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Negative regulation of cytoplasmic protein tyrosine kinase activity by adaptor proteins

Review Article

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Summary

Adaptor proteins are cytoplasmic signaling molecules that lack intrinsic catalytic activity but they are nevertheless crucial for signal transduction. They bear homology domains (SH2, SH3, PH, PTB, …) important for protein-protein interactions and for function. The first adaptors identified were positive regulators of cell responses, with some even having oncogenic activities. More recently, a new subfamily has emerged that negatively regulates signaling responses. This review will focus on adaptors of this genre and specifically, those that inhibit cytoplasmic tyrosine kinase function and which define a new mechanism for *in vivo* kinase regulation. They include the inhibitors of the Jak, Syk, Fak and Src kinase family and their mechanism for inhibition as well as their possible function in cellular regulation will be discussed.

I. Cytoplasmic protein tyrosine kinases

Tyrosine phospshorylation corresponds to less than 0.1% of the protein phosphorylation content in mammalian cells and yet protein tyrosine kinases play a pivotal role in cell regulation. These enzymes are classified in two distinct families: receptor and non-receptor tyrosine kinases. The first includes receptors for growth factors and ligands involved in neuronal axon guidance. The later comprises cytoplasmic proteins grouped into 8 subfamilies, Src, Fak, Jak, Btk, Syk, Csk, Abl and Fps tyrosine kinases (Courtneidge, 1994). While having a striking homology in their catalytic sequences, they diverge greatly in their regulatory, non-enzymatic sequences, attributing to distinct kinase regulation, substrate phosphorylation and function. These kinases play important roles in cellular signaling and are activated by a large number of stimuli, including hormones, neurotransmitters, growth factors, cytokines, activation of T and B cells, cellular stress, adhesion and migration. In fact, most stimuli that use protein tyrosine phosphorylation for signaling activate two types of tyrosine kinases, one invariably being a member of the Src family. It is proposed that Src functions to phosphorylate and activate a further enzyme responsible for substrate specific phosphorylation. For example, during T cell activation, the Src family member Lck, phosphorylates and activates Zap-70 which in turn phosphorylates downstream effectors for interleukin 2 (IL-2) gene expression (Courtneidge, 1994). Further evidence implicating cytoplasmic tyrosine kinases are genetic studies in mice: gene knockouts of *fak* (Ilic et al., 1995), *csk* (Imamoto and Soriano, 1993) or triple disruption of *src*, *fyn* and *yes* genes (Klinghoffer et al., 1999) all display an embryonic lethal phenotype. Finally, deregulation of these kinases has been linked to several human diseases: for example, oncogenic forms of Abl, Jak, and Src kinases have been identified in human cancers and have also been involved in leukemia or carcinoma development (Roche and Courtneidge, 1997). Moreover, a *btk* gene deficiency causes human X-linked agammaglobulinemia and murine X-linked immunodeficiency (Wen and Van Etten, 1997).

Not surprisingly, their kinase activity is tightly regulated *in vivo* and much effort has gone towards unravelling the molecular mechanism(s) involved. Kinase activation generally begins with the phosphorylation of a tyrosine residue present in an activation loop (Courtneidge, 1994). However, less in known about the additional

mechanisms required for full kinase activation. The best examples studied to date are the Src family members. These enzymes are negatively regulated by phosphorylation of another regulatory tyrosine present at the C-terminus (refered to as pY527 in the chicken Src sequence). The importance of this conserved residue is confirmed by frequent residue deletions observed in oncogenic alleles (Roche and Courtneidge, 1997). Recent structural and mutagenic studies have revealed an even more elaborate model for regulation that in fact involves two intramolecular interactions: the SH2 domain associates with the pY527 and the SH3 complexes with a sequence between the SH2 domain and the kinase domain called the "SH2-linker" and the small lobe of the kinase domain itself. Regulated Src is in a closed or "off" conformation which when opened or turned "on", renders the kinase active (Superti-Furga and Gonfloni, 1997). Several mechanisms for activation can be proposed based on this model, for example, by dephosphorylating the C-terminal tyrosine and/or by interactions with ligands that disrupt either interaction (Superti-Furga and Gonfloni, 1997). While the 3-dimentional structure for Abl is not yet known, mutagenic studies already hint to a similar intramolecular regulation involving the SH3 domain, implying a general mechanism (Barila and Superti-Furga, 1998). In this review, we propose a further mechanism for *in vivo* kinase regulation involving adaptor molecules which can prevent enzymatic activation and/or subtrate accessibility.

II. Adaptors

Adaptors are cytoplasmic proteins that do not possess any intrinsic catalytic activity but do contain known homology domains (SH2, SH3 , PTB, PH,…) important for protein interactions and signaling. For example SH2 domains interact with proteins through the recognition of phosphotyrosine residues and SH3 domains through association with proline rich sequences (van der Geer et al., 1994). The first members identified were either novel regulators of cell growth as induced by growth factors or oncogenes (Grb2 and Shc), or were transduced by transforming retroviruses (vCrk or vCbl) (see **Table 1**). They are thought to signal *via* complexing with their cognate effectors with subsequent targeting to the plasma membrane for activation. For example, the adaptor Grb2 is constitutively associated with the activator of the small GTP-binding protein Ras in the cytoplasm and upon growth factor stimulation, the complex is directed to the membrane enabling signaling (van der Geer et al., 1994). More recently, a new family of protein adaptors has emerged that inhibit cell responses. One of the first examples was Cis (Yoshimura et al., 1995) which was discovered by searching for new genes activated by cytokines. Cis is a small molecule that contains an SH2 domain which bears some homology with the transcription factor Stat, and when overexpressed the cytokine response is inhibited. Since then several adaptors with similar properties have been identified. These include TGFβ signaling proteins of the Smad family (Heldin et al., 1997), gene products related to Cis (the SOCS or SSI family) (Yoshimura, 1998), Cbl (Ota and Samelson, 1997), APS (Yokouchi et al., 1999), FRNK (Richardson and Parsons, 1996), Grb14 (Kasus-Jacobi et al., 1998) and Slap (Roche et al., 1998) (see **Table 1**). Smad (Heldin et al., 1997) and APS (Yokouchi et al., 1999) act by associating with other adaptors to inhibit cellular function, Grb14 (Kasus-Jacobi et al., 1998) appears to inhibit signaling by at least repressing the activity of targeted tyrosine kinase receptors and the others function by inhibiting cytoplasmic tyrosine kinase activities, *in vivo*. The molecular mechanism(s) and the function of the last group is discussed below.

Adapter	Cell response	Cytoplasmic tyrosine kinase regulated by adapters
Grb2	+	
Shc	+	
Crk	+	
Nck	+	
Grb10	+	
SOCS/CIS	-	Jak
Slap	-	Src
FRNK	-	Fak
Cbl	-	Syk
Grb14	-	
Smad	-	
APS		

Table 1. Adapter proteins that activate (+) or inhibit (-) cell response and the cytoplasmic tyrosine kinases regulated

A. Jak inhibition by the SOCS adaptors

The cytoplasmic tyrosine kinases of the Janus kinase family (Jak) are important regulators of the biological effects exerted by cytokines in haematopoietic and immune cells. Jak are constitutively associated with cytokine receptors and undergo tyrosine phosphorylation and activation following ligand binding. Important effectors of these kinases are the transcription factors of the Stat family. Upon cytokine stimulation, Stat associates with the cytoplasmic tail of the receptor through its SH2 domain and becomes phosphorylated by Jak. As a consequence, phosphorylated Stat dimerizes and translocates to the nucleus for target gene activation (Starr and Hilton, 1999). A functional approach was utilized in order to isolate inhibitors of cytokine signaling and led to the identification of the Supressor Of Cytokine Signaling family (SOCS)

(Starr and Hilton, 1999). Additional members were further identified while searching for Jak interactors and new gene products with Stat SH2 homology. The SOCS family comprizes at least 8 members (SOCS1–7 and Cis) (Starr and Hilton, 1999) and are composed of a variable N-terminus, a central SH2 domain with Stat SH2 homology and a conserved sequence of about 40 amino acids called the "SOCS box" (**Figure 1**). This new domain has also been found in several unrelated proteins and is thought to participate in protein degradation. SOCS adaptors inhibit Jak function in two ways: either by inhibiting the kinase activity (SOCS-1/JAB/SS-1) or by preventing association with and phosphorylation of the Stat proteins (Cis) (see **Figure 2A**). Mechanistically, SOCS-1 interacts with the phosphotyrosine in the activation loop of the Jak2 catalytic core preventing kinase activation both *in vitro* and *in vivo* (**Figure 2A**). While association involves the SOCS-1 SH2 domain, efficient inhibition also requires an additional 12 amino acid stretch present in the N-terminus (the "kinase inhibitory region"). SOCS-1 is activated at the transcriptional level by Stat and may define a negative regulatory loop as it is activated within the first minutes of cytokine stimulation (Yasukawa et al., 1999). Recently, an additional mechanism has been proposed in SOCS regulation, namely protein degradation. Elongin B and C were identified as interactors of the "SOCS box" domain and which potentiated Jak inhibition and increased SOCS protein stability (Kamura et al., 1998; Yasukawa et al., 1999). Interestingly, elongin B displays some structural homology with Skp1, a protein involved in the proteasomal degradation pathway and so SOCS may also target elongin to Jak for subsequent degradation.

The physiological function of SOCS-1 is not known and still awaits gene disruption in the whole animal, however when overexpressed, SOCS-1 is able to inhibit a number of cytokine responses suggesting that it plays a central role in the regulation of the Jak/Stat pathway. Thus far, the cytokine signaling pathways affected include oncostatin M, thrombopoietin, growth hormone, leukaemia inhibitory factor and IL-6 (Starr and Hilton, 1999). Finally, it should be noted that SOCS-1 function may not be solely restricted to the Jak tyrosine kinases since it was recently shown to reduce tyrosine kinase activity of the Tec family (Starr and Hilton, 1999). However, the mechanism and role of SOCS in Tec signaling is currently not known.

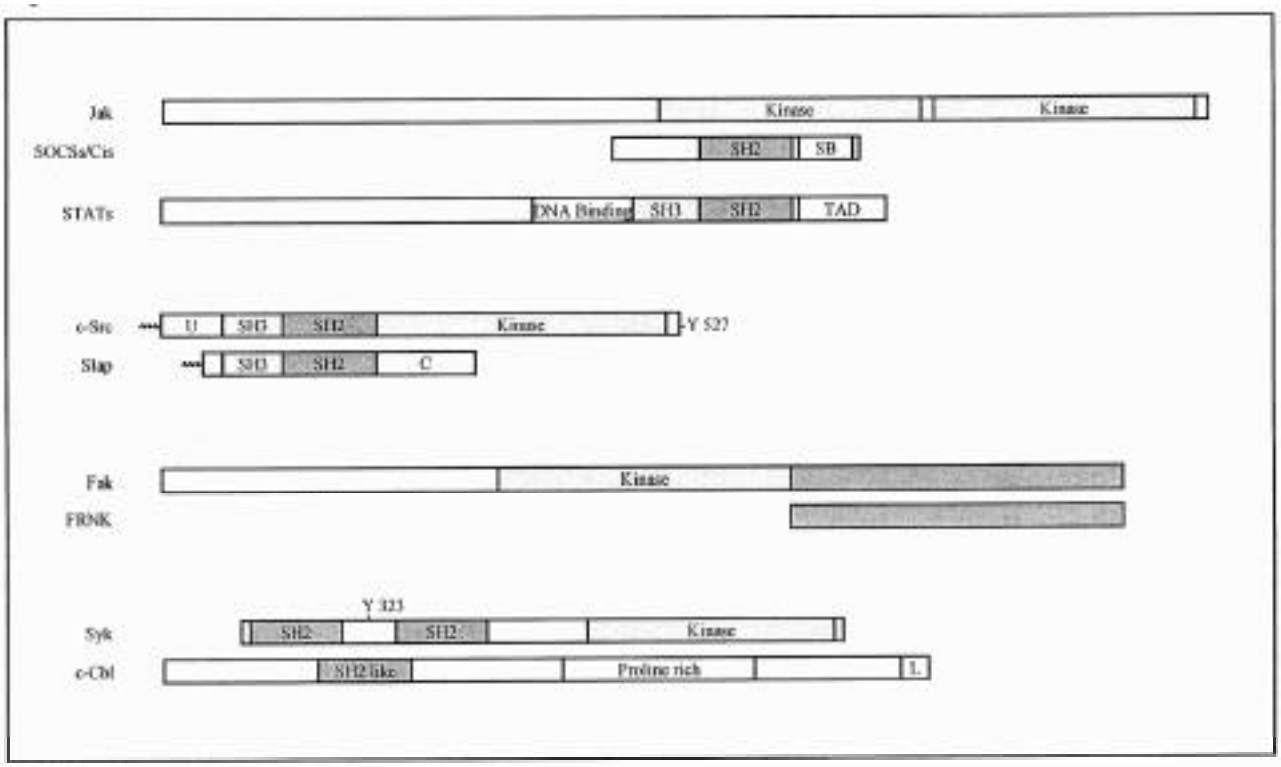

Figure 1. Structure of various cytoplasmic protein-tyrosine kinases, transcription factor and adapter proteins involved in negative regulation. The presence of the kinase region, the SH2, SH3 and unique (U) domains, as well as the DNA binding domain, the transactivation domain (TAD), the SOCS box domain (SB), the leucine zipper domain (L) and the proline rich region are indicated. (✹) indicates the presence of a myristyl group.

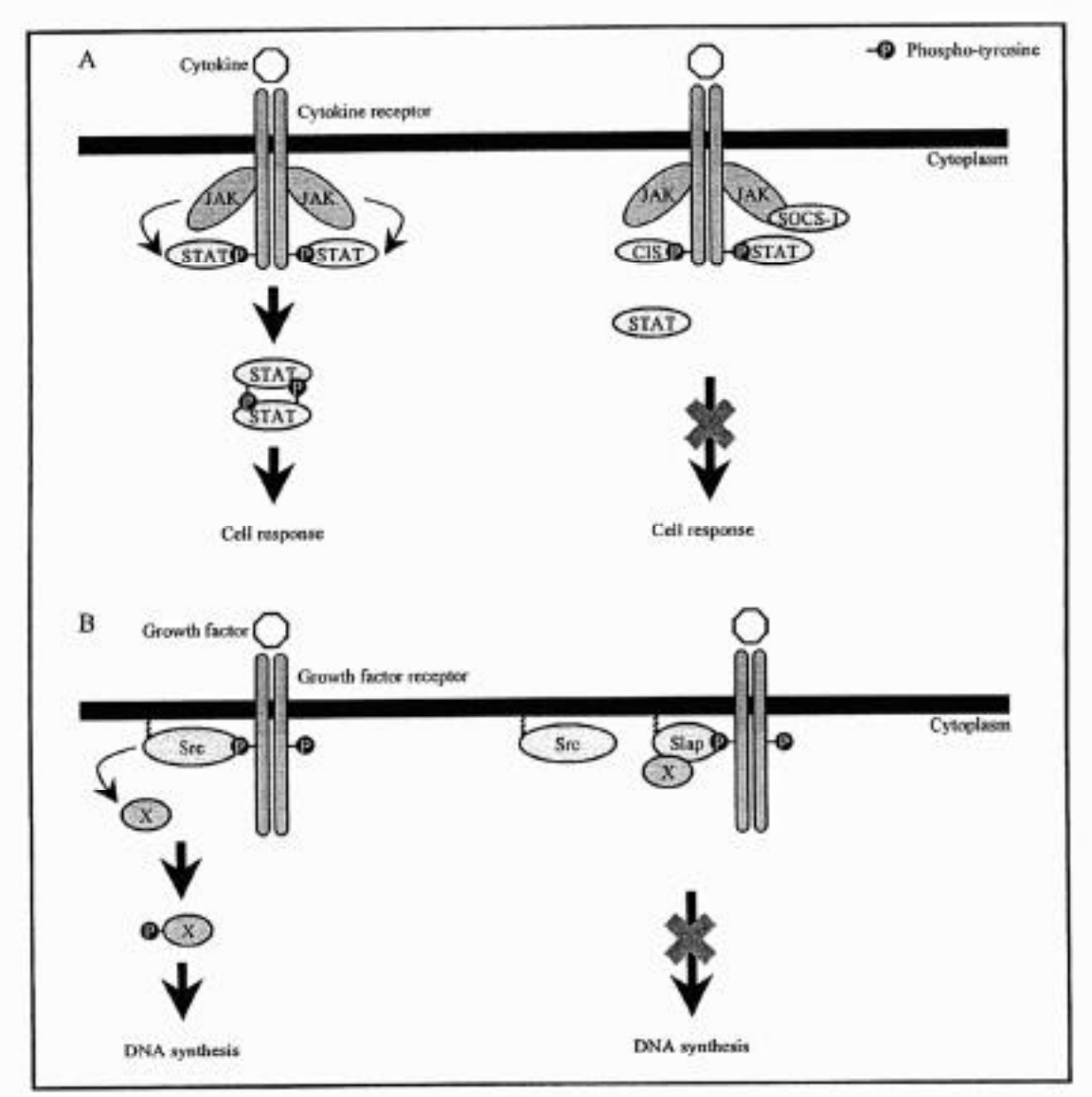

Figure 2. Mechanisms for negative regulation of the Jak/STAT (A) and Src (B) pathways. A: Inhibition of the Jak activity by SOCS-1 and inhibition of STAT receptor association and phosphorylation by CIS. B: Inhibition of Src activation by growth factor receptor and substrate phosphorylation by Slap.

In contrast to SOCS-1, Cis does not affect Jak kinase activity but rather acts by preventing substrate phosphorylation *in vivo* (**Figure 2A**). The Cis SH2 domain bears strong homology with the Stat SH2 domain and competes with Stat for cytokine receptor association, thus preventing Stat membrane translocation and Jak2 association. Like SOCS-1, Cis is activated at the transcriptional level by IL-3 and Epo. However, it again differs from SOCS-1 in the time course of activation since *cis* expression persists after 24h of ligand exposure (Yoshimura, 1998). Therefore, various SOCS may regulate different aspects of cellular responses induced by cytokines and a combination of adaptors may define a fine tuning mechanism for the strength and length of signaling. This aspect of signal transduction can have dramatic consequences on cell response; for example, it has been shown that a prolonged activation of the serine/threonine Map kinase switches the cell from a growth response to a differentation process in the neuronal precursor cell line, PC12 (Dikic et al., 1994; Traverse et al., 1994).

B. Syk inhibition by the adaptor Cbl

Cbl is the second example of an adaptor that negatively regulates cytoplasmic tyrosine kinases *in vivo*. Cbl is a cellular proto-oncogene with a viral homolog v-Cbl originally found in a rodent leukemia retrovirus (Langdon et al., 1989). In contrast to the later, Cbl inhibits cell growth and transformation (Liu and Altman, 1998) and targets the tyrosine kinase Syk of the ZAP-70 family, which is important for lymphoid T and B cell activity and mast cell degranulation. Syk is activated by translocating to the membrane and associating with the T (or B) cell antigen receptor. This complex formation involves tyrosine phosphorylated sequences (ITAMs) in the receptor and the two Syk SH2 domains. Membrane associated Syk is then phosphorylated by Lck leading to maximal kinase activation. Moreover, Syk can be inactivated by phosphorylation of tyrosine 323 present in the linker region between the two SH2 domains and which prevents receptor association (Meng et al., 1999). When overexpressed, Cbl associates with phosphorylated Syk and is able to inhibit mast cell activation (Ota and Samelson, 1997). This adaptor also contains an SH2-like (SHL) domain at the N-terminus (see **Figure 1**) that has recently been shown to function as a phosphotyrosine binding domain (Meng et al., 1999). Cbl can inhibit Syk by association with pY323 through its SHL, leaving the kinase in a repressed form. Therefore, Cbl may regulate Syk signaling by preventing kinase activation rather than inhibiting the catalytic activity as proposed for SOCS-1. Just how the Cbl-Syk complex formation is regulated is not known.

In addition to Syk, Cbl also regulates receptor tyrosine kinase function including the receptors for growth factors (Liu and Altman, 1998). However, while its inhibitory mechanisms are still unclear, several reports suggest that it induces receptor down regulation and degradation (Lee et al., 1999; Levkowitz et al., 1998). In contrast, microinjection data suggests that Cbl may specifically affect the DNA synthesis signaling pathway activated by cytoplasmic tyrosine kinases of the Src family (Broome et al., 1999). Finally, this adaptor becomes tyrosine phosphorylated *in vivo* creating binding sites for various signaling proteins including phosphoinositide 3-kinase and the adaptor Grb2, which all may influence signaling (Liu and Altman, 1998). Overall, the data suggests that Cbl may be a general inhibitor of cell activation and may affect several intracellular events. A physiological role for Cbl was first demonstrated in *C. elegans* where the orthologue, SLI-1, reduces Let23 (EGF receptor) signaling (Jongeward et al., 1995). In mammals, its function has also been addressed by gene disruption in mice ; while a number of negative functions have been attributed to Cbl using tissue culture models, $cbl^{-/-}$ mice are viable suggesting that this adaptor is not mandatory for development. Rather, a marked change in haematopoietic profile was observed including lymphoid hyperplasia and enhanced T cell signaling (Murphy et al., 1998). This led to the postulation that adaptors with negative function may define a subtle mechanism for cell regulation rather than an « on/off » process. Finally, an *in vitro* study attributed a positive function for Cbl during bone resorption (Tanaka et al., 1996) indicating that these adaptors could also have positive functions in some circumstances.

C. FRNK as a negative regulator of Fak

The Focal adhesion kinase, Fak, is an important regulator of cell adhesion and migration. In contrast to the other subfamilies, it does not bear any known homology sequences like SH2 or SH3 domains. Instead, it has two proline-rich regions important for binding SH3-containing proteins and a C-terminal sequence called FAT, which mediates cellular focal contact association (Parsons, 1996) (**Figure 1**). While the mechanism for kinase activation is not fully understood, Fak tyrosine phosphorylation creates an SH2 binding site for Src (or Fyn) leading to enzymatic stimulation that in turns phosphorylates Fak further for maximal stimulation (Parsons, 1996). The product of an alternative spliced variant of the *fak* gene has been isolated which encodes the pp125FAK-related non-kinase, FRNK. This adaptor is composed of the C-terminal, non catalytic sequence of Fak and includes both the proline-rich and FAT sequences (**Figure 1**). When overexpressed, FRNK was shown to inhibit Fak *in vivo* preventing focal adhesion formation on fibronectin and substrate phosphorylation (Richardson and Parsons, 1996). Like Cis, FRNK may act by a competitive mechanism since Fak over expression reverses the FRNK inhibitory effect (Richardson et al., 1997). However, neither the importance of this spliced variant *in vivo* nor how its activity is regulated are known. Endogeneous protein has been detected in non transformed rodent embryo fibroblasts (Richardson and Parsons, 1996) suggesting that FRNK could define a signaling threshold but the relative protein levels of FRNK and Fak need to be investigated.

D. Negative regulation of Src by the adaptor Slap

The Src family tyrosine kinases have important functions in a number of cell responses including cell growth, differentiation and migration. Moreover, when deregulated, they display oncogenic activity (Roche and Courtneidge, 1997). They are anchored at the membrane *via* myrsitoylation at the N-terminus and include in addition to a catalytic sequence an SH3 and SH2 domain important for kinase regulation (**Figure 1**). In non stimulated cells, endogenous Src kinases are inactive but are transiently activated by a number of extracellular stimuli including growth factors (Roche and Courtneidge, 1997). In the later case, activated receptors associate with Src members

through their SH2 domains leading to kinase activation. While substrates important for growth factor response are ill-defined, several groups including our own suggest a requirement for these kinases during mitogenesis (Broome and Hunter, 1996; Roche et al., 1995). A microinjection approach has also indicated that they act by upregulating the transcription factor, *c-myc* (Barone and Courtneidge, 1995). In contrast to most cytoplasmic kinases, Src members show a broad range of substrate phosphorylation. Nevertheless, they require intact SH3 and SH2 domains for signaling, probably for specific susbtrate association and phosphorylation. For example, despite a high kinase activity, deregulated Src lacking an SH3 domain fails to be transforming (Erpel et al., 1995) but is able to inhibit the mitogenic response induced by PDGF in fibroblasts (Erpel et al., 1996).

The Src-Like Adaptor Protein (Slap) was originally identified in a 2-hybrid screen while searching for new interactors of the Eck tyrosine kinase receptor (Pandey et al., 1995) and has an SH2 and SH3 domain with high identity to those of Src. In addition, Slap is myristoylated and largely colocalizes with Src *in vivo* (Manes et al., 2000) and also bears a unique C-terminus sequence of about 100 amino acids that is involved in protein-protein interaction (**Figure 1**). We have previously shown that Slap inhibits growth factor-induced DNA synthesis when overexpressed (Roche et al., 1998) and mutagenesis analyses together with the microinjection studies have allowed us to conclude that Slap inhibits Src function probably by associating with and competing for Src signaling substrates (Manes et al., 2000). The Src and Slap SH2 domains show strong functional homology and are essential for their respective functions. As a consequence, Slap associates with growth factor receptors through the same phosphotyrosine binding site used by Src. Therefore it may act by preventing association with and activation of Src by the receptor. However, Slap does not act solely by a competition mechanism as described for FRNK ; indeed, Src overexpression does not reverse the Slap inhibitory effect and further analyses pointed to an involvement of the C-terminus (Manes et al., 2000). We therefore propose a model where Slap utilizes its SH2 domain primarily for proper cell localisation (binding to the PDGF receptor for example) whereas the whole Slap molecule would additionally titrate Src effectors implying the C-terminus sequence, thus preventing signaling despite Src overexpression (**Figure 2B**). From this we initially predicted two mechanisms of Src inhibition: a competitive (*via* the SH2 domain) or a non competitive (*via* both the SH2 and the C-terminus) mechanism. However, another important difference between Src and Slap lies in the SH3 domain. Despite the strong homology, three critical residues important for ligand binding specificity in Src SH3 are replaced in Slap SH3. As a consequence, they display distinct binding specificities suggesting that Slap will not inihibit Src SH3-dependent signaling. Indeed, Slap over expression is unable to reverse cell transformation induced by oncogenic Src (Manes et al., 2000), probably by its inability to interfere with Src SH3 effectors required for oncogenic events such as cytoskeletal rearrengement. These latter observations predict that Slap SH3 may also have specific functions and binding partners over and above being a simple Src competitor.

How Slap itself is regulated is not known although it should be noted that a proline-rich «SH2 linker » is present int the Slap C-terminus. It is enticing to speculate that a similar intramolecular regulation exists for Slap as does for Src. In contrast to SOCS inhibitors, its protein level does not change dramatically during the cell cycle, suggesting that it does not define a negative regulatory loop. Also, Slap is not tyrosine phosphorylated like the adaptor Cbl. We feel that Slap could also define a signaling threshold for cell response modulation and to support this hypothesis, inhibition of endogeneous Slap function by antibody microinjection led to an increased cell response toward growth factors in fibroblasts (Roche et al., 1998). Furthermore, SLAP may transduce a signal for cell differentiation as its gene was found upregulated in cells treated by a differentiation agent.

III. Conclusion

The identification of this new family of adaptors with negative function in cellular processes provides a new mechanism for cytoplasmic tyrosine kinase regulation *in vivo*. These adaptors clearly involve several mechanisms including inhibition of the catalytic activity, prevention of activation by competitive association with receptors and inhibition of phosphorylation by substrate association. The use of adaptors for tyrosine kinase regulation may be a general phenomenon and additional members are likely to be discovered. For example, kinase inhibitors of Btk (Yamadori et al., 1999) and Abl (Wen and Van Etten, 1997) have recently been identified but have not been discussed in this review since they have no obvious homology domains. Regulation of their activity is not clearly established. Some, such as SOCS, define a negative regulatory loop and are activated by gene expression while others like Slap may orchestrate a signaling threshold response and could also be involved in cell differentiation processes. Regulation of their activity is an important issue that needs to be addressed, as it may have important consequences on cell responses and human diseases linked to cytoplasmic tyrosine kinases. For the most part, their physiological function *in vivo* are not known and this is another important issue that needs to be addressed. However, given the fact that they are involved in the fine tuning of signaling for optimal cell responses, one can comfortably predict that they will not be mandatory for embryogenesis.

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