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Minireview

Negative regulation of receptor tyrosine kinase signals

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Abstract In Metazoans a number of cellular functions are controlled by receptor tyrosine kinases (RTKs) during development and in postnatal life. The execution of these programs requires that signals of adequate strength are delivered for the appropriate time within precise spatial boundaries. Several RTK inhibitors have been identified in invertebrate and mammalian organisms. Because they are involved in tuning and termination of receptor signals, negative regulators of RTK activity fulfill a fundamental function in the control of receptor signaling.

Key words: Receptor tyrosine kinase; Signal inhibition; Feedback inhibitor; Negative signaling; Cell cycle

1. Introduction

In multicellular organisms receptor tyrosine kinases (RTKs) are involved in the control of fundamental aspects of cell physiology, ranging from cell migration to survival, growth, proliferation and differentiation. Once bound by their cognate ligand, RTKs dimerize and become catalytically active. The earliest consequence of dimerization-based RTK activation is receptor trans-phosphorylation on specific tyrosine residues: this process generates recognition codes for signaling proteins containing PTyr binding modules (SH2 and PTB domains) [1,2] (Fig. 1A,B).

The nucleation of these complexes onto activated receptors allows transmission of receptor signals to specific pathways, due to the fact that PTB and SH2 domains are present in either enzymes (e.g. c-src, PLC-γ, PI-3K, Ras-GAP etc.) or adapter proteins (e.g. GRB-2). Recruitment of enzymes onto RTKs leads to their activation by virtue of three mechanisms: (a) direct phosphorylation on specific tyrosine residues; (b) allosteric changes induced by PTyr-directed protein–protein interactions; (c) recruitment to the plasma membrane, i.e. to a subcellular compartment where substrates are available. Adapter proteins are devoid of intrinsic catalytic activities but regulate downstream enzymes via further protein–protein interactions [1,2] (Fig. 1C). A variation of this paradigm is the extensive phosphorylation by RTKs of membrane-located scaffold proteins (e.g. IRS proteins and GAB-1) which, similarly to autophosphorylated RTKs, serve as platforms for the assembly of complexes of signaling proteins containing PTB and SH2 domains [1].

These networks of protein–protein interactions allow for rapid, spatially controlled and reversible activation of signaling pathways downstream to RTKs. They also allow for quantitative control of signaling by providing for either amplification or insulation of signals. Last, but not least, the presence of several protein–protein interaction modules in multi-domain signaling proteins is instrumental in regulating integration of signals among different pathways [3].

Despite structural diversification, most RTKs activate broadly overlapping sets of signaling pathways. Thus, a major issue in the field of cell signaling is how different RTKs instruct cells to adopt specific fates [4]. The identity of the responding cell, i.e. its developmental history, is a major factor in determining the outcome of a given signal or set of signals. Accordingly, activation of Trk-A, the high affinity receptor for nerve growth factor (NGF), elicits mitogenic and transforming signals in murine fibroblasts whereas drives neuronal differentiation of pheochromocytoma-derived PC12 cells. In such an example Trk-A signals impinge on qualitatively different cellular machineries, e.g. transcription factors, which are geared to execute fundamentally different programs. In a more refined version of this theme it is the integration of a 'generic' RTK signal with that emanating from another receptor, e.g. Notch, which generates the specific cellular responses that fate the cell [4].

More challenging to be resolved at the molecular level is the scenario in which different biological responses may be induced in the same cell by the same signal, depending on the magnitude and duration of the latter. Cultured PC12 cells serve again as a paradigm to understand cell behavior in molecular terms. A short wave of ERK activation, as elicited by epidermal growth factor (EGF) stimulation, drives PC12 cells to proliferate, whereas lengthy ERK activation, as obtained with NGF treatment, causes PC12 cells to differentiate [5]. Along the same line, graded levels of ERK activity pattern the posterior termini of *Drosophila melanogaster* embryos. Here the Torso RTK is activated by its ligand Trunk. As Trunk is produced in limiting amounts by a localized source, its diffusion establishes a gradient, which leads to different levels of Torso occupancy and to correspondingly graded

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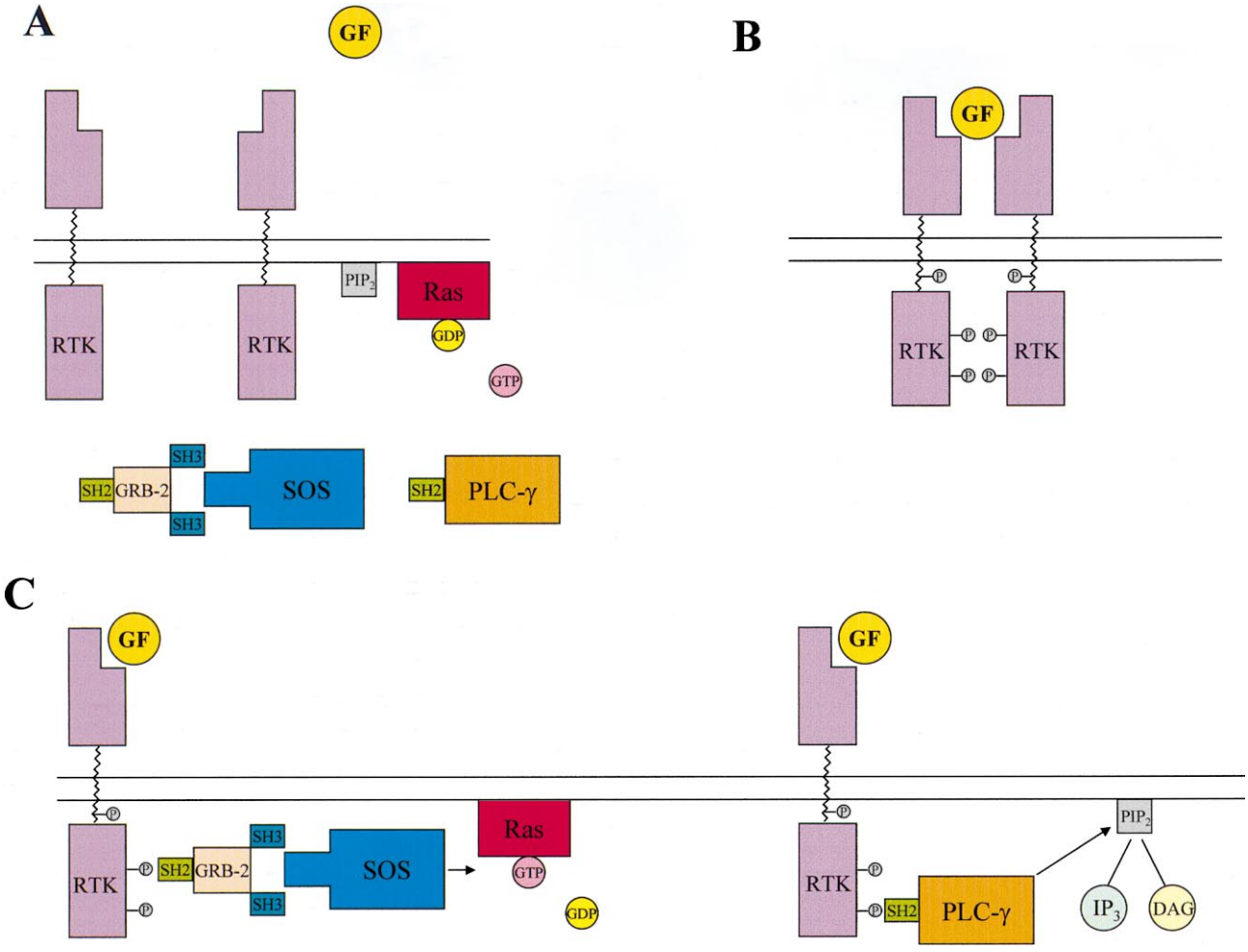

Fig. 1. Signaling by RTKs. A: In the absence of ligand stimulation RTKs are present as catalytically silent monomers. B: Ligand binding induces productive dimerization of RTKs which is followed by catalytic activation and receptor cross-phosphorylation on specific tyrosine residues. C: Phosphotyrosine residues on activated RTKs serve as docking sites for effectors such as the enzyme phospholipase C-γ (PLC-γ). Binding to activated RTKs induces translocation of PLC-γ from cytosol to the plasma membrane and enhances tyrosine phosphorylation of PLC-γ by RTKs: both steps are necessary for full catalytic activation of PLC-γ which leads to hydrolysis of PIP_2 to IP_3 and DAG. Phosphotyrosine residues on activated RTKs may serve as docking sites also for adapters such as GRB-2. This leads to translocation of the cytosolic GRB-2/SOS complex to the plasma membrane, thus allowing the guanyl-nucleotide exchanger SOS to target its substrate Ras.

levels of Torso-dependent ERK activity [6]. At the posterior pole of the embryo this is translated into different transcriptional responses: *huckebein* is expressed where ERK activity is greatest, whereas the domain of expression of *tailless* extends into regions of lower ERK activity. Gradients of *hkb* and *tll* in turn regulate downstream target genes which are thought to implement distinct morphogenetic responses [7,8].

Because variations in strength and duration of a given signal are means to convey different instructions to a cell, mechanisms have evolved which ensure that appropriate thresholds of signal are achieved and maintained for the right time. It follows that generation, tuning and termination of signals must be viewed as integrated processes which organize in time and space the correct output of any given signal.

The purpose of this review is to discuss the role of negative regulators of RTK signaling in establishing appropriate thresholds of biochemical responses to receptor activation. Protein tyrosine phosphatases (PTPs) reverse RTK signals by dephosphorylating RTK substrates: hence the activity of PTPs is an effective measure of counteracting RTK signals. The role of PTPs in RTK signaling has been the subject of recent reviews to which the reader is therefore referred [9,10].

Here we will briefly discuss how negative regulators of RTK signals influence developmental processes in invertebrates. We will then focus on structural and functional features of molecules which interact physically with mammalian RTKs and attenuate receptor signaling.

2. Lessons from developmental biology of invertebrates

Developmental studies in *Caenorhabditis elegans* and *D. melanogaster* have provided compelling evidence that negative regulation of RTK signaling is required to ensure proper fate determination and patterning.

In *C. elegans* induction of the vulva depends on the localized activity of Lin-3, a member of the EGF family of growth factors, which is produced by the anchor cell (AC) [11]. Lin-3 instructs three out of six vulva precursor cells (VPCs), which lay beneath the AC, to proliferate and differentiate, thus forming the mature vulva (Fig. 2). Several lines of evidence

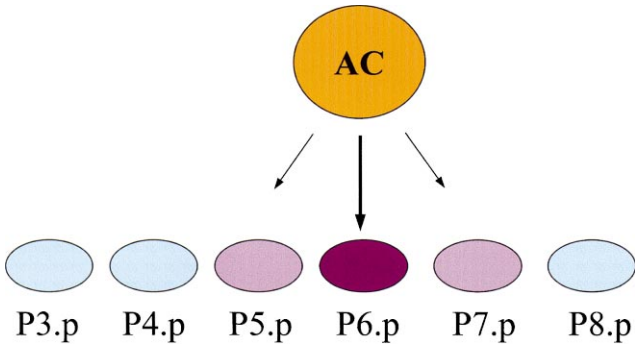

Fig. 2. Model of vulva development in *C. elegans*. The AC produces the EGF-like factor Lin-3. VPC receive different doses of Lin-3, depending on their distance from the AC. Under normal conditions, P6.p receives the highest dose (long and thick arrow) and adopts a 1° fate, whereas P5.p and P7.p receive a lower dose (short arrows) and adopt a 2° fate. The mature vulva is eventually generated by three rounds of proliferation of 1° and 2° fate VPCs. Reduction of function mutations of negative regulators of the Lin-3→LET-23→LET-60 pathway cause recruitment to vulval fate of extra VPCs (i.e. P3.p or P4.p or P8.p).

indicate that the distance of the AC from VPCs establishes a gradient of Lin-3 activity, with P6.p receiving a stronger signal than P5.p and P7.p [12]. As a consequence, P5.p and P7.p adopt a 2° fate, whereas the highest dose of Lin-3 instructs P6.p to adopt a 1° fate. Lin-3 activates LET-23, a *C. elegans* homolog of EGFR; LET-23 binds Sem-5/GRB-2 which signals to LET-60/Ras [11,13].

Restricted availability of Lin-3 is instrumental in generating a spatially defined pattern of VPC fates. Disruption of this balance by excess Lin-3→LET-23→LET-60 activity leads to a multivulva phenotype, caused by recruitment of extra VPCs to the vulval fate. The implication of these findings is that LET-23 activity must be held in check. Genetic studies indicate that negative regulators of receptor signals, such as sli-1/cbl [14], gap-1/RasGAP [15] and ARK-1 [16], are key players in the modulation of LET-23 activity. Hence, the activity of negative regulators of the LET-23→LET-60 pathway integrates the restricted availability of Lin-3 to establish precise thresholds of signals.

In mammalians, c-Cbl has ubiquitin E3 ligase activity and interacts physically with the EGFR to promote its ubiquitinilation and subsequent degradation ([17–19], for review see Waterman and Yarden, this issue of FEBS Lett.). By analogy, sli-1 is thought to dampen LET-23 signaling by promoting its down-regulation. Gap-1 inhibits LET-60 by accelerating its GTPase activity, thus locking LET-60 in the inactive GDP-bound state. ARK-1 is related to the mammalian tyrosine kinase ACK [20]; how ARK-1 signals to LET-23 has not been determined, due also to the fact that the function of ACK in mammals remains elusive. It is clear however that ARK-1 requires Sem-5/GRB-2 for negative signaling to LET-23 [16]. The genetic interaction between ARK-1 and Sem-5 may be explained by the finding that the two proteins form a physical complex in yeast two hybrid experiments. Therefore it is thought that ARK-1 may inhibit LET-23 activity by being recruited to the receptor in a complex with Sem-5/GRB-2 [16]. This model implies that Sem-5 can exert opposite effects on the LET-23→LET-60 pathway, presumably by forming complexes with different effectors.

As in *C. elegans*, a homolog of EGFR in *D. melanogaster* also regulates a number of developmental processes by triggering a conserved signaling cassette which leads to Ras activation [21]. Since DER (*Drosophila* EGF receptor) is ubiquitously expressed, restricted availability of its ligands (Gurken, Vein and Spitz) and tight regulation of its activity in time and space are required to ensure accurate execution of developmental programs. Genetic studies have revealed that a crucial role in the regulation of DER activity is played by inhibitors of receptor function which are expressed in the context of transcriptional responses triggered by DER itself (Fig. 3).

Argos is a soluble molecule which carries a modified EGF domain and binds the extracellular region of DER. Once bound to Argos, DER is prevented from binding Spitz and dimerizing (Fig. 3): hence Argos inhibits crucial steps leading to catalytic activation of DER [22].

The role of Argos in patterning the embryo ventral ectoderm is an example of how Argos expression contributes to the spatial and temporal control of signaling by the DER RTK. Regulated cleavage of membrane-bound Spitz along the ventral midline of the *Drosophila* embryo activates DER. Because soluble Spitz is produced in limiting amounts, its diffusion leads to graded DER activation: in turn, graded DER activity patterns the ventral ectoderm [23]. *Argos* is transcribed in cells in which DER itself is activated most strongly. Diffusion of Argos from its site of production leads to inhibition of DER signaling in neighboring cells. If Argos expression is ablated, cells in which DER is normally activated at intermediate to low levels end up receiving a stronger Spitz signal for a longer time. This leads to ventralization of the ectoderm, i.e. the expansion of the domain in which cells adopt the ventralmost fate [23]. Thus, by diffusing in the domain of Spitz activity, Argos refines the Spitz gradient and maintains the graded levels of DER activity required for patterning the ventral ectoderm.

Kekkon1 typifies a different class of feedback inhibitors of DER. During oogenesis, localized production of Gurken in the dorsal-anterior region of the oocyte leads to activation of DER in the surrounding follicular cells. DER signaling leads to expression of *rhomboid* whose product enhances Spitz cleavage: this establishes an autocrine loop which boosts DER signaling and broadens the domain of DER activity [24]. Cells located in the initial domain of Gurken and Spitz activity receive the highest signal and produce Argos which, in turn, acts locally to inhibit DER signaling. As a consequence, the domain of DER activity is split into two symmetric peaks which pattern the dorsal appendages [24]. *Kekkon 1* was identified as a gene transcriptionally activated by DER signaling during oogenesis [25]. Overexpression of *kekkon 1* leads to loss of dorsal appendages (as observed upon loss of *gurken* and *egfr*), whereas its genetic ablation causes lateral expansion of dorsal appendages, i.e. broadening of the domain of DER activity. These phenotypes coupled to epistatic analysis indicate that Kekkon 1 inhibits DER signaling at the receptor level [25]. Kekkon 1 is a transmembrane protein which is able to interact with DER and has features similar to those of cell adhesion molecules. Overexpression of a truncated Kekkon 1 mutant spanning the extracellular and transmembrane domains is sufficient to cause inhibition of DER. Hence it has been proposed that Kekkon 1 exerts its function by virtue of a physical interaction with DER, which leads to direct or indirect inhibition of DER signaling (Fig. 3) [25].

A third feedback inhibitor of DER is *sprouty*. Sprouty was

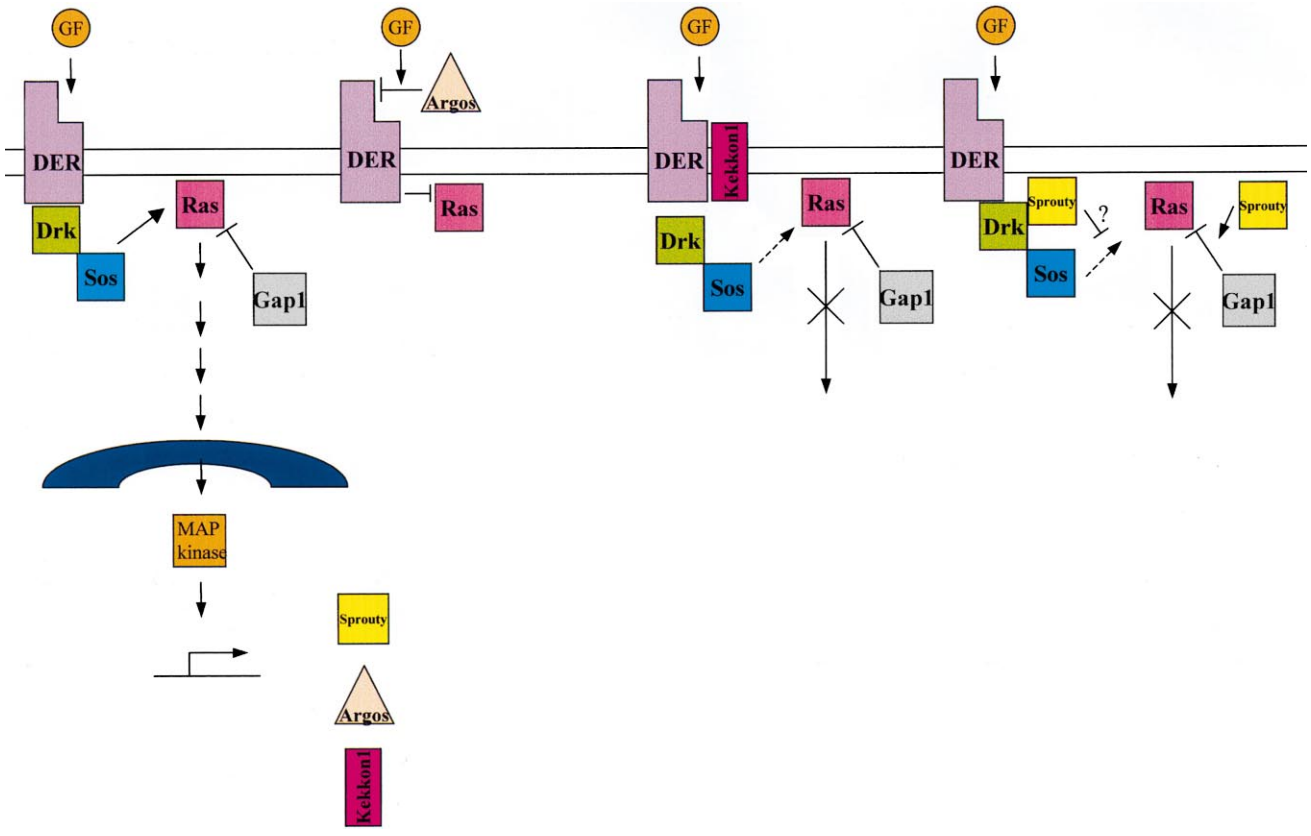

Fig. 3. Feedback inhibition of EGFR signaling in *Drosophila*. DER signals through the Ras/ERK pathway and induces expression of Argos, Kekkon1 and Sprouty. Argos is a secreted molecule which binds to the extracellular domain of DER and prevents DER activation by Spitz. Kekkon1 is a transmembrane protein which is thought to interfere with DER activation and/or signaling by binding to the receptor. Sprouty is anchored to the inner surface of the plasma membrane and binds to signal regulators such as Gap and Drk: it is speculated that Sprouty either acts as a sink for positive effectors or enhances the function of negative regulators of RTK signaling (e.g. Ras GAP).

originally cloned as a feedback inhibitor of *breathless*, a *Drosophila* FGF receptor [26]. During branching morphogenesis of the respiratory tree, *sprouty* expression is induced in cells receiving the highest level of Branchless, an FGF-like ligand which acts locally to induce branching of tracheal epithelium. The *sprouty* product, in turn, acts non-cell autonomously to repress Breathless signaling in neighboring cells. This spatial restriction of Breathless activity ensures that only cells which are closest to the source of activating ligand are induced to branch; this allows the reproduction of precise patterns of airway branching [27].

Sprouty expression is induced also by DER activity, although not in all tissues. Loss of *sprouty* expression in the eye causes increased recruitment of photoreceptor and non-photoreceptors cells, a phenotype associated with excess DER signaling [28]. At variance with tracheal branching, the *sprouty* phenotype in the eye is cell autonomous. Loss of *sprouty* causes hyperactivation of DER also in the egg, leading to expansion of the dorsal appendage fate laterally and into the inter-appendage region [29]. The reduction of inter-appendage region upon *sprouty* loss is interesting, because *argos* is still expressed under these conditions: this implies that Argos and Sprouty act cooperatively in repressing DER activity in this domain [29].

At variance with Argos and Kekkon 1, which appear to be specific for DER, Sprouty is promiscuous in that it inhibits also signaling from other RTKs such as Breathless, Torso and possibly Sevenless [28,29]. The promiscuity of Sprouty as an inhibitor of RTK signaling to the Ras→ERK pathway may be explained by its structural features. Sprouty is an intracellular protein (Fig. 3) which localizes to the inner surface of the plasma membrane via a Cys-rich domain and binds to Drk/GRB-2 and Gap-1/RasGAP [28]. The current model holds that Sprouty constitutive location in the plasma membrane may regulate the availability and/or function of cytosolic signal transducers recruited by activated RTKs to signal to Ras (Fig. 3).

3. Negative regulation of RTK signaling in mammalian cells

As in the case of nematodes and flies, inhibitory molecules in mammalian organisms may also target RTK signaling cassettes at different steps.

Herstatin is a secreted protein encoded by an alternatively spliced form of the *HER-2/erbB-2* mRNA [30]. This splicing event generates a 274 nt insertion contiguous to exon 9 sequences. The resulting reading frame causes translation termination at the end of a 79 aa in frame insertion which is fused to the first 340 residues of ErbB-2: thus, Herstatin encodes a soluble protein which contains subdomain I and II of ErbB-2 extracellular domain [30] (Fig. 4). Herstatin binds to $gp185^{ErbB-2}$ with high affinity and inhibits dimerization and catalytic activation of ErbB-2. Consistently with these biochemical observations, addition of Herstatin to the culture

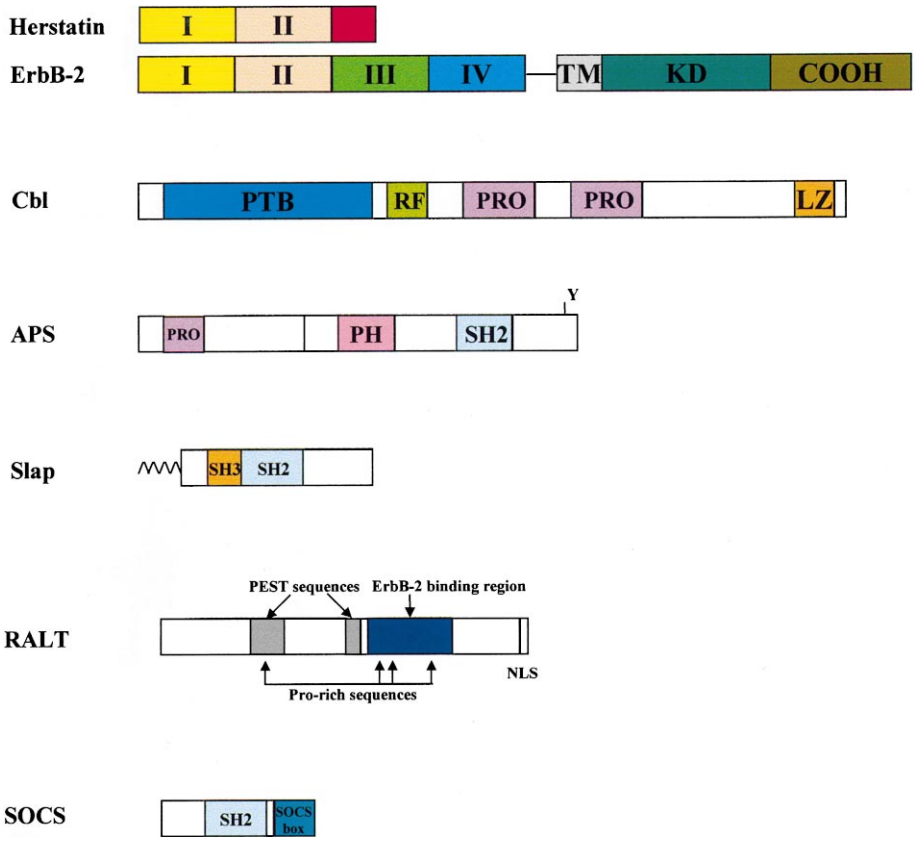

Fig. 4. Structural features of mammalian proteins involved in negative regulation of RTK signaling. The extracellular domain of ErbB-2 comprises sub-domains I to IV. TM, KD and COOH indicate the transmembrane, kinase and C-terminal domains, respectively. Herstatin is encoded by an alternatively spliced ErbB-2 mRNA which generates a secreted protein containing sub-domains I and II of ErbB-2 and a C-terminal novel sequence of 79 residues. c-Cbl contains a phosphotyrosine binding domain (PTB), a RING finger (RF) domain, two Proline-rich regions (PRO) and a leucine zipper (LZ). APS contains PH and SH2 domains, in addition to a Pro-rich region. Slap contains a myristoylation signal at its NH_2-terminus and consists of SH3 and SH2 domains followed by a COOH-terminal tail of about 100 residues. RALT contains two blocks of PEST sequences, an ErbB-2 binding region and a potential nuclear localization signal (NLS); several Pro-rich sequences targeted by SH3 domains are also present in the RALT protein. SOCS proteins consist essentially of a SH2 domain and a C-terminal SOCS box.

medium causes inhibition of anchorage-independent growth of NIH-ErbB-2 transfectants [30]. Although it has not been reported whether Herstatin inhibits binding of NDF (or other ligands) to ErbB heterodimers containing ErbB-2, it seems likely that Herstatin may have functions similar to those of Argos (Fig. 5A). It is not clear how the expression of the alternative transcripts encoding Herstatin is regulated. However it appears that expression of Herstatin may be modulated during development: Herstatin transcripts were detected in fetal kidney but not in fetal brain and lung. ErbB receptors are implicated in branching morphogenesis of the kidney [31]. Because branching morphogenesis requires reiterated and tightly regulated cycles of migration, proliferation and differentiation, one can imagine that Herstatin expression may contribute to spatial regulation of ErbB receptor activity during kidney morphogenesis.

As mentioned above, *sli-1*, a homolog of mammalian c-*cbl*, was discovered as a negative regulator of Let-23 activity in *C. elegans*. Cbl contains an NH_2-terminal phosphotyrosine binding domain, a RING finger domain, Pro-rich sequences and a COOH-terminal leucine-zipper (Fig. 4). Biochemical studies have shown that the RING finger domain of c-Cbl (Fig. 4) has E3 ubiquitin ligase activity [17,18]. Cbl is recruited to the EGFR upon receptor activation and becomes phosphorylated on a tyrosine residue adjacent to the RING finger domain. This leads to activation of the ubiquitin E3 ligase function of c-Cbl [18] (Fig. 5B). The ensuing polyubiquitination of EGFR targets the receptor to the internalization and degradation pathway, thus contributing to signal attenuation (reviewed in Waterman and Yarden, this issue of FEBS Lett.). Overexpression of c-Cbl has been shown to also inhibit mitogenic activity of PDGF-R [32]; conversely microinjection of anti-Cbl antibodies enhanced mitogenic response to sub-optimal doses of PDGF [32]. Cbl is also implicated in internalization of the CSF-1 receptor [33]. Thus c-Cbl is involved in negative regulation of several RTKs.

Herstatin and c-Cbl are examples of negative regulators of RTK activity that are likely to dampen the entire breadth of receptor signaling. In contrast, a number of adapters have been identified in the past couple of years that appear to be involved in negative regulation of specific pathways triggered by RTKs.

APS, Lnk and SH2-B represent a family of structurally and functionally related adapters involved in negative regulation of RTKs and cytokine receptors [34]. All three family members share a common structural organization, consisting of an NH_2-terminal region containing potential binding sites for SH3 domains, a PH domain, an SH2 module and a COOH tail containing a conserved tyrosine phosphorylation site [34]. Signaling by APS (adapter containing PH and SH2 domains,

see Fig. 4) has been studied in some detail in the context of PDGF-R activation [35]. Upon PDGF stimulation, APS binds via its SH2 domain to PTyr 1021 of the βPDGF-R, is phosphorylated on a tyrosine residues in its COOH tail and is relocated to the plasma membrane. Tyrosine-phosphorylated APS in turn binds to GRB-2 and to c-Cbl. Overexpression of APS in NIH 3T3 fibroblasts inhibited mitogenic signaling induced by PDGF. The biological effects of APS overexpression correlated with inhibition of *fos* and *myc* transcription induced by PDGF; importantly, PDGF-R signaling to the PI-3 kinase complex was not altered by APS overexpression [35]. An APS mutant lacking the COOH tail was not phosphorylated on tyrosine upon PDGF-R triggering, did not bind c-Cbl and did not inhibit PDGF-dependent mitogenesis when overexpressed in 3T3 cells. Binding of c-Cbl to APS enhanced the inhibitory activity of the latter on *fos* transcription; this effect was reversed by deletion of the Tyr residue involved in the recruitment of c-Cbl onto APS. Thus it appears that the inhibitory function of APS on PDGF-R signaling requires recruiting of c-Cbl in a PTyr-dependent complex with APS itself [35] (Fig. 5B). It is possible that recruitment of APS to the plasma membrane is initially mediated by its interaction with the PDGF-R and subsequently stabilized by the binding of its PH domain to PIP products generated by PDGF-R activity. Such a two-step process may explain the slow kinetics of phosphorylation of APS by PDGF-R and the delayed inhibition of *fos* transcription by overexpressed APS [35]. This also raises the possibility that APS nucleates the generation of inhibitory signals only once it becomes membrane-bound, perhaps by targeting receptors and/or RTK effectors for ubiquitin-dependent degradation enforced by c-Cbl activation.

Slap (Src-like adapter protein) provides another example of an adapter involved in negative regulation of a specific RTK pathway. Slap consists of an SH3 domain, an SH2 module and a COOH-terminal tail of about 100 residues [36] (Fig. 4). Slap overexpression in quiescent fibroblasts inhibits progression to S phase upon PDGF or serum stimulation. Conversely, microinjection of anti-Slap antibodies enhances the mitogenic response of quiescent NIH 3T3 cells to either serum or PDGF [36]. Slap binds via its SH2 domain to PTyr 579 and 581 of PDGF-R, i.e. to the sites to which c-Src also binds. In a straightforward model, Slap may inhibit PDGF-R signaling by competing out c-Src binding to the PDGF-R. Accordingly, ablation of Slap SH2 domain should cause loss of Slap inhibition on PDGF-R signaling. Furthermore, because a dominant negative *src* allele inhibits PDGF-R activity in a Myc-dependent, Ras-independent fashion, the phenotype caused by Slap overexpression should be rescued by ectopic expression of Myc, but not Fos and Jun. Both predictions are validated by experimental data. The competitive inhibition model, however, can not account for the inability of overexpressed Src or Fyn to rescue the Slap phenotype. Further genetic analysis indicated that COOH-terminal sequences of Slap are responsible for non-competitive inhibition of PDGF-activated Src [37]. The emerging picture is that recruitment of Slap to the PDGF-R not only inhibits Src binding but also recruits specific inhibitors of Src via the Slap COOH tail (Fig. 5B).

APS and Slap are examples of negative regulators of PDGF-R signaling whose expression has not been reported to be modulated by receptor function. On the other hand, feedback inhibitors, i.e. negative regulators whose expression is transcriptionally activated by receptor signaling, are involved in attenuation of RTK functions in a restricted window of time. Here we will discuss the role of SOCS and RALT as RTK feedback inhibitors.

The family of SOCS (suppressor of cytokine signaling) proteins consists of eight members which share a common structural organization, i.e. a NH_2-terminal SH2 domain and a

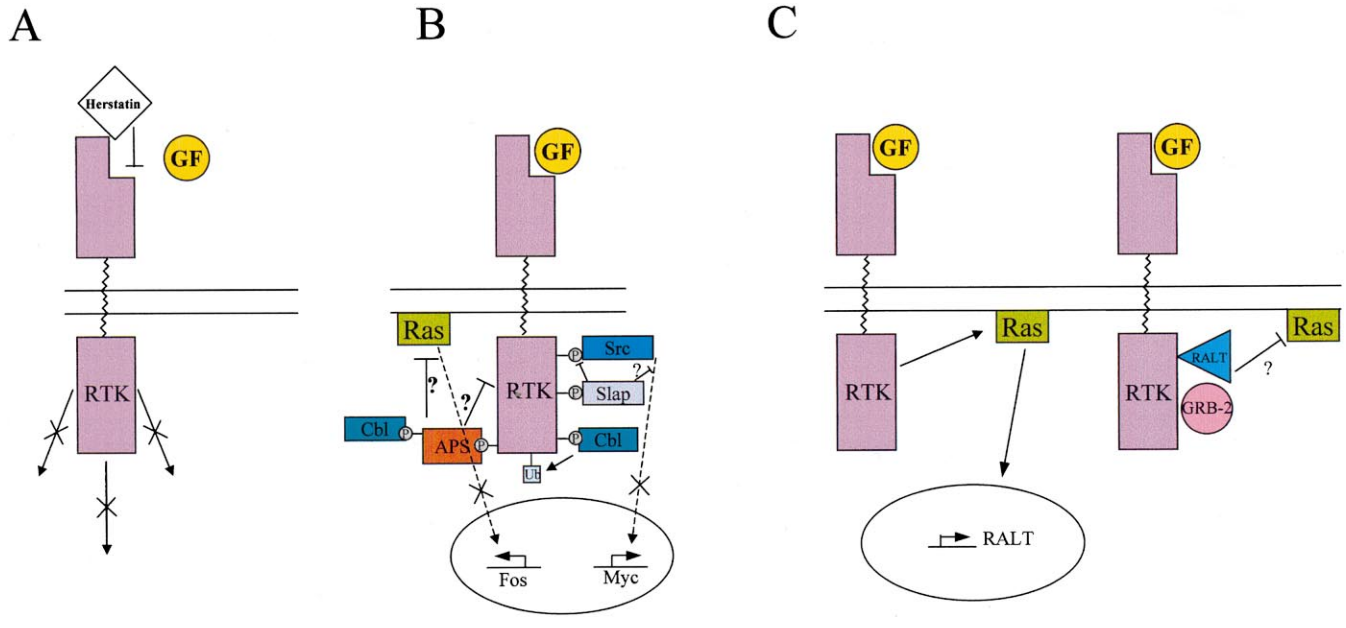

Fig. 5. Inhibitors of mammalian RTKs target different steps of receptor signaling cascades. A: Soluble inhibitors such as Herstatin interact with the extracellular domain of RTKs thereby inhibiting the initial steps of receptor activation (ligand binding and/or receptor dimerization). B: Following ligand activation, inhibitors such as c-Cbl, Slap and APS-1 are recruited onto RTKs. c-Cbl enhances RTK ubiquitination, thereby driving activated RTKs to the internalization/degradation pathway. APS-1 is phosphorylated on tyrosine, binds c-Cbl and inhibits PDGF-R signals leading to *fos* transcription. Slap inhibits PDGF-R signals which lead to Myc transcription via Src activation. C: Signals from ErbB RTKs induce transcription of *ralt*, whose product binds to activated ErbB receptors and inhibits signaling to the Ras/ERK pathway.

COOH-terminal conserved region denominated SOCS box [38] (Fig. 4). SOCS proteins are thought to inhibit cytokine receptors signaling by binding to receptors themselves and/or Jak kinases via their SH-2 domain in a PTyr-dependent fashion [38].

Recent studies have implicated SOCS proteins also in negative regulation of a number of RTKs. SOCS-3 was identified as a feedback inhibitor of the Stat5b pathway activated by the insulin receptor (IR). In this study it was shown that insulin stimulation of 3T3-L1 adipocytes induced expression of SOCS-3 via Stat5b; SOCS-3 in turn bound to PTyr 960 of the IR [39]. Because Stat5b itself binds to PTyr 960, it is likely that SOCS-3 competes with Stat5b for binding to the IR, thus inhibiting receptor signaling to this pathway [39].

SOCS-1 has been shown to bind to ligand-activated c-Kit in a PTyr-dependent fashion, as well as to GRB-2 and to the Rho family exchange factor Vav in a PTyr-independent fashion [40]. In cells of the hematopoietic lineage *socs-1* mRNA expression is turned on by c-Kit signaling with kinetics typical of immediate–early genes [40]. Constitutive expression of SOCS-1 in hematopoietic cells inhibits stem cell factor (SCF, the c-Kit ligand)-dependent proliferation, while affecting viability only to a limited extent. Hence SOCS-1 overexpression ablates proliferative but not survival signals propagated by the c-Kit RTK [40]. Because its overexpression has no consequences on c-Kit enzymatic function, it is thought that SOCS-1 may compete with c-Kit effector/s for binding to the receptor. Alternatively, SOCS-1 may inhibit the activity of transducers, such as Vav and GRB-2, which SOCS-1 itself interacts with [40].

An attractive scenario is depicted by the recent observation that the COOH-terminus of SOCS proteins binds Elongins B and C. The elongin B/C complex was initially co-purified with Elongin A and was shown to enhance transcription by RNA pol II. More recently Elongin B/C was also detected in a complex with the VHL (Von Hippel-Lindau) tumor suppressor protein [41]. Binding of the Elongin B/C complex to either Elongin A or VHL is mutually exclusive, because both Elongin A and VHL contain a BC box which recognizes Elongin C. Independently of its binding to Elongin A or VHL protein, Elongin C can bind to Cullin-2, a putative ubiquitin E3 ligase [41]. Elongin B, on the other hand, has a ubiquitin-like sequence which in other proteins is involved in the recognition of components of the proteasome. Hence the product of the VHL locus is thought to divert cellular proteins to the proteasome machinery: mutations found in VHL patients compromise this function, because of the inability of mutant VHL products to bind to the Elongin B/C complex [41,42].

Recently, the BC box has been found in the COOH-termini of several other proteins, including the SOCS family members as well as signaling proteins containing either WD40 or ankyrin repeats [43]. By analogy with the VHL proteins it has been proposed that the BC box allows these signaling proteins to assemble complexes which have ubiquitin E3 ligase activity and target-associated proteins for protein degradation. SOCS-1 can indeed be recovered in a complex with Elongin B and C; importantly this interaction requires integrity of the SOCS-1 BC box [43,44]. A recent report by De Sepulveda et al. provides tantalizing evidence that SOCS-1 may direct Vav to the ubiquitin/proteasome degradation pathway, thus inhibiting Vav transforming signals [45]. Vav could be found in a complex with SOCS-1; SOCS-1 overexpression, in turn, reduced steady state levels of co-expressed Vav by enhancing its degradation [45]. Significantly, inhibition of proteasome function led to stabilization of Vav protein co-expressed with SOCS-1 and allowed the detection of ubiquitin-conjugated Vav. Because Vav acts as an exchange factor for the Rac GTPase, SOCS-1 may dampen c-Kit signaling to Rac. Furthermore, recruitment of SOCS-1 to c-Kit may supply in *trans* a ubiquitin E3 ligase activity directed toward c-Kit itself and/or other effector molecules bound to the receptor.

RALT (receptor-associated late transducer) is a recently described feedback inhibitor of mitogenic responses propagated by the ErbB-2 receptor [46] (Fig. 4). RALT mRNA and protein are barely detectable in growth-arrested murine fibroblasts. However strong activation of *ralt* transcription is observed upon activation of the ErbB-2 kinase or serum stimulation; because *ralt* mRNA accumulation is enhanced by cycloheximide and abolished by actinomycin D, *ralt* is considered to be an immediate–early gene. RALT protein accumulates during the G_1 phase of the cell-cycle but disappears abruptly as cells approach the S phase [46]. In cells treated with proteasome inhibitors the RALT protein is stabilized (M.F. and O.S., unpublished observations). Significantly, RALT contains two blocks of PEST sequences (Fig. 4), usually found in proteins with rapid turn-over rates. This integrated control of RALT expression (transcriptional and post-translational) allows RALT to be expressed in a window of the G_1 phase which follows early biochemical events triggered by mitogenic stimulation and precedes transition into S phase. Cells engineered to express ectopic RALT at levels exceeding 2–3 fold those of the endogenous protein are impaired in their ability to proliferate in response to ErbB-2 activation. This is caused by accumulation of cells in the G_1 phase, without any loss of cell viability [46]. It is noteworthy that mitogenic responses to serum and PMA were not altered by RALT overexpression, despite the ability of both agents to induce RALT expression. Hence RALT is not a general suppressor of mitogenic signaling, but appears to target specifically receptors of the ErbB family ([46], M.A., L.F. and O.S., unpublished observations). Disappearance of RALT upon entry into S phase likely allows cycling cells to escape from a priori inhibition of ErbB-2 when entering the next G_1.

The ability of RALT to interfere with ErbB-2 signaling may depend on its ability to interact with ligand activated ErbB-2 molecules. RALT interacts also with SH3 domains in vitro and can be co-immunoprecipitated with GRB-2. Thus, RALT behaves as an adapter: its relocation from cytosol to the membrane compartment upon ErbB-2 activation may allow for regulated recruitment of inhibitors to ErbB-2 [46]. In a search for pathways targeted by RALT inhibitory activity, it was found that in murine fibroblasts overexpressing RALT activation of ERKs is altered upon ErbB-2 triggering but not following serum or PMA stimulation. It is noteworthy that RALT overexpression did not alter the initial profile of EGFR/ErbB-2-dependent ERK activity (i.e. up to 1 h of EGF stimulation), whereas inhibited late ERK activation (i.e. that observed after 3–9 h of ErbB-2 activation). This observation is interesting because it indicates that ectopic RALT intercepts ERK activation in the same temporal window in which also endogenous RALT is expressed [46]. Furthermore, because induction of RALT expression by ErbB-2 signaling is largely controlled by the Ras/ERK pathway (O.S. and S. Alemà, unpublished observations), it appears that RALT is part of

a feedback loop which modulates the output of signals generated by the Ras/ERK pathway (Fig. 5C).

4. An emerging picture of negative regulation of RTK signaling in mammalian cells

The examples discussed above indicate that negative regulators of RTK signaling exert their function by engaging in protein–protein interactions. This comes as no surprise, since the activity of RTK effectors is regulated via a complex network of molecular interactions [3]. The emerging role of negative regulators of RTK signaling also suggests that in many instances we will have to refer to signals initiated by RTKs as circuits rather than vectorial pathways.

Our understanding of the biochemistry and biology of RTK inhibitors is still quite rudimentary. Yet, a few paradigms emerge (Fig. 5) which are worth to be discussed: (1) more strategies of negative signaling may be coordinately used by a cell to modulate the output of a given RTK; (2) different modes of interference with RTK signaling may lead to global dampening of RTK activity or selective ablation of a defined signaling pathway; (3) inhibitors may target different temporal windows of RTK signaling; (4) the level of expression of a given inhibitor relative to the level of receptor occupancy (i.e. ligand availability) critically defines whether an inhibitor is involved in fine tuning of RTK signaling or radical termination of receptor activity.

1. The EGFR is an example of an RTK which is sequentially targeted by different inhibitors. Following ligand activation, the EGFR is bound by c-Cbl which promotes receptor internalization and degradation [47]. EGFR signaling, in turn, induces expression of RALT [46] which feeds back to the receptor to inhibit its mitogenic signaling. Internalized EGFR molecules are still competent for signaling to the Ras pathway [48] but, at variance with receptors located at the plasma membrane, are unable to promote PI hydrolysis via PLC-γ [49]. Interestingly, RALT was shown to co-localize with internalized EGFR/ErbB-2 chimeric receptors and to inhibit late ERK activation by ErbB-2 [46]. Hence it is possible that the sequential recruitment of c-Cbl and RALT onto the EGFR ensures that receptors signals are quenched in all the subcellular compartments in which active receptors are localized.
2. Different strategies of interference with RTK activation are likely to have evolved in order to provide flexibility of regulation. Soluble molecules such as Herstatin [30] can block the early steps of receptor activation and attenuate signaling to the entire repertoire of effector pathways utilized by the ErbB-2 receptor. Because ErbB-2 is expressed mainly on epithelial cells and ligands for which ErbB-2 acts as a co-receptor are synthesized by surrounding stromal cells, production of Herstatin by cells expressing ErbB-2 may represent an effective measure to counteract ligand activity and block receptor function. Soluble RTK antagonists appear to be important to impose a spatial constraint to the activity of RTK ligands in scenarios such as vectorial cell migration and proliferation. On the other hand, intracellular inhibitors which block specific pathways give the cell the option to attenuate just one of the many signals propagated by a receptor. This strategy may be useful to regulate kinetics and/or strength of a given signal (when considering a linear pathway) or the activity of a network (when considering interconnected pathways). Relatively small differences in signal output may have far reaching consequences in terms of cellular responses. In the *Drosophila* embryo a two fold difference in the dosage of activated Ras can have a striking impact on the development of posterior structures [8]. Likewise, transcriptional responses under combinatorial control may be tuned by varying the input of just one of the different RTK signals which act combinatorially to modulate the activity of gene promoters.
3. The rate limiting step for the activation of inhibitors such as Cbl, Slap and APS is essentially represented by the availability of cognate docking sites on activated RTKs (see above). If this event is not temporally regulated, one can assume that this class of inhibitors acts coordinately with positive effectors to set thresholds of RTK signaling at any given time of receptor activity. On the other hand, RTK feedback inhibitors such as RALT and SOCS-1 epitomize temporally restricted regulation of RTK activity. In this scenario, transcriptional activation of genes encoding feedback inhibitors is a means for the cell to gauge the accumulation of RTK signals; in turn, the ensuing accumulation of RTK inhibitors is used to hold signals in check. Such a mechanism is therefore geared to ensure an accurate reproduction of precise patterns of signals. Accumulation of feedback inhibitors may also protect cells from perturbation, should conflicting signals be generated by simultaneous activation of more receptors. For instance, IL-10 treatment of monocytes induces refractoriness to subsequent signaling by INF-α and γ, an effect which has been attributed to the induction of SOCS-3 by IL-10 [50]. RALT is induced by many mitogenic stimuli in murine fibroblasts, although RALT overexpression inhibits only signaling by ErbB receptors [46]. Such a seemingly redundant transcriptional response may ensure that cells stimulated by non-ErbB ligands become refractory to coincident but inappropriate triggering of ubiquitously expressed ErbB receptors.
4. Typically, ablation of the function and/or expression of RTK inhibitors causes excess RTK signaling; conversely overexpression of RTK inhibitors leads to severe reduction of receptor activity. The physiological function of RTK inhibitors may often lay in between these extreme experimental conditions. Levels of receptor occupancy set the initial threshold of signaling, which is then continuously refined by the interplay between ongoing receptor activity, triggering of effector molecules and recruitment of negative regulators. The developmental genetics of *C. elegans* and *Drosophila* tell us that ultimately it is the tissue context which adjusts this balance to its own needs: in domains where RTK activity is required, negative regulators may simply tune signal output to make sure that the latter is commensurate to the developmental logic. In domains in which ligand activity is still present, and yet RTK function would be detrimental, the job of negative regulators is to extinguish signaling and help to delineate boundaries of receptor activity.

5. Perspectives

Work on negative regulation of RTK signaling has lagged

behind the exponential increase of studies dealing with the biochemistry and biology of RTK effectors. It may be safe to assume that strategies specifically aimed at the identification of RTK inhibitors may yield new members of this functional class of signal regulators. The availability of the complete sequence of the genomes of *C. elegans*, *D. melanogaster* and *Homo sapiens* along with the use of the EST database will allow the thorough characterization of families of RTK inhibitors. In particular, newly identified adapter molecules containing PTB and/or SH2 domains will have to be considered as potential signal inhibitors. Screenings for interactors of RTKs are also likely to yield novel information about molecules involved in inhibition of RTK function. Particular attention will have to be given to the identification of novel feedback inhibitors: here the identification of the full spectrum of RTK-dependent transcriptional responses allowed by cDNA array technologies will be particularly helpful. Finally, the SOCS/Elongin connection and the finding that c-Cbl has E3 ubiquitin ligase activity imply that regulated intracellular protein degradation is an expanding paradigm in negative signaling to RTKs.

Most of the studies on negative regulators of mammalian RTKs have been carried out in cultured cells and are based on overexpression experiments. It is certainly encouraging that nullizigous mice for either *socs-1* or *socs-3* have phenotypes compatible with some of the activities of these gene products defined in tissue culture experiments. *Socs-1* −/− mice die perinatally due to unbalanced cellular responses to INF-γ [51,52]. The *socs-3* nullizigous state is embryonically lethal at day 12–16, due to excess erythropoiesis and massive erythrocytosis; the likely cause of this pathology is thought to be unrestrained signaling by the EPO receptor [53].

There are also indications that tumor cells may exploit the genetic or epigenetic removal of negative regulators of RTK signaling to acquire unrestrained proliferative ability: APS [35] and Herstatin [30] were found to be expressed in some tumor samples at lower levels than in normal tissues. Furthermore, v-Cbl oncoproteins are generated by loss of E3 ubiquitin ligase activity: thus, v-cbl transforming activity may depend on its ability to dominantly suppress c-Cbl and promote unabated signaling by receptors with oncogenic potential [17,54].

Future studies will have to systematically characterize the phenotypes of mice carrying null copies of negative regulators of RTK function. Studies aimed at the ablation of the function of RTK inhibitors in cultured cells will still be very valuable, as redundancy of function within families of related proteins may mask phenotypes in KO mice.

The mechanistic basis of the function of most of the negative regulators of RTK signaling so far described is still far from being elucidated. Ultimately it will be important to assess, in reconstitution experiments, the thresholds of expression of inhibitory molecules which are required to attenuate and possibly terminate RTK signals and compare these experimental thresholds to physiological levels of expression. These notions will have to be integrated with the in situ analysis of the expression of RTK inhibitors during development and in adult life. Different cell types, for instance, may adopt different strategies for regulating signaling by a given RTK, thus allowing for tissue specific differences in the intensity and duration of RTK signals.

Despite our fundamental ignorance of many aspects of the biology and biochemistry of RTK inhibitors, this rapidly evolving field of cell signaling holds great promises for fostering a better understanding of how RTK function is modulated. Eventually, given the pathogenic role of deregulated RTK activity in pathologic conditions such as cancer, it will be possible to harness RTK inhibitors to devise novel approaches for treatment of human diseases.

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