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Review

Commentary: The carboxyl-terminal Crk SH3 domain: Regulatory strategies and new perspectives

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ABSTRACT

Since their discovery as cellular counterparts of viral oncogenes more than two decades ago, enormous progress has been made in unraveling the complex regulatory pathways of signal transduction initiated by the Crk family of proteins. New structural and biochemical studies have uncovered novel insights into both negative and positive regulation of Crk mediated by its atypical carboxyl-terminal SH3 domain (SH3C). Moreover, SH3C is tyrosine phosphorylated by receptor tyrosine kinases and non-receptor tyrosine kinases, thereby permitting assemblages of other SH2/PTB domain containing proteins. Such non-canonical signaling by the Crk SH3C reveals new regulatory strategies for adaptor proteins.

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Named for their ability to elevate cellular tyrosine phosphorylation and transform primary chicken embryo fibroblasts (CEFs) [1], CT10 regulator of kinase (Crk) is the prototypical member of a class of Src Homology-2 (SH2) and Src Homology-3 (SH3) domain-containing proteins that lack intrinsic enzymatic activity but instead function to promote protein–protein interactions [2,3]. Over the past two decades, enormous progress in our understanding of signal transduction has emerged from studies on Crk. The canonical signaling pathway, defined by recognition of specific phosphotyrosine motifs by the SH2 domain (in the context of *p*-Tyr.X.X.Pro) [4] and Polyproline Type II motifs by the N-terminal SH3 domain {in the context of Pro. X. X. Pro. X. (Lys/Arg)} [5], identified Crk as a connector between Receptor Tyrosine Kinases (RTKs) and/or their substrates and GTPase effector pathways (Fig. 1). Along with efforts on Grb2 [6], these insights were instrumental in defining missing links between integrins and/or tyrosine phosphorylated growth factor receptors and downstream effector pathways that regulate growth and differentiation, and also more generally for defining elements of modular domains in signaling. In recent years, new layers of regulation for Crk have emerged, pointing to not only unusual levels of auto-inhibition mediated by its atypical carboxyl-terminal SH3 domain, but also unexpected features of SH3 domain signaling that activate non-canonical pathways.

CrkII and CrkL each contain an atypical C-terminal SH3 domain (SH3C), defined by its inability to bind to Polyproline Type II (PPII) motifs [7,22]. In both proteins, the atypical SH3 domain retains core structural characteristics of SH3 domains, consisting of a five-stranded β-barrel which forms the standard SH3 fold. However, the aromatic amino acids – F141, W169, Y186 which line the canonical PPII binding pocket on the Crk SH3N and are conserved in conventional SH3 domains, are replaced by polar residues – Q244, Q274 and H290 on the SH3C surface (Fig. 2), thereby rationalizing the drastically reduced affinity of this domain for proline rich sequences.

The carboxyl-terminal region of Crk is considered inhibitory, since it contains elements that constrain binding to the N-terminal SH2 and SH3 domains. Phosphorylation at Tyr221 in the inter-SH3 linker region facilitates binding to the Crk SH2 in *cis* to form a closed structure that prevents the SH2 domain from binding in *trans* to tyrosine phosphorylated proteins [8–10] (Fig. 3a). Another layer of negative regulation appears to operate to prevent binding in *trans* to the SH3N. This is mediated via *cis*–*trans* isomerization about the Gly237-Pro238 peptide bond (in gallus CrkII) [11,12]. Intriguingly, only the *cis* conformer adopts an autoinhibited state whereby the hydrophobic surface of the canonical PPII binding site on the SH3N (composed of Phe142, Phe144, Trp170, Tyr187, Pro184 and Pro186) is occupied by Pro238, Phe239 and Ile270 of the SH3C in a manner similar to a PPII peptide. This arrangement in essence allows the SH3C to act as a reversible lid that caps the SH3N from binding PPII ligands.

In contrast, the *trans* conformer adopts an open state where the PPII binding site on the SH3N is not occluded by the SH3C as Phe239 undergoes a major shift to the interior of the SH3C and Ile270 moves to face the center. In the ligand unbound form, the *cis* conformer is favored thereby resulting in 90% of the SH3N-lin-

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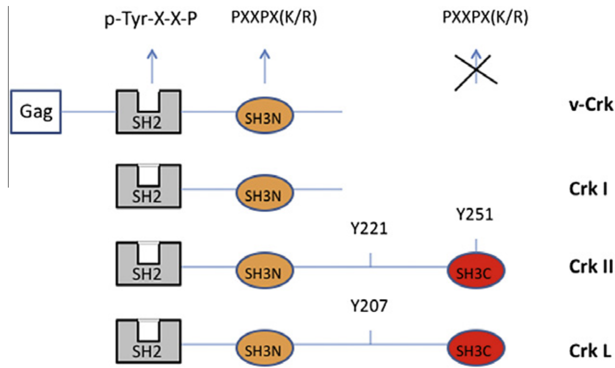

Fig. 1. Overview of the Crk family of adaptor proteins. Domain organization and binding preferences of the Crk family of adaptor proteins. Note that the C-terminal SH3 domain is atypical in that it does not recognize PPII motifs.

ker-SH3C molecules to be in the auto-inhibited state (Fig. 3b). While CypA can affect the kinetics of conversion between the auto-inhibited and open state by catalyzing the inter-conversion between the *cis* and *trans* conformers, CypA does not appear to be the driving force that regulates the ratio of these conformers. Ligand binding to the subset of molecules in the open state is predicted to shift the equilibrium towards the open state and "activate" Crk SH3N mediated signaling. This is likely mediated by the cellular localization and availability of the natively expressed proteins with PPII motifs.

Adding to the complexity, the sequence around Pro238 is not conserved in human Crk and it remains to be determined if *cis–trans* isomerization operates to negatively regulate the human Crk SH3N. However, the solution structure of human Crk has revealed the existence of a modified mode of auto-inhibition conferred by the linker in between the SH3 domains [10], the same general region identified to control *cis–trans* isomerization in the chicken Crk protein (Fig. 3c). Residues 224–237, called the Inter – SH3 core (ISC), form contacts with the SH2 and the SH3 domains to assemble CrkII into a compact structure. Mutation of prolines and hydrophobic residues in the ISC to glycine and serine respectively, enhances the affinity of the SH3N for a PPII peptide in the context of full length CrkII. Also, mutation of W275 in the SH3C, which would disrupt the core fold of this domain, also enhances the affinity of the SH3N suggesting that the structural integrity of the SH3C is required to maintain crucial contacts with the ISC for SH3N auto-inhibition. Although the mode of auto-inhibition of the SH3N may not be conserved between chicken and human Crk, the function of the linker and the atypical SH3 to regulate SH3N mediated signaling nonetheless appears to be maintained.

This notion that assembly of inter-domain interactions has significant functional consequences is further highlighted by insights into the structure of CrkL, a closely related protein to CrkII. These studies have brought to light distinct features that are regulated by structural organization of the SH2 and SH3 domains [22]. Although there is a high level of conservation within the modular domains between the two proteins and the binding preferences are very similar, the linker and the SH3C domain are organized very differently in CrkL compared to CrkII (Fig. 3d). While in CrkII, the linker and the SH3C have been shown to play a key role in auto-inhibition of the SH3N, there appears to be no such contribution by the respective elements in CrkL. Also strikingly, the SH2 and the SH3N interact directly by predominantly polar contacts to occlude the binding pocket of the SH2 domain in CrkL whereas such occlusion of the SH2 binding pocket is not observed in CrkII. Interestingly, phosphorylation at the negative regulatory tyrosine 207 in CrkL that results in binding to its own SH2 does not reduce the affinity of the SH3N domain for its ligands. In contrast, the CrkII SH3N has been shown to be partially occluded upon phosphorylation at Y221 and binding to its own SH2 domain. These new insights have impressed upon the idea that although the typical folds of these modular domains determine specificity of ligand recognition, the linkers have evolved to bear key regulatory features that determine the binding affinities of these modular domains for their respective ligands in the context of full length proteins.

While the atypical SH3C domain is intrinsically negative regulatory in nature, new data also suggests that it can have an affirmative role in signaling courtesy of a non-canonical role mediated by tyrosine phosphorylation. The Crk SH3C, in contrast to conventional SH3 domains, has a tyrosine residue in the RT-loop structure. Recently, this residue, Y251, was shown to be phosphorylated by Abl and downstream of EGFR in addition to the negative regulatory tyrosine 221 [13]. Phosphorylation at Y251 enhances Abl activation by virtue of binding to the Abl SH2 domain and unclamping the

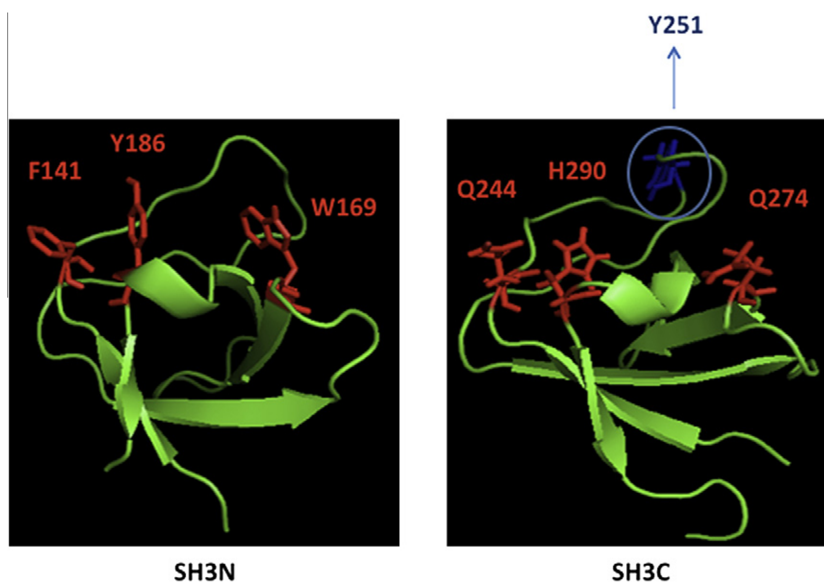

Fig. 2. Comparison of the conventional and atypical SH3 domains in Crk. The atypical C-terminal SH3 domain is presented side by side with the conventional N-terminal SH3 domain. Note that the hydrophobic residues lining the canonical PPII binding site on the SH3N are replaced by polar residues on the SH3C.

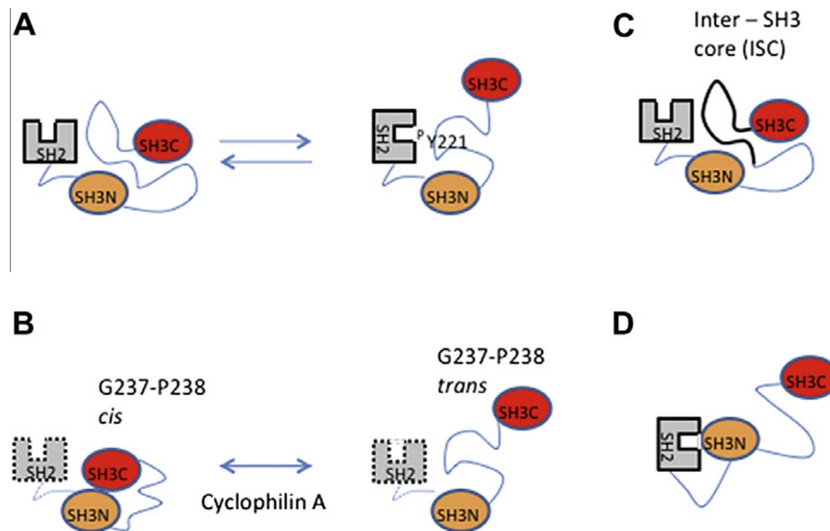

Fig. 3. Modes of auto-inhibition in Crk II. (A) Phosphorylation at Y221 on the linker between the Crk SH3 domains results in binding to the Crk SH2 in *cis* thereby resulting in the formation of a closed structure and disassembly of Crk SH2-mediated complexes. (B) In gallus CrkII, binding of PPII ligands to the SH3N is negatively regulated by *cis–trans* isomerization about the Gly237-Pro238 peptide bond whereby, the SH3C masks the PPII binding site on the SH3N in the *cis* conformation but the PPII binding site is not occluded by the SH3C in the *trans* conformation. (C) In human CrkII, the linker assembles individual domains by means of an inter-SH3 core (ISC, in bold) in a way that packs the SH3N which results in reduced affinity for PPII ligands in the context of full length CrkII compared to the isolated domain. The interactions of the SH3C with ISC stabilize the core. (D) Contrastingly in CrkL, the SH2 and the SH3N domains interact directly with each other whereby the phosphotyrosine motif binding pocket of the SH2 is occluded by the SH3N. However, neither the linker nor the SH3C domain occludes the PPII binding site on the SH3N.

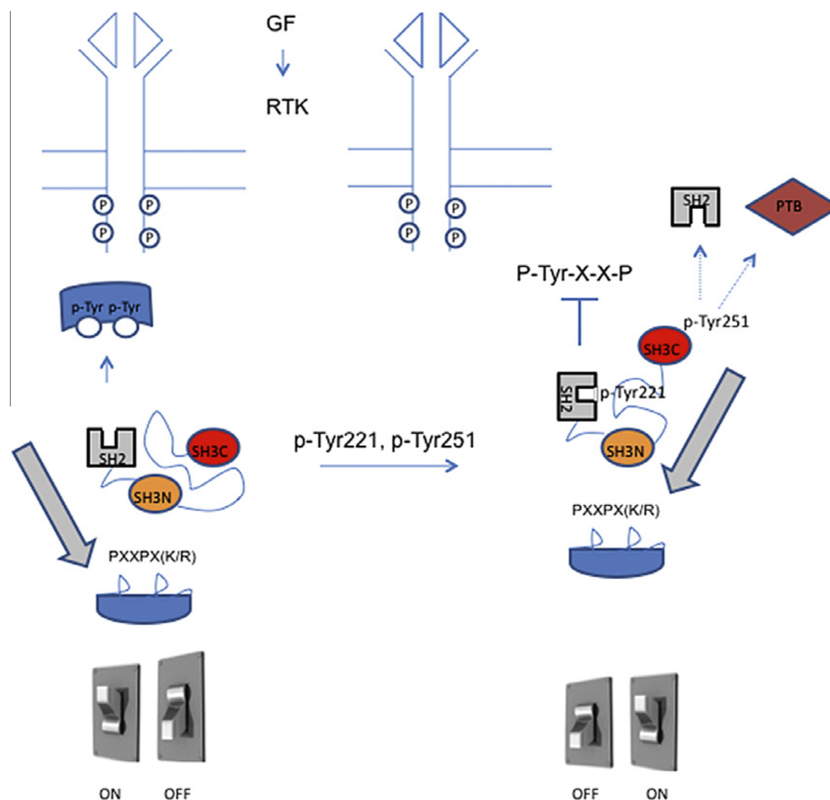

Fig. 4. Binary switch mechanism of signaling by Crk II via tyrosine phosphorylation. Downstream of activated receptor tyrosine kinases (RTK), Crk is recruited to specific tyrosine phosphorylation sites at the plasma membrane via its SH2 domain and in turn recruits downstream effectors such as DOCK180, C3G or Abl to the membrane via binding to PPII motifs through its SH3N domain. Recent evidence that Crk is phosphorylated at Y251 in addition to Y221 downstream of EGFR suggests that tyrosine phosphorylation at these two sites could now switch localization and/or signaling from the SH2–SH3N axis to an SH3C mediated pathway as indicated. GF stands for growth factor.

auto-inhibited Abl kinase structure. Further, phosphorylated SH3C provides binding sites for SH2/PTB domain containing proteins thereby predicting non-canonical signaling pathways. Such a modification confers a gain of function that immediately highlights an interesting scenario involving a binary switch mechanism (Fig. 4). By this mechanism, phosphorylation at Y221 on Crk would result

in binding to its own SH2 domain to down modulate signaling via the SH2–SH3N axis while simultaneous phosphorylation at Y251 would re-localize Crk by virtue of binding to another SH2/PTB domain containing protein thereby switching to an SH3C mediated signaling pathway. This scenario also predicts that upon receptor tyrosine kinase activation (such as EGFR), localization of Crk would be elegantly 'switched' by tyrosine phosphorylation at Y221 and Y251 from being SH2-dependent to being SH3C dependent for an appropriate biological response such as cell migration towards a growth factor gradient.

Although post-translational modification of conventional SH3 domains by tyrosine phosphorylation has been documented, for example for Btk, Abl and recently for the CAS/BCAR1 SH3 domain, in contrast to the Crk SH3C, this occurs on the conserved tyrosine in the hydrophobic ligand binding pocket and thereby reduces the affinity of the SH3 domain for its ligands [14–18]. The SH3 domain of CAS/BCAR1, a focal adhesion protein shown to be associated with resistance to anti-estrogens in breast cancer, is phosphorylated on this tyrosine in Src-transformed cells [19]. Phosphorylation on this tyrosine reduced association with FAK and PTP-PEST, decreased cell spreading and enhanced cell migration [18], implying this modification within the CAS SH3 domain likely serves as a switch to promote turnover of focal adhesions. The unifying theme that emerges from the above findings is that SH3 domain–ligand complexes can be turned over by tyrosine phosphorylation of the SH3 binding pocket thereby permitting re-assembly at a different location in the case of scaffold proteins. Affirmative recruitment of SH2 domains reflects another variation in this theme, where this would allow phosphorylated Crk to re-engage signals when p-Tyr221 is occupied intra-molecularly with its SH2 domain.

The duplication of SH3 domains in Crk and the divergence in biological function of the SH3C is reminiscent of the duplication in other simple adaptor proteins such as Grb2 and Nck. The Grb2 SH3C has been shown to recognize a sequence with an RxxK core binding motif in Gab2 which can be embedded within a PPII motif [20,21]. Although this is removed from a conventional SH3 binding sequence, the Grb2 SH3C retains the hydrophobic amino acids that line the canonical PPII binding pocket, and has therefore not diverged as much as the Crk SH3C from conventional SH3 domains. It is likely that a unique combination of an atypical SH3 domain and a typical SH3 domain have co-evolved for synergistic and cooperative signaling, for example as seen for the capping of the SH3C to the SH3N in Crk II. It should be of interest to assign other co-operative functions where combination of one conventional SH3 and one atypical SH3 exist in single proteins.

The emergence of functional insight into the role of the atypical SH3 domain in Crk provides new details into the biology of Crk proteins, particularly in the differential regulation of Crk I and Crk II and also in defining species specific variations in regulation. Crk signaling is often upregulated in cancers because of increased coupling with overexpressed or deregulated growth factor receptors and non-receptor tyrosine kinases. Further interrogation of the Crk family will likely provide additional information about their modes of regulation as well as their contributions to disease and potential drug targets.

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