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Crk at the Quarter Century Mark: Perspectives in Signaling and Cancer

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ABSTRACT

The Crk adaptor protein, discovered 25 years ago as the transforming gene (*v-crk*) product encoded by the CT10 avian retrovirus, has made a great impact on the field of signal transduction. By encoding an oncoprotein that contained a viral gag protein fused to only SH2 and SH3 domains, v-Crk demonstrated the significance of SH2 and SH3 domains in oncogenic signaling by their virtue of binding in a sequence-specific context to organize and assemble protein networks. In more recent years, the cellular homologs of Crk (Crk II, Crk I, and CrkL) have been extensively studied, and shown to have critical functions in a wide spectrum of biological and pathological processes that include cell motility, invasion, survival, bacterial pathogenesis, and the efferocytosis of apoptotic cells. Clinically, Crk proteins are implicated in the aggressive behavior of human cancers, including adenocarcinomas of the lung, breast, and stomach, as well as in sarcomas and gliomas. Over-expression of Crk proteins in human cancers has led to a renewed interest in both their signal transduction pathways and mechanisms of up-regulation. This prospect summarizes recent developments in Crk biology, including new structural and biochemical roles for the atypical carboxyl-terminal SH3 (SH3C) domain, revelations regarding the molecular differences between Crk II and Crk L, and the significance of Crk expression in stratified human tumor samples. J. Cell. Biochem. 115: 819–825, 2014.

KEY WORDS: Crk; SH2 and SH3 DOMAIN; TYROSINE PHOSPHORYLATION; CANCER GENETICS

In an era of oncogene discovery, when many viral gene products with dysregulated tyrosine kinase activity were routinely identified, the discovery of the CT10 oncogene product v-*crk* was particularly interesting. At the molecular level, v-Crk retained non-catalytic regulatory sequences similar to Src (the so-called Src homology 2 [SH2] and Src homology 3 [SH3] domains), but lacked intrinsic tyrosine kinase activity [Mayer et al., 1988]. Instead, v-Crk was demonstrated to regulate tyrosine kinases in *trans* [Mayer & Hanafusa, 1990a; Akagi et al., 2000; Shishido et al., 2001] (and aptly named the *C*T10 *r*egulator of *k*inase) as opposed to *cis*-acting type of regulation seen for Src and Abl, where the SH2 and SH3 domains regulated kinase activity intramolecularly [Gonfloni et al., 1997; Sicheri et al., 1997; Hantschel et al., 2003; Nagar et al., 2003]. The fact that v-Crk could transform fibroblasts or induce tumors in chickens with short latency demonstrated that SH2 and SH3 domains alone could function as oncogenes. The search for the "transforming property" of v-Crk paved the way for conceptualizing signaling mechanisms in terms of protein modules, as evident by the fact that the SH2 domain of v-Crk could directly bind to phosphotyrosine-containing proteins [Matsuda et al., 1990, 1991; Mayer and Hanafusa, 1990a] and the SH3 domain bound in the context of a proline-rich PxxPxK,R element (PPII) [Knudsen et al., 1995; Wu et al., 1995]. As a result, reductionist approaches to understand functions of signaling proteins in terms of modular domains became universally accepted [Birge and Hanafusa, 1993; Birge et al., 1996; Pawson and Nash, 2003; Pawson, 2004].

The cellular homologs, Crk II, and Crk I, and the related CrkL are ubiquitously expressed and highly conserved in metazoans (Fig. 1). Crk I and Crk II are splice variants; while Crk I has a structure similar to v-Crk, Crk II, and CrkL each possess an inter-SH3 linker and an C-terminal atypical SH3 (SH3C) domain that does not bind to proline-rich motifs [Reichman et al., 1992; Reichman et al., 2005; Muralidharan et al., 2006]. Over the past two decades, many signaling proteins have been identified that bind to the SH2 and SH3N domains,

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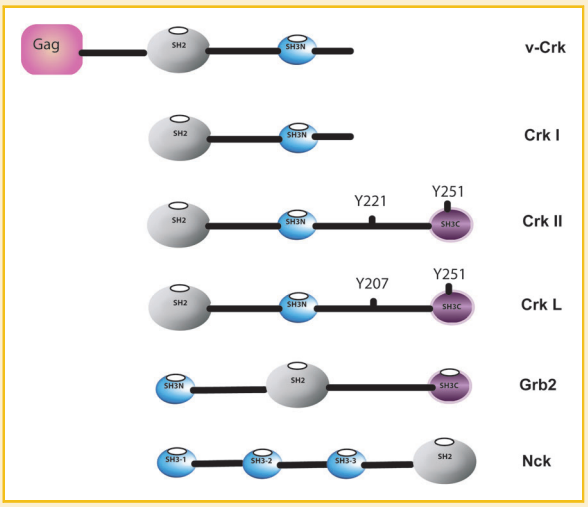

Fig. 1. *Domain organization of Grb2, Nck and the Crk family of adaptor proteins.* Note the atypical SH3C domains in Crk II, Crk L, and Grb2. The Grb2 SH3C has been shown to bind unconventional RxxK motifs while the Crk II and Crk L SH3C domains have no known binding pockets.

and their significance has been discussed in many reviews [Feller, 2001; Birge et al., 2009]. In more recent years, there is a growing appreciation that both Crk and CrkL are dysregulated in human malignancies, and studies have suggested that the relative level of Crk expression correlates with the aggressiveness and disease progression. This has brought on a renewed interest to study Crk, not only to understand how expression is regulated, but also to mechanistically dissect out the roles of each modular domain individually and in the context of other domains in the full length Crk/CrkL proteins. Despite the remarkable conceptual advances in Crk biology over the past two decades, there are still several areas under active investigation that include (i) the biological role of the Crk SH3C domain (ii) differences in signaling mechanisms between Crk and CrkL and (iii) clinical relevance of Crk expression in human cancers. Here, we elaborate on each of the above points.

ROLE OF THE ATYPICAL CARBOXYL-TERMINAL SH3 DOMAIN (SH3C)

Crk II and Crk L have two SH3 domains, named SH3N and SH3C based on their proximity to the N- and C-termini. Only the SH3N binds to canonical PxxPxK,R motifs (Polyproline type II or PPII), while the inability of the SH3C to bind to proline-rich motifs distinguishes it as an atypical SH3 domain. Although historically understudied, research on the Crk SH3C has picked up over the past several years, revealing novel insights and functions at both the structural and biochemical levels.

At the structural level, using NMR approaches to study the three-dimensional structure of chicken Crk II, the solved structure of the SH3N-linker-SH3C unit revealed that the SH3C participates in an elegant form of negative regulation whereby residues on the surface of the SH3C (Pro238, Phe239, and Ile270) cap the PPII binding site on the SH3N (Phe142, Phe144, Trp170, Tyr187, Pro184, and Pro186) by an intramolecular snap lock, a process that is regulated by *cis–trans* isomerization at a glycine-proline ($G_{237}P_{238}$) peptide bond located at the SH3C boundary [Sarkar et al., 2007; Sarkar et al., 2011]. Consequently, mutations that disrupt the SH3C enhance binding of ligands to the SH3N implying a dynamic negative regulatory role for the SH3C in the adaptor function of Crk II [Akakura et al., 2005].

Adding complexity, the SH3C of Crk II can also be phosphorylated at Tyr251 on the surface-exposed RT-loop by several kinases including, but not limited to, the epidermal growth factor receptor (EGFR) to initiate an affirmative signal transduction pathway by post-translational modification. Phosphorylated Tyr251 (pTyr251), in turn, binds *in trans* to the Abl SH2 and promotes Abl kinase transactivation [Sriram et al., 2011]. The fact that phosphorylation at Tyr251 and Tyr221 occur concomitantly following EGFR activation provides new evidence for a non-canonical role for Crk II. In this model, following Tyr221 and Tyr251 dual phosphorylation, Crk II would divert from an SH2 → SH3N module to a (pTyr251)-SH3C ↔ SH3N module in a binary fashion (Fig. 2). The identification of additional physiologically relevant tyrosine kinases that phosphorylate Tyr251 will help determine the context in which this switch occurs. Equally important will be to determine the entire repertoire of SH2 and PTB domain containing proteins that interact with pTyr251. Since Abl and Arg (the only non-receptor tyrosine kinases so far identified to bind to Crk) have recently been shown to promote invadopodia formation in cancer cells [Smith-Pearson et al., 2010; Mader et al., 2011; Gil-Henn et al., 2013], an important initial step in metastasis, one attractive hypothesis is that pTyr251-Crk II colocalizes with active Abl/Arg at invadopodia, and contributes to invasion.

CRKII AND CRKL HAVE DISTINCT 3-D STRUCTURES WHICH MAY EXPLAIN SPECIALIZED FUNCTIONS

Since both Crk II and CrkL share ~60% homology overall, an even higher degree of homology within their SH2 and SH3 domains, and both are ubiquitously expressed, it has been somewhat enigmatic as to why Crk II and CrkL do not biologically compensate during embryonic development. Indeed, both Crk (−/−) and CrkL (−/−) mice present with developmental defects that include cardiovascular and craniofacial defects for Crk and defects in cranial and cardiac neural crest derivatives for Crk L [Guris et al., 2001; Park et al., 2006]. Notwithstanding the high degree of homology in the SH2 and SH3 domains, a possible molecular interpretation for these non-overlapping functions of Crk II and CrkL has been recently put forward [Jankowski et al., 2012]. Comparative 3D solution structures of Crk II and CrkL revealed unexpected differences, whereby each had unique inter-domain organizations. For example, in its native state, the CrkL SH2 pTyr-binding pocket is auto-inhibited by the SH3N, while the Crk II SH2 presents an exposed binding surface, suggesting differential recruitment to pTyr motifs downstream of tyrosine kinases. Moreover, the Crk II SH2 has an internal PxxP motif, lacking in the CrkL SH2, that binds to the Abl SH3 domain [Donaldson et al., 2002] suggesting differential interaction with Abl kinases with potentially different outcomes on modulation of kinase activity.

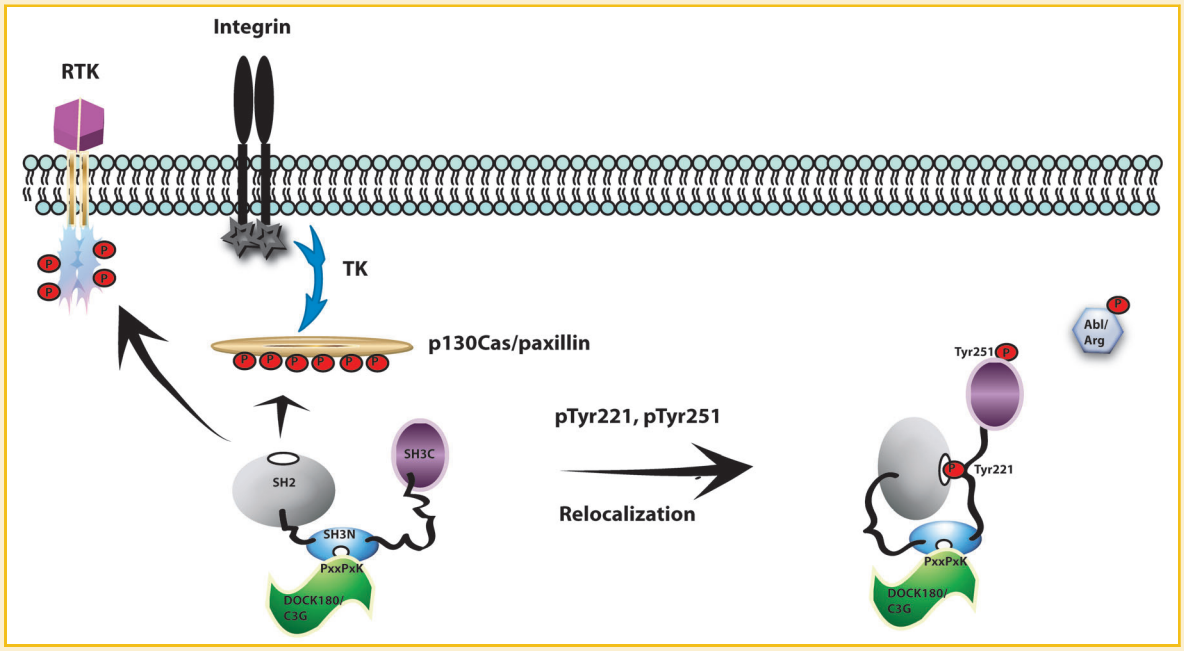

Fig. 2. *Binary switch model for signal transduction by Crk facilitated by phosphorylation at Tyr221 and Tyr251.* The SH2–SH3N axis of Crk is engaged downstream of receptor tyrosine kinases (RTKs) or integrins by recruiting the Crk SH2 domain. Phosphorylation of Crk at Tyr221 and Tyr251 induces on one hand, an intramolecular SH2–pTyr221 interaction that shuts off the SH2–SH3N axis. Simultaneous phosphorylation at Tyr251 on the SH3C, on the other hand, promotes transactivation of Abl kinases and potentially engages other SH2/PTB containing proteins, facilitating a switch to a pSH3C–SH3N signaling axis.

In addition to differences in structural elements and inter-domain communications, there is also a lack of sequence conservation in the region surrounding pTyr251. While both sites (on Crk II and CrkL) are phosphorylated as evidenced by mass-spectrometry data from several cancer cell lines (Phosphosite.org), the pTyr motif diverges from PNAY^{251} DKTALALE in Crk II to PCAY^{251}DKTALALE in CrkL. As alluded to above, while the pTyr251 motif on Crk II binds the Abl SH2 and promotes transactivation of Abl by Crk II, future studies are required to ascertain if the same is true of CrkL. Such studies could shed light on differential activities or mechanisms of action of Crk II and CrkL in cancer cell invasion. Further, experiments to elucidate binding partners of the phosphorylated SH3C of Crk II and CrkL side-by-side should provide insight into differences in phosphorylated SH3C-dependent downstream signaling.

CLINICAL RELEVANCE OF CRK IN HUMAN CANCER

While clinical studies have shown general association between Crk expression and tumor progression, a complete understanding of the relevance of Crk in specific human cancers is not yet realized. Paradoxically, querying the Cancer Genome Atlas database using cBioPortal (Memorial Sloan Kettering Cancer Center) for Crk expression in 30 of the most common human cancers uncovered that Crk or CrkL mRNA expression was not significantly upregulated when compared to three sets of 200 randomly selected genes in any of the unstratified cancer tissue samples (Fig. 3A), whereas driver oncogenes, such as EGFR and MYC (frequently up-regulated) and classic tumor suppressors PTEN and RB1 (frequently down-regulated) have expected profiles (Fig. 3B). Curiously, when comparing each of the adaptor proteins (Crk, CrkL, Grb2, and Nck), both the Crk and CrkL mRNA are globally unchanged or down-regulated in a pattern more typical of the tumor suppressor genes while only Nck showed a consistent pattern of up-regulation. At the mutational level, neither Crk nor CrkL show regular mutations, and never at sites that negatively regulate Crk II (Y221) or CrkL (Y207).

Such analyses suggest that Crk/CrkL proteins are not likely driver mutations for human cancers, but impress that meaningful data mining must rely on stratification by cancer staging within data sets. For example, when lung adenocarcinoma data sets are stratified into stage I versus stage III, or well differentiated versus poorly differentiated, there is a clear correlation of Crk expression with disease severity and outcome [Miller et al., 2003] (Table I). Consistent with the Cancer Genome Atlas, when these tumor samples are non-stratified, differences in expression of Crk that are associated specifically with the advanced staging of the disease are masked. A similar conclusion has been demonstrated in grade I versus grade III human breast cancer [Fathers et al., 2012], and glioblastoma multiforme (normal brain vs. non-stratified glioblastoma) [Takino et al., 2003] (Table I). In this latter case, the investigators also compared Crk I versus Crk II, suggesting that in some tumors, it may be important to distinguish between Crk splice variants. Importantly, these studies demonstrate that comparison of unstratified pooled tumor samples to normal tissue may not uncover association of Crk with advanced disease in specific cancers and highlight the importance of stratifying cancer tissues to exploit meaningful biological data relating Crk and cancer.

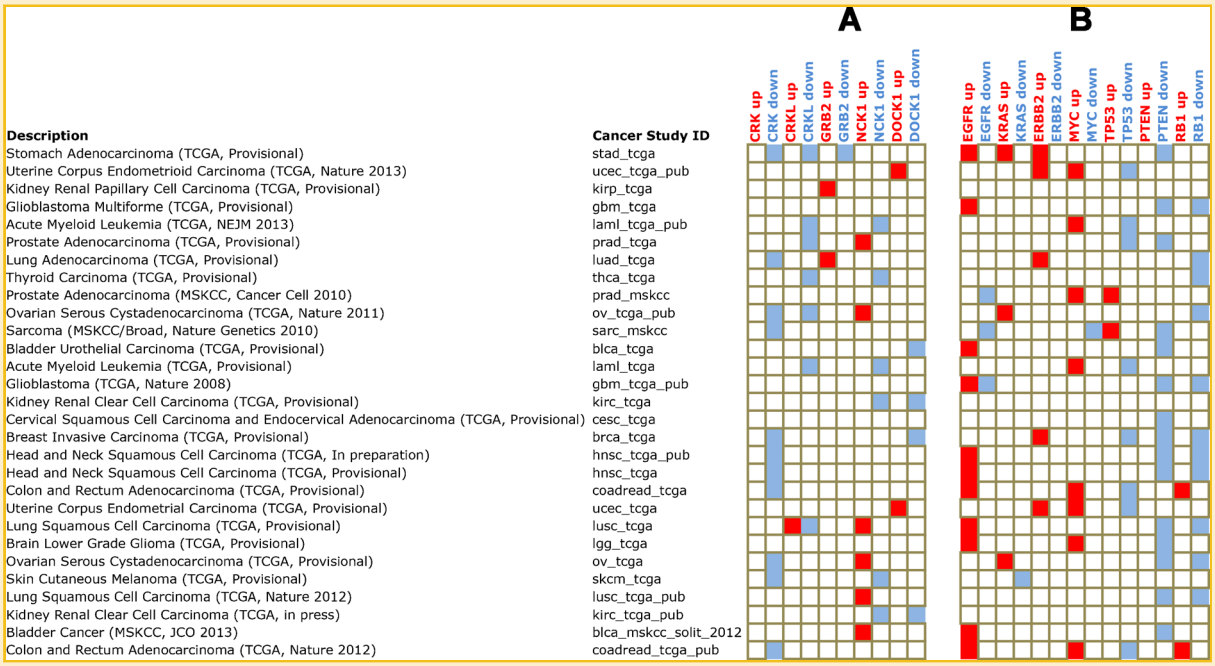

Fig. 3. *Summary of clinical data for the EGFR, KRAS, ERBB2 and C-MYC proto-oncogenes, PTEN, TP53 and RB1 tumor suppressor genes (panel B) and the adaptors, Crk, CrkL, Grb2, and Nck (panel A).* For each gene of interest, all genetic profiles with mRNA expression data in the cBioPortal for Cancer Genomics [Cerami et al., 2012; Gao et al., 2013] were queried using the API provided (http://www.cbioportal.org/public-portal/web_api.jsp), for each of the 43 cancer studies available. The percentage of samples with up- or down-regulation of a given gene in a profile was determined using a z-value threshold of ±2. A background distribution for the percentage of regulated samples was built with a set of 200 randomly selected protein-coding genes from Refseq (extracted from http://hgdownload.cse.ucsc.edu/goldenPath/hg19/database/refGene.txt.gz). A 95% confidence interval for the percentage of regulation was built for the 29 studies that have more than 10 mRNA expression samples, and the value obtained for each gene of interest was then compared with this interval. Genes showing more up- or down-regulation than the background are shown with a red or blue filled square, respectively.

TABLE I. Summary of Relative Crk Expression Levels in Three Different Studies With/Without Stratification

Classification	Relative Crk mRNA expression
Lung cancer [Miller et al., 2003]	
Normal tissue (10)	+
Pooled tumor samples (non-stratified) (86)	+
Stage I tumors (19)	+
Stage III tumors (67)	++
Well differentiated tumors (23)	+
Poorly differentiated tumors (20)	++

Classification	Relative Crk protein expression
Breast cancer [Fathers et al., 2012]	
Grade I breast tumors (15)	+
Grade III breast tumors (74)	++

	Relative Crk mRNA expression	
Classification	Crk I	Crk II
Glioblastoma [Takino et al., 2003]		
Normal brain	+	+
Glioblastoma (non-stratified)	++	+

Above, '+' and '++' refer to relative levels of expression within each study. '++' denotes over-expression. In the lung cancer study, stratified tumor samples displayed correlation of Crk mRNA expression with advanced staging while expression in the non-stratified pooled tumor samples was not significantly different from the normal lung tissue. Similarly CrkI (but not CrkII) expression was significantly different between normal brain and pooled glioblastoma samples. Akin to the lung cancer study, correlation of Crk protein expression with tumor grade was observed in stratified breast tumor samples.

TRANSLATION OF HUMAN CANCER DATA BACK TO MOUSE MODELS

Based on the prospects that Crk and CrkL are not likely driver mutations in human cancers, future studies that cross Crk transgenic mice with relevant Crk co-activators would be meritorious. Studies by Park and colleagues have provided an elegant start to this endeavor and showed that MMTV driven over-expression of Crk II in a mouse model induced atypical mammary gland architecture and tumor formation with a long-latency (17.6% tumor incidence compared to 4% in the controls) [Fathers et al., 2010]. These studies suggest that while elevated CrkII levels may predispose mammary epithelial cells to transformation, the absence of metastatic lesions highlights the need for studies on gain-of-function effects of Crk downstream of tyrosine kinase oncogenes. Relevant data mining of genes that co-stratify with elevated Crk expression can identify physiologically relevant systems to be exploited in conjunction with typical oncogenes using transgenic and conditional knockout mouse models.

More recently, the elevated levels of CrkI/II were observed in a cohort of breast cancer patients by tissue microarray [Fathers et al., 2012]. By identifying genes upregulated by CrkII over expression in T47D breast cancer cells, the investigators identified a "Crk gene signature" that significantly correlates with basal breast cancers of high grade and poor prognosis. Akin to this study, systems analysis of tumors driven by specific Crk isoforms may lend new mechanistic insight into how distinct regulatory networks are engaged. In this light, examining transcriptomes and phosphoproteomes in metastatic cells driven by Crk or CrkL holds promise, particularly if they can be directed back into the development of mouse models that mimic the human expression data.

HOW IS CRK REGULATED IN MALIGNANT CELLS?

Based on the aforementioned arguments, an important future goal will be to define the specific mechanisms that govern Crk up-regulation in malignant cells. While it has been realized for many years that forced over-expression of Crk promotes metastatic properties of invasion and migration in patient derived cancer cell lines, the mechanisms of up-regulation (i.e., at the mRNA and protein level) in human tumors is still not well understood. At the mRNA level, Crk up-regulation in cancers could be due to enhanced transcriptional activity, mRNA stabilization or miRNA dysregulation. The proximal promoter segment reveals several potential transcription factor-binding sites, although the various physiological contexts in which specific subsets of these factors influence Crk expression have not been well studied.

On the other hand, analysis of the Crk-3′UTR reveals miRNAs that could potentially target the Crk mRNA for degradation/translational inhibition. Among these, the role of miR-126 in post-transcriptional regulation of the Crk mRNA is the best studied and has been validated in several human cancers. Levels of miR-126 inversely correlate with Crk expression levels and moreover, miR-126 functionally attenuates Crk-mediated phenotypes of invasion and migration in patient derived cancer cell lines [Crawford et al., 2008; Feng et al., 2010]. Interestingly, Src transformed cells show suppression of miR126 expression in a manner dependent on Src kinase activity [Li et al., 2009]. Further, this enhances Crk expression and migration of cells. The fact that miR-126 interfaces between Src and Crk in a way that Src can influence Crk expression is quite interesting, and raises the question whether oncogenes can crosstalk with Crk via microRNAs. To elucidate how general these oncogene networks are will be highly meritorious.

Another interesting aspect of Crk biology concerns the regulation of the half-lives of tyrosine phosphorylation on Crk SH2 binding partners as well as regulation of Crk protein stability by post-translational modifications. One attractive idea is that the binding of Crk to phosphotyrosine containing proteins may stabilize the tyrosine phosphorylated state of Crk SH2 binding partners. Clearly, many studies have shown that over-expression of Crk results in the elevation of cellular phosphotyrosine [Mayer & Hanafusa, 1990b; Iwahara et al., 2004], but more recently, that knockdown of Crk causes decreased p130Cas phosphorylation [Fathers et al., 2012], supporting the aforementioned model that Crk acts as a pTyr trap. Taking this a step further, it is attractive to posit that the SH2 may act as a molecular "sensor" of the level of phosphorylated tyrosines in cells. This can be conceived to be intertwined with the half-life of Tyr221 phosphorylation on Crk as the status of Tyr221 would govern the availability of the Crk SH2 to bind phosphorylated tyrosines *in trans*. The half-life of pTyr221 on Crk has been shown to be extremely short [Tunceroglu et al., 2010] highlighting the significance of high phosphatase activity towards this site in controlling the dynamic turnover and reformation of Crk-mediated complexes. However, the spectrum of Crk pTyr221–specific phosphatases and their roles in cancer have also been historically understudied.

CRK AT THE QUARTER CENTURY MARK: CONCLUSIONS AND FUTURE PERSPECTIVES

The discovery of v-Crk 25 years ago has accelerated our understanding of signal transduction, and provided a roadmap for how cells detect intracellular phosphotyrosine status and relay downstream signals. The logic of modular protein interacting domains and intracellular communication learned from Crk highlighted many important principles in signal transduction. Going forward, new studies on Crk suggest a two-tiered mechanism of response, wherein initial complex formation is followed by simultaneous negative regulatory (Tyr221) and gain-of-function tyrosine phosphorylation (Tyr251 being one such site) downstream of tyrosine kinases for dynamic re-organization of protein complexes. Conceptually, the versatility of modular domains in Crk, which can in turn be tuned by tyrosine phosphorylation (Fig. 2), hints at an elegant system to elicit specific and distinct responses to tyrosine kinases. Future studies to identify novel binding partners of the pTyr251 motif on the Crk SH3C are poised to open up new avenues of research in signal transduction. Likewise, it will be important to understand, at the genetic and epigenetic levels, how Crk is up-regulated in human cancers, and which genes are co-regulated with Crk. If the current advances continue at an accelerated pace, agents that target Crk pathways may eventually provide useful therapies in human cancer.

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