFR1 was shown by immunoblotting with anti-FGFR1.

FIGURE 3

Tyrosine phosphorylated hSpry2 binds to the SH2-like domain of c-Cbl

A, a schematic representation of HA-tagged full-length c-Cbl (FL) and truncation constructs Cbl-NR, Cbl-NO, Cbl-NS and Cbl-CR. 4H, four-helix bundle; EF, calcium-binding motif; PRO, proline-rich domain; LZ, leucine zipper motif; RF, Ring Finger motif. B, the SH2-like domain of c-Cbl interacts with tyrosine phosphorylated hSpry2. HA-tagged c-Cbl truncation constructs were coexpressed with FGFR1 in HEK293T cells in the absence (left panel) or presence (right panel) of FLAG-hSpry2. The lysates were immunoprecipitated (IP) with M2 beads and immunoblotted (IB) with PY20 and HA antibodies. Expression of hSpry2 and FGFR1 proteins were confirmed by FLAG and FGFR1 antibodies respectively. Asterisk
refers to the pertinent band. C, a schematic representation of HA-tagged Cbl-C. D, the Ring Finger domain of c-Cbl interacts with hSpry2 constitutively. HA-tagged c-Cbl truncation constructs were expressed in HEK293T cells in the presence or absence of FLAG-hSpry2. The lysates were immunoprecipitated (IP) with M2 beads and immunoblotted as before. Asterisk refers to the pertinent band.

FIGURE 4

hSpry2 modulation of EGFR endocytosis and ubiquitination is dependent on Y55

A, hSpry2, but not hSpry2Y55F, inhibits c-Cbl-mediated EGFR downregulation. COS-7 cells were transfected with 5 µg plasmid encoding c-Cbl alone (∇, open triangles), or together with either 4 µg hSpry2 (●, closed circles) or hSpry2Y55F (▲, open squares) per 100mm dish before sub-culture into a 24-well plate. At 48 h post-transfection, duplicate wells were incubated with EGF (100 ng/ml) at 37 °C for various time intervals as indicated. Unbound EGF was then washed off, and the levels of surface EGFR determined by a competitive binding assay with 125I-EGF (see Experimental Procedures). Control cells were not exposed to EGF (■, closed squares). The average of duplicate determinations ± standard error mean was expressed as the percentage of total radioactivity at time = 0 min. The experiment was repeated twice. B, a western-blot analysis demonstrating the various protein expression. C, hSpry2Y55F fails to inhibit c-Cbl ubiquitination of EGFR. 1 µg HA-Ubiquitin and 1 µg myc-tagged EGFR cDNA were transfected into 60mm dishes of HEK293T cells together with hSpry2/Y55F constructs (1 µg) or c-Cbl (1 µg) or alone (control). 48 h post-transfection, 3 µg myc antibody and protein A/G beads were used to immunoprecipitate EGFR from the cell lysate. The immunoprecipitates were resolved on 7.5 % SDS-PAGE and immunoblotted for Ubiquitin using HA antibody. Expressions of the various transfected plasmids were shown using the indicated antibodies. D, hSpry2Y55F translocates to the cell membrane upon EGF
FLAG-tagged hSpry2 and hSpry2Y55F were transfected into COS-7 cells and serum-starved for 16 h. EGF (50 ng/ml) was added to designated wells to stimulate the endogenous EGFR for 10 min. Subsequently, the ligand was washed off with PBS and the cells fixed and stained for hSpry2 using FLAG antibody as stated in Experimental Procedures.

FIGURE 5
Ubiquitination of FGFR1 and FRS2α are not affected by hSpry2 expression

A, ubiquitination of FGFR1 is not affected by hSpry2. 1 µg HA-Ubiquitin was transfected into 100mm dishes of HEK293T cells together with hSpry2/Y55F constructs (3 µg) or c-Cbl (3 µg) in the absence or presence of FGFR1 (1 µg). 48 h post transfection, 3 µg FGFR1 antibody and protein A/G beads were used to immunoprecipitate FGFR1 from the cell lysates. The immunoprecipitates were resolved on 7.5 % SDS-PAGE and immunoblotted for Ubiquitin using HA antibody. Expression levels of the various transfected plasmids were compared using the indicated antibodies. B, FRS2α shows a basal ubiquitination level that is not modulated by hSpry2 overexpression. 1 µg HA-Ubiquitin and 1 µg FRS2α cDNA were transfected into 100mm dishes of HEK293T cells together with hSpry2/Y55F constructs (1 µg) or c-Cbl (1 µg) with and without FGFR1 (1 µg). FRS2α antibody was used for precipitation and the samples were treated as before.

FIGURE 6
c-Cbl interaction is required for hSpry2 inhibition of FGFR-stimulated ERK activity

A, effect of hSpry2 alanine mutants on the basal ERK2 activity. hSpry2 wild type (WT) and alanine mutants (52A to 59A) were cotransfected with ERK2 into HEK293T cells. 48 h post-transfection the cell lysates were resolved by SDS-PAGE and probed for levels of phospho-
ERK. Equal expression of ERK2 and hSpry2 and were determined by immunoblotting the SDS-PAGE-separated cell lysates with antibodies against ERK2 and FLAG, respectively. B, *inhibition of FGFR1-activated ERK2 activity by hSpry2 alanine mutants.* hSpry2 wild type (WT) and alanine mutants (52A to 59A) were cotransfected with FGFR1 and ERK2 into HEK293T cells and the lysates treated as before. Equal expression of FGFR1 was determined by immunoblotting the cell lysate with antibodies against FGFR1. The maximum level of ERK2 activation by FGFR1 is shown in lane 1 (Control) without hSpry2 coexpressed. C, a histogram of the phospho-ERK2 levels from 3 independent experiments from Fig. 6B is presented here as % average ERK2 stimulation ± standard error mean (control, C = 100% stimulation). The basal (unstimulated) level of ERK activity was found to be below 5% from 3 independent experiments.
Figure 1

C

FGFR1:

IB:
- α-GST (c-Cbl)
- PY20
- α- FGFR1
- α-FLAG (hSpry2)

hSpry2
- +
hSpry2 Y55F
- +
Control
- +

Cell Lysate

IP: FLAG
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Figure 3

C

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\begin{array}{c}
C_o & 437 & \text{PRO} & \text{PRO} & \text{LZ} & 906 \\
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D

IB:

- \( \alpha\text{-HA (c-Cbl)} \)

- \( \alpha\text{-FLAG (hSpry2)} \)

IP: FLAG

Cell Lysate
Figure 4

C

IB:

α-HA (Ub)

α-Myc (EGFR)

α-c-Cbl

α-FLAG (hSpry2)

EGF 10 min

Unstimulated Control c-Cbl hSpry2 hSpry2Y55F

IP: Myc

Cell Lysate

D

Unstimulated EGF 10 min

hSpry2

hSpry2Y55F
Figure 6

A

IB:

Unstimulated

Control  WT  52A  53A  54A  55A  56A  57A  58A  59A

α-phospho-ERK2

α-ERK2

α-FLAG (hSpry2)
Figure 6

B

IB:

α-phospho-ERK2
α-ERK2
α-FGFR1
α-FLAG (hSpry2)

C

Relative % ERK2 stimulation
Tyrosine phosphorylation of sprouty2 enhances its interaction with c-Cbl and is crucial for its function
Chee Wai Fong, Hwei Fen Leong, Esther Sook Miin Wong, Jormay Lim, Permeen Yusoff and Graeme R. Guy

J. Biol. Chem. published online June 18, 2003

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