REVIEW

Sprouty proteins: modified modulators, matchmakers or missing links?

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Abstract

Sprouty proteins are involved in organogenesis, particularly during the branching of endothelial tubes, and existing evidence suggests that Sprouty's point of action lies downstream of receptor signaling to inhibit the activation of the central Ras/Erk pathway. How Sprouty proteins accomplish their inhibitory action and whether they interact with other signaling pathways are significant questions. Sprouty proteins are devoid of any recognizable protein interaction domain, and clues as to how they function have been mainly derived from screening for interacting partners. Conserved across all the Sprouty proteins are three sequences: a Cbl-tyrosine kinase-binding (TKB) binding motif centered on an obligatorily phosphorylated tyrosine (Y55 in Sprouty2), a serine-rich motif (SRM) and a cysteine-rich domain (CRD). With the exception of a handful of proteins that bind to the N-terminus, most of the binding to Sprouty occurs via the

CRD, predominantly by serine/threonine kinases that target sites within the SRM on Sprouty. Some of the resultant increase in phosphorylation is opposed by activated protein phosphatase 2A that binds to the N-terminal Cbl-TKB binding motif. Significantly, two ubiquitin E3 ligases also bind to the N-terminus of Sprouty: c-Cbl binds with high affinity to the TKB binding motif and SIAH2 binds constitutively to a different site; both proteins are able to direct the ubiquitination of Sprouty proteins and its destruction. The collective evidence points to Sprouty proteins as being substantially covalently-modified to control its location, stability, association, and destruction. With such stringent control of the Sproutys, the main question is what key proteins does this facilitator bring together?

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Introduction

The nature and number of intracellular pathways responsible for transmitting cues from the external environment into a physiological response was literally a 'black box' of knowledge deficiency, 20 years ago. Since then, the field of cell signaling has occupied 30–50% of the cell biology research literature, and is gaining an ever-expanding insight into the complexity of transduction pathways employed by the cell. Marrying information garnered from *Caenorhabditis elegans* and *Drosophila*, the first signaling pathway – where a rational and continuous connection was made between growth factor/receptor engagements to the transcription of a subset of genes downstream – was the receptor tyrosine kinase (RTK)/Ras/ERK pathway. Although this pathway achieved fame as the first to be delineated, it still maintains a high profile in signaling (Pearson *et al.* 2001, Roux & Blenis 2004, Murphy & Blenis 2006), and ensuing research has demonstrated that each of the core components in the pathway is subjected to multiple positive and negative signals and can be clustered together by a range of docker and scaffold proteins in a temporal or spatial

manner within the cell (Brown & Sacks 2009). Moreover, many aberrations in this pathway are most epitomized by cancer, particularly in three of the central players, RTKs (e.g. epidermal growth factor receptor, EGFR), Ras and Raf. Each of these proteins has a high gain-of-function mutational incidence (Dhillon *et al.* 2007) and therefore, inhibitors of the RTK/Ras/ERK pathway are strategically placed to control this indisputably key pathway in cellular function. Two families of inhibitors of the pathway have emerged in the last decade, termed Sprouty and Sprouty-related Ena/VASP homology 1 (EVH1) domain containing protein (SPRED), and are the focus of research for a number of groups.

Sprouty and Spred

The founding member of the Sprouty (Spry) family was discovered in a screen for genes in *Drosophila* that were responsible for shaping the developing trachea (Hacohen *et al.* 1998). The sole *Drosophila* Spry protein was demonstrated to inhibit the RTK/Ras/ERK pathway downstream of several

different growth factors (Casci *et al.* 1999). Subsequent studies revealed four mammalian SPRY proteins with one of them, SPRY2, playing a similar role to *dSpry* in modeling the branching of the mammalian lung (de Maximy *et al.* 1999, Tefft *et al.* 1999). There were few clues from the sequences of the SPRY proteins as to their likely mode of action, with initial analyses indicating no likely enzyme motifs and no conserved protein–protein interaction domains. The family members contained a conserved but novel cysteine-rich domain (CRD) that was later found reiterated in the SPRED family of proteins (Wakioka *et al.* 2001).

Sprouty proteins are ubiquitously expressed in the developing embryo as well as in adult tissues, with the exception of SPRY3, which has a more restricted distribution in the brain and the testis (Minowada *et al.* 1999, Leeksa *et al.* 2002). Evidence from gene knock-out studies demonstrate SPRY's role in the formation of a number of tissues and organs during mammalian development (summarized in Supplementary Table 1, available in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/cgi/content/full/JOE-09-0110/DC1>). Presently, the major role assigned to SPRY is to inhibit Ras/Raf/ERK signaling and SPRY proteins have been shown to do this downstream of a wide range of growth factor stimuli, including fibroblast growth factor (FGF), vascular-endothelial growth factor, platelet-derived growth factor (PDGF), hepatocyte growth factor, glial-derived growth factor, and nerve growth factor (NGF; reviewed in Guy *et al.* 2003, Li *et al.* 2003, Kim & Bar-Sagi 2004, Mason *et al.* 2006, Cabrita & Christofori 2008). Interestingly, the inhibitory function of SPRY is both cell and ligand specific, as SPRY does not inhibit MAPK activation in response to EGF signaling (Sasaki *et al.* 2001). Despite a mound of literature dedicated to SPRY's activity, a universal mechanism as to how SPRY accomplishes its action is yet to be confirmed, and most of the evidence comes from cell culture and over-expression-derived data. While SPRY proteins exercise their effects by directly interacting with other proteins, their own levels can also be controlled at various points of transcription and translation. Indeed, SPRY1 levels in myocardial fibroblasts can be controlled at the level of translation by microRNA-21 leading to increased MAP kinase levels concomitant with the onset of cardiovascular disease (Thum *et al.* 2008).

Owing to its shared interaction domain with Sprouty proteins, a brief introduction of the SPRED protein family is necessary (a more comprehensive review can be found elsewhere (Bundschu *et al.* 2007)). Prior to the discovery of *dSpry*, a gene called *ae33* was discovered in a screen for *Drosophila* eye development (DeMille *et al.* 1996). The predicted protein from this transcript showed strong homology to enabled (Ena) and vasodilator-stimulated phosphoprotein (VASP) in its N-terminus, and a novel cysteine-rich (cys-rich) region in its C-terminus. Some years later, in a yeast two-hybrid screen for interacting partners to the active kinase domain of c-Kit and c-Fms receptors,

Wakioka *et al.* (2001) isolated a novel protein from a mouse osteoclast cDNA library that they designated SPRED1 in reference to its N-terminal EVH1 domain and its Cys-rich C-terminus reminiscent of the CRD that was at the time unique to Sprouty.

There are four mammalian SPRED proteins: SPRED1–3 and EVE-3 (a splice variant of SPRED3; Wakioka *et al.* 2001, Kato *et al.* 2003, King *et al.* 2006). Common to both SPRED1 and SPRED2 are the EVH1 and CRD, separated by a highly divergent sequence encompassing the c-Kit binding domain (KBD) to which the kinase domain of c-Kit receptor binds. SPRED3 lacks a functional KBD and consequently cannot bind to c-Kit. SPRED1 and 2 are tyrosine phosphorylated by stem cell factor, PDGF, and EGF and, like SPRY, SPRED can inhibit ERK phosphorylation stimulated by NGF, and FGF (Wakioka *et al.* 2001, Bundschu *et al.* 2007). It is presently unclear how SPRED proteins differ from SPRY proteins in exercising their respective inhibitory effects on the Ras/ERK pathway, however, studies have shown that, under certain conditions, both the EVH1 and the CRD are indispensable for this function (King *et al.* 2005). SPRED3 shows less inhibitory activity than SPRED1 and 2, suggesting that a functional KBD may also be required. Interestingly, the liver-restricted EVE3 contains a single EVH1 domain and, like SPRED, is capable of inhibiting Ras/ERK signaling (King *et al.* 2006). This emphasizes the relative importance of the EVH1 domain for SPRED proteins in their inhibitory function. As yet, no *bona fide* interacting partner for the EVH1 domain of SPRED has been identified, while a number of proteins bind to the common CRD of both SPRED and SPRY family members. Complicating matters further, both SPRED proteins can homodimerize and heterodimerize via the CRD (King *et al.* 2005), and the possibility remains that SPRED and SPRY proteins can heterodimerize via the same mechanism.

The major mode of action of the SPRY family of proteins in cell signaling remains elusive, and while interacting partners for SPRY are constantly unveiled, we are still at a loss to locate some 'universal rules' on how such complexes work in a physiological context. Here, rather than simply summarizing the present SPRY literature, we endeavor to surmise the likely function of the SPRY proteins, particularly SPRY2, using the relevant findings presently put forward in the SPRY field to support these assumptions and predictions. The review is primarily limited to assess the effect of proteins that have substantially been shown to bind directly to SPRY proteins or directly modify them.

Common sequence motifs delineating SPRY functions

For signaling proteins that lack a presently-recognized interaction domain, their function may be implied by identifying proteins that associate directly with them: the 'guilty-by-association' approach. This is usually matched with

a detailed analysis of common sequence motifs within the family of proteins and, if such a motif has been conserved throughout evolution, it can be assumed that it is vital to the function of the proteins in which it is found. Such an analysis of the *Drosophila* and four mammalian SPRY proteins, combined with prediction and experimental evidence, aided in the identification and characterization of several conserved sequences, of which three are now well-defined (Fig. 1): 1) the canonical Casitas B-lineage lymphoma (c-Cbl) tyrosine kinase-binding (TKB) motif centered around a key tyrosine residue (Y55), 2) the serine-rich motif (SRM) and 3) the cysteine-rich domain (CRD; also referred to elsewhere as the SPR domain or the translocation domain).

The canonical Cbl-TKB binding site

Early experimental evidence indicated that SPRY2 was tyrosine phosphorylated upon stimulation and that the tyrosine residue at amino acid position 55 was the main target (Sasaki *et al.* 2001, Fong *et al.* 2003, Mason *et al.* 2004). Prior to this,

SPRY2 was observed to bind to the c-Cbl E3 ubiquitin ligase as a consequence of FGFR and EGFR activations (Wong *et al.* 2001) and it later emerged that this same Y55, when phosphorylated, was in the center of the canonical Cbl TKB motif: N-X-Y(p)-S/T-X-X-P (Lupher *et al.* 1997). The TKB domain is structurally an SH2 domain in association with a 4H domain and an EF-hand, and completes its unique binding capabilities through the interactions of these subdomains with the substrate protein (Meng *et al.* 1999). This motif was one of three known Cbl-TKB binding motifs, and proteins were classified into groups based on these derivative consensus sequences. Crystallographic evidence now suggests that Cbl in fact recognizes a less-radical binding motif, with N-X-Y(p) or R-Y(p) essential for binding, and the other conserved residues required to enhance the binding affinity (Ng *et al.* 2008). It is interesting to note that the targeted proteins are either receptor and non-RTK kinases (e.g. EGFR and Lck) or adaptor proteins such as the members of the APS family. Some of the binding proteins are Cbl-mediated ubiquitin targets (c-Met; Peschard *et al.* 2001)

Figure 1 The conserved motifs/domains of Sprouty proteins. The three conserved sequences on Sprouty proteins are indicated: the cysteine-rich domain (shaded in light and dark blue), the serine-rich motif (conserved S/T residues are shaded in green), and residues from the canonical Cbl TKB binding site (shaded in red). Several other sequences that are conserved without any assigned function are shaded in grey. The proline motif on Sprouty2 that binds SH3 domains is shaded in pink.

Table 1 Cbl tyrosine kinase-binding (TKB)-binding motifs

	-2	0	+4
	NX	Yp	SXXP
	D		T
ZAP-70	TLNSD	Gyp	TPEPA
P75NTR	KGDGN	LYp	SSLPL
SRC/FYN	LIEDN	Eyp	TARGQ
CSF-1R	LLQPNN	Yp	QFC
SYK	TVSFPN	Yp	PEPELA
mSPRY2	IRNTN	Eyp	TEGP
mSPRY1	IRGSN	Eyp	TEGPS
mSPRY4	SHVEND	Yp	VDTPL
dSPRY	ERLTN	Eyp	VDTPL

The amino acid motifs on various proteins have been shown experimentally to bind to the TKB domain of Cbl protein. The consensus motif is shown on the top of the table with the phosphorylated tyrosine designated by convention as the zero reference point. The motifs in the top half of the table all come from tyrosine kinases and those in the bottom half from Sprouty family proteins. There are two other groups of TKB-binding motifs (not shown) that have derivative binding sequences.

while others apparently utilize Cbl as a docker protein (APS; Hu & Hubbard 2005).

With respect to the original, ‘ideal’ canonical binding sequence, Table 1 shows that four proteins – ZAP70, p75NTR, SPRY1 and SPRY2 – each retain the four conserved amino acids. This being the ‘ideal’ motif is reflected in the strength of binding ascertained for SPRY2 over other TKB binding peptides that dispensed with one or more of the conserved residues (Ng *et al.* 2008). The crystallographic results demonstrated that the phosphorylated tyrosine and the proline in the +4 position (relative to the central tyrosine) occupies a positively-charged pocket and a shallow hydrophobic cleft on the TKB domain of Cbl respectively (Fig. 2). However, this proline residue was deemed non-essential for

binding; rather it contributes to the overall affinity of the phosphorylated peptide for the TKB domain. Similarly, the conserved serine/threonine residue at the +1 position is also employed only to increase the binding affinity, as verified by SPRY2’s higher affinity for Cbl over SPRY4 which lacks this residue. Interestingly, there is recent evidence to suggest that this threonine residue in SPRY is phosphorylated in cultured cells (Sweet *et al.* 2008); how this phosphorylation event affects the phosphorylation of the conserved tyrosine or the binding affinity of SPRY to Cbl will be interesting to assess.

Unlike the APS protein that employs c-Cbl as an adapter protein, our group and others have demonstrated that, in over-expression systems, the binding of SPRY2 to c-Cbl results in the ubiquitination and subsequent destruction of SPRY2 in the endosomal compartment, and it was interpreted that the level of SPRY2 in cells is controlled in this manner (Hall *et al.* 2003, Rubin *et al.* 2003). In other experiments, when SPRY2 was over-expressed in cultured cells, EGF stimulation failed to cause the Cbl-directed downregulation of the EGFR and instead led to sustained ERK signaling (Wong *et al.* 2002b, Hall *et al.* 2003, Rubin *et al.* 2003). At the time, this was presumed to be the result of the selective sequestration of Cbl by the high levels of SPRY2, and our later work comparing the binding affinities of SPRY2 and EGFR peptides with the TKB domain suggests that this could theoretically be achieved (Ng *et al.* 2008).

While this possibility stands, in our reasoning, it seems unlikely that SPRY2 would have such high affinity for c-Cbl simply to sequester it from acting in other systems, particularly since Cbl tightly controls itself in order to ensure that excessive signaling does not ensue from RTKs, such as FGFR and possibly EGFR. Therefore, we envisage that the binding of c-Cbl to SPRY proteins has one of the three likely physiological outcomes: 1) SPRY is a Cbl-directed ubiquitin

Figure 2 Binding of SPRY2 to the c-Cbl TKB domain. Crystal structure and electrostatic surface representations of the TKB complexed with SPRY2^{49–61} from two perspectives: looking down (left) and cut-away (right). The longer arrow indicates the position of the phosphorylated tyrosine in a positively-charged pocket and the shorter arrow indicates the proline residue (+4) in a shallow hydrophobic cleft. Hydrogen bonds between the TKB domain and the peptide are shown as grey dotted lines (derived from Ng *et al.* 2008).

target, 2) SPRY targets Cbl to other proteins for ubiquitination, and/or 3) SPRY functions as an adaptor protein for Cbl, utilizing its scaffolding but not its ubiquitin E3 ligase function. In the first scenario, SPRY proteins would be the front-line targets for c-Cbl directed ubiquitination as their presence in the cell is required only in a time-restricted manner. Furthermore, in our unpublished and published work, it has been noted that high affinity binding does not necessarily correlate with ubiquitination: APS binds Cbl strongly but is not a substrate for ubiquitination (Hu & Hubbard 2005) and the converse applies to c-Met (Peschard *et al.* 2001). However, it does appear somewhat counterintuitive that one down-regulator of the ERK pathway itself is targeted for destruction by another down-regulator of the same pathway.

Sprouty proteins may also act as facilitators, targeting Cbl to other proteins to which Cbl does not directly bind, resulting in the ubiquitination of these targeted proteins. A similar example of targeting of Cbl exists between c-Cbl and the adaptor protein GRB2 in FGF signaling (Wong *et al.* 2002a). GRB2 mediates the binding of c-Cbl to the FRS2/FGFR complex following ligand stimulation and results in the ubiquitination and down-regulation of both FRS2 and receptor. More recently, the ubiquitination of the β -subunit of the interleukin-6 receptor (also known as gp130) was shown to be mediated by c-Cbl via its association with SHP2 (listed as PTPN11 in the Hugo Database) acting in its capacity as an adaptor protein (Tanaka *et al.* 2008). In either case, by binding to both Cbl and SHP2, SPRY2 may be a necessary link to bring Cbl into contact with its substrates via SHP2.

The third potential role for SPRY with respect to its interaction with c-Cbl is as an adaptor protein. SPRY could then make functional use of Cbl's wide range of associate proteins as opposed to its ubiquitin E3 ligase ability (Schmidt & Dikic 2005, Thien & Langdon 2005). There is precedence for this in insulin signaling, where Cbl is targeted to the insulin receptor via the APS adaptor protein. Neither APS nor the insulin receptor is ubiquitinated by Cbl in the resulting complex; rather Cbl facilitates GLUT4 translocation within the cell (Hu & Hubbard 2005).

While a case for Cbl can be put forward, another ubiquitin E3 ligase also binds to the N-terminal of SPRY2. In a yeast two-hybrid screen, Nadeau *et al.* (2006) identified human seven in absentia homolog 2 (SIAH2) as a SPRY2 interacting protein. The N-terminal half of Spry2 was demonstrated to interact with the ring finger domain of SIAH2 in a tyrosine phosphorylation-independent manner. Over-expressed SIAH2 initiated the ubiquitination and subsequent degradation of SPRY2, SPRY1, and to a lesser degree SPRY4. It is possible that much of the predictions and speculation made for Cbl in the preceding text could also apply to SIAH2.

In addition to acting as a binding site for Cbl, the Y55 residue has also been shown to be essential for the binding of the protein phosphatase 2A (PP2A; Lao *et al.* 2007). DaSilva *et al.* (2006) demonstrated that the degree of Y55 phosphorylation and the stability of Spry2 were affected by the serine/threonine phosphorylation status of certain

residues in the SRM of the protein. In support of this, Lao *et al.* (2007) provided evidence that, under stimulated conditions, SPRY2 requires the dephosphorylation of certain serine residues within the SRM to change its tertiary structure and permit GRB2 binding to a canonical SH3 binding motif on the C-terminus. The trimeric PP2A phosphatase was shown to be responsible for this dephosphorylation, binding to a motif on SPRY2 surrounding the Y55 residue via the scaffolding A subunit (Lao *et al.* 2007). It is presently not known whether phosphorylation of Y55 favors this interaction, and similarly, the necessity of a phosphorylation event on the +1 threonine is also yet to be established.

Regulating the Y55 residue

The accumulated evidence clearly indicates that the phosphorylation status of the Y55 residue plays a major role in the physiological function of SPRY, particularly for its activity as an inhibitor of the Ras/ERK/MAPK signaling cascade. The phosphorylation status of Y55 is dependent on the relative activity of the Y55 kinase(s) and Y55 phosphatase(s) with which it interacts. Present evidence indicates that c-Src (or a related kinase) is the tyrosine kinase responsible for phosphorylating the Y55 residue (Li *et al.* 2004). Interestingly, the c-Src autophosphorylation site, E-N-E-Y-T (Bjorge *et al.* 2000), is almost identical to the motif surrounding the Y55 residue in SPRY1 and 2, with SPRY3, SPRY4 and *dSpry* lacking the threonine in the +1 position. It has been noted in several labs that while SPRY1 and 2 become strongly phosphorylated upon FGFR stimulation, the phosphorylation of SPRY4 is relatively weak. These differing degrees of phosphorylation parallel the relative degrees of binding of SPRY proteins to c-Cbl both *in vivo* and *in vitro*, with SPRY4 showing weak binding compared with SPRY2 (Fong *et al.* 2003, Ng *et al.* 2008).

The activation of the Ras/ERK pathway downstream of a number of RTKs has been demonstrated to require the phosphatase activity of SHP2 (Araki *et al.* 2003). In fibroblasts, SHP2 undergoes phosphorylation in two C-terminal tyrosyl residues in response to FGF and PDGF but not other growth factors, such as EGF or insulin-like growth factor. Downstream of the FGFR, SHP2 binds to two phosphorylated tyrosines on FRS2, to which the SOS-GRB2 complex is also linked via GRB2, and thus facilitates the activation of membrane-located Ras and downstream ERK signaling (Hadari *et al.* 1998). The substrate(s) for SHP2 requiring dephosphorylation in this context has been sought for some time. It was reasoned that SPRY proteins, being tyrosine phosphorylation-dependent negative regulators of the ERK pathway, would enable activation of the pathway when functionally inactivated by the dephosphorylation of Y55. Several groups have provided evidence that SPRY1 and SPRY2 bind to SHP2, and Hanafusa *et al.* (2004) demonstrated that the expression of an activating SHP2 mutant (Shp2E76A) leads to enhanced dephosphorylation of

Figure 3 Activation versus degradation of SPRY2. A stylized diagram to illustrate the dynamic nature of Sprouty's fate following tyrosine phosphorylation of Y55. The TKB domain of c-Cbl binds the consensus motif and directs the ubiquitination and subsequent destruction of Sprouty2. PP2A subunits compete for binding with c-Cbl and form a platform for the selective dephosphorylation of serine residues within the serine-rich motif. During this process, Sprouty2 undergoes a change in tertiary structure that exposes the otherwise cryptic SH3-binding motif on the C-terminus enabling interaction with GRB2 and other SH3-containing proteins. In this 'activated' form, Sprouty2 is able to inhibit the phosphorylation (and activation) of ERK.

domain of protein kinase C, binds to Ras with relatively low affinity, and works in concert with the Ras binding domain to fully augment the Ras/Raf functional association and facilitate the downstream signaling (Mott *et al.* 1996, Okada *et al.* 1999). By contrast, the CRDs of other proteins play varying roles: the CRD of the calcium-sensing receptor (hCaR) is necessary for signal transmission from the N-terminal venus-flytrap domain to the seven-transmembrane domain (Hu *et al.* 2001, Tan *et al.* 2003); the mannose receptor uses the CRD as a carbohydrate recognition motif (Fiete *et al.* 1998); the CRD from a rubella virus non-structural protein is essential for viral protease activity and virus replication (Zhou *et al.* 2009); the scavenger receptor MARCO utilizes the CRD for ligand recognition (Ojalo *et al.* 2007); and the disintegrin-like/CRD of ADAM12 functions as a cell adhesion domain (Zhao *et al.* 2004).

Unsurprisingly, the CRD in the various SPRY proteins shows no functional similarity to any of those previously described. Intuitively, it might then be assumed that this

shared domain is central to the mode of inhibition of the SPRY and SPRED families with respect to ERK inhibition, but evidence for this hypothesis is presently lacking. The CRD of SPRY was shown to mediate both homo- and heterodimerization between its family members (Ozaki *et al.* 2005). The CRD was also shown to influence the function of SPRY by localizing them to the cell membrane, both in *Drosophila* and in vertebrates (Casici *et al.* 1999, Lim *et al.* 2000). Membrane targeting was attributed to palmitoylation of the unusually large number of cysteine residues in this region (Impagnatiello *et al.* 2001), and its binding to the lipid PtdIns(4,5)P₂, predominantly found on the plasma membrane of activated cells (Lim *et al.* 2002). The functional importance of the latter observation was validated by the finding that a mutant of SPRY2 (R242D) that was defective in binding PtdIns(4,5)P₂, not only failed to translocate to the membrane, but also lost its ERK inhibitory capacity. The CRD domain of the SPRED family of proteins share these features including membrane translocation, binding to

PtdIns(4,5)P₂, and the loss of these functions when the implicated arginine residue is mutated, and thus it has been speculated that the function of the CRD in SPRED also lay in directing the localization of the protein.

However, a second role for this domain in protein–protein interactions later came to light when binding to Raf1 was reported for both SPRY and SPRED by virtue of the CRDs (Sasaki *et al.* 2003). Later, two independent yeast two-hybrid screens for two kinases demonstrated an interaction with the SPRY family members via the CRD: the testicular protein kinase (TESK) and the dual-specificity tyrosine-phosphorylation-regulated protein kinase (DYRK). Leeksmma *et al.* (2002) showed that the CRD of SPRY4 binds directly to TESK1, a serine/threonine kinase related to LIM kinases, and later demonstrated that the complex inhibits the kinase activity of TESK1, suppressing cofilin phosphorylation and the subsequent formation of stress fibers and focal adhesions via the C-terminal 100 amino acids of TESK1 (that are absent in the non-binding TESK2; Tsumura *et al.* 2005). Chandramouli *et al.* (2008) later showed that all SPRY and SPRED proteins bind to TESK1, but not TESK2, via their respective CRDs. Like the case of SPRY4, SPRY2 binding to TESK1

suppressed cofilin phosphorylation, and also suppressed the inhibitory action of SPRY2; the latter is likely caused by sequestering SPRY2 away from interacting partners such as the PP2A-A and C subunits. The TESK1–SPRY2 interaction caused an increase in the slower migrating band of SPRY on SDS-PAGE gels, indicating that the kinase affects the phosphorylation of serine (or threonine) residues within the SRM of SPRY2; whether this is direct phosphorylation by the kinase or via indirect mechanisms is yet to be ascertained.

A more recent yeast two-hybrid screen using the dual-specificity tyrosine phosphorylation-regulated kinase (DYRK1A) as bait identified SPRY2 as a binding partner. Aranda *et al.* (2008) demonstrated that DYRK1A interacts with and regulates the phosphorylation status of SPRY2, and identified Thr75 on SPRY2 as a DYRK1A phosphorylation site *in vivo* and *in vitro*. The site appeared to be functional in that its mutation modestly enhanced the repressive function of SPRY2 in FGF-induced ERK signaling, and the two proteins were shown to co-localize in several structures in the mouse brain.

While there is commonality in the binding of the CRD of SPRED and SPRY, there is also variation. For instance, it has

Figure 4 The covalent modifiers of SPRY proteins. Sprouty's presentation within the cell and its likely function are controlled by covalent modification: predominantly phosphorylation and ubiquitination. The location of the binding and targeting for the Ser/Thr kinases is indicated. The binding location of MNK has not been specified, while the target of DYRK appears to be on the N-terminus, outside the serine-rich motif (SRM). Two ubiquitin E3 ligases, Cbl, and SIAH2, bind to the N-terminus of SPRY, whereas the SHP2 tyrosine phosphatase and Raf kinase bind to the cysteine-rich domain (CRD). Although capable of modifying targets on Sprouty, there is presently no firm evidence that SHP2 and Raf do so; SHP2 likely has a scaffolding function. It presently appears that covalent modifications that decide the activity and fate of SPRY occur on the N-terminus while the majority of binding occurs on the CRD.

been observed that although binding of Raf1 is mediated by the CRD of SPRED1, the binding of SPRED2 and most of the SPRY isoforms to Raf1 is minimal (our unpublished data). In addition, replacing the CRD of the relatively weak inhibitor SPRED3 with that of SPRED1 results in the chimera inhibiting ERK phosphorylation as strongly as SPRED1 itself (Kato *et al.* 2003). Furthermore, there is an apparently functional variation among the CRDs of the SPRED family; King *et al.* (2005) provided evidence that while the CRD mediates heterodimer formation, the CRD of SPRED2 is required for ERK inhibition, whereas the equivalent domain of SPRED1 is not, indicating that the two isoforms use different mechanisms for the inhibition of the ERK pathway. A recent report implicated the CRD of SPRY in mediating its binding to Caveolin1 (Cabrita *et al.* 2006), another protein that also binds to SPRED (Nonami *et al.* 2005) and, although the region for mediating this binding was not demonstrated, they revealed that various SPRY2 mutants (including the CRD mutation at R242D) failed to bind to caveolin and concomitantly showed a compromised ability to downregulate ERK phosphorylation.

It is apparent that a substantial fraction of the CRD-interacting proteins are kinases. Our laboratory has also recently characterized an interaction with kinases from the PKC family that associate with the CRD of Spry proteins in conjunction with other regions of the protein (Chow *et al.* 2009). The interaction occurs upon stimulation and results in the inhibition of the downstream substrate PKD1 that was also involved in a trimeric complex with SPRY2 and PKC δ . In the early phase of this study, another PKC isoform, PKC ζ , was also shown to bind the CRD domain of all the SPRY isoforms (our unpublished data).

A summary of the binding location and target sites of various enzymes that covalently modify SPRY proteins is shown in Fig. 4.

Sprouty function: the unanswered questions

Integrating the present information, it emerges that SPRY is fulfilling a role as a consummate integrator. It is stringently controlled both at the expression level and by a number of covalent modifications. The expression occurs as a result of the stimulation of at least one of the pathways that it feeds back on. Once expressed, evidence suggests that SPRY is in an inactive/inaccessible conformation and requires the appropriate dephosphorylation of key residues within a specialized hinge region for its activation.

The evidence that SPRY proteins are targets for ubiquitination and subsequent destruction by at least two ubiquitin E3 ligases (Cbl and SIAH2) indicates that it is in the cell's interest to limit the duration of the presence of SPRY. There also appears to be some form of competition between activators (PP2A) and downregulators (c-Cbl). Being responsive to a number of kinases, and the pathways they represent, would enable SPRY proteins to sense the intracellular environment via the activation status of the associating kinases. If the various kinases and phosphatases described above are altering the state of readiness of SPRY proteins, in particular SPRY2, a major question emerges: what proteins are being brought into a complex by SPRY to specify its major function?

As described above, there is strong evidence that c-Cbl may be one of these key proteins. If we assume that SPRY1 or 2 is acting as an adaptor rather than being a substrate for c-Cbl

Figure 5 Sprouty binding proteins. A summary of the proteins that have been shown to bind to Sprouty1 and 2 and the site to which they bind. The proteins depicted between the Sprouty proteins are common to both Sprouty1 and 2, whereas those on the outside are specific to the adjacent binding partner. Kinases are in aqua, phosphatases in green, and Ubiquitin E3 ligases in pink and other proteins in shades of brown. PKC δ binds to several sites, and this is reflected in its ambiguous location.

mediated ubiquitination, such as APS in insulin-mediated signaling, it is possible that these SPRY proteins bring c-Cbl into close proximity of potential ubiquitination targets. Wong *et al.* (2002a) provided evidence that GRB2 recruits the c-Cbl E3 ligase and its associated ubiquitination machinery into a complex with both FRS2 and FGFR1 to silence the activity of both signaling proteins. Recently, Tanaka *et al.* (2008) demonstrated that the tyrosine phosphatase SHP2, acting in its adaptor/dock role, associated with c-Cbl and the gp130 cytokine co-receptor and this resulted in the ubiquitination and downregulation of the latter protein. From these data, it may be reasoned that Cbl could be targeted towards any protein to which GRB2 or SHP2 binds via their respective SH2 domain. It is notable that SPRY2 has been shown to bind to both FRS2 and SHP2 without GRB2 mediation and in both the above-mentioned cases SPRY1 or 2 could be the protein that complexes c-Cbl with FRS2 (and FGFR1) and SHP2 and hence gp130. Such connections would only involve SPRY1 and 2 as the other SPRY proteins do not bind well to the TKB targeting sequence of Cbl. If this hypothesis was true, it would mean that each of the SPRY proteins would have distinctly different coordination tasks albeit with common modes of control.

There is also evidence that SPRY proteins are involved in the process of endocytosis of receptors; the association of SPRY2 with CIN85 (Haglund *et al.* 2005) and with Hrs (Kim *et al.* 2007). Direct binding in the latter case was not ascertained but it was demonstrated that hSPRY2 interferes with the ordered progression of ERK signaling from the early to late endosomes. This type of inhibition was similar in effect to that observed with Sef, which resides in the Golgi apparatus and inhibits MEK/ERK signaling specifically in this location (Torii *et al.* 2004). It was indicated that the interaction with Hrs offered an explanation for the observation that, contrary to expectations, SPRY2 up-regulated ERK signaling with EGF stimulation when genetic evidence indicated that SPRY proteins were downregulators of signaling (Kim *et al.* 2007). It could be postulated that SPRY2, CIN85 and Hrs cooperate in a complex to enhance the downregulation of certain RTKs. This aspect of SPRY function appears to warrant deeper investigation.

Summary

Presently, it appears that SPRY (and SPRED) proteins are integrators and sensors of cell signaling; some aspects of their function are relatively well-established and some questions are still to be determined:

- 1) The SPRY proteins have conserved the c-Cbl TKB binding site implicating Cbl as an important binding partner.
- 2) The SPRY proteins have a relatively well-conserved serine-rich domain that appears to have a function in the stability of the three-dimensional disposition of the protein by functioning as a structural 'hinge'.

- 3) SPRY2 has a canonical SH3 domain interacting proline-rich sequence. This sequence may be expected to interact with a number of SH3-containing proteins beyond GRB2.
- 4) The CRD remains enigmatic with evidence that it is involved in cellular location, dimer formation, and the binding of the majority of interacting proteins. While this domain is well-conserved, there is enough sequence variation to enable a degree of discrimination in binding among each of the SPRY and SPRED isoforms.

We are left with several major questions that will need to be addressed in order to fully comprehend the function of SPRY proteins in a broader context: 1) are SPRY proteins the major targets for Cbl-directed ubiquitination? If they are, then this suggests that it is desirable to confine the activation of SPRY to a defined temporal window. This is closely linked to the second question: 2) does SPRY direct c-Cbl to an extended range of potential substrates for ubiquitination and down-regulation? Presently, Cbl-directed ubiquitination is believed to be directed by Cbl-TKB motifs on proteins that require phosphorylation on a motif-central tyrosine residue. The SPRY-directed Cbl could extend targeting to other SPRY-binding proteins or even those associated with these proteins. There are no SPRY proteins in *C. elegans*, one in *Drosophila* and four in mammals, and the mechanisms of SPRY action are gradually being linked to signaling molecules that are derived from other signaling pathways, thus raising the question: 3) are SPRY proteins involved in signaling pathways other than the Ras/ERK pathway? There is strong genetic evidence that SPRY is involved in organogenesis, and it could be assumed that multiple isoforms of SPRY arose in higher mammals to enable specific functions in our more complex architecture, as compared to *C. elegans* and *Drosophila*. It would be reasonable to expect SPRY to be involved in other signaling pathways given the nature of the associated proteins.

The assortment of protein types that are interacting with the SPRY proteins shows a number of kinases, two ubiquitin E3 ligases and several phosphatases (summarized in Fig. 5). There is a recent realization that ubiquitin addition and removal in its various combinations is likely to play a similar role to phosphorylation in cell signaling. The two pathways indulge in cross-talk, cooperation, and augmentation, and it is possible that SPRY proteins integrate both of these signals.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this work.

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