MINIREVIEW

Negative regulation of receptor tyrosine kinases: unexpected links to c-Cbl and receptor ubiquitylation

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ABSTRACT

Intracellular signals mediated by the family of receptor tyrosine kinases play pivotal roles in morphogenesis, cell fate determination and pathogenesis. Precise control of signal amplitude and duration is critical for the fidelity and robustness of these processes. Activation of receptor tyrosine kinases by their cognate growth factors not only leads to propagation of the signal through various biochemical cascades, but also sets in motion multiple attenuation mechanisms that ultimately terminate the active state. Early attenuators pre-exist prior to receptor activation and they act to limit signal propagation. Subsequently, late attenuators, such as Lrig and Sprouty, are transcriptionally induced and further act to dampen the signal. Central to the process of signaling attenuation is the role of the E3 ubiquitin ligase c-Cbl. While Cbl-mediated processes of receptor ubiquitylation and endocytosis are relatively well understood, the links of Cbl to other negative regulators are just now beginning to be appreciated. Here we review some emerging interfaces between Cbl and the transcriptionally induced negative regulators Lrig and Sprouty.

Keywords: endocytosis, growth factor, oncogene, signal transduction, ubiquitin, tyrosine kinase.

INTRODUCTION

Receptor tyrosine kinases (RTKs) have evolved as primary mediators of cell-to-cell communication in multicellular organisms. These membrane-spanning proteins mediate a variety of cellular responses ranging from cell migration to survival, cell proliferation and differentiation. Ligand binding to the cognate receptor triggers receptor dimerization and activation of the kinase domain, rendering the receptor catalytically active. Receptor trans- and auto-phosphorylation on tyrosine residues located within the cytoplasmic tail create docking sites for proteins containing phosphotyrosine-binding modules. In turn, the recruited proteins initiate various signaling cascades [1]. Tuning of signaling amplitude and duration is crucial for induction of the correct physiological outcome [2]. For example, the patterning of the *Drosophila* embryonic ventral ectoderm relies on different levels of epidermal growth factor receptor (EGFR) signaling for the induction of various cell fates [reviewed in [3]]. In this vein, ventral midline cells provide the source for Spitz, a soluble ligand for the *Drosophila* EGF-receptor (DER). Cells in close proximity to midline cells experience high levels of DER activation and express a specific set of markers, including orthodenticle. More distal cells exhibit lower DER activation and express other markers such as fasciclin III.

It is becoming apparent that a large set of inhibitory proteins act to attenuate the signal emanating from activated receptors (Tab. 1). These negative regulators can either exist prior to receptor activation or they are newly synthesized following signaling initiation (see Fig. 1). Indeed, receptor activation not only instigates multiple positively acting pathways, such as the Ras-mitogen-activated protein kinase (MAPK); and the phosphatidylinositol 3-kinase (PI3K)-Akt cascades, but also sets in motion mechanisms that will ultimately terminate signaling. The induction of negative feedback loops has been well characterized in insects. Examples of genes that are transcriptionally induced following receptor activation include the intracellular membrane-bound protein Sprouty [4], the membrane spanning Kekkon proteins [5] and the antagonistic ligand of the insect EGFR, namely Argos [6]. Similarly, mammalian RTK attenuators such as the pan-ErbB inhibitor RALT/Mig-6 [7] and the fibroblasts-derived growth factor receptor (FGFR) inhibitor, Sef [8], are also newly synthe-

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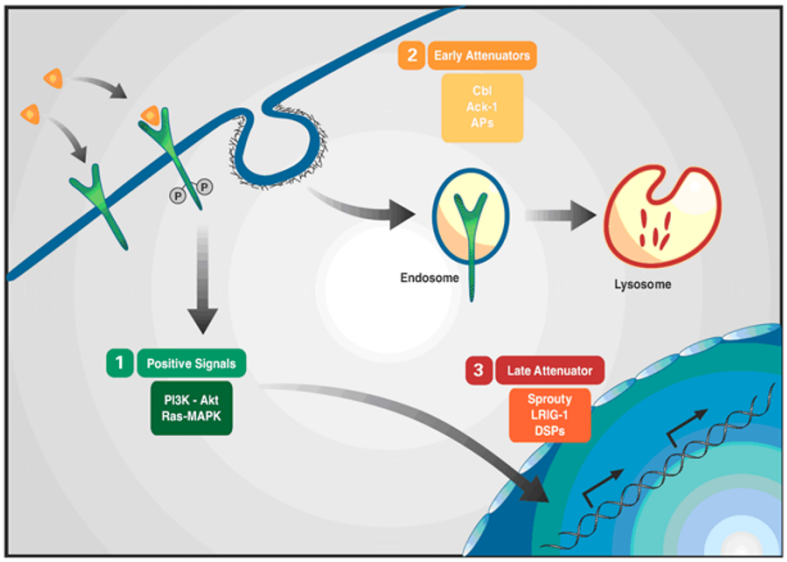

Fig. 1 Activation-dependent mechanisms of signal attenuation. Growth factor binding to a receptor tyrosine kinase induces receptor auto-phosphorylation, followed by simultaneous activation of multiple positive signaling pathways. Cbl-mediated receptor ubiquitylation marks the onset of attenuation, starting with pre-existing molecules involved in endocytosis and cytoskeleton rearrangement (early attenuators), and culminating in transcription-dependent negative regulatory pathways (late attenuators). The list of up-regulated genes includes those encoding MAPK-specific phosphatases, various adaptors, transcription factors and secondary growth factors. DSPs, dual-specificity phosphatases; APs, clathrin-associated proteins.

Tab. 1 Negative regulators of RTKs and their modes of action

Protein name	Domain structure	Inhibitory targets	Mechanism of action
Ack1	TK, SH3, CRIB, UBA	EGFR	Unknown
Argos	EGF	DER	Ligand sequestration
c-Cbl	SH2, RF, UBA	Several RTKs	Mediates receptor ubiquitylation
Echinoid	Ig-like, FN3, TM	EGFR	Unknown [49]
Kekkon	LRR, Ig-like, TM	DER	Inhibits growth factor binding
LRIG1	LRR, Ig-like, TM	ErbB family members	Enhances receptor ubiquitylation
Nedd4	C2, WW, HECT	IGF1R, VEGFR	Mediates receptor ubiquitylation [50,51]
Sef	None identified	Multiple RTKs	Retains Erk in the cytoplasm [8,52]
Senseless	Zinc fingers	DER	Represses pointed- mediated transcription [53]
Socs 1 and 3	SH2, SOCS box	Insulin and cytokine receptors	Mediate IRS1/2 ubiquitylation and block access of substrates [54]
Spred	EVH-1, CRD	RTKs	Inhibits Ras-mediated Raf activation [55]
Sprouty	CRD	Several RTKs	Unknown
Ralt/Mig6	None identified	EGFR, ErbB-2	Unknown [7]

sized in response to growth factors.

REGULATION OF RECEPTOR ENDOCYTOSIS

Of the negative regulators, which are independent of new transcription, the E3 ubiquitin ligase c-Cbl/Sli-1 is relatively well understood. Screens for suppressors of hypomorphic mutations of EGFR/LET-23 in *C. elegans* identified SLI-1 as a negative regulator of the EGFR signaling pathway [9]. In line with genetic evidence indicating that SLI-1 acts downstream to the receptor and upstream of Ras, c-Cbl, a mammalian ortholog of SLI-1, was shown to be rapidly phosphorylated and to complex

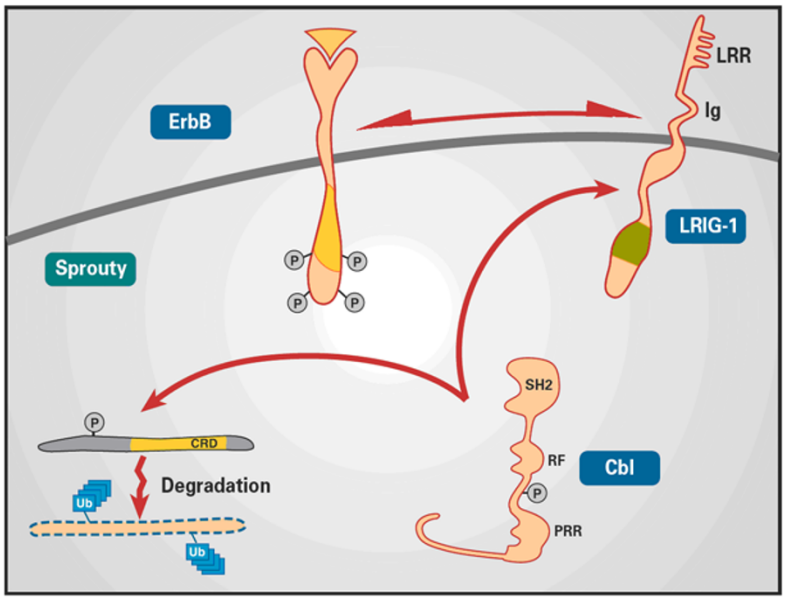

Fig. 2 Modulators of ErbB signaling. The domain structures of LRIG-1, Cbl and Sprouty proteins are schematically presented. Arrows indicate direct or indirect interactions. Thus, the extracellular domains of ErbB and LRIG proteins physically interact in a ligand-independent manner, and ErbB increases tyrosine phosphorylation of Sprouty. Cbl proteins bind in a phosphorylation-dependent manner to ErbB and Sprouty proteins, but the interaction with LRIG-1 is phosphorylation-independent. All three targets of Cbl undergo ubiquitylation, which dictates degradation by lysosomes or proteasomes. CRD, cysteine-rich domain; Ig, immunoglobulin-like domain; LRD, leucine-rich region; PRR, proline-rich domain; RF, RING finger.

with the EGFR following EGF stimulation [10]. The domain structure of c-Cbl consists of a tyrosine kinase binding (TKB) domain through which it binds tyrosine-phosphorylated targets, a RING finger domain, which binds E2-conjugating enzymes, and a C-terminal ubiquitin-associated (UBA) domain. Additional studies demonstrated that c-Cbl facilitates ligand-induced ubiquitylation of the EGFR [11,12], as well as several other RTKs (reviewed in [13]) by means of RING finger-dependant recruitment of E2 ubiquitin-conjugating enzymes to the receptor's vicinity. Receptor ubiquitylation results in accelerated removal from the cell surface and to subsequent receptor degradation in the lysosomal compartment, thereby terminating RTK signaling.

Recent studies have linked RTK ubiquitylation to the process of receptor endocytosis [14]. This phenomenon entails the internalization of the receptor from the plasma membrane and its routing through several intracellular compartments. Multiple sorting steps, along the endocytotic course, ultimately determine whether the receptor will be destined for degradation or recycle back to the cell surface. Although receptor degradation is a possible consequence of endocytosis, this process cannot be solely considered as a signal termination mechanism. Emerging data indicate that the specificity, kinetics and magnitude of receptor's responses may be regulated by the locationof the activated receptor in the endocytotic pathway [15].

Ligand binding initiates receptor auto-phosphorylation, which is followed by the recruitment of the Cbl TKB domain to a specific phosphotyrosine, such as tyrosine 1045 of the EGFR [11]. This proximity facilitates Cbl-mediated receptor mono-ubiquitylation [16,17], which commences at the plasma membrane [18,19]. It is believed that mono-ubiquitylation of the receptor on multiple lysine residues robustly generates docking sites for endocytotic adaptor proteins possessing ubiquitin-binding domains. Adaptors, such as Eps15, may recruit receptors to clathrin-coated pits as they comprise both ubiquitin interacting motifs (UIMs) [20] and DPF motifs that couple to clathrin adaptors [AP2; [21]]. Recruited receptors are thus linked to AP2 complexes that drive the assembly of clathrin-coated vesicles (CCVs). The CCVs shed clathrin and fuse with internal vesicles to form early endosomes that proceed along the endocytic pathway to the multi-vesicular body (MVB). At the MVB, endocytotic adaptors sort receptors for destruction in the lysosome or for recycling vesicles,

which eventually fuse with the plasma membrane [22]. This final sorting step is also regulated by the E3 ligase activity of Cbl; sustained ubiquitylation of the EGFR in endosomes was shown to be necessary for the sorting to lysosomal compartments [18], implying that Cbl-mediated ubiquitylation serves as a switch shunting activated receptors for degradation

The primordial form of Cbl appeared relatively early during evolution in nematodes such as *C. elegans*. Two negative regulators of RTKs, namely Sprouty and Kekkon, which have subsequently evolved in insects, retain complex relationships with Cbl both in insects and in mammals [23-26]. In addition, the association of these inhibitors with Cbl leads to the ubiquitylation and degradation of Sprouty and LRIG in the proteasome, which subsequently limits their inhibitory capacity (see Fig. 2). Hence, it seems that evolution has coupled its more recent inventions to the more ancient ones, designating Cbl not only as a regulator of RTKs but also as master regulator of secondary regulators, as described below.

SPROUTY PROTEINS: DUAL REGULATORS OF RTK SIGNALING

Sprouty was originally identified in *Drosophila* as an antagonist of Breathless, the insect equivalent of the fibroblast growth factor receptor (FGFR; [27]). Additional studies demonstrated that Sprouty also inhibits the *Drosophila* EGFR (DER), as well as other RTKs [28]. Four orthologs of the *Drosophila* sprouty (dspry) have been identified in mammals. Of these, spry2 exhibits the highest level of homology to the ancestral gene. Functionally, human Spry2 has retained its regulatory role, impeding activation of the MAPK by some, but not all RTKs. The negatively regulated mammalian RTKs include the receptors for FGF, the vascular endothelial growth factor and the hepatocyte growth factor [29,30]. In contrast, the effect of Spry2 on signaling downstream to the epidermal growth factor receptor (EGFR) seems more complex [31]. Likewise, it is still unclear at which level Sprouty proteins regulate MAPK signaling. Spry2 appears to physically interact with multiple components of the Ras-MAPK pathway, including Grb2, FRS2, Raf1 the Ras GTPase activating protein (Ras-GAP; [32]) and c-Cbl [33]. Several regulatory mechanisms have been proposed, including inhibition at the levels of Grb2 [34], GAP [4], or the Raf1 kinase [35,36]. In contrast, the interactions with c-Cbl may positively impact on EGFR signaling [26,37].

In addition to its role as a regulator of RTK signaling, Spry2 itself is subject to tight regulation by RTKs on several levels. First, the activation of RTKs triggers an induction in spry2 transcription in epithelial cells [35]. Regulation of the mature protein is mediated by ligand-induced phosphorylation of Spry2 on a tyrosine residue located at position 55 [24, 26, 34, 38]. This evolutionarily conserved tyrosine is essential for the inhibitory activity of Spry2 [35], although the exact mechanism remains unknown. In addition to its role as an operational switch, phosphorylation of tyrosine 55 creates a docking site for the E3 ubiquitin ligase c-Cbl. Following RTK activation, c-Cbl binds phosphorylated Spry2 through its TKB domain. This association results in the ubiquitylation and proteasomal degradation of active Spry2, thus limiting its inhibitory effects [24, 26]. On the other hand, the association between Sprouty and c-Cbl may sequester the E3 ligase in a way that prevents ubiquitylation of target proteins. In summary, the interaction between Sprouty and Cbl emerges as a focal point not only in Sprouty's role in restraining RTK signals but also in the life cycle of Sprouty proteins themselves. We speculate that Cbl plays a dual role in this web; along with ubiquitylating Sprouty-associated proteins and sorting them for lysosomal or proteasomal degradation, Cbl's function as a multivalent adaptor capable of engaging more than 50 different proteins [39] may underlie the ability of Sprouty to interfere with signaling pathways.

LRIG1 AS A NEGATIVE REGULATOR OF MAMMALIAN RTKS

Shortly following the identification of Sprouty as an inducible inhibitor of FGF-signaling, another feedback regulator was shown to inhibit the activity of DER during oogenesis [40]. This gene, named *kekkon-1*, encodes a transmembrane protein that physically binds to and directly inhibits EGFR molecules [41]. The six leucine-rich repeats (LRRs) of Kekkon-1 are necessary for recognition of EGFR, and for consequent inhibition of activation by growth factors [41,42]. The multiple Kekkon proteins of insects have no clear orthologs in mammals [43]. On the other hand, a clear ortholog of mammalian LRIG proteins exists in flies and nematodes. Nevertheless, the three mammalian LRIG genes share domain organization with Kekkons [44-47]. The extracellular regions of both the murine Lrig1/Lig-1 [47] and the human LRIG1 [46] share 15 LRRs followed by three Ig domains. Interestingly, disruption of the *LRIG1* gene in mice resulted in fertile animals that developed defects in skin [48], a major site of EGFR action.

Similar to kekkon-1 and to other ligand-dependent negative feedback regulators of ErbB signaling, Lrig1 is transcriptionally up-regulated upon EGF stimulation [23]. The structural similarity of LRIG1 to Kekkons predicted that LRIG1 would interact with and restrict ErbB signaling in mammals. Indeed, LRIG1 localizes to the basolateral surface of epithelial cells, the site of ErbB function, and physically interacts with all members of the ErbB family [23,25].

This recognition involves both LRIG1's and the receptor's ectodomains and it requires no stimulation by the respective ErbB ligand. However, whereas only the LRRs of Kekkon-1 are necessary for recognition of the *Drosophila* EGFR ectodomain [41,42], the LRRs and the immunoglobulin- (Ig-) like domains of LRIG1 are each sufficient for receptor binding [23].

Ligand-induced up-regulation of LRIG-1 expression shortens the half-life of ErbB-1/EGFR, due to enhancement of ligand-induced receptor ubiquitylation and an associated sorting of ligand-activated receptors to intracellular degradation, thus restricting growth factor signaling [23]. Conclusions derived from both *in vivo* and *in vitro* assays suggest that the N-terminal half of c-Cbl directly binds to the juxtamembrane region of LRIG1 [23]. By recruiting c-Cbl to the vicinity of EGFR, both LRIG1 and the receptor undergo ubiquitylation and subsequent degradation [23,25]. Remarkably, Kekkon1-EGFR interaction inhibits EGFR signaling in an apparently different mechanism, which involves inhibition of growth factor binding, receptor auto-phosphorylation and MAPK activation in response to EGF [41]. In summary, both *Drosophila*'s Kekkon proteins and mammalian LRIG family members are induced upon receptor activation and in both cases receptor signaling is subsequently blocked. However, LRIG and Kekkon proteins have no common genetic origin and their modes of action differ, implying evolutionary convergence of negative feedback mechanisms.

PERSPECTIVES

The growing interest in negative regulation of RTK signaling has led to the identification of multiple proteins involved in the process of signal attenuation. Although in many cases the mechanism of action has proven elusive, the overall picture is of regulation at numerous levels, including the level of the ligand, the receptor, the downstream signaling components and the respective target transcription factors (Tab. 1). Of these mechanisms, receptor downregulation by means of endocytosis emerges as a major player in signaling attenuation. Central to this process is the role of the E3 ligase Cbl, which directs ligand-induced receptor ubiquitylation and subsequent degradation. Interestingly, additional negative regulators such as Sprouty and LRIG, which evolved later in the course of evolution, fine-tune Cbl activity and are themselves subjected to Cbl-mediated regulation. Unlike Cbl, both Sprouty and LRIG are transcriptionally induced upon receptor stimulation, thus participating in a large-scale negative feedback program. Future studies will help untangling the underlying genetic program and uncovering the many layers of receptor regulation.

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