# STRUTURAL AND DYNAMIC NMR STUDIES OF THE CRK PROTO-ONCOGENE

By

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## **ABSTRACT OF THE DISSERTATION**

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#### **PROTO-ONCOGENE**

#### **By PARAMITA SARKAR**

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**Professor Charalampos G. Kalodimos** 

The Crk family of adaptor proteins is ubiquitously expressed in most tissues and mediates the timely formation of protein complexes elicited by a variety of extracellular stimuli, including various growth and differentiation factors. This class of proteins lacks an apparent catalytic domain and may serve as adaptors, coupling different proteins of a signal transduction cascade. Crk adaptor proteins are made up of one modular Src homology 2 (SH2) and two Src homology 3 (SH3) domains. SH2 domains bind to phosphotyrosine (pTyr) containing sequence, while SH3 domain binds to proline rich motifs. The two SH3 domains are separated by long linker containing highly conserved proline reisdues. Although the role of SH2 and N-terminal SH3 (SH3<sup>N</sup>) domains of Crk

has been generally delineated, the role of C-terminal SH3 (SH3<sup>C</sup>) domain remains entirely unknown. There is, however, increasing evidence that the SH3<sup>C</sup> domain along with the linker act as a regulatory element. Despite the fact that Crk has provided a model system for understanding how adaptor proteins mediate signal transduction, currently the mechanistic basis for the regulation of its function remain elusive. Such an understanding is now rendered evev more urgent because of Crk has been found to be overexpressed in many human cancers.

Here, using an integrated NMR, thermodynamic, and biochemical approach, we show the presence of a unique regulatory mechanism in Crk. We have shown hat the  $SH3^{C}$  domain serves to regulate the binding activity of the  $SH3^{N}$  domain through an intramolecular interaction that is controlled by a prolyl *cis/trans* isomerization. Proline isomeerization toggles rk between two conformations: an autoinhibitory one, stabilized by the intramolecular association of the two SH3 domains in the *cis* form, and an unhibited, activated one prompted by the *trans* form. The data provides atomic insight into the mechanisms that underpin the functionality of this binary switch and elucidate its remarkable efficiency. The results also demonstrate the interactions are mediated by novel SH3 binding surface. Also, the phosphorylation of the regulatory Tyrosine (Y222) by c-Abl is regulated by another set of prolyl-peptidyl bond, which also serve as a substrate for Cyclophilin A (CypA).

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# **Chapter 1 : Introduction**

#### **1.1. Crk Adaptor Proteins**

## 1.1.1. Crk

The Crk family of adaptor proteins is ubiquitously expressed in most tissues and mediates the timely formation of protein complexes elicited by a variety of extracellular stimuli, including various growth and differentiation factors (Birge et al., 2009; Feller, 2001). Crk was first identified by Bruce Mayer in 1988 as an oncogene (Gag-Crk) from the genome of a defective avian sarcoma retrovirus called CT-10 (chicken tumor Virus-10) with no catalytic activity (Mayer et al., 1988). The gene consisted of a viral gag sequence encoding major structural proteins of the virion and a cellular gene encoding for a sequence of two protein domains which have homology to other signaling proteins like non-receptor tyrosine kinase and phopholipase C-y. A year later an independent avian retrovirus was isolated named avian sarcoma virus 1 (ASV-1) (Tsuchie et al., 1989), which was found to contain a nearly identical oncogene, expressing proteins different only in few amino acids. Even though Crk lacks a catalytic domain it remarkably increases tyrosine phosphorylation of proteins pp70 and pp130 in chicken embryo fibroblast transformed by this virus. This observation suggest that Crk can activate cellular tyrosine kinases and was hence named crk (CT10 regulator of kinase) (Mayer et al., 1988). Following the isolation of v-Crk, Reichman et al, reported the first cellular homolog of Crk (c-Crk) by screening a chicken brain library using a protein of the v-Crk probe devoid of the retrovirus-derived gag sequence (Reichman et al., 1992). This class of proteins lacks an apparent catalytic domain and may serve as regulatory subunits of enzymes, and as adaptors, coupling different proteins of a signal transduction cascade. Crk proteins play important role in cell growth regulation, cytoskeletal reorganization required for cell adhesion, spreading and phagocytosis(Feller, 2001).

# 1.1.2. Crk Family

The *c-crk* gene encodes three alternatively spliced translational products: c-CrkI, c-CrkII (also referred to as Crk), and the recently identified c-CrkIII (Chodniewicz and Klemke, 2004; Matsuda et al., 1992). v-Crk and its cellular homologs, CrkII, CrkI, CrkIII and the paralog CrkL are made up of modular Src-homology 2 (SH2) and Src-homology 3 (SH3) domains (**Figure1.1**).



Figure 1.1 Domain organizations of Crk family members. Y222 (chicken numbering) becomes phosphorylated by c-Abl. The two prolines P221 and P238 that we have identified to exhibit *cis*-*trans* isomerization are highlighted.

While the Crk family members share similar overall structure, the biological differences between each member is not entirely clear. Subsequent studies lead to the realization that even proteins containing only modular units, which have no intrinsic

catalytic or transcriptional activity, have the ability to transform cells and to modulate the activity of kinases (Birge et al., 1996; Feller, 2001; Feller et al., 1998). It is now well established that the function of adaptor proteins are indeed a consequence of the SH2 and SH3 domains (Pawson and Scott, 1997), whose mere presence can trigger oncogenic transformation as well as normal cellular functions. The structure and binding specificities of the two ubiquitous adaptor modules, SH2 and SH3, have been extensively investigated and are well understood (Pawson and Nash, 2003). SH2 domain are made up of ~ 100 amino acids present in Src oncogene and various other signaling proteins and bind to phosphotyrosine (pTyr) containing sequences (Pawson, 2004). SH3 domains are made up of ~60 amino acids and bind polyproline type II (PPII) sequence. The Crk SH3<sup>N</sup> domain binds specifically to P-x-x-P-x-K motif.



**Figure 1.2** Various steps involved in the regulation of Crk proteins via phosphorylation of Y222 in chicken (or Y221 in human Crk-II and Y207 in CrkL) by c-Abl. Intramolecuar arrangement

formed by binding of the SH2 domain to pY-x-x-P. Masks the binding site of the SH3<sup>N</sup> domain of Crk and signaling is aborted.

CrkL is highly homologus to CrkII but is encoded by a distinct gene made up of one SH2 domain followed by two SH3 domains with a molecular weight of 40kDa (Figure 1.1). Unlike CrkL, c-Crk is expressed as two different proteins of 28 and 40Kda, respectively (Matsuda et al., 1992). c-CrkI is consists of one SH2 domain followed by one SH3 domain, whereas c-CrkII has an additional C-terminal SH3 domain (SH3<sup>C</sup>) attached by a ~50 amino acid proline-rich linker to SH3<sup>N</sup>. Structurally c-CrkI is more similar to v-Crk and has a higher transformation activity than c-CrkII (Feller et al., 1998). The difference in transforming activities of CrkI and CrkII, can be explained, at least in part, by a specific post-translational modification of Crk that involves phosphorylation of Y222 in chicken (Y221 in mouse and human) (Feller et al., 1994). CrkL is also neatively regulated by the Y207 phosphorylation(Birge et al., 2009). Phosphorylation of Tyr (207 in CrkL and 222 in CrkII) causes intramolecular binding SH2 domain to the linker region, masking both the SH2 and SH3<sup>N</sup> domain binding pockets and thus decreasing their accessibility to their binding partners and thereby shutting off Crk signaling (Figure1.2) (Kobashigawa et al., 2007; Rosen et al., 1995).

## 1.1.3. Crk in signaling

The Crk family of adaptor proteins have central roles in a vast number of cellular processes, including from cell proliferation, cell adhesion and migration, phagocytic and endocytic pathways for apoptotic cells and parasitic organisms respectively, apoptosis, and regulation of gene expression (Feller, 2001). Various biochemical and biophysical

techniques, such as GST (glutathione S transferase)-pull-down assays, Far Western blotting, yeast two hybrid approaches were used for the initial characterization of various Crk binding partners and their role in the participating signaling pathway. The experiments were facilitated by the modular nature of the SH2 and SH3 domains, which maintain their binding specificity and affinity even when expressed as isolated domains (Feller et al., 1995b; Matsuda et al., 1991). During the last two decades, over 40 cellular proteins have been characterized as binding partners of Crk family proteins clearly suggesting the complexity of Crk signaling transduction (Figure 1.3) (Feller, 2001). Still Crk function can be subdivided into two major pathways: first the input pathway, where in the Crk SH2 domain binds to phosphotyrosine-containing motif in an inducible onand off- switch mechanism that involves regulated tyrosine phosphorylation and dephosporylation; Second, the output pathway involving SH3<sup>N</sup>-domain complexes, which are generally regulated without post-translational modifications, with only few exceptions where tyrosine phosphorylation regulates the SH3<sup>N</sup> complex formation (Birge et al., 2009; Kirsch et al., 2001).



Figure 1.3 CrkII and their identified binding partners of all three respective domains.

Inspite of, the considerable homology of the SH2 and SH3 domains of CrkII and CrkL, development of knockout mouse models clearly show these gene products have distinct, non-overlapping roles, during embryonic development as both Crk knockout and CrkL knockout mice die perinatally with different developmental defects (Birge et al., 2009). Further, in CrkII (-/-) fibroblasts, CrkL proteins are not over-expressed to compensate for CrkII and vice versa in CrkL (-/-) cells. This observation suggests little molecular crosstalk at the level of expression in embryonic cells between CrkII and CrkL (Isakov, 2008). Interestingly, mice homozygous for a null mutation of CrkL show a phenotype characteristic of DiGeorge/velocardiofacial syndrome, in which neural crest derived cells fail to differentiate and migrate leading to defects in cranial and cardiac development (Guris et al., 2006; Guris et al., 2001; Moon et al., 2006). Whereas, Crk null mice lacking both CrkII and CrkI die perinatally due to defects in cardiac and skeletal development (Park et al., 2006). Imaizumi *et.al* generated another mouse using an exon trap method that lacks CrkII but still expresses CrkI. Although, mice expressing CrkI show no obvious developmental abnormalities, these mice might develop age-dependent malignancies, since they die within a few days after birth by unknown mechanisms (Imaizumi et al., 1999).

Although the above mentioned studies shows that CrkL and CrkII have independent roles during developmental process, many recent studies suggest that CrkII and CrkL are co-expressed in somatic cells and can compensate for each other in signaling(Birge et al., 2009). The Reelin signaling pathway in cortical pyramidal cells of the hippocampus provides an example for the compensatory nature of CrkII and CrkL (**Figure 1.4**).



**Figure 1.4** Schematic diagram of the role of Crk and CrkL in the Reelin signaling pathway. Upon inititation of the Reelin signaling pathway, Crk and CrkL leads to activation of different effector protein resulting int different cellular activity (Park and Curran, 2008).

Reelin, a secreted glycoprotein that binds to receptors on hippocampal neurons such as ApoER2, VLDLR, and  $\alpha 3\beta 1$  integrin, controls cell positioning during adult neurogenesis via Src-dependent tyrosine phosphorylation of the scaffold protein Dab1 (D'Arcangelo et al., 1999). Upon receptor activation, Dab1 becomes tyrosine phosphorylated at multiple sites and binds a variety of SH2 domain-containing proteins that include both Crk II and CrkL (Huang et al., 2004; Yip et al., 2007). Studies using shRNA or cre-lox deletion of Crk and CrkL showed that knockdown of both proteins are required for the defects in neuronal positioning, and both proteins appear to converge on the activation of Rap1 through C3G (**Figure 1.4**) (Feng and Cooper, 2009; Matsuki et al., 2008; Park and Curran, 2008).

Crk family proteins are also required in platelet-derived-growth-factor receptor  $\beta$  (PDGF $\beta$ R) signaling for actin-cytoskeleton rearrangement and cell migration (Antoku et al., 2008). Another example of overlapping function CrkII and CrkL is the PDGF-

stimulated formation of dorsal-membrane ruffles (Antoku and Mayer, 2009). These studies have shown that PDGF stimulation induces tyrosine phosphorylation of Crk proteins by Abl family (Abl and Arg) tyrosine kinases, leading to disruption of Crk-p130Cas complex and decrease in p130Cas phosphorylation which results in disassembly of FA and finally termination of reorganization of actin cytoskeleton. In contrast cells in expressing CrkI, which lacks the regulatory Y221 or CrkIIY221F mutant, PDGF stimulated FA persisted longer than cells expressing CrkII/CrkL (Antoku et al., 2008). In this study Antoku *et al.* also suggest a novel role for the SH3<sup>C</sup> domain in directing Crk adaptors to activate Rac1 at the expense of Rap1. How universal the CrkII and CrkL compensation is in signal transmission remains an active area of investigation.

#### 1.1.3.1. The SH2 Domain

SH2 domains are structurally conserved modular protein motifs of ~100 amino acids present in various signaling proteins (**Figure1.5**). According to one study there are over one hundred and nineteen (119) genes in the human genome containing SH2-domain (Lander et al., 2001). A different study predict a minimum of one hundred forty one (141) by SMART program, EMBL website (Schultz et al., 2000). The SH2 domains are so called because it was first discovered in Src tyrosine kinases with SH1 refereing to their catalytic domains.

SH2 domains are hemispherical in shape, consist of a central  $\beta$ -pleated sheet flanked by two  $\alpha$ -helices, and bind to phosphotyrosine (pTyr)-containing sequences (**Figure 1.6**)(Pawson, 2004). The N- and C-terminal residues of SH2 are in close proximity on the surface opposite the peptide binding site, indicating that this domain can be inserted between any two surface residues on a protein without greatly disturbing its fold (**Figure1.6**).



**Figure 1.5** Example of Src homology 2 (SH2)-domain-containing proteins and their modular primary structure (Pawson et al., 2001)



**Figure 1.6** Structure of the Crk SH2 domain bound to the Crk phosphopeptide (residues 221–224). Peptide is colored in blue and residues of SH2 make contacts are represented by red (Donaldson et al., 2002).

Phosphotyrosine-containing sequences for SH2 domains, have been classified into four main groups based on the specificity for the three to six amino acids C-terminal to the phosphotyrosine residue (Songyang and Cantley, 1995; Turk and Cantley, 2003). In the case, of Crk SH2 the preferred binding motif is pY-x-x-P. This preference was first recognized by *in vitro* approaches (Songyang et al., 1993) and subsequently confirmed by mutational analyses of several Crk SH2 domain binding partners. Most of the Crk SH2 binding proteins, such as paxillin (p70) (Birge et al., 1993), p130Cas (Bouton et al., 2001; Sakai et al., 1994) and its homologues, the ubiquitin ligase c-Cbl (p120) (Smit and Borst, 1997; Tsygankov et al., 2001) and the large docking protein Gab1 (Garcia-Guzman et al., 1999; Sakkab et al., 2000) contain the pY-x-x-P binding motif.



**Figure 1.7** Sequence comparison of SH2 domain of Crk-II with homologues v-Crk and CrkL. Residues at the binding pocket are highlighted (Donaldson et al., 2002).

The highly homologous SH2 domains of both Crk and CrkL have a very similar binding specificity towards pTyr containing motifs (**Figure1.7**) (Feller et al., 1995a). This was further supported by co-immunoprecipitation of various v-Crk and c-Crk SH2 binding proteins with CrkL (Feller, 2001; Salgia et al., 1996; Salgia et al., 1995). The Xenopus Crk SH2 domain binds somewhat different motif with specificity for Y-x-x-L in the apoptosis-inducing cell cycle regulator XWee-1 (Smith et al., 2000).

## 1.1.3.2. The SH3 domain

More than 300 genes have been identified to date containing the SH3 domain in the human genome (Lander et al., 2001). The SH3 domain consists of ~ 60 amino acids and are made up of two perpendicular anti-parallel  $\beta$ -sheets and SH3 recognizes proline-rich sites adopting a left-handed polyproline type II (PPII) helix conformation (**Figure 1.8** and 1.9) (Mayer, 2001).



Figure 1.8 SH3 domain of CrkII (PDB107)

The PPII helix has three residues per turn making a roughly triangular crosssection, with the base of this triangle siting on the surface of the SH3 domain. Two of the three ligand-binding pockets of the SH3 domain are occupied by two hydrophobicproline (x-P) dipeptides on two adjacent turns of the helix, while the third 'specificity' pocket in most cases interacts with a basic residue (mostly Arg) in the ligand distal to the xPxxP core. Depending upon the position of the two invariant proline dipeptides (xP) on the surface of the SH3 domain, with respect to the axis of the PPII helix have been identified two classes. Looking from the PPII helix axis along specificity pockets of the SH3 domain, in class I the prolines will be lying on the right side and in class II prolines will be lying on the left side (**Figure 1.9A and 1.9B**).

The ligand binding site on the SH3 domain is comprised of conserved aromatic residues. Additionally, there are two variable regions, named the RT- and n-src loops, that participate in ligand binding. A P-x-x-P-x-K motif is crucial for highly selective binding of Crk SH3<sup>N</sup> (Knudsen et al., 1994; Wu et al., 1995).Further studies showed that the lysine following in position +2 of the P-x-x-P motif is crucial for the high affinity

interaction with SH3<sup>N</sup>. The positive charge of the side chain of Lys is balanced by three negative residues in the SH3 domain, while the hydrophobic side chain of Lys is positioned against the hydrophobic tryptophan (Wu et al., 1995).



Figure1.9A.

Figure1.9B.

**Figure 1.9** Interaction of SH3 domains with their PPII ligands. (A) Binding of class I (top) and class II (bottom) ligands to the surface of the SH3 domain is depicted diagrammatically. The orientation of the ligand is indicated beneath each diagram. Approximate positions of the variable RT and N-Src loops of the SH3 domain are indicated. (B) Schematic view of an X-P dipeptide of a class I (top) or class II (bottom) ligand on the surface of a SH3domain. The surface of the SH3 domain is represented in green, and the portions of the X-P dipeptide that contact the surface are represented in red(Mayer, 2001).

The first identified binding partner for Crk SH3<sup>N</sup> was C3G, a guanine nucleotide releasing protein for the small GTPase Rap1 (**Figure1.10**). C3G contains several P-x-x-P-x-K motifs in its central region (Knudsen et al., 1994). Other prominent Crk SH3<sup>N</sup> binding partners are DOCK 180 (Feller et al., 1995a), c-Abl (Feller et al., 1994), Bcr-Abl and Tel-Abl (Voss et al., 2000).


**Figure 1.10** The interface between the Crk SH3<sup>N</sup> domain and the C3G peptide. (a) Important hydrophobic and polar interactions between Crk SH3<sup>N</sup> and the C3G peptide. The polypeptide backbone of the SH3 domain is represented by a green ribbon, and the SH3 side chains that interact with the peptide are colored red for acidic residues and white for hydrophobic residues. The RT and Src loops flanking the C-terminal region of the C3G peptide are labeled. The C3G peptide is colored yellow, except for Lys8 and Lys9, which are colored blue. The van der Waals surfaces of Lys8 (in C3G) and Trp169 (in Crk) are indicated by small dots. Hydrogen bonds associated with the side chains of the C3G peptide are indicated by dashed white lines, and hydrogen bonds made to the backbone of C3G are drawn in blue. (b) The molecular surface of the the Crk-SH3<sup>N</sup>/C3G complex is displayed. The surface is colored according to the local electrostatic potential calculated in the absence of peptide, assuming a 0.10 M NaCl concentration in the solvent. Note the negatively charged pocket (red) surrounding the Lys8 side chain of the C3G peptide and the neutral region (gray) interacting with Pro2–Pro6 of C3G. The hydrophobic side chains underneath the SH3 surface are displayed in green. The orientation in (b) is the same as in (a), for ease of identification of the residues. Figure was adapte from (Wu et al., 1995).

Despite the fact that several proteins have been identified to bind to the SH3<sup>N</sup> domain, no cellular molecules have been shown to interact with the Crk SH3<sup>C</sup> domain, with the single exception of the nuclear export factor crm1 (Smith et al., 2002).

#### 1.1.4. Binding partners (adaptors and enzymes) of Crk Proteins

#### 1.1.4.1. p130Cas family

p130Cas (Crk associated kinase substrate) is a 130kDa protein and is a substrate to retroviral tyrosine kinase oncoproteins like v-Src. p130Cas was discovered as a result of its elevated phophotyrosine level observed in Crk transformed cells (Birge et al., 1992; Matsuda et al., 1990; Sakai et al., 1994).



**Figure 1.11** p130Cas under mechanical coupling and stress undergo phosphorylation at multiple Y-x-x-P site by the Src kinase, followed by recruitment of Crk through its SH2 domain, which in turn initializes various signaling pathways by recruiting of other effector proteins by Crk SH3<sup>N</sup> such as SOS, DOCK180, Abl and C3G (Birge et al., 2009).

p130Cas importance arises as previous studies showes that even tyrosine kinases and Ras oncogenes fail to transform p130Cas (-/-) fibroblast and that p130Cas is necessary and sufficient to transform cells (Auvinen M, 1995). Overexpression of p130Cas also renders breast cancer cells more resistant to cytotoxic chemotherapies like anti-estrogen, suggest that p130Cas activates survival and metastatic pathways when phosphorylated (Schrecengost et al., 2007; Ta et al., 2008).

p130Cas is a multidomain docking protein including an N-terminal SH3 domain that binds FAK and Pyk2, an interior 260 amino acid long substrate domain consisting of 15 Y-x-x-P motifs served as binding sites for the Crk SH2 domain, a C-terminal Src binding domain, followed by a highly conserved C-terminal region that binds to the Nsp family of proteins (Nsp 1, And-34, and Chat) (Defilippi et al., 2006). The precise nature of the multiprotein complexes formed on the p130Cas family protein scaffolds most probably varies appreciably, depending on cell type and stimulus. Various studies using different cell lines have shown that p130Cas family proteins along with Crk and other enzymes play an important role in the cytoskeleton reorganization during cell migration and shape change during engulfment and adhesion (Cheresh et al., 1999; Klemke et al., 1998). Previous studies have shown that when bound to Src kinase p130Cas undergoes processive phosphorylation at the substrate region in a particular pattern to initiate a particular signaling cascade (Pellicena and Miller, 2001). Recent studies have shown that phosphorylation does not follow obligatory sequence and is stochastic (Patwardhan et al., 2006) suggesting that repetitiveness of the Y-x-x-P motif in p130Cas would mainly function in signal amplification along with diversifying signaling (Figure 1.11) (Birge et al., 2009).

#### 1.1.4.2. Paxillin

The Paxillin protein family consists of ~70kDa proteins acting as a substrates to various normal and oncogenic tyrosine kinases, including Src family kinases (SFK) and Focal Adhesion kinases (FAK). Paxillin, like p130Cas, also acts as a scaffold protein to organize signal transduction proteins in large complexes at 'focal adhesions' (Feller, 2001). Paxillin regulates signaling pathways involving cytoskeletal reorganization and

cellular growth (Schaller, 2001; Turner, 2000). Paxillin also regulates cell morphology and motility (Petit et al., 2000). Paxillin (Dawid, 1998) is attached to the cell membrane via its C-terminal LIM domains (Dawid, 1998) and binds to  $\alpha$ 4-integrins (Liu et al., 1999). It also binds to the Arf-GAP protein p95PKL (paxillin ± kinase linker) via a leucine-rich repeat (LD4 motif), which leads to its indirect interaction with PAK (p21 GTPase-activating kinase), Nck and PIX (PAK-interacting exchange factor) (Feller, 2001; Turner et al., 1999). Paxillin also have two Y-x-x-P (Y31 and Y118) motifs that function as SH2-binding sites for Crk/CrkL proteins (Petit et al., 2000; Schaller and Parsons, 1995). This binding further leads to accumulation of effector proteins via the SH3<sup>N</sup> domain at paxillin sites. Hyperphosphorylated Paxillin is found in v-Crk transformed cells and serves as a binding partner for v-Crk through by SH2 domain (Birge et al., 1993).

#### 1.1.4.3. Other Crk SH2 binding partners of Crk

The Crk SH2 domain binds to many other proteins including Dab1 (Chen et al., 2004b), IRS1 (Beitner-Johnson et al., 1996), p120c-Cbl (Feller et al., 1998) Gab1(Watanabe et al., 2006), and CasL/Efs(Defilippi et al., 2006), all containing tandem Y-x-x-P motifs as p130Cas family proteins. These interactions are initiated by various kinds of stimuli, including growth factors, cytokines to ECM, microbial products, immune complexes, endogenous metabolic products and apoptotic cells.

#### 1.1.4.4. C3G

C3G (Crk SH3-domain-binding guanine – nucleotide releasing factor) was the first identified SH3<sup>N</sup> domain binding partner of Crk (Knudsen et al., 1994; Tanaka et al.,

1994). C3G is a guanine nucleotide releasing protein that removes GDP from the small GTPases Rap1, Rap2, and R-Ras, allowing their spontaneous reloading with GTP (Gotoh et al., 1995; van den Berghe et al., 1997). GTPases have an intrinsic enzymatic activity that hydrolyses GTP to GDP and phosphate. The duration of Rap GTPase activity depends on the rate of hydrolysis of GTP. C3G catalyzes this reaction by releasing bound GDP from these small GTPases. C3G contains Crk SH3<sup>N</sup> binding motif P-x-x-P-x-K at its center and binds to both Crk and CrkL. Various stimuli, such as insulin receptor activation, integrin stimulation and along with many others regulate this complex formation (Okada and Pessin, 1997). Crk-C3G complex get dissociated on phosphorylation of Y222 (in chicken or Y 221 in human/mouse) on insulin-stimulation in chineese hamster ovary cells (Feller et al., 1994; Okada et al., 1998). Binding of Crk to activated receptors and their substrate proteins on external stimulation via its SH2 domain leads to relocalization of C3G within the cells, presumably to Rap1 sites. C3G is phopshporylated at Y504 by SFK's (Ichiba et al., 1999), which leads to its binding to p130Cas and resulting to cell adhesion to extra cellular matrix (ECM) upon stimulation with certain cytokines and also stretch-inducible mechanical force. In neuronal cells p130Cas/Crk/C3G-induced activation of Rap1 leads to activation of B-Raf in neurons. Complex formation of B-Raf with Rap1 converges to a signaling pathway to sustainably activate the mitogen-activated protein kinase (MAPK) pathway to control neuroblast differentiation (York et al., 1998). Studies also showed that C3G play an important role in protin-trafficking and secretion (Radha et al., 2004).

#### 1.1.4.5. DOCK180 (DOCK1)

DOCK180, is a 180kDa protein first identified by 'Far Western' overlay blots (Feller et al., 1995b) (Hasegawa et al., 1996), to bind to the Crk SH3<sup>N</sup> domain. Dock180 is also a guanine nucleotide exchange factor (GEF) for small GTPase Rac-1. Proper activation of Rac-1 can only take place when DOCK180 is bound to ELMO via its DHR2/CZH2 domains. Rac plays important role in regulation of various cellular activities like controlling actin dynamics, integrin mediated cell adhesion and cell shape changes along with engulfment of dead cell bodies (Albert et al., 2000; Bishop and Hall, 2000; Cheresh et al., 1999; Giancotti, 1997; Reddien and Horvitz, 2000; Savill and Fadok, 2000; Schmitz et al., 2000; Tosello-Trampont et al., 2001). Although DOCK180 was identified as SH3<sup>N</sup> binding partner of CRK, it is not entirely clear whether this interaction directly mediates Rac1 activation (Albert et al., 2000; Kiyokawa et al., 1998). Rather it appears that Crk has a secondary function in the regulation of Rac1 activation (Tosello-Trampont et al., 2007) mediated by the SH3<sup>C</sup> domain (Akakura et al., 2005). Mutations of the SH3<sup>C</sup> domain prevent the regulated turnover of DOCK180/ELMO complex, suggesting that Crk also controls assembly of the ELMO/DOCK180 complex. More recent functional studies have shown that a direct interaction of CrkII with DOCK180 was dispensable for both engulfment of apoptotic cells as well as for the recruitment of DOCK180 since expression of DOCK180 mutant lacking the proline-rich sequence were sufficient for Rac1 activation (Tosello-Trampont et al., 2007). Moreover, mutant Crk protein that dosen't interact with DOCK180 blocks Rac1 activation (Gu et al., 2001). Many recent studies indicate that DOCK5, another member of DOCK family, which participate in Caco-2 cell motility by interacting with CrkII and CrkL SH3<sup>N</sup> domain via atypical

proline-rich motifs (Sanders et al., 2009). Similar interaction between DOCK and Crk proteins via the atypical proline rich motif have been reported for DOCK2, which lacks the classic C-terminal region for binding Crk, and appears to bind CrkL via regions of the DOCKER domain (Nishihara et al., 2002a). Recent studies showes that DOCK5 cooperates with DOCK180 for the differentiation of myofibers (Laurin et al., 2008).

#### 1.1.4.6. Abl Kinase

Abl and its homologous Arg (Abl related gene) are large non-receptor tyrosine kinases. Abl was first identified as a cellular homolog of the v-Abl oncogene, product of the Abelson murine leukemia virus. Abl plays important roles in cell growth, transformation, cell stress, apoptosis, and remodeling of the actin cytoskeleton. Both proteins contain four tandem proline-rich motifs, C-terminally to the kinase domain (Feller et al., 1994; Ren et al., 1994) that mediates binding to the Crk SH3<sup>N</sup> domain. Systematic mutagenesis studies showed that the 1<sup>st</sup>, 2<sup>nd</sup>, and 4<sup>th</sup> region (from the N-terminus to the C-terminus) proline-rich motifs predominantly bound Crk, whereas the 3<sup>rd</sup> region bound to SH3 domains of Nck, Ponson/CAP, and ArgB2 (Antoku et al., 2008). In focal adhesion Abl regulates the formation and maintenance of adherens junctions via a Crk and Rac1 pathway that regulates cadherin-catenin adhesion complex (Zandy et al., 2007). Abl has also been shown to tyrosine phosphorylate SOS1, which associates with Abl via Crk (Sini et al., 2004).

The interaction between Crk and Abl is suggested to be bidirectional: Crk transactivates Abl and Abl transinhibits CrkII and CrkL (**Figure1.12**). Shishido and Hanafusa (Shishido et al., 2001) first reported transactivation of Abl by Crk on basis of their observation of increased levels of tyrosine phosphorylated cellular protein upon co-

expression of Crk and Abl in Abl expressing cells. Reichman *et al* showed that expression of Crk along with Abl enhances autophophorylation of Abl at its major sites (Tyr 245 and Tyr 412), suggesting that Crk-mediated enhances Abl enzymatic activity (Reichman et al., 2005). The level of Crk transactivation of Abl increases when Y221 and P225 Crk motifs are mutated, in which case Crk phosphorylation is prevented leading to autoinhibitory intramolecular rearrangement (Reichman et al., 2005; Shishido et al., 2001). Biochemical studies have shown that transactivation of Abl by Crk is regulated by the SH3<sup>C</sup>-linker (SH3<sup>C</sup>-L) in Crk and the PNAY motif in the RT loop of SH3<sup>C</sup> domain. Other studies also showed that association of Crk with Abl increases steady state levels of Abl (Reichman et al., 2005), possibly to prevent degradation. *In vitro*, addition of Crk SH3<sup>N</sup> domain to Abl can reverse the inhibition of Abl activity by F-actin, further suggesting that Crk transactivation may occur locally in the focal adhesions (Woodring et al., 2005).



Figure 1.12 Association between c-Abl and Crk (Birge et al., 2009)

Subsequent binding of Abl to Crk SH3<sup>N</sup> domain and Abl transactivation leads to Crk Y222 (chicken) phosphorylation. How the Abl-Crk assembles and functions remains an important avenue of future investigation to understand the various transition states involved in this interaction. One important function of Y222 phosphorylation is to regulate transactivation of complexes involving Crk and Abl. Abl mediated Crk Y222 phosphorylation actes as a molecular switch to dissociate a p130cas/Crk/DOCK1 ternary complex, in order to block motility and invasive behavior of human pancreatic cancer cells (Cho and Klemke, 2000). Another recent study show that phosphorylation of Crk Y222 by Abl leads to dissociation of C3G from Crk which leads to decrease activation of Rap1-GTP, decreased affinity of  $\beta$ 1-integrin to ECM, and finally loss of cell adhesion (Huang et al., 2008). Crk Y222 phosphorylation by Abl also have a negative effect on HGF-stimulation by inhibiting Crk-Abl complex formation (Cipres et al., 2007). Tyrosine phosphorylation of Crk by Abl in tumor cells may underscore a novel antioncogenic pathway. EphB2 receptor tyrosine kinase act as a tumor suppressor in breast cancer cell and depletion of EphB2 or its ligand and epherinB2 prediposes breast cancer cells to invasion and metastasis (Noren et al., 2006). It was also showed that epherinB2 induces Crk Y222 phosphorylation and decreases Rac1 activation. Paterson and Long identified an important functional role of Crk and Abl complet downstream of human killer cell Ig-like receptor (KIR) that inhibits Natural Killer (NK) cell activation and cytotoxicity (Peterson and Long, 2008). They showed that killing phenotype correlates with the Cbl/Crk/C3G complex, whereas the inhibitory phenotype correlates with the Crk-Abl complex, where phosphorylation of Crk Y222 by Abl dissociates the Cbl/Crk/C3G complex (Peterson and Long, 2008). Crk pY222 has been sown to localize activated Rac1 to the membrane fraction (Abassi and Vuori, 2002). Crk Y221F mutant prevents regulated turnover of Crk/Abl/p130Cas or Crk/Abl/paxillin complexes, leading to impaired cell adhesion and migration (Escalante et al., 2000). All these studies showed that phosphorylation of Crk Y222 by Abl and Arg are just not a simple -on and -off mechanism, but rather a dynamic process to execute Crk-dependent signaling involving Rac1 activation and cell migration.

# 1.1.5. The Crk SH3<sup>C</sup> domain

# 1.1.5.1. The role of SH3<sup>C</sup> domain in Crk

Despite the fact that several proteins have been identified to bind to the SH3<sup>N</sup> domain, no cellular molecules have been shown to interact with the Crk SH3<sup>C</sup> domain, with the single exception of the nuclear export factor crm1 (Smith et al., 2002). Currently, the function of the Crk SH3<sup>C</sup> domain is poorly understood. There is, however, increasing evidence that the SH3<sup>C</sup> domain functions as a regulatory element. It has been shown that the SH3<sup>C</sup> domain negatively reulates tyrosine phosphorylation of focal adhesion kinase (FAK) and focal adhesion proteins p130Cas and paxillin and disrupted EGF-induced Ras activation (Kizaka-Kondoh et al., 1996). More recently, it was determined that Crk SH3<sup>C</sup> has unique function in phagocytosis and Rac activation. Mutation that disrupts the SH3<sup>C</sup> domain of Crk results in the stabilization of a ternary complex of Crk, DOCK180, and ELMO that stimulates Rac activation, but strongly abrogates engulfment of apoptotic cells (Akakura et al., 2005). These data indicate that the assembly and disassembly of the complex is regulated by Crk through its unconventional SH3<sup>C</sup> domain. Interestingly, the SH3<sup>C</sup> domain has been proposed to enhance the ability to activate c-Abl (Reichman et al., 2005; Shishido et al., 2001). It was hypothesized that SH3<sup>C</sup> binds to c-Abl following the essential binding of the SH3<sup>N</sup> domain of Crk to c-Abl, thereby enhancing the affinity of the complex. On the other hand, it was found that a point mutation in SH3<sup>C</sup> or its truncation increased c-Abl binding to SH3<sup>N</sup> domain of Crk and increased Y222 phosphorylation (Reichman et al., 2005). Crk SH3<sup>C</sup>, in particular the RT loop residues, is

required for the transient activation of Abl by Crk, possibly by mimicking and destabilizing the negative rregulatory PTIY motif in Abl. Some of the results suggested that the SH3<sup>C</sup> and the linker region perform different functions: the linker may directly inhibit transactivation, perhaps by binding to the kinase domain in a manner similar to the Abl kinase domain activation loop, and SH3<sup>C</sup> may serve to modulate the affinity of CrkII for Abl. These results have led to the suggestion that the SH3<sup>C</sup> domain of CrkII could serve as a negative regulatory element and as an activator of Abl. Neverthless, how these seemingly diverse functions are accomplished is not understood.

# 1.1.5.2. Solution structure of SH3<sup>C</sup>

The solution structure of the  $\text{SH3}^{\text{C}}$  domain of Crk murine was solved in recent by using NMR (Muralidharan et al., 2006). Although the Crk  $\text{SH3}^{\text{C}}$  domain is highly conserved from C.*elegans* to mammals, there are notable differences in between the Crk  $\text{SH3}^{\text{C}}$  and other canonical SH3 binding domains that bind P-x-x-P motifs. Crk  $\text{SH3}^{\text{C}}$  also has the canonical SH3 domain fold comprising of five-stranded  $\beta$ -barrel but with a highly divergent PPII binding surface (Muralidharan et al., 2006; Reichman et al., 2005).



**Figure 1.13** Overlaid structures of the SH3<sup>N</sup> and SH3<sup>C</sup> domains of CrkII are shown (left). The polyproline ligand for the SH3<sup>N</sup> domain is also shown (in blue). The aromatic residues that line the ligand-binding pocket in the SH3<sup>N</sup> domain (in green) and the corresponding residues occupying the same position in the SH3<sup>C</sup> domain (in red) are also indicated. The residues that form the entire canonical ligand-binding pocket in the SH3<sup>C</sup> domain are shown in a surface representation and are indicated (right) (Muralidharan et al., 2006).

The solution structure of the SH3<sup>C</sup> domain of Crk murine was solved in recent by using NMR (Muralidharan et al., 2006). Although the Crk SH3<sup>C</sup> domain is highly conserved from C.*elegans* to mammals, there are notable differences in between the Crk SH3<sup>C</sup> and other canonical SH3 binding domains that bind P-x-x-P motifs (**Figure 1.14**). Crk SH3<sup>C</sup> also has the canonical SH3 domain fold comprising of five-stranded  $\beta$ -barrel but with a highly divergent PPII binding surface (Muralidharan et al., 2006; Reichman et al., 2005). The binding pocket of SH3 domains are mainly made up of aromatic residues, such as Phe141, Tyr186, and Trp169 in case of CrkII SH3N, whereas, in case of CrkII SH3C domain these residues are replaced by more polar, nonaromatic residues, such as Gln244, H290, and GLn274 (**Figure1.13**). No obvious binding groove could be inferred from the solution structure of SH3<sup>C</sup>, suggesting that Crk SH3<sup>C</sup> may not bind to target proteins (Muralidharan et al., 2006).

	<i>B</i> 1	RT-Loop	<i>β</i> 2	nSrc β3	Distal \$4	3 <sub>10</sub> \$5
	237	247	257	267	278	286 294
c-Crk cSH3	PIYARVIQKR	VPNAYDKTAL	ALEVGELVKV	TKINVSOWEO	-E-CNGKRGH	FPFTHVRLL
c-Crk nSH3 (lcka)	AEYVRALFDF	NGNDEEDL	PFKKGDILRI	ROKPEEOWWNA	-EDSEGKRGM	IPVPYVEKYG
Csk SH3 (ljeg)	GTECIAKYNF	HGTAEQDL	PFCKGDVLTI	VAVTKDPNWY	AKNKVGREGI	IPANYVQKR
a-spectrin SH3 (1shg)	KELVLALYDY	QEKSPREV	TMKKGDILTL	LNSTNKDWWK	-EVND-ROGF	VPAAYVKKLD
Sem5 SH3 (lsem)	TKEVQALEDE	NPQESGEL	AFKRGDVITL	INKODPNWE	-QLNN-RRGI	<b>FPSNYV</b> CPYN
Fyn SH3 (1shf)	VTLEVALYDY	EARTEDDL	SFHKGEKFQI	LNSSEGDWE	RSLTTGETGY	IPSNYVAPVD S
Src SH3 (1fmk)	VTTFVALYDY	ESRTETDL	SFKKGERLQI	VNNTEGDWIL	HSLSTGOTGY	IPSNYVAPSD S
Lck SH3 (11ck)	DNLVIALHSY	EPSHDGDL	GFEKGEQLRI	LEQS-GEWKA	QSLTTGQEGF	IPFNFVAKAN S
Hck SH3 (lad5)	DITIVVALYDY	EAIHHEDL	SFOKGDOMVV	LEES-GEWKA	RSLATRNEGY	IPSNYVARVD S
Abl SH3 (labo)	DPNLEVALYDE	VASGDNTL	SITKGEKLRV	LGYNHNGEWCE	AQTENGQ-GW	VPSNYITPVN S

**Figure 1.14** Sequence and secondary-structure alignment of SH3 domains. Conserved residues involved in ligand binding are highlighted in oranges boxes, and served residues of the hydrophobic core are highlighted in blue boxes. The PDB codes are in parentheses. Figure adapted from (Muralidharan et al., 2006)

To date, Crm1 is the only putative binding partner of Crk SH3<sup>C</sup> domain. Crm1 bind to a LALEVGELVKV sequence in the CrkSH3<sup>C</sup>, and has large similarity to a nuclear export sequence (Smith et al., 2002). However, according to the solution structure of murine Crk SH3<sup>C</sup> this LVK motif is buried in the hydrophobic core of the SH3<sup>C</sup>. In the case of CrkL SH3<sup>C</sup>, partial unfolding of the SH3<sup>C</sup> domain that may occur during the transition from monomer to dimer, was suggested then expose the LVK motif, thereby providing a surface for Crm1 binding (Harkiolaki et al., 2006). This suggests that regulated unfolding of Crk may be a novel pathway for regulating Crk efflux in context of nuclear Crk.

#### 1.1.6. Solution Structure of full length Crk

The CrkI and CrkII (human) solution structures were recently reported by Inagaki and colleagues (**Figure 1.15**) (Kobashigawa et al., 2007).



**Figure 1.15** Ribbon representation of CRKII. Magenta, SH2 (residues 10–120); green, SH3N (134–191); blue, SH3C (238–293); yellow, ISC (220–237); gray, other regions.

Their result shows that CrkI has an elongated flexible structure. In contrast, CrkII adopts a compact structure. With all three SH domains held together in a compact assembly via a short central sequence lying in the inter SH3 region (224-237) called the inter SH3 core region (ISC). The interface between ISC and the SH domains can be subdivided into two regions. The first is a hydrophobic core formed between Pro224 and Val226 of ISC and Val184, Lys189, Trp170, and Lys164 of SH3<sup>N</sup> (**Figure 1.16**). A second set of interaction comprises a more extensive hydrophobic core involving Leu230, Pro231, Ile236 and Pro237 of the ISC and Ile269, Trp275, Val266, Lys265, Tyr239 and Ile263 of SH3<sup>C</sup> (**Figure 1.16**). A third set of hydrophobic contacts within the ISC region among Thr228, Pro229, and Ile236 stabilizes the ISC in a bent conformation.

The structure of human CrkII also showed that the SH3<sup>N</sup> binding pocket is masked by SH2, partially mimicking the binding mode of P-x-x-P motif to SH3<sup>N</sup>. They

also showed that the mutation of highly conserved Pro residues within the ISC region destabilizes the CrkII compact structure. These structural data are in agreement with previously reported data that the affinity of CrkII SH3<sup>N</sup> domain for the target polyproline peptide is lower than the affinity of CrkI.

CrkII is phosphorylated at Y222 in the ISC region by c-Abl, acting as a regulatory mechanism for Crk activity. The structure of phosphorylated CrkII (pCrkII) shows that along with the intramolecular interaction between the SH2 domain and Y222 as reported previously, the SH3<sup>N</sup> binding pocket also is blocked by a linker region (Arg122-Gu133) between the SH2 and SH3<sup>N</sup> domains. These data suggest that pCrkII can neither bind to SH2 nor SH3<sup>N</sup> binding targets.



**Figure 1.16** Ribbon model of hydrophobic core of CRKII, between the ISC and the SH domains, Magenta, SH2 (residues 10–120); green, SH3N (134–191); blue, SH3C (238–293); yellow, ISC (220–237); gray, other regions.

In summary, the structures of CrkI, CrkII and pCrkII showed that CrkI is constitutively active, CrkII is regulated by intramolecular interactions between its SH domains and pCrkII completely prevents CrkII from binding to its partner. All this observations are in accordance with previously reported biochemical and biophysical data.

#### 1.1.7. Crk in diseases

#### 1.1.7.1. Crk in cancer

Crk has been found to be overexpress in many human cancers including various carcinomas and sarcomas (Miller et al., 2003; Nishihara et al., 2002b; Takino et al., 2003). Crk is a key component of focal adhesion and involved in cell growth, invasion, and dissemination of human ovarian cell line MCAS (Linghu et al., 2006). Deregualtion of CAS/Crk signaling also contributes to cancer progression and developmental defects in humans (Chodniewicz and Klemke, 2004). Additionally, the levels of c-CrkI oncoprotein and phosphorylated c-CrkII were significantly increased in clinically Immunohistochemical aggressive lung adenocarcinomas. analysis of c-CrkII demonstrated that the levels of c-CrkII were significantly elevated in most of the tumors, particularly in the colon and lung cancers. Furthermore, immunobloting analysis using human lung cancer cell lines revealed that the expression level of the c-CrkII were correlated to growth rates of cells and that the elevated expression levels of c-CrkII might be related to the development of human cancers (Nishihara et al., 2002b). Thus, there is now evidence that all Crk family members are capable of transforming cells and are direct targets of several oncogenes, which strongly implicates these adaptor proteins in cancer. Notably, Crk family adaptors also appear to play a role in mediating the action of the Bcr-Abl protein (Kolibaba et al., 1999), which causes chronic myelogenous leukemia and Philadelphia-chromosome positive acute lymphoblastic leukemia (Deininger et al., 2000). Multiple independent studies have implicated CrkL in transduction of oncogenic signals of Bcr/Abl and CrkL tyrosine-phosphorylation is used as a diagnostic tool for Philadelphia-positive leukemia (Hemmeryckx et al., 2002).

#### 1.1.7.2. Crk in bacterial infectious diseases

Many recent studies provide evidence suggesting that Crk may be involved in bacterial pathogenesis by influencing bacterial entering into cells or by serving as targets of bacterial toxins that upset important cellular function through Crk-dependent mechanisms. Weidow et al demonstrated for the first time the role of Crk in bacterial uptake pathway by showing that Yersinia pseudotuberculosis infection into human epithelial cells activated the p1130Cas-Crk-DOCK180-Rac1 pathway via binding of the bacterial invasion to the  $\beta$ 1-integrin receptor (Bruce-Staskal et al., 2002; Weidow et al., 2000). Also Burton and colleagues found that Crk is involved in Shigela flexneri infection into NIH 3T3 cells using a intriguing pathway involving Abl-Arg-mediated Crk Y222 phosphorylation, whereby Y222F mutation disrupt S.Flexneri infection (Burton et al., 2003). A novel role for Abl and Crk phosphorylation was found to be essential for Pseudomonas aeruginosa internalization (Pielage et al., 2008). Also, in case of Helicobacter pylori Crk associates with tyrosine phosphorylated CagA, and in turn CagA/Crk complex regulates Erk signaling and Rac-1 mediated cytoskeletal assemblages (Suzuki et al., 2005). Finally, exoenzyme T (ExoT) of P. aeruginosa ADP-ribosylates CrkI and CrkII at specific residues in SH2 domain (Arg20) that impairs binding of Crk to p130Cas (Deng et al., 2005). Also, CrkL has been identified to bind to NS1 proteins in Influenza A infected cells (Heikkinen et al., 2008).

#### **1.2 Nuclear Magnetic Resonance (NMR) spectroscopy**

#### **1.2.1 NMR as a tool in biochemistry.**

X-ray crystallography and NMR spectroscopy are the two most powerful biophysical techniques that are in use to determine the structure of various biomolecules at atomic resolution.Nuclear magnetic resonance (NMR) spectroscopy is based on the physical concept of nuclear spin angular momentum of nuclei. Protein NMR was pioneered by Richard R. Ernst and Kurt Wüthrich (Wuthrich, 1990). Nowadays, major advancements in the bio NMR field have been achieved by the use of highly advanced hardware and methodologies, which have significantly helped NMR spectroscopists to overcome two major problems associated with NMR of biomolecules: signal-to-noise (S/N) ratio (sensitivity) and spectral overlap (resolution). Moreover, the development of multidimensional NMR, specific labeling schemes, and the introduction of TROSY-type pulse sequences, have enabled NMR structural investigation of large proteins(Ikura et al., 1990c).

The major advantage of NMR over X-ray crystallography is that one can study biomolecules in solution. Moreover, NMR is the only technique that provides information on protein motion on different time scales (pico seconds –hours) at residue level along with the structure at atomic resolution. Thus, biomolecular NMR spectrocopy is indispensable in providing insight into the conformational dynamics and exchange processes of biomolecules and their complexes with ligands. NMR methods can be used to characterize denatured states of proteins, folding intermediates and even lowelypopulated transition states (Sattler, 2004). NMR can also provide useful insights into the kinetics of folding and unfolding as well as detailed structural, dynamic, and kinetic information of enzymatic reactions (Sattler, 2004).

NMR is also very useful for mapping protein/protein, protein/ligand and protein/nucleic acid interactions. Chemical shift mapping can be used to localize ligand binding sites and to determine the energetics of protein/ligand interaction (Sattler, 2004), thus becoming one of the most important technique in the field of drug discovery.

Currently, NMR spectroscopy can be routinely applied for the *ab initio* structure determination of biomolecules up to ~30 kDa. Major steps involved in protein structure determination are as follows:

- 1) Protein sample preparation.
- 2) NMR data collection
- 3) Sequence specific resonance assignment
- 4) Collection of structural constraints
- 5) Elucidation of secondary structure
- 6) 3D structure calculation
- 7) 3D structure refinement

#### **1.2.1. Protein assignment.**

#### 1.2.1.1. Backbone assignment.

The first step in the NMR characterization of a protein usually involves acquisition of the heteronuclear single quantum coherence (HSQC) spectrum (Kanelis et al., 2001). In a typical <sup>1</sup>H-<sup>15</sup>N HSQC experiment, the amide (NH) resonances are observed - the magnetization is transferred from <sup>1</sup>H to the attached <sup>15</sup>N nucleus via the J-coupling and

then transferred back to <sup>1</sup>H for detection. The number of the obtained resonance peaks roughly corresponds to the number of amino acids in the protein molecule.

In the next step, assignment of the backbone chemical shifts is performed (Kay, 2005). The most commonly used approach to obtain chemical shift assignment involves uniform labeling of the protein under study with <sup>13</sup>C and <sup>15</sup>N isotopes and use of a series of multidimensional heteronuclear NMR experiments. The assignment protocols that are routinely used are based on the complimentary sets of HNCA, HN(CO)CA, HNCO, HN(CA)CO, HBHA(CBCA)NH, HBHA(CBCACO)NH, CBCANH, CBCA(CO)NH and/or HNNHN(C)N, three-dimensional heteronuclear experiments. These experiments are based on magnetization transfer between <sup>13</sup>C and <sup>15</sup>N nuclei through bonds, facilitated by the large J-couplings (**Figure 1.17**).



**Figure 1.17** Spin system of the peptide backbond and size of the <sup>1</sup>J and <sup>2</sup>J coupling constants that are used for magnetization transfer in <sup>13</sup>C-, <sup>15</sup>N-labelled proteins.

To accomplish sequential backbone assignment, two-set of experiments are recorded that provide complementary information. The first set of experiments gives both intra- and inter-residue correlations, and the second one provides only inter-residue correlations (Kanelis et al., 2001). For example, in the HNCO experiment (first set), the magnetization is transferred from <sup>1</sup>H to <sup>15</sup>N and then selectively to the carbonyl group of the residue *i-1* through the <sup>15</sup>NH-<sup>13</sup>CO J-coupling (Grzesiek and Bax, 1992; Muhandiram and Kay, 1994; Yamazaki et al., 1994a). As a result, the chemical shift for all three nuclei yields a three-dimensional spectrum (**Figure 1.18 A**).



Figure 1.18 The magnetization transfer pathway in HNCO (A) and HN(CA)CO experiment (B).

The second set, the HN(CA)CO experiment correlates two carbonyl (CO) groups (*i* and *i*-*1*) with respect to each amide (NH) group (Clubb et al., 1992; Kay et al., 1994; Matsuo et al., 1996). As shown in **Figure 1.18 B**, the magnetization transfer occurs from <sup>1</sup>H to <sup>15</sup>N and then to the <sup>13</sup>CO (via <sup>13</sup>Ca) through the N-<sup>13</sup>Ca and <sup>13</sup>Ca-<sup>13</sup>CO J-couplings. Importantly, the chemical shift only evolves on <sup>1</sup>H, <sup>15</sup>N and <sup>13</sup>CO, but not on <sup>13</sup>Ca. Due to the stronger N<sub>i</sub>-Cai coupling, with respect to one between N<sub>i</sub> and Ca<sub>i-1</sub>, the observed H<sub>i</sub>-N<sub>i</sub>-CO<sub>i</sub> peak is usually more intense than the H<sub>i</sub>-N<sub>i</sub>-CO<sub>i-1</sub> peak. Similarly, the <sup>13</sup>Ca nuclei in residues *i* and *i*-1 can be correlated via HNCA and HN(CO)CA experiments (Grzesiek and Bax, 1992; Ikura et al., 1990b; Ikura et al., 1990c; Yamazaki et al., 1994a; Yamazaki et al., 1994b). In the HNCA experiment, the peak intensity for the directly bonded <sup>13</sup>Ca nucleus is stronger due to the larger J-coupling. In the HN(CO)CA experiment the obtained spectrum is selective for the <sup>13</sup>Ca of the *i*-1 residue. In the HNCACB experiment, both C $\alpha$  and C $\beta$  are observed for each amide group. The chemical shift evolution occurs simultaneously on <sup>13</sup>C $\alpha$  and <sup>13</sup>C $\beta$ .

#### 1.2.1.2. Side chain assignment

After completion of the backbone assignment, the next step involves assignment of the side-chain signals (Marintchev et al., 2007). The assignment of the side-chain <sup>1</sup>H and <sup>13</sup>C resonances can be performed by a combination of 3D H(C)(CO)NH, (H)C(CO)NH, <sup>15</sup>N and <sup>13</sup>C NOESY-HSQC experiments.

#### **3D H(CCO)NH and C(CO)NH experiments**

In general, the sequential connectivity along with the side-chain assignments can be performed using the H(C)(CO)NH and (H)C(CO)NH experiments (Lin and Wagner, 1999), as these experiments correlate the <sup>1</sup>H and <sup>13</sup>C side-chain atoms of the residue *i*-1 with the amide <sup>1</sup>H and <sup>15</sup>N of the residue *i*.



Figure 1.19 The magnetization transfer pathway in H(C)(CO)NH experiment.

In the H(C)(CO)NH experiment (**Figure 1.19**), the magnetization is transferred from the peripheral side chain proton and carbon nuclei using the isotropic  $^{13}$ C mixing to the carbonyl  $^{13}$ C. Afterwards, the magnetization is transferred onto the amide nitrogen

and finally to the amide proton for detection. As a result, a 3D spectrum with one <sup>15</sup>N and two <sup>1</sup>H dimensions is produced (Montelione et al., 1992).

In the (H)C(CO)NH experiment, the evolution of the chemical shift occurs through a mechanism similar to H(C)(CO)NH experiment; however, the observed nucleus is  $^{13}$ C (Grzesiek et al., 1993).

#### **1.2.1.3.** Nuclear Overhauser Effect Spectroscopy (NOESY)

The importance of nuclear Overhauser effect (NOE) spectroscopy in the structure determination of biomolecules cannot be overestimated. In simple terms, it provides the correlations between spatially proximate nuclei (usually within 5-6 Å) and, therefore, provides true gold standard for protein structure calculation (Kanelis et al., 2001). The NOE originates from dipolar interactions between different nuclei. The magnitude of the NOE is inversely dependent on the sixth power of the internuclear distance (1/r6) (Wuthrich, 2003). In a typical NOE-based experiment (e.g., <sup>15</sup>N-NOESY-HSQC), the magnetization is exchanged between all protons. Afterwards, it is transferred to the neighboring <sup>15</sup>N nuclei and back to <sup>1</sup>H for detection. For each NH group, all <sup>1</sup>H within 5-6 Å will provide NOE correlations and will appear in the spectrum. NOEs between side chain protons can be similarly obtained from <sup>13</sup>C-NOESY-HSQC spectra. In addition, 3D HMQC-NOESY-HMQC experiment can be used to extract NOEs from extremely overcrowded spectral regions (Andersson et al., 1998; Frenkiel et al., 1990; Ikura et al., 1990a).

#### **1.2.1.4.** Residual Dipolar Couplings (RDC)

The relative orientations of interacting biomolecules, or separate domains of a large biomolecule, can be derived by measuring residual dipolar couplings (RDCs) between NMR active nuclei in partially oriented media (Foster et al., 2007; Tjandra et al., 1995).

RDC is a very useful phenomenon because it appears to be sensitive to distance, orientation, and dynamics, and it can be defined as a through-space interaction between any two magnetically active nuclei. In solution, the dipolar couplings average to zero due to the Brownian motion effects. As a result, they can be only observed under anisotropic conditions. For a pair of dipole-coupled nuclei, A and B, their observable dipolar coupling in solution, DAB, can be estimated as follows:

$$D_{AB}(\theta,\phi) = A_a^{AB} \left\{ (3\cos^2\theta - 1) + \frac{3}{2}R(\sin^2\theta\cos 2\phi) \right\}$$

$$A_a^{AB} = -\left(\frac{\mu_0 h}{16\pi^3}\right) S\gamma_A \gamma_B \langle r_{AB}^{-3} \rangle A_a$$

where  $A_a^{AB}$  and R are the axial and rhombic components, respectively, of the molecular alignment tensor;  $\theta$  is the angle between the internuclear bond vector and the z-axis of the alignment tensor;  $\varphi$  is the angle between the projection of the internuclear bond vector onto the x-y plane and the x-axis;  $\mu \theta$  is the permeability in vacuum, *h* is Planck's constant (6.626\*10-34 J\*s); S is the generalized order parameter;  $\gamma_A$ ,  $\gamma_B$  are the gyromagnetic ratios of two nuclei; *r* is the time-averaged internuclear distance; and Aa is the aforementioned axial component of the molecular alignment tensor (Lipsitz and Tjandra, 2004b). To obtain RDCs in solution, a co-solute is required to induce the partial alignment of protein molecules (**Figure 1.20**). Dipolar couplings in solids are typically tens of kHz, while in partially oriented solutions they are usually under 100 Hz. RDCs can be observed in molecules with large magnetic susceptibility anisotropy, such as metalloproteins and DNA, that causes a field-dependent alignment of molecules (Gayathri et al., 1982; Lipsitz and Tjandra, 2004b).



**Figure 1.20** The ordering of protein molecules in solution (left) and in partially aligned media (right).

The degree of alignment can be measured from the  ${}^{2}$ H quadrupolar splitting in the HDO resonance. The splitting originates from the exchange between isotropic bulk H<sub>2</sub>O and aligned H<sub>2</sub>O molecules associated with the aligned media (Lipsitz and Tjandra, 2004b).

For most biomolecules dissolved in orienting media, the mechanism of alignment relies on weak interactions because the induced ordering must retain the molecular folding while maintaining the high spectral resolution (Prestegard et al., 2000).

Numerous alignment media have been developed, such as bicelles, phage particles, poly (ethyleneglycol)/alcohol mixtures, purple membrane fragments, helfrich phases, strained gels(Tjandra et al., 1995). The choice of a proper system depends on the properties of the protein under study. For instance, for highly charged proteins, a neutral system has to be considered, such as poly (ethylene glycol)/alcohol or strained gels.

Furthermore, for weakly charged proteins, one may select a system that is oppositely charged (Fleming and Matthews, 2004).

There are two general categories of NMR methods for measuring the dipolar coupling constants: (i) frequency resolved methods where the peak center separation is measured in a frequency domain (Montelione and Wagner, 1989), and (ii) intensity-based experiments where the coupling is extracted from the resonance intensity (Bax et al., 1994; Prestegard et al., 2000; Vuister and Bax, 1993). N-HN dipolar couplings can be measured using IPAP <sup>15</sup>N-HSQC experiment where the components of the multiplet are separated (Ottiger et al., 1998), while the 3D (HA)CA(CO)HN and HNCO can be used to detect C $\alpha$ -H $\alpha$ , and C $\alpha$ -C' couplings (Ferentz and Wagner, 2000; Ottiger and Bax, 1998; Permi et al., 2000).

In our study, the IPAP <sup>15</sup>N-HSQC method has been used to measure N-HN dipolar couplings. This method is based on the acquisition of two spectra, one where the coupling evolves in-phase (IP) and the other one where the coupling evolves anti-phase (AP). The subsequent addition and subtraction of these spectra yields two spectra, each with one of the doublet components (Prestegard et al., 2000).

With a large number of RDCs in hand, the principal components of the alignment tensor can be precisely calculated. Several methods have been developed to determine  $A_a$  and R parameters. For example, in the histogram method proposed by Clore et al. (Clore et al., 1998a), the RDCs are measured, normalized and plotted in a histogram (**Figure 1.21**).



Figure 1.21 A histogram of RDC.(Lipsitz and Tjandra, 2004a)

The values for  $A_{zz}$ ,  $A_{yy}$  and  $A_{xx}$  can be obtained from the three extrema of the histogram as shown on (**Figure 1.21**). These values can be then used to calculate  $A_a$  and R (Lipsitz and Tjandra, 2004a).

A-B lies along 
$$D_{zz}$$
:  $\theta = 0^{\circ}$   $A_{zz} = 2A_a$ ,  
A-B lies along  $D_{yy}$ :  $\theta = 90^{\circ}$ ,  $\phi = 90^{\circ}$   $A_{yy} = -A_a \left\{ 1 + \frac{3}{2}R \right\}$ ,  
A-B lies along  $D_{xx}$ :  $\theta = 90^{\circ}$ ,  $\phi = 0^{\circ}$   $A_{xx} = -A_a \left\{ 1 - \frac{3}{2}R \right\}$ .

#### 1.2.1.5. TALOS

TALOS is a database system used to empirically predict  $\varphi$  and  $\psi$  backbone torsion angles for a given protein sequence (**Figure 1.22**) using as a source a set of several chemical shifts (H $\alpha$ , C $\alpha$ , C $\beta$ , CO, N) (Cornilescu et al., 1999).



Figure. 1.22 Backbone (dihedral) angles in the protein structure.

TALOS searches the database for triplets of adjacent residues with secondary chemical shifts and sequence similarity in order to provide the best match to the query triplet of interest. The database contains  ${}^{13}C\alpha$ ,  ${}^{13}C\beta$ ,  ${}^{13}C'$ ,  ${}^{1}H_{\alpha}$  and  ${}^{15}N$  chemical shifts for 20 proteins with available high-resolution X-ray structure. The goal of TALOS is to make quantitative predictions for the protein backbone angles (*phi* and *psi*) and to estimate the uncertainty for these predictions. The output of TALOS is 10 triplets that have the closest similarity in the secondary chemical shift and amino acid sequence to those of the query sequence. If the central residues in these 10 triplets show similar backbone angles, their averages can be reliably used as angular restraints for the protein under study.

#### **1.2.2. Protein structure calculation**

Once all the experimental data (distance restraints from NOE and PRE, dihedral angle restrains, RDCs) have been collected, it is used as an input for the protein structure calculation. The structure calculation software produces a set (ensemble) of structures consistent with the experimental NMR data. Then, the coordinate precision, defined as the root-mean-square deviation (rmsd) for the atomic coordinates between the structures of the NMR ensemble, is used to assess the precision of the calculated structures.

The rmsd from experimental distance, dihedral angle should be small. The calculated distance restraints have to be within 0.5 Å. In addition, the Ramachandran plot is a good quality measure. The Ramachandran plot is based on a statistical analysis of high-resolution crystal structures and is often used to specify the fraction of backbone  $\varphi$  and  $\psi$  angles corresponding to favored, additionally allowed, generally allowed and disallowed conformations (Sattler, 2004).

In our case, ARIA, XPLOR-NIH and (Schwieters et al., 2006) programs were employed for the protein structure calculation.

#### **1.2.4. Protein Dynamics**

Proteins undergo various types of dynamic motions covering a wide range of amplitudes and time-scales (**Figure 1.23**) (Bruschweiler, 2003). All these movements are necessary to carry out various biological functions by proteins. Solution NMR is a unique and powerful tool for characterizing the internal dynamics of proteins. For large biomolecules, the motions that affect the relaxation rates can be divided into three major groups: (1) overall rotation of the molecule (ns time scale); (2) fast (ps-ns) internal fluctuations of individual groups of atoms within the molecular frame of reference; and (3) motions on the slow time scale ( $\mu$ s-ms) that affect only transverse relaxation rates. In terms of biological function, the internal molecular motions are the most important (Lee, 2001).



Figure 1.23 NMR time scales and dynamics in biology (Sattler, 2004).

The order parameter  $(S^2)$  is a value that is a characteristic of the angular fluctuation of a chemical bond vector, such as NH bond, and therefore reflects the flexibility of the polypeptide chain at a particular site (Ishima and Torchia, 2000). The order parameter,  $S^2$ , is a measure of the amplitude of internal motions on the pico-tonanosecond (ps-ns) timescale and may vary from  $S^2=1$ , for a bond vector having no internal motion, to  $S^2=0$ , for a bond vector rapidly sampling multiple orientations(Mittermaier and Kay, 2006).

In general, order parameters are calculated by recording three different relaxation components: (i) the longitudinal relaxation rate ( $R_1$ ), (ii) the transverse relaxation rate ( $R_2$ ), and (iii) the heteronuclear NOE. The transverse relaxation rate ( $R_2$ ) is sensitive to motions occurring on both ps-ns and µs-ms time scales. However, only the ps-ns motions are related to the order parameter. The fluctuations on the ps-ns time scale usually tend to decrease the relaxation rate. Accurate determination of order parameters critically depends on the identification and measurement of chemical exchange phenomena,  $R_{ex}$  (Evenas et al., 1999).

The conformational changes can alter the neighborhood of a nuclear spin and, consequently, modulate its chemical shift. When these changes occur on the  $\mu$ s-ms timescale, the resulting chemical shift changes are large and the observed NMR signal broadens, due to an increase in R<sub>2</sub> (Kroenke et al., 1998). This process can be monitored by measuring R<sub>2</sub> as a function of the effective radiofrequency (ERF) field strength using spin-lock and Carr-Purcell-Meiboom-Gill (CPMG) methods (Ishima and Torchia, 2000).

The stochastic exchange processes often contribute to the dephasing of the coherent magnetization. The coherent magnetization can be refocused by applying either a train of 180° radio-frequency (RF) pulses separated by delay of length  $\tau_{cp}$ , as in the CPMG experiment, or a continuous-wave (CW) "spin-lock" RF field of strength  $\omega_{eff}$ , as in the rotating-frame relaxation (R<sub>1</sub>) experiment (Cavanagh and Akke, 2000). The dependence of the transverse relaxation rate (R<sub>2</sub>) on the strength of the refocusing RF field ( $\omega_{eff}$  or  $1/\tau_{cp}$ ) determines a relaxation dispersion profile that can be subsequently used to obtain the exchange rates, populations and differences in chemical shift (Akke, 2002). Depending on the exchange timescale, it may be beneficial to combine CPMG and R<sub>1</sub> experiments in order to improve the sampling of the dispersion profile (Meekhof and Freund, 1999; Mulder et al., 1999).

#### **1.3.** Isothermal titration calorimetry (ITC).

Isothermal titration calorimetry (ITC) is one of the principal calorimetric techniques used in biomolecular studies. ITC measures the heat evolution (or absorption) during the course of molecular association. It is one of the most powerful and accurate techniques to measure all the thermodynamic parameters involved in a biomolecular ineraction (Perozzo et al., 2004).

Various physical parameters of the system under study can be obtained, such as the standard free energy change ( $\Delta G$ ), the enthalpy change ( $\Delta H$ ), the entropy change ( $\Delta S$ ), and the stoichiometry (*n*) of the association event. Moreover, the heat capacity change ( $\Delta Cp$ ) of the binding reaction can be obtained by performing experiments at variable temperature. The Gibbs free energy ( $\Delta G$ ) is the most important thermodynamic parameter of the binding event, as it determines the stability of any given biological complex. It can be calculated from the following equation:

$$\Delta G = -RT \ln K_B$$

Where *R* is a universal gas constant (1.987 cal\*K-1\*mol<sup>-1</sup>), *T* is temperature (in K), and  $K_B$  is the equilibrium binding constant.

The enthalpy change of binding ( $\Delta H$ ) is a measure of the disruption of protein-solvent hydrogen bonds and van der Waals interactions, formation of protein-ligand bonds, salt bridges and van der Waals contacts, and solvent reorganization near protein surfaces (Fisher and Singh, 1995).

The binding entropy ( $\Delta S$ ) is usually derived from the following equation:

$$\Delta G = \Delta H - T \, \Delta S$$

Hydration effects have been believed to be the main factors contributing to  $\Delta S$  of complex formation. A negative entropy change ( $\Delta S < 0$ ) does not always indicate increased or unchanged hydration interfaces, whereas a positive entropy change ( $\Delta S > 0$ ) is a strong indication that water molecules have been released from the surface of the complex (Jelesarov and Bosshard, 1999).

For the binding reaction, the heat capacity change ( $\Delta Cp$ ) is almost always negative when the complexed state of the biomolecule is treated as a reference state.  $\Delta Cp$  becomes substantial when water is released from the surface and is proportional to the surface area involved. Therefore, the heat capacity can translate thermodynamic data into the structural information (Baker and Murphy, 1998; Luque and Freire, 1998). The determination of binding stoichiometry (n) is of central importance for the characterization of the underlying binding mechanism. It is usually derived from the molar ratio of the interacting species at the equivalence point (Perozzo et al., 2004).

#### **1.1.** Western Blot

Western blot, also known as protein immunoblot, is an analytical technique used to identify and locate specific proteins based on their ability to bind to specific antibodies in a given sample of tissue homogenate or extract. The method was primarily developed the laboratory of George Stark at Stanford. This technique is extensively used in the fields of molecular biology, biochemistry, immunogenetics and other molecular biology disciplines. Western blot analysis can detect protein of interest from a mixture of a large number of proteins but also can analyze recombinant proteins synthesized *in vitro*. Western blotting provides information about the size of the protein, as well as about protein expression levels. The technique makes use of gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane, commomly, nitrocellulose or Polyvinylidene fluoride (PVDF) membranes are used, where they are probed (detected) using antibodies specific to the target protein.

The proteins are first separated by using gel-electrophoresis using polyacrylamide gel and buffer containing sodium dodecyl sulphate (SDS), a denaturing agent. On SDS polyacrylamide gel proteins are separated on basis of molecular weight. The next step involves protein transferring to nitrocellulose or PVDF membranes by elctroblotting. The proteins move from the gel onto the membrane while maintaining the organization they had within the gel. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. After completion of protein transfer to membrane, the membrane is places in a blocking solution, typically 3-5% bovine-serum albumin (BSA) or non-fat dry milk in Tris-buffered saline (TBS) with a minute percentage of detergent (Tween-200 ot Triton-X 100). These prevent any nonspecific binding between antibody and the membrane. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

After blocking, the membrane is incubated with a dilute solution of primary antibody (generally between 0.5 and 5 micrograms.ml<sup>-1</sup>) under rocking motion for 30 mins to overnight. The primary antibody binds to the protein of interest during the incubation period. Then the membranes are washed 3-4 times with TBST buffer to remove excess of unbound primary antibody, followed by one hour incubation of the membrane with the secondary antibody. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. Several secondary antibodies can bind to one primary antibody and enhance the signal. The secondary antibody is directed at a species-specific portion of the primary antibody.

Commonly used horseradish peroxidase-linked secondary antibodies cleave a chemiluminescence agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot.

### **Chapter 2 : Research Outline**

Despite the fact that Crk has provided a model system to for understanding how adaptor proteins mediate signal transduction, currently the mechanistic basis for the regulation of its functions remain elusive. Such an understanding is now rendered even more urgent because of the many recent studies highlighting the important role played by Crk family adaptor proteins mediating the action of human oncogenes. The goal of the current research is as follows:

# Identify the role of the SH3<sup>C</sup> domain and linker region in the regulation of c-CrkII activity.

Although the role of the SH2 and SH3N domains in Crk II has been generally delineated, the role of the SH3C domain remains elusive. Many biochemical and biophysical studies have shown that SH3C domain along with the linker region serve as a negative regulatory element in Crk. We have used NMR combines with biochemical and biophysical approaches, to identify the mechanism underlying SH3C-mediated negative regulation of CrkII.

# 2) Delineate the structural and dynamic features of both the *cis* and *trans* conformers Crk SH3<sup>C</sup>.

We have described that the inter-SH3 linker interacts with the  $SH3^{C}$  domain and this interaction is modulated by a *cis-trans* isomerization about the Gly237-Pro238 imide bond. We aimed to determine the solution structure of the linker-SH3<sup>C</sup> fragment in both *cis* and *trans* forms. We also aimed to determine the backbone relaxation rates of the Crk
fragments to obtain a better understanding of how protein motions adjust during proline isomerization and ligand binding.

# 3. Determine the structural and dynamic basis of the SH3<sup>N</sup>-SH3<sup>C</sup> intramolecular interaction.

Our results showed that the two SH3 domains interact in an intramolecular fashion leasding to an autoinhibitory state of CrkII. Interestingly, this interaction is mediated by only by one of the two (*cis* and *trans*) conformations of the Gly27-Pro238 peptide bond. We aimed to determine the structure of the inhibition conformation of Crk, which will provide us with the molecular details involving this interaction.

We also aimed to determine the dynamic properties of autoinhibited and activated Crk, which will give an idea of how motions adjust as the protein witches from one conformational state to another (open to close form or viceversa).

### 4. Discern the role of CypA as a PPIase in regulation of Crk activity.

Our studies have identified Crk as a substrate for cyclophilinA (CypA), which belongs to the highly conserved family of cycophilins (peptidyl-prolyl *cis-trans* isomerases, PPIases). We will use NMR and biochemical studies to understand the role of CypA in regulating Crk II biological activity.

# Chapter 3 : Proline *cis-trans* isomerization at Gly237-Pro238 controls autoinhibition of the Crk adaptor protein

### **3.1 Introduction**

In cell signaling, autoinhibition is present not only in enzymatically active proteins, such as kinases (Huse and Kuriyan, 2002), but also in non-catalytic adaptor proteins (Aghazadeh et al., 2000; Kim et al., 2000; Tan et al., 1999). The precise regulation of protein activity is essential for the normal functioning of essentially all cellular processes. Over the last years, it has been recognized that intramolecular interactions within a single polypeptide may provide a common regulatory strategy to modulate protein function (Schlessinger, 2003) (Pufall and Graves, 2002). Such interactions typically result in negative regulation of the activity and form the basis of an autoinhibitory mechanism. Truncations or mutations that disrupt the autoinhibitory interactions often result in a constitutively active functional form, wherein no activity control is possible leading to aberrant function (Blume-Jensen and Hunter, 2001; Reindl and Spiekermann, 2006). Autoinhibition is increasingly seen in proteins involved in a diverse set of biological phenomena, including signaling, transcription, and transport (Aghazadeh et al., 2000; Dueber et al., 2004; Huse and Kuriyan, 2002; Kobe and Kemp, 1999; Pufall and Graves, 2002). In the absence of a counteracting signal, these systems exist always in the "off" state since they do not have the intrinsic capacity to control their own autoinhibition. Mechanisms that are known to relieve autoinhibition include post-translational modification, proteolysis, and addition of proteins or small ligands (Groemping et al., 2003; Kim et al., 2000; Nagar et al., 2003; Otomo et al., 2005).

Proline isomerization is emerging as a critical component of certain important biological processes, as it has been shown to modulate activity in cell signaling (Brazin et al., 2002; Wulf et al., 2005; Zhou et al., 2000), control channel gating (Lummis et al., 2005), regulate lysine methylation (Nelson et al., 2006), direct conformer-specific ligand recognition (Breheny et al., 2003; Pletneva et al., 2006; Santiveri et al., 2004), regulate amyloidogenesis (Eakin et al., 2006), open and close flaps over active sites (Grochulski et al., 1994), and function as a timer in phage infection (Eckert et al., 2005). In contrast to covalent modifications afforded by post-translational processes, proline isomerization is an intrinsic conformational exchange process that has the potential to control protein activity without altering the covalent structure of the protein. Additionally, proline isomerization has two distinct and unique features: first, because the difference between the *cis* and *trans* conformational states of the dihedral angle ( $\omega$ ) of the prolyl bond (X-P) is large (180°), it can introduce dramatic effects on the protein structure and, second, the process can be modulated by both intra- and inter-molecular interactions (Andreotti, 2003, 2006).

In oligopeptides and unfolded polypeptides, the general preference of secondary amide peptide bonds for the *trans* conformation with an equilibrium constant  $K=[cis]/[trans]=(k_{trans to cis}/k_{cis to trans}) <<10^{-2}$  (Schiene-Fischer and Fischer, 2001), where k (s<sup>-1</sup>) stands for the rate constants of the *cis-trans* interconversion, is somewhat reduced for prolyl bonds. Depending upon the amino acids flanking the proline residue, K is in the range of 0.1-1.0 (Reimer et al., 1998; Yao et al., 1994).

This unique property of proline to adopt either *cis* or *trans* isomers of the backbone torsion angle  $\omega$  is due to its five-membered ring in the peptide backbone. Depending upon the local environment of the proline in a given polypetide or protein, the relative free energy of the *cis* and *trans* isomers can differ resulting into wide variation in the ratio of *cis* to *trans*. In the case of native protein folding most structures require proline to adopt only one of the conformation. Nevertheless, there is growing eveidence for the presence of both *cis* and *trans* conformers in a given polypeptide (Andreotti, 2003).

Prolyl *cis-trans* isomerization is a slow process, in the time scale region of minutes at 25°C, due to the relatively high enegy barrier ( $\varepsilon^u$ =14-24kcal/mol) (Nicholson and Lu, 2007). This makes it unsuitable to carry out biological process, which are usually very fast. Interestingly, this slow isomerization process can be catalyzed by a highly conserved and ubiquitous group of enzymes known as peptidyl-prolyl *cis-trans* isomerases (PPIases), including cyclophillins, FK50-binding proteins and parvullins (**Figure3.1**) (Schiene and Fischer, 2000). PPIases reduce the *cis-trans* interconversion ratedown to millisecond regimeby reducing the activation energy. Thus, the action of PPIases bring the kinetics of the isomerizationit to a more meaningful timescale for the regulation of biological process (**Figure 3.1**).



**Figure 3.1** (A) Interconversion between cis and trans isomers of the peptide bond (C'-N) between proline and its preceding residue involves a 180° rotation about the C'-N bond. (B) The difference in free energy between the cis and trans states ( $\Delta G$ ) determines the cis:trans population ratio at

thermal equilibrium, while the energy barrier separating the states (ɛu, uncatalyzed, or ɛcat, PPIase catalyzed) determines the rate of interconversion, as shown in Crk (Nicholson and Lu, 2007).

Cellular Crk (c-CrkII) consists of a single SH2 domain, an N-terminal SH3  $(SH3^N)$  and a C-terminal SH3 domain  $(SH3^C)$  (Figure 3.2). The SH3<sup>N</sup> and SH3<sup>C</sup> domains are tethered by a 50-residue long linker, which contains a Tyr residue (Y222 in chicken) that becomes phosphorylated by c-Abl(Feller et al., 1994). Crk-protein complex formation is mediated by the SH2 and SH3<sup>N</sup> domains of Crk, which selectively bind to pY-x-x-P and P-x-L-P-x-K motifs, respectively (Songyang et al., 1993; Wu et al., 1995). On the other hand, it has been shown that SH3<sup>C</sup>, which adopts the structure of a classical SH3 domain (Muralidharan et al., 2006), does not bind to conventional polyproline II (PPII)-type sequences because of the lack of aromatic residues at its binding surface as described in the first chapter (**Figure 1.13 and 3.3**) (Muralidharan et al., 2006; Reichman et al., 2005).



**Figure 3.2** Schematic diagram of the domain organization of Crk. The various Crk fragments used in this study are indicated. Highly conserved proline linker residues are indicated in bold. Pro238, which undergoes cis-trans isomerization, is highlighted. The tyrosine residue (Y222) that becomes phosphorylated by Abl is also indicated.



Figure 3.3 Ribbon digram of SH3<sup>C</sup>.

The highly conserved nature of SH3<sup>C</sup> throughout all organisms suggests an important role of this domain; nevertheless, hitherto its biological function has remained elusive. As stated in the first chapter, there is increasing evidence that point mutations or deletions in the SH3<sup>C</sup> domain may have a strong effect on a variety of cellular processes, including adhesion, proliferation and phagocytosis through negative regulation of Crk activity (Akakura et al., 2005; Kizaka-Kondoh et al., 1996; Ogawa et al., 1994; Zvara et al., 2001).

### **3.2. Results**

# **3.2.1.** Proline isomerization induces conformational heterogeneity at the linker-SH3<sup>°</sup> Crk polypeptide

The proposed regulatory role of  $\text{SH3}^{\text{C}}$  could stem from its interaction with other regions within Crk. The linker that tethers the two SH3 domains (**Figure 3.2**) is such a potential site because of the presence of many highly conserved proline residues. To test the hypothesis that  $\text{SH3}^{\text{C}}$  interacts with the linker we characterized the hl-SH3<sup>C</sup> construct



**Figure 3.4** <sup>1</sup>H-<sup>15</sup>N HSQC of hl-SH3<sup>C</sup>. Representative assignment of both *cis* (c) and *trans* (t) conformations is included.

The <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of hl-SH3<sup>C</sup>, which is monomeric at concentrations used for NMR studies (~0.6 mM), is very well resolved, indicating that hl-SH3<sup>C</sup> exists in a well folded form (**Figure3.4**). Notably, backbone assignment revealed that about 50 of the amide signals are duplicated indicating the presence of two conformations in slow exchange. Volume integration of isolated cross-peaks corresponding to the two conformations indicates that the populations of the two forms are approximately equal at room temperature.

The chemical shift difference between the  ${}^{13}C_{\beta}$  and the  ${}^{13}C_{\gamma}$  nuclei for the *cis* and *trans* conformations of residue Pro238 are 9.2 and 4.6 ppm respectively, which, is in agreement with the statistical analysis of  ${}^{13}C$  chemical shifts of proline residues in proteins in both conformations (Schubert et al., 2002). Characteristic NOE correlations (Wüthrich, 1986) between Gly237 and Pro238 (**Figure 3.5**) further corroborates the fact that the two forms correspond to the *cis* and *trans* conformations of the Gly237-Pro238 bond. In case of *cis* conformation there exist a NOE between H<sub>a</sub> of Pro238 and H<sub>a</sub> of the preceding residue, here it is Gly237. In the *trans* conformer the characteristic NOE is between H<sub>b</sub> of Pro 238 and H<sub>a</sub> of preceding residue Gly 237.



**Figure 3.5** Characteristic NOE crosspeaks from <sup>13</sup>C-edited NOESY spectra between the Gly237-H $\alpha_1$  and Pro238-H $\alpha$  protons for the *cis* conformation, and the Gly237-H $\alpha_1$  and Pro238-H $\delta_{1,2}$ protons for the *trans* conformation.

As an independent test of the validity of the Pro238 cis-trans isomerization process, we mutated Pro238 residue to Ala. The <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of hl-SH3<sup>C</sup>-P238A mutant (**Figure 3.6**) shows the presence of only one set of peaks for each residue; thus, the mutant adopts a unique conformation in contrast with the two conformations adopted by wild type hl-SH3<sup>C</sup>. Because peptidyl-prolyl cis-trans isomerization is slow with respect to the NMR chemical shift scale, two resonance frequencies are observed for each nuclear spin affected by the isomerization process. We conclude that the source of the conformational heterogeneity in hl-SH3<sup>C</sup> is the presence of cis-trans isomerization about the Gly237–Pro238 prolyl bond and the two conformational states have similar stability.



**Figure 3.6** Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of hl-SH3<sup>C</sup> (blue) and hl-SH3<sup>C</sup>-P238A (orange).

Interestingly, a comparison of the hl-SH3<sup>C</sup> and hl-SH3<sup>C</sup>-P238A spectra demonstrates that the chemical shift of the amide resonances in the mutant is generally

different from either the *cis* or *trans* form of wild-type hl-SH3<sup>C</sup> (Figure 3.7). This observation suggests that the *cis* and *trans* conformations of Pro238 are unique and substitution of Ala residue for Pro cannot even mimic the *trans* conformation of the Gly237–Pro238 prolyl bond.



**Figure 3.7** Chemical shift difference( $\Delta\delta$ ) between hl-SH3<sup>C</sup>-P238A and the *cis* (blue) and *trans* (red) conformation of hl-SH3<sup>C</sup>.

# **3.2.2.** The linker interacts with the hl-SH3<sup>C</sup> and the interaction is modulated by *cis*-*trans* isomerization about the Gly237-P238238 prolyl bond

The consequences of the Gly237–Pro238 prolyl bond isomerization are pronounced, with the majority of SH3<sup>C</sup> signal amides affected (**Figure 3.8 and 3.9**). This observation is rather intriguing as Pro238 is located outside the boundaries of the SH3<sup>C</sup> domain (**Figures 3.2 and 3.8**).



**Figure 3.8** Effect of *cis-trans* isomerization at Pro238 on SH3<sup>C</sup>, assessed by chemical shift mapping. Chemical shift difference ( $\Delta\delta$ ) between the two conformationsis mapped by continuous-scale color onto the structure of SH3<sup>C</sup> (PDB entry 2GGR). Proline residues are colored white.

For some of the SH3<sup>C</sup> residues the differences in chemical shift values between the *cis* and *trans* conformers are large (>3 ppm in <sup>15</sup>N and >1 ppm in <sup>1</sup>H dimension) indicating significant differences in the chemical environment of nuclei in the two conformations (**Figure 3.8 and Figure 3.9**). When the chemical shift difference ( $\Delta\delta$ ) is mapped on the structure of the SH3<sup>C</sup> domain, it becomes apparent that the residues mostly affected form a contiguous region that faces the linker (**Figure 3.8**).



**Figure 3.9** Effect of *cis-trans* isomerization on SH3<sup>C</sup> conformation. Chemical shift difference  $(\Delta \delta)$  between the *cis* and *trans* conformations of hl-SH3<sup>C</sup>. The (o) symbols indicate Pro residues.

These data suggest that the presence of the linker affects the conformation of the SH3<sup>C</sup> domain, most likely through a direct linker-SH3<sup>C</sup> interaction. Indeed, a comparison of the NMR spectra of SH3<sup>C</sup> in the presence (hl-SH3<sup>C</sup>) and absence (isolated SH3<sup>C</sup>; residues 239-297; **Figure 3.2**) of the linker shows that removal of the linker has a dramatic effect on the spectrum of the SH3<sup>C</sup> (**Figure 3.10**), supporting the existence of interaction between the linker and the SH3<sup>C</sup> domain. Interestingly, the amide signals of isolated SH3<sup>C</sup> do not match with those from either the *cis* or the *trans* conformation in hl-SH3<sup>C</sup>. This observation argues that the linker interacts with the SH3<sup>C</sup> domain both in the *cis* and the *trans* conformation. Chemical shift difference ( $\Delta\delta$ ) between the isolated SH3<sup>C</sup> and the conformers of hl-SH3<sup>C</sup> plotted as a bar graph shows that there a stronger effect is observed for the *trans* conformer (**Figures 3.11 and 3.12**). Overall, our results suggest that the heterogeneous Pro238 residue acts as a hinge that modulates the linker-SH3<sup>C</sup> interaction, presumably by controlling the relative orientation of the linker.



Figure 3.10 Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of isolated SH3<sup>C</sup> (magenta) and hl-SH3<sup>C</sup> (blue).



**Figure 3.11** Chemical shift difference ( $\Delta\delta$ ) between isolated SH3<sup>C</sup> and the *cis* (blue) and *trans* (red) conformers of hl-SH3<sup>C</sup> plotted as a bar graph. (o) symbols indicate proline residues.

As a consequence of altered linker-SH3<sup>C</sup> interactions, the effect of *cis-trans* isomerization at Pro238 is not confined to SH3<sup>C</sup>, but extends to the linker as well,

wherein large chemical shift differences between the two conformations are observed (Figure 3.9).



**Figure 3.12** Chemical shift difference ( $\Delta\delta$ ) between isolated SH3<sup>C</sup> and the *cis* and *trans*) conformers of hl-SH3<sup>C</sup> is mapped (for the *trans* conformation) using a continuous-scale color on the structure of SH3<sup>C</sup>.



**Figure 3.13** Effect of *cis-trans* isomerization on SH3<sup>C</sup> conformation. Residues that are affected by the isomerization at Pro238 are colored pink.

NMR analysis of the fl-SH3<sup>C</sup> construct (**Figure 3.2**), which comprises the full linker, shows that crosspeaks in the hl-SH3<sup>C</sup> spectrum is an exact subset of those in the spectra of fl-SH3<sup>C</sup> (**Figure 3.14**). The residues at the N-terminal half of the linker (residues 190-209) are not affected by the isomerization process as they show only single peaks. The combined results indicate that only residues at the C-terminal half of the linker linker participate in the interaction with the SH3<sup>C</sup> domain.



**Figure 3.14** Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of hl-SH3<sup>C</sup> (blue) and fl-SH3<sup>C</sup> (green).

# **3.2.3.** The two SH3 domains interact intramolecularly occluding the SH3<sup>N</sup> binding site

To test the hypothesis emerging from biochemical experiments that SH3<sup>C</sup> regulates the activity of SH3<sup>N</sup>, and to explore how *cis-trans* isomerization at Pro238 may

be involved in the regulation, we characterized the SH3<sup>N</sup>-fl-SH3<sup>C</sup> polypeptide (**Figure 3.2**). The <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of SH3<sup>N</sup>-fl-SH3<sup>C</sup>, which is in a monomeric state at the concentration used for the NMR studies (~0.6 mM), (**Figure 3.15**). To see if there is indeed any kind of intramolecular interaction between the two SH3 domains, we overlaid the <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of SH3<sup>N</sup>-fl-SH3<sup>C</sup> polypeptide with the spectra of the isolated SH3<sup>N</sup> domain and the fl-SH3<sup>C</sup> polypeptide. Analysis of the spectra shows that the amide signals of the SH3<sup>N</sup>, SH3<sup>C</sup> and part of the linker change dramatically their chemical shift when compared isolated and in the context of the SH3<sup>N</sup>-fl-SH3<sup>C</sup> polypeptide (**Figures 3.16 and 3.17**). Although the two SH3 domains are tethered with a long linker, they do not adopt a dumbbell-like conformation in solution. Instead, the two SH3 domains interact with each other in an intramolecular fashion.



**Figure 3.15** Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of isolated SH3<sup>N</sup> (cyan), fl-SH3<sup>C</sup> (green), and SH3<sup>N</sup>-L-SH3<sup>C</sup> (red). Representative assignment exemplifying the large chemical shift change upon intramolecular interaction for the SH3<sup>N</sup> (cyan) and SH3<sup>C</sup> (magenta), as well as the selection of only the *cis* conformer upon binding, is indicated.

Chemical shift analysis of the SH3<sup>N</sup> domain isolated and in the context of SH3<sup>N</sup>-fl-SH3<sup>C</sup> shows that the SH3<sup>N</sup>/SH3<sup>C</sup> interaction is mediated by residues lining the conventional PPII-binding site at SH3<sup>N</sup> (**Figures 3.17 and 3.18**).



**Figure 3.16** Chemical shift difference ( $\Delta\delta$ ) between SH3<sup>N</sup>-fl-SH3<sup>C</sup> and its isolated components SH3<sup>N</sup> and fl-SH3<sup>C</sup> plotted as a bar graph, (o) symbols indicate proline residues.

This site, which specifically interacts with short sequences encompassing a P-x-L-P-x-K motif (Knudsen et al., 1995; Wu et al., 1995), is used by Crk to form a variety of protein complexes (Chodniewicz and Klemke, 2004). Therefore, such an intramolecular structural arrangement would be particularly important because the binding sites on SH3<sup>N</sup> for PPII ligands and SH3<sup>C</sup> would be overlapping.



**Figure 3.17** Chemical shift difference ( $\Delta\delta$ ) between SH3<sup>N</sup>-fl-SH3<sup>C</sup> and its isolated components SH3<sup>N</sup> and fl-SH3<sup>C</sup> mapped using a continuous-scale color on the structures of SH3<sup>N</sup> and SH3<sup>C</sup>. SH3<sup>N</sup> is displayed with a P-x-L-P-x-K peptide bound (brown sticks; PDB entry 1CKA) to simply indicate the PPII-binding site.

To directly test this hypothesis, we titrated SH3<sup>N</sup>-fl-SH3<sup>C</sup> with a peptide derived from the guanine nucleotide exchange factor C3G containing the consensus motif sequence (P-x-L-P-x-K). The NMR spectrum of SH3<sup>N</sup>-fl-SH3<sup>C</sup> bound to the PPII peptide is compared to the spectrum of isolated SH3<sup>N</sup> bound to the same PPII peptide and the spectrum of the fl-SH3<sup>C</sup> polypeptide (**Figure 3.19**). The NMR data clearly show that addition of the PPII peptide outcompetes SH3<sup>C</sup>, resulting in SH3<sup>C</sup> being dissociated from SH3<sup>N</sup>. At a protein: PPII peptide ratio of 1:1, the SH3<sup>N</sup> is found exclusively bound to the PPII peptide suggesting that the affinity of the consensus peptide for the SH3<sup>N</sup> is much greater than the affinity of the SH3<sup>C</sup> domain. We, thus, conclude that the binding sites on SH3<sup>N</sup> for PPII ligands and SH3<sup>C</sup> are mutually exclusive. The residues in  $SH3^{C}$  that are mostly affected by its interaction with  $SH3^{N}$  are 239-242, 266-271, and 290-296, and form a contiguous surface that most likely serves as the binding interface for the  $SH3^{N}$  domain (**Figure 3.17 and 3.18**).



**Figure 3.18** Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of isolated SH3<sup>N</sup> in complex with the PPII peptide (cyan), fl-SH3<sup>C</sup> (green), and SH3<sup>N</sup>-fl-SH3<sup>C</sup> in complex with the PPII peptide (red).

NMR analysis of several mutations of residues located at these regions indicate significant effect on the conformation of the  $SH3^N / SH3^C$  arrangement. Nevertheless, only the F239A mutations appear to completely abolish the intramolecular  $SH3^N / SH3^C$  interaction resulting in the complete opening of the  $SH3^N$ -fl- $SH3^C$  structure (**Figure 3.19**). Hence, it is very likely that the  $SH3^N / SH3^C$  binding is mediated primarily by the interaction of Phe239 and Tyr240 with the highly conserved aromatic cluster at the  $SH3^N$  binding site (**Figure 3.20**).



**Figure 3.19** Effect of the F239A mutation on the autoinhibitory conformation of SH3<sup>N</sup>-fl-SH3<sup>C</sup>. Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of SH3<sup>N</sup>-fl-SH3<sup>C</sup> (red), SH3<sup>N</sup>-fl-SH3<sup>C</sup> -F239A (orange), and isolated SH3N (cyan). F239A mutation results in all resonances of the SH3<sup>N</sup> domain in SH3<sup>N</sup>-fl-SH3<sup>C</sup> shifting to the corresponding chemical shifts of the isolated domain (characteristic shifts are indicated in the figure).



**Figure 3.20** Intramolecular interaction between SH3<sup>N</sup> and SH3<sup>C</sup>. Red-colored regions are the ones mostly affected by the interaction based on  $\Delta\delta$ . The highly conserved aromatic residues that most likely mediate the binding are highlighted. The PPII peptide is shown bound to SH3N to simply indicate the binding site.

# **3.2.4.** The SH3<sup>N</sup>/SH3<sup>C</sup> intramolecular interaction provides an autoinhibitory mechanism

Our NMR results raise the intriguing possibility that this intramolecular arrangement may inhibit the binding activity of SH3<sup>N</sup> for PPII ligands, thereby forming the basis of an autoinhibitory mechanism. To test this hypothesis, we used isothermal titration calorimetry (ITC) to directly measure the energetics of the PPII peptide binding to SH3<sup>N</sup>, isolated and in the context of SH3<sup>N</sup>-fl-SH3<sup>C</sup>. The PPII peptide binds to isolated SH3<sup>N</sup> with a moderate affinity ( $K_d \sim 2 \mu M$ ), and the interaction is enthalpically favored (**Figure 3.21, blue curve**). The affinity of PPII peptide binding to SH3<sup>N</sup> in the context of the SH3<sup>N</sup>-fl-SH3<sup>C</sup> construct, however, is lowered by a factor of ~10 (**Figure 3.21, red curve**).



**Figure 3.21** Binding isotherms of the calorimetric titration of PPII peptide to isolated SH3<sup>N</sup> (cyan) and SH3<sup>N</sup>-fl-SH3<sup>C</sup> (red).

	K <sub>d</sub> μM	∆G kcal mol <sup>-1</sup>	∆H kcal mol <sup>-1</sup>	-T∆S kcal mol <sup>-1</sup> 2.2
SH3 <sup>N</sup>	2	-7.7	-9.9	
SH3 <sup>N</sup> -fl-SH3 <sup>C</sup>	20	-6.3	0.5	-6.8

**Table.1**.Thermodynamic parameters of calorimetric titration of PPII peptide to isolated SH3<sup>N</sup> and SH3<sup>N</sup>-fl-SH3<sup>C</sup>.

Interestingly, the interaction now becomes enthalpically unfavorable, apparently because of the disruption of the SH3<sup>N</sup> / SH3<sup>C</sup> interface required for the accommodation of the peptide on SH3<sup>N</sup>. The binding is now driven by a very favorable change in the entropy due to, at least partially, the release of the SH3<sup>C</sup> domain induced upon PPII peptide binding to SH3<sup>N</sup> (**Figure 3.21**). Overall, the thermodynamic results further corroborate the NMR data that SH3<sup>N</sup> is intramolecularly inhibited by SH3<sup>C</sup> structurally occluding the PPII binding site.

# **3.2.5.** The prolyl *cis* conformer stabilizes the autoinhibitory conformation whereas the *trans* conformer destabilizes it

A very interesting observation from the NMR data is that only one set of peaks for the linker- SH3<sup>C</sup> region is present in the spectrum of SH3<sup>N</sup>-fl-SH3<sup>C</sup> (**Figure 3.15**). This is in contrast to the conformational heterogeneity seen for fl- SH3<sup>C</sup> (**Figures 3.4 and 3.15**). Chemical shift and NOE data demonstrate that the Gly237–Pro238 prolyl bond adopts only the *cis* conformation in SH3<sup>N</sup>-fl-SH3<sup>C</sup>. Interestingly, once the SH3<sup>C</sup> is displaced from SH3<sup>N</sup> by PPII peptide binding, the Gly237–Pro238 prolyl bond adopts again both the *cis* and *trans* conformations (**Figure 3.18**). Notably, a more detailed analysis of the SH3<sup>N</sup>-fl-SH3<sup>C</sup> spectra revealed the presence of a minor conformation corresponding to ~10% of the population of the major, autoinhibited conformation (**Figure 3.22**). The chemical shifts of the minor conformation resonances correspond to the isolated SH3<sup>N</sup> and fl- SH3<sup>C</sup> domains (**Figure 3.22**). It is of particular interest that only the *trans* conformer of the Gly237-Pro238 prolyl bond is present for the fl- SH3<sup>C</sup> peaks region in the minor conformation.



**Figure 3.22** Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of isolated SH3<sup>N</sup> (cyan), fl-SH3<sup>C</sup> (green), and SH3<sup>N</sup>fl-SH3<sup>C</sup> (red) indicating the presence of a minor conformation of SH3<sup>N</sup>-fl-SH3<sup>C</sup> in an open, uninhibited conformation. In this conformation the SH3<sup>N</sup>-binding site is completely accessible and the Gly237-Pro238 prolyl bond adopts only the *trans* conformation. SH3<sup>N</sup> residues in the minor conformation are primed.

Because the chemical shift of the resonances of the minor conformation corresponds to the characteristic chemical shifts of the unoccupied SH3<sup>N</sup> domain, the data suggest that the minor conformer may adopt an open, uninhibited conformation. To further corroborate these results, we used NMR relaxation methodologies (Popovych et al., 2006). We measured the longitudinal  $(T_1)$  and transverse  $(T_2)$  relaxation rates of the backbone amide moiety of both the major and minor conformations of SH3<sup>N</sup>-fl-SH3<sup>C</sup> (**Figure. 3.22 and 3.23**). Both relaxation rates are sensitive to and thus report on changes of the overall motional properties of the protein.

If only residues located at rigid parts of the molecule are considered, then the ratio  $T_1/T_2$  provides a good estimate of the correlation time. Relaxation analysis demonstrates that the overall tumbling of the SH3 domains takes place much faster in the minor than in the major conformation, suggesting that they correspond to an "open" and "closed" conformation, respectively (**Figure 3.23**). These data establish that the autoinhibitory conformation, which is stabilized only in the *cis* conformation, exists in equilibrium with a low-populated uninhibited conformation favored by the *trans* conformation.



**Figure 3.23** Ratio of  $T_1$  over  $T_2$  relaxation rates of the major (red) and minor (blue) conformation of Crk SH3<sup>N</sup>-fl-SH3<sup>C</sup> polypeptide. The major and minor conformations correspond to a closed and open intramolecular conformation, respectively. The points for the minor conformation are much fewer than for the major conformation because of the lower intensity of the minor crosspeaks. The  $T_1/T_2$  ratio provides information about the correlation time (tumbling) of the molecule. Higher values indicate slower tumbling.

# The presence of a significant percentage of molecules in the extended conformation will have strong implications for the activation process. Because the SH3<sup>N</sup> binding site is accessible in the uninhibited conformation, the PPII ligand is expected to preferentially bind to this conformation over the autoinhibited one. Direct evidence for this is provided by the ITC titration profile. The data show that in the first two injections the PPII peptide binds indeed preferentially to the open conformation as enthalpy change ( $\Delta$ H) is negative and similar to $\Delta$ H released upon peptide binding to isolated SH3<sup>N</sup> (**Figure 3.21**). As additional PPII ligand is titrated, the SH3<sup>N</sup> / SH3<sup>C</sup> interaction needs to be disrupted to accommodate the PPII binding, and, thus, the binding now becomes enthalpically unfavorable. Therefore, the biphasic nature of the ITC titration curve and the switch of the $\Delta$ H sign from negative to positive as more PPII ligand is titrated confirm the presence of both the closed and open conformations and the preferential binding of the PPII ligand to the open one.

**3.2.6.** Crk is activated by PPII ligand binding to a low-populated open conformation

### 3.3. Discussion

We have provided here the first example of a protein wherein autoinhibition is controlled by an internal conformational switch afforded by proline isomerization. We found that part of the linker tethering the two SH3 domains in Crk interacts with the SH3<sup>C</sup> domain. A single prolyl bond (Gly237-Pro238) undergoes cis-trans isomerization, thereby regulating the linker- SH3<sup>C</sup> interaction giving rise to two distinct conformations. Although the vast majority (~95%) of peptidyl prolines in folded proteins adopt the energetically most favorable *trans* conformation (Jabs et al., 1999; Weiss et al., 1998),

the *cis* conformer of Gly237-Pro238 prolyl bond in the uninhibited state of Crk is equally populated to the *trans* conformer (50%). The reason for that being the linker- SH3<sup>C</sup> interaction, which favors dual prolyl bond conformers. The presence of a significant population of the *cis* conformer is of extreme importance as it is only this conformer that yields the SH3<sup>N</sup> / SH3<sup>C</sup> autoinhibitory conformation. In contrast, the *trans* conformer favors an open structure wherein the PPII binding site on SH3<sup>N</sup> is accessible to ligands. Because the topology of the SH3<sup>C</sup> binding surface is directly regulated by the cis-trans isomerization about the Gly237-Pro238 prolyl bond, two alternative surfaces are presented for binding to SH3<sup>N</sup>, which selectively interacts with the *cis* conformer. Because the *cis* and *trans* conformations are in equilibrium, SH3<sup>N</sup> / SH3<sup>C</sup> binding will strongly shift the equilibrium towards the conformation with the highest affinity for SH3<sup>N</sup>, that is, the *cis* conformation. Therefore, *cis-trans* isomerization of a single prolyl imide bond affords this region the ability to control its binding affinity for SH3<sup>N</sup>, thereby providing a mechanism for intrinsically regulating the intramolecular conformational arrangement.

Although Crk SH3<sup>C</sup> does not function as a classical SH3 domain (Mayer, 2001), since it does not bind PPII-type ligands (Muralidharan et al., 2006; Reichman et al., 2005), it does participate in critical intramolecular binding interactions. It is of particular interest that SH3<sup>C</sup> employs alternative surface patches to interact with the adjacent linker residues and the SH3<sup>N</sup>. The binding surfaces used by SH3<sup>C</sup> consist of exposed residues located at  $\beta$ -sheets a, b, and c (**Figures 3.17**). These surfaces are structurally separate from the canonical PPII binding site located between the RT and n-Src loops in typical SH3 domains. Such non-canonical binding sites have been previously found in other SH3

domains (Barnett et al., 2000; Chan et al., 2003; Groemping et al., 2003; Kami et al., 2002; Vaynberg et al., 2005) and underscore the binding versatility of SH3 domains.

Because the tyrosine residue (Y222 in chicken) that becomes phosphorylated by c-Abl is part of the linker region that interacts with the SH3<sup>C</sup> domain (**Figure 3.2**), it is possible that phosphorylation of this residue may modulate the linker- SH3<sup>C</sup> interaction, thereby providing an additional mechanism for regulating the autoinhibitory conformation. Interestingly, the Crk SH2 domain was shown to interact intramolecularly with the pY222-x-x-P225 region (Rosen et al., 1995). It is intriguing that multiple layers of regulation are present in a simple adaptor protein and it will be important to ultimately understand how all these regulatory mechanisms integrate within the same protein molecule and how they respond to the presence of the various Crk partners.

The mechanisms and, especially, the thermodynamics and kinetics of the activation process in autoregulated proteins are generally poorly understood. Since the active site is buried in the inhibited system, ligand binding requires conformational changes that render the site more accessible. Very often, this is accomplished by allosteric effector binding to regulatory sites that are distinct from the binding site (Kobe and Kemp, 1999). Another potential mechanism is that the ligand binds to a less-populated excited state where the binding site is more accessible. Nevertheless, direct evidence of the presence of such excited states is sparse. Here, we have shown that ~10% of the total population of the Crk molecules exist in an open conformation wherein the PPII binding site on SH3<sup>N</sup> is completely unoccupied (**Figure 3.22 and 3.23**). Each Crk molecule will dynamically sample both conformations (**Figure 3.24**). Ligand will bind preferentially to the ~10% of the Crk molecules with accessible SH3<sup>N</sup> binding site,

as evidenced by the thermodynamic data (**Figure 3.21**), resulting in equilibrium shifting towards the extended, uninhibited conformation. Thus, a ligand does not need to induce specific conformational changes in the SH3<sup>N</sup>/SH3<sup>C</sup> binding interface, but instead may drive Crk activation by biasing the dynamic conformational ensemble. The availability of a significant number of Crk molecules in the extended conformation may contribute significantly to the rapid activation kinetics that autoinhibited systems should exhibit in response to ligand binding. In this case, acceleration of the intrinsically slow prolyl *cis/trans* isomerization rates would be required, which could be accomplished by formation of a H-bond to the proline amide nitrogen (Cox and Lectka, 1998) or the catalytic action of peptidyl-prolyl isomerases (Fischer and Aumuller, 2003).



**Figure 3.24** Model of the equilibrium of conformational States of Crk SH3<sup>N</sup>-fl-SH3<sup>C</sup> polypeptide, its autoinhibition, and its activation. The intramolecular inhibitory SH3<sup>N</sup>/SH3<sup>C</sup> interaction is stabilized by the *cis* conformer of the Gly237-Pro238 prolyl bond, whereas the *trans* conformer favors an uninhibited state. Activation occurs by PPII ligand binding to a low population of uninhibited states wherein SH3<sup>N</sup>-binding site is accessible, thereby shifting the equilibrium

toward the SH3<sup>N</sup>-PPII ligand bound state. Pro238 acts as a molecular switch that has the intrinsic capacity to regulate the autoinhibition of Crk.

Because the relative ratio of the "closed" and "open" conformations is 10:1, the free energy ( $\Delta G$ ) of the intramolecular SH3<sup>N</sup>/SH3<sup>C</sup> interaction is ~1.4 kcal mol<sup>-1</sup> (**Figure 3.24**). Thus, PPII ligand binding to SH3<sup>N</sup>-fl-SH3<sup>C</sup> is expected to be suppressed, relative to the binding to the isolated SH3<sup>N</sup> domain, by a factor of 10, which is in excellent agreement with the present thermodynamic data (**Figure 3.21**). Such a moderate degree of autoinhibition is a fine balance between the requirement for the protein to be self-activating, as no extrinsic factors act to relieve inhibition, and the requirement that the process will be executed only in the presence of an activating signal that is sufficiently strong to overcome the inhibition. Thus, this intramolecular interaction may safeguard against inappropriate function by acting as a selectivity barrier, but the same time allowing for a fast response to signal by providing a significant percentage of protein molecules in an uninhibited conformation.

### 3.4. Conclusion

We have shown that prolyl *cis/trans* isomerization may function as a particularly effective and reversible molecular switch that controls autoinhibition of a signaling molecule. Isomerization at a single proline modulates the structural features of one of the domains of the Crk adaptor protein in such a way so that the two isomeric forms are conferred different binding properties, rendering only one of them capable of inducing the autoinhibitory conformation. This novel role of prolyl isomerization further extends the list of known biological functions of this phenomenon (Andreotti, 2006),

underscoring its unique capacity and versatility in regulating important biological processes.

# **Chapter 4 : Structural basis for autoinhibition and activation of the Crk adaptor protein by a proline switch**

### **4.1. Introduction**

As described in the previous chapter, the binding activity of chicken Crk is modulated by proline isomerization at highly conserved residue, Pro238. In this chapter we will describe the structural basis of the intramolecular SH3<sup>N</sup>-SH3<sup>C</sup> interaction in Crk and how this is regulated by the proline isomerization. As mentioned before, in contrast to covalent modifications afforded by post-translational processes, proline isomerization is an intrinsic conformational exchange process that has the potential to control protein activity (Andreotti, 2003, 2006; Lu et al., 2007) without altering the covalent structure of the protein. Proline isomerization may exert its function through multiple, non-mutuallyexclusive mechanisms, involving (i) significant conformational changes caused by the 180° rotation about the prolyl bond, (ii) slow kinetics of isomerization affording a molecular timer, (iii) and the recruitment of prolyl cis-trans isomerase enzymes (PPIases). For these reasons proline *cis-trans* isomerization is emerging as a critical component of an ever increasing number of important biological processes, including cell signaling (Brazin et al., 2002; Lu and Zhou, 2007; Sarkar et al., 2007; Wulf et al., 2005; Yaffe et al., 1997; Zhou et al., 2000), neurodegeneration (Pastorino et al., 2006), amyloidogenesis(Calabrese et al., 2008; Eakin et al., 2006), channel gating(Lummis et al., 2005), gene regulation(Nelson et al., 2006; Wang et al.), phage and virus infection (Eckert et al., 2005; Franke et al., 1994), enzyme function(Grochulski et al., 1994; OuYang et al., 2008), and ligand recognition(Santiveri et al., 2004; Severin et al., 2009). However, despite the importance of *cis-trans* isomerization, there is a surprising paucity

of high-resolution structural data elucidating the effect that this process may elicit within a protein.

Here we present high-resolution structures of both the *cis* and *trans* conformers of Pro238 in Crk. The data show that the linker interacts extensively with the SH3<sup>C</sup> domain and the interaction is strongly modulated by proline isomerization. The remarkable conformational rearrangement induced by *cis-trans* isomerization endows the conformers with distinct binding properties.

Interestingly, all of these interactions are mediated by novel SH3 binding surface. As described in the first chapter, SH3 domains of  $\sim 60$  amino acids are made up of two perpendicular anti-parallel  $\beta$ -sheets and bind to proline-rich ligands that adopt a lefthanded polyproline type II (PPII) helix conformations. Proline rich sequences are widely distributed in the proteomes, from prokaryotes to eukaryotes (Ravi Chandra et al., 2004; Rubin et al., 2000). Many recent studies have shown that SH3 domain also binds to motifs lacking PxxP core element. The SH3 domains from the tyrosine kinase substrate Eps8 and related proteins bind selectively to the PxxDY motif (Mongiovi et al., 1999). The SH3 domains of Fyn and the Fyn-binding protein, Fyb or SLAP130 [SLP-76 (SH2domain-containing leucocyte protein of 76 kDa)-associated protein], engages a site in SKAP55 (Src kinase associated protein of 55 kDa) bearing a consensus sequence RKxxYxxY that is devoid of proline residues (Kang et al., 2000). The SH3 domain of the yeast protein Pex13p is capable of binding, using distinct surfaces, two proteins of the peroxisomal import pathway that are related in neither sequence nor structure. Whereas it interacts with Pex14p through a PPII helix in a conventional manner, it binds independently to an  $\alpha$ -helix formed by a novel sequence motif WxxxFxxLE present in Pex5p through a site that is removed from the conventional surface (Barnett et al., 2000; Douangamath et al., 2002).

Domain	Target sequence or motif	Affinity	Ligand	References
		(Kd, µM)	strucutre	
SH3	(R/K)xxPxxP (class I)	1–200	PPII	(Sparks et al., 1996; Sparks et al., 1998)
	PxxPx(R/K) (class II)	1–200	PPII	(Sparks et al., 1996; Sparks et al., 1998)
	RxxK (class III)	0.1–30	3 <sup>10</sup> helix	(Berry et al., 2002; Harkiolaki et al., 2003; Kaneko et al., 2003; Lewitzky et al., 2004; Lewitzky et al., 2001; Liu et al., 2003; Liu et al., 2001)
	RKxxYxxY	20-60	NA	(Kang et al., 2000)
	PxxDY	NA	NA	(Mongiovi et al., 1999)
	(R/K/G)XXPPGX(R/K)	10-200	PPII	(Freund et al., 2002)
	R/K-rich	10-100	N/A	(Jia et al., 2005; Kojima et al., 2004)
	WQTDFEKLEKE	NA	a-Helix	(Douangamath et al., 2002)
	RPSADLILNRCSESTKRKL ASA	2-13	α-Helix	(Kami et al., 2002)
	PWTDQFEKLEKEVAEN	NA	a-Helix	(Barnett et al., 2000)
	PX domain	50	PPII*	(Hiroaki et al., 2001)
	SH2 domain	1-5	Tertiary	(Chan et al., 2003; Li et al., 2003)
	LIM domain	3000	Tertiary	(Vaynberg et al., 2005)

 Table 4.1. Non-conventional binding motifs of SH3 domain

Our results provide (i) unprecedented insight into the drastic, long-range conformational effect that proline isomerization may afford providing an elegant

mechanism for protein activity regulation and (ii) highlight the versatile and unique binding properties of the SH3 modular domain.

### 4.2. Results

# **4.2.1.** Structure Determination of the *trans* and *cis* conformers and SH3-SH3 fragment of Crk



**Figure 4.1** (a)Schematic diagram of the domain organization of Crk. Pro238, which undergoes *cis-trans* isomerization, and the tyrosine residue (Tyr222) that becomes phosphorylated by Abl are indicated.1 (b) Schematic of *cis-trans* isomerization about the prolyl Gly237–Pro238 bond.

In the present study we solve the structure of SH3<sup>C</sup>-hl in both *cis* and *trans* conformer along with the Crk<sup>SLS</sup> fragment by using solution NMR (**Figure 4.1 a**). <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of SH3<sup>C</sup>-hl and Crk<sup>SLS</sup>, is in a monomeric state at the concentration (~0.6 mM) (**Figure 4.2**), is used for all NMR studies.



Figure 4.2 <sup>1</sup>H-<sup>15</sup>N HSQC spectrum of SH3<sup>C</sup>-hl

Squential assignment of the <sup>1</sup>H,<sup>13</sup>C, and <sup>15</sup>N protein backbone chemical shifts were achieved in each case using a U-<sup>13</sup>C/<sup>15</sup>N sample by means of through-bond heteronuclear scalar correlations using the following 3D pulse sequences: 3DHNCO, 3DHN(CA)CO, 3DHNCA, 3DHN(CO)CA, 3DHNCACB, and 3D HN(CO)CACB, 3DHN(CCO)NH, 3DC(CO)NH, and 3D HCCH-TOCSY spectra have been used to assign side chain chemical shift.

Distance restrains for all the three structures were obtained by  $3D^{15}N$ -edited NOESY-HSQC (mixing time 100 ms),  $3D^{13}C$ -edited NOESY-HSQC (mixing-time 80 ms), and 2D NOESY in D<sub>2</sub>O buffer (mixing-time 80 ms). All of the above mentioned spectra were processed by using NMRPipe software package (Delaglio et al., 1995) and analyzed with NMRView (Johnson and Blevins, 1994). Residual dipolar couplings

(RDCs) were used in the final stages of structure calculations of the Crk<sup>SLS</sup> for getting a better understanding of the orientation of the two SH3 domains with respect to each other in the autoinhibited form. <sup>1</sup>H-<sup>15</sup>N residual dipolar couplings were measured using the IPAP HSQC experiment.

## 4.2.2. Structure Determination of the trans and cis conformers of Crk

As described earlier (chapter 3rd) that Pro238 in Crk undergoes *cis-trans* isomerization giving rise to two distinct, equally populated conformational states of the linker-SH3<sup>C</sup> (L-SH3<sup>C</sup>) polypeptide (**Figure 4.1 a, b** and **Figure 4.2**). Because of the intrinsically high activation energy for rotation about the prolyl bond (~20 kcal mol<sup>-1</sup>) (Andreotti, 2003), the two conformational states interconvert at a very slow rate. Moreover, the *cis* and *trans* conformers in Crk L-SH3<sup>C</sup> are equally populated (~50% each; **Figure 4.2**). These favorable conditions enabled the complete NMR characterization and structure determination for both conformers (**Figure 4.3** and **Figure 4.4**).


## 4.2.3. Proline isomerization modulates the interaction between the linker and SH3<sup>C</sup>

**Figure 4.3** Overlay of the 20 lowest-energy conformers of (a) *trans* Crk L-SH3<sup>C</sup>, (b) *cis* Crk L-SH3<sup>C</sup>



**Figure 4.4** Structural characterization of the *cis* and *trans* Crk L-SH3<sup>C</sup> conformers. The SH3<sup>C</sup> domain is colored light blue and the linker is colored grey. Pro238 is shown in red.

In both conformers the SH3<sup>C</sup> domain adopts a typical SH3 fold; however, because of the lack of aromatic residues at its canonical binding site, SH3<sup>C</sup> does not bind polyproline II (PPII)-type sequences (Muralidharan et al., 2006; Reichman et al., 2005). Superposition of the structures of the *cis* and *trans* conformers show that isomerization about the Gly237–Pro238 prolyl bond results in notably distinct conformations of the linker with respect to SH3<sup>C</sup> (**Figure 4.5**).

In the *trans* form the  $SH3^{C}$  n-src loop adopts a typical conformation seen in the majority of the SH3 domains. However, in the *cis* form the n-src loop rearranges significantly to accommodate the linker, which, as a result of the *cis* conformation adopted by the Gly237–Pro238 prolyl bond, is pushed further closer to  $SH3^{C}$  (**Figure 4.5 and Figure 4.6**). Interestingly,  $SH3^{C}$  uses the same surface to interact with the linker in both the *cis* and *trans* conformers but distinct sets of interactions stabilize them.



**Figure 4.5** Superposition of the *trans* and *cis* conformers of L-SH3<sup>C</sup> on the SH3<sup>C</sup> domain residues (residues 239–295). The SH3<sup>C</sup> domain in the *trans* and *cis* conformation is in light and dark grey, respectively. The linker adopts a very different structure in the *trans* (green) and the *cis* (purple) conformation. Representative residues at the linker are indicated. The conformation of the n-src loop is distinct in the *trans* (green) and the *cis* (purple) conformer.



**Figure 4.6** Effect of *cis-trans* isomerization on the structure of  $SH3^{C}$ . The side-chain conformation of select residues is shown. The side chain of Phe239 is solvent exposed in the *cis* isomer (purple) whereas it is buried in the *trans* isomer (green).

The interaction of the linker with  $SH3^{C}$  in the *trans* conformer is mediated primarily by hydrophobic contacts. More specifically, Ala223, Ile227, Pro230 and Pro232 at the linker interact with Phe239, Lys269, Ile270, Asn271, Leu294, and Trp276 at  $SH3^{C}$  (**Figure 4.7**). The linker– $SH3^{C}$  interaction is further enhanced by hydrogen bonds formed between Asn233 and Lys269 and between Asn236 and Qln297. Overall, the linker– $SH3^{C}$  interaction buries a remarkable ~1,150 Å2 of surface (**Figure 4.8**). The last 10 C-terminal residues of the linker (Ile227 to Asn236) interact intimately with the  $SH3^{C}$  domain. In contrast, residues preceding Ile227, other than Ala223, do not appear to form strong and persistent contacts with  $SH3^{C}$ , an observation that is further corroborated by NMR relaxation analysis showing that indeed the N-terminal linker residues are very flexible on the fast (pico-to-nanosecond,ps-ns) time scale.



**Figure 4.7** Structure of the Crk L-SH3<sup>C</sup> polypeptide in the trans conformation. The SH3<sup>C</sup> domain is displayed as a semi-transparent surface and residues involved in the linker-SH3<sup>C</sup> interactions are highlighted. Linker and SH3<sup>C</sup> residues are colored green and yellow, respectively. Dotted lines denote hydrogen bonding.



**Figure 4.8** Structure of Crk L-SH3<sup> $^{C}$ </sup> in the *trans*. The SH3<sup> $^{C}$ </sup> domain is shown as a solvent-accessible surface and the linker as tube cartoon. Linker residues that interact with SH3<sup> $^{C}$ </sup> are shown as sticks and are labeled.

In contrast to the *trans* conformer, the linker–SH3<sup>C</sup> in the *cis* conformer is mediated exclusively by hydrophobic contacts (**Figure 4.9**). In fact, only three residues from the linker (Pro230, Leu231, and Leu234) interact with SH3<sup>C</sup> (**Figure 4.9** and **Figure 4.10**).



**Figure 4.9** Structure of Crk L-SH3<sup>C</sup> in the *cis*. The SH3<sup>C</sup> domain is shown as a solvent-accessible surface and the linker as tube cartoon. Linker residues that interact with SH3<sup>C</sup> are shown as sticks and are labeled.

Nevertheless, the linker–SH3<sup>C</sup> interaction in the *cis* conformer buries ~1,140 Å2 of surface, which equals the amount of surface buried in the *trans* conformer. In the *cis* conformer the side chain of Leu231 is completely buried into a hydrophobic pocket in SH3<sup>C</sup> lined by residues Ala241, Val267, Trp276, Phe289, and Val292 (**Figure 4.10**). The strong linker–SH3<sup>C</sup> hydrophobic contacts result in significant stabilization of the interface and thus of the *cis* conformer, which is equally populated to the *trans* conformer.



**Figure 4.10** Structure of the Crk L-SH3<sup>C</sup> polypeptide in the *cis* conformation. The SH3<sup>C</sup> domain is displayed as a semi-transparent surface and residues involved in the linker-SH3<sup>C</sup> interactions are highlighted. Linker and SH3<sup>C</sup> residues are colored green and yellow, respectively. Dotted lines denote hydrogen bonding.

The linker in the *trans* conformer interacts extensively with the SH3<sup>C</sup> domain whereas in the *cis* conformer only three linker residues interact with SH3<sup>C</sup> (**Figure 4.7**, **4.8**, **4.9**, and **4.10**).

However, in the *cis* conformer the N-terminal part of the linker (residues Pro221 to Ile227) folds over and packs against the C-terminal part of the linker (residues Pro230 to Asn236) (**Figure 4.11**). Whereas in the *trans* conformer Pro230 and Pro232 are facing and interact with SH3<sup>C</sup> (**Figure 4.7**), in the *cis* conformer the two Pro residues form a hydrophobic pocket facing the linker with Pro225 and Ile227 fitting snugly into it (**Figure 4.11**).



Figure 4.11 Detailed view of the interactions within the linker in the *cis* form of L-SH3<sup>C</sup>.

# **4.2.4.** SH3<sup>C</sup> adjusts its surface to interact with the proline-induced alternative linker conformations

Prolyl *cis–trans* isomerization at Pro238 results in a large conformational rearrangement of the linker in the two conformers (**Figure 4.5**). As a consequence, the linker presents an entirely different surface to  $SH3^{C}$  for binding [**Figure 4.12 (a) and (b)**]. Notably, the  $SH3^{C}$  surface that interacts with the linker adjusts drastically between the *cis* and *trans* conformers in order to optimize binding contacts with the alternative linker conformations (**Figure 4.6**). The  $SH3^{C}$  n-src loop undergoes a significant conformational change between the *cis* and *trans* conformers with a maximum translation of ~8 Å (**Figure 4.6**). As a result, many of the n-src and neighboring side chains rearrange, with notable examples being residues Trp276, Phe289 and Ile270. In the *trans* conformer, the linker residues Ile227, Pro230 and Pro232 mediate all of the hydrophobic contacts with  $SH3^{C}$ . In the *cis* conformer, Ile227 and Pro232 swing out of the linker– $SH3^{C}$  interface

(Figure 4.5, 4.8 and 4.9) in order to form, together with Pro230 and Pro225, a hydrophobic core within the linker (Figure 4.11).



Figure 4.12 Solvent-accessible surface presentation of the *trans* (a) and *cis* (b) conformations.

To compensate for the loss of these linker–SH3<sup>C</sup> hydrophobic contacts, as well as for the complete lack of polar interactions, the linker in the *cis* conformer uses Leu231 to interact firmly with SH3<sup>C</sup> (**Figure 4.13**). Ile270, Trp276 and Phe289 of SH3<sup>C</sup> adjust their side chain conformation to form a deep hydrophobic pocket wherein Leu231 side chain enters into and completely buries itself (**Figure 4.13**). Interestingly, the conformation adopted by Phe239 in the *trans* conformer is not compatible with Leu231 binding to the deep pocket and as result of the *trans*-to-*cis* isomerization Phe239 is expelled from the interior of the protein (*trans*) to become completely solvent exposed (*cis*) (**Figure 4.6 and 4.13**). The drastically different SH3 and linker conformations induced by *cis–trans* isomerization give rise to distinct surfaces of the L-SH3<sup>C</sup>, potentially conferring the two conformers with distinct binding and physicochemical properties (**Figure 4.12 a and b**).



**Figure 4.13** Leu231 becomes completely buried in the *cis* conformation into a hydrophobic pocket in SH3<sup>C</sup>.

#### 4.2.5. Dynamic changes caused by *cis-trans* isomerization

*Cis-trans* isomerization causes a drastic structural change in both the linker and the SH3<sup>C</sup> domain. Does the isomerization process also bring about changes in the dynamics of the protein? To address this question we measured backbone relaxation rates (Mittermaier and Kay, 2006; Palmer, 2004) to obtain sitespecific information about the N-H bond order parameters (**Figure 4.14**). The order parameter, S<sup>2</sup>, is a measure of the amplitude of internal motions on the pico-to-nanosecond (ps-ns) timescale and may vary from S<sup>2</sup>=1, for a bond vector having no internal motion, to S<sup>2</sup>=0, for a bond vector rapidly sampling multiple orientations(Mittermaier and Kay, 2006).

*Cis–trans* isomerization at Pro238 results in significant changes in  $S^2$  throughout Crk L-SH3<sup>C</sup> (Figure 4.15). In the region around Pro238 there is significant enhancement

in the flexibility on going from the *trans* to the *cis* isomer presumably as a result of the increase in solvent exposure of Phe 239 (**Figure 4.15**).



**Figure 4.14** Relaxation rates  $[T_1, T_2 \text{ and } (^1\text{H})^{-15}\text{N-NOE}]$  and N-H order parameters  $(S^2)$  for Crk L-SH3<sup>C</sup> in the trans (blue) and cis (red) forms as a function of residue number.



**Figure 4.15** Dynamic characterization of the *trans* and *cis* isomers. (a) N-H bond order parameters,  $S^2$ , plotted as a function of the primary sequence, for the *cis* and *trans* conformers of L-SH3<sup>C</sup>. (b) Changes in order parameters,  $\Delta S^2$ , between the *trans* and the *cis* conformers mapped using a color code on the structure of *cis* L-SH3<sup>C</sup>.  $\Delta S^2$  is given as  $S^2$  (*trans*) –  $S^2$  (*cis*), so positive  $\Delta S^2$  values denote enhanced rigidity of the protein backbone in the *trans* over the *cis* conformation.

A similar trend is also seen for the region around Ile270 for the same reason. In contrast, *trans-to-cis* isomerization causes significant rigidification in the linker, especially nearby Leu231, the residue that is completely buried into a hydrophobic pocket in the SH3<sup>C</sup> domain in the *cis* isomer (**Figure 4.13**). Moreover, there is significant rigidification in most of the n-src loop as a result of the better packing between this loop and the linker. In addition, most the linker residues appear to be slightly more rigid in the *cis* conformer than in the *trans* one primarily because of the better packing within the linker in the *cis* conformer (**Figure 4.11**). Thus, the data indicate that *cis-trans* isomerization causes not only significant structural changes (**Figure 4.15**).

## 4.2.6. Structural basis for the Crk<sup>SLS</sup> intramolecular interaction

Combined chemical shift and relaxation rate analyses have indicated11 that Crk SH3<sup>N</sup>linker-SH3<sup>C</sup> (Crk<sup>SLS</sup>) exists in two conformations in solution: a major one (90%), wherein Gly237–Pro238 adopts the *cis* conformation and the two SH3 domains (SH3<sup>N</sup> and SH3<sup>C</sup>) interact in an intramolecular fashion (closed conformation), and a minor one (10%), wherein Gly237–Pro238 adopts the *trans* conformation and the two SH3 domains do not interact (open conformation) (**Figure 4.17**).



**Figure 4.16 (a)** Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of isolated SH3<sup>N</sup> (cyan), L-SH3<sup>C</sup> (green), and Crk<sup>SLS</sup> (red) indicating the presence of a minor (~10%) conformation of Crk<sup>SLS</sup> in an open, uninhibited conformation(Sarkar et al., 2007). In this conformation the canonical binding site in SH3<sup>N</sup> is completely accessible and the Gly237–Pro238 prolyl bond adopts only the *trans* conformation. SH3<sup>N</sup> residues in the minor conformation are primed. (b) Model of the equilibrium of conformational states of Crk<sup>SLS</sup>, its autoinhibition, and its activation.

We now provide atomic insight into this very interesting binding process by having determined the structure of the closed conformation of Crk<sup>SLS</sup> (**Figure 4.17** and **4.18**).



**Figure 4.17** Overlay of the 20 lowest-energy conformers of Crk<sup>SLS</sup>. The entire linker is displayed only in one conformer as there is very poor overlap because of its intrinsic flexibility.



**Figure 4.18** Lowest-energy structure of Crk<sup>SLS</sup> shown as cartoon with SH3<sup>N</sup> in pink, SH3<sup>C</sup> in light blue, and linker in grey.

The structural data show, in agreement with the chemical shift analysis data, that the two SH3 domains interact extensively in the closed Crk<sup>SLS</sup> (**Figure 4.18**). The SH3<sup>N</sup> domain uses its canonical binding site, typically interacting with PPII ligands, to interact with SH3<sup>C</sup>. Specifically, a cluster of SH3<sup>N</sup> aromatic residues (Phe142, Phe144, Trp170 and Tyr187) along with Pro184 and Pro186 form an elongated hydrophobic solventexposed surface comprising its canonical ligand-binding site (**Figure 4.19**). This site is well suited to interact with hydrophobic sequences, such as proline-rich sequences. In Crk, SH3<sup>C</sup> uses the hydrophobic residues Pro238, Phe239 and Ile270 to interact with the canonical binding-site of SH3<sup>N</sup> (**Figure 4.19**). The interaction is further enhanced by the formation of two polar contacts: a hydrogen bond between Qln169 and Qln297 and a salt bridge between Asp143 and Lys269. Overall, the SH3<sup>N</sup>–SH3<sup>C</sup> interaction buries a total surface of ~800 Å2.



**Figure 4.19** Close-up view of the SH3<sup>N</sup>–SH3<sup>C</sup> interface. SH3<sup>N</sup> and SH3<sup>C</sup> residues are colored green and orange, respectively.

The Crk SH3<sup>N</sup> domain binds selectively to ligands carrying the P-x-L-P-x-K sequence motif (Knudsen et al., 1995), such as the Abl kinase (Ren et al., 1994). Pro1, Leu3 and Pro4 residues of the PPII ligand are closely packed against the hydrophobic residues in SH3<sup>N</sup>, whereas Lys6 forms salt bridges with conserved acidic residues10 (**Figure 4.19**). SH3<sup>C</sup> uses a similar set of interactions to bind specifically to SH3<sup>N</sup>: Pro238 and Phe239 of SH3<sup>C</sup> occupy similar positions, relative to SH3<sup>N</sup>, to Pro4 and Leu3, respectively, of the PPII peptide. Qln297, which forms a hydrogenbond to SH3<sup>N</sup>, is found in a similar position to Lys6, whereas Lys269 forms a salt-bridge to Asp143 (**Figure 4.19 and 4.20a**), a highly conserved residue that does not participate though in

the interaction between SH3<sup>N</sup> and the PPII peptide (**Figure 4.20 b**). Interestingly, the juxtaposition of SH3<sup>N</sup> with the PPII ligand appears to be more optimal than with SH3<sup>C</sup> since complex formation buries ~1,120 Å2 in the first case but only ~800 Å2 in the latter. The rather small interface between SH3<sup>N</sup> and SH3<sup>C</sup> is consistent with the low free energy ( $\Delta G$ ) of SH3<sup>N</sup>–SH3<sup>C</sup> interaction, which is only ~1.4 kcal mol-1 (**Figure 4.16 b**). This is the reason why the SH3<sup>N</sup>–SH3<sup>C</sup> interaction occurs only in an intramolecular fashion.



**Figure 4.20** (a) View from above  $SH3^{C}$ , which is shown as transparent blue cartoon.  $SH3^{N}$  is displayed as pink solvent-accessible surface.  $SH3^{C}$  residues interacting with  $SH3^{N}$  are shown as orange sticks. (b) Structure of  $SH3^{N}$  in complex with a consensus PPII peptide (P-x-P-L-x-K)(Wu et al., 1995). Peptide shown in blue and  $SH3^{N}$  is displayed as pink solvent-accessible surface.

# **4.2.7.** The SH3<sup>N</sup>–SH3<sup>C</sup> intramolecular interaction is mediated only by the *cis* conformer

Analysis of the closed Crk<sup>SLS</sup> structure shows that Pro238 adopts uniquely the *cis* conformation in the SH3<sup>N</sup>–SH3<sup>C</sup> complex. Interestingly, Pro238 adopts the *trans* conformation in the minor, open conformation of Crk<sup>SLS</sup>. Thus, in agreement with our

previous chemical shift analysis (Sarkar et al., 2007), the structural data demonstrate that the intramolecular interaction between the two SH3 domains is mediated exclusively by the *cis* conformer. The present data provide now the structural basis for this observation. **Figure 4.21a** displays the overlay of the structures of  $Crk^{SLS}$  and the *cis* and *trans* conformers of L-SH3<sup>C</sup> superimposed on the SH3<sup>C</sup> domain. The SH3<sup>N</sup>–SH3<sup>C</sup> interaction is primarily mediated by SH3<sup>C</sup> residues Pro238, Phe239, and Ile270. The structural data clearly show that all these three residues are well poised to interact with the SH3<sup>N</sup> domain in the *cis* conformer. In contrast, in the *trans* conformer Phe289 is located in the interior of the SH3<sup>C</sup> domain and Ile270 swings towards the center of the SH3<sup>C</sup> domain as a result of the drastic rearrangement that n-src loop undergoes triggered by *cis–trans* isomerization (**Figure 4.6** and **4.21a**). Thus, neither Phe289 nor Ile270 are poised to interact with the SH3<sup>N</sup> domain in the *trans* conformation.



**Figure 4.21** Structural basis for conformer-specific SH3<sup>N</sup>–SH3<sup>C</sup> interaction in Crk<sup>SLS</sup>. (a) Superposition of the *cis* and *trans* L-SH3<sup>C</sup> and Crk<sup>SLS</sup> on the SH3<sup>C</sup> domain. The side chains of Pro238, Phe239, and Ile270, residues primarily mediating the SH3<sup>N</sup>–SH3<sup>C</sup> interaction, are shown in green for trans and yellow for cis L-SH3<sup>C</sup> and orange for Crk<sup>SLS</sup>. SH3<sup>N</sup> is shown as solvent-

accessible surface. (b) Effect of single amino acid substitution on the stability of the closed conformation of  $Crk^{SLS}$  as assessed by measuring the population of the closed and open conformations of  $Crk^{SLS}$  by NMR.  $\Delta G$  for P238A and L231G is a lower-bound limit since populations less than ~5% would be beyond the detection limit.

Phe239 undergoes the most dramatic conformational change upon cis-trans isomerization (Figure 4.6 and 4.21a) and seems to play a key role in mediating SH3<sup>N</sup>–SH3<sup>C</sup> binding. Indeed, substitution of Phe239 by Ala abolishes the intramolecular interaction and Crk<sup>SLS</sup>-F239A adopts predominantly the open conformation (Figure 4.21b and 4.22). Similarly, the P238A substitution also abrogates the closed conformation (Figure 4.21b). To test the effect of destabilizing the cis conformation on the integrity of the closed conformation we prepared and characterized substitutions of Pro232 and Leu231, two residues that are important for stabilizing the *cis* conformer through hydrophobic interactions (Figure 4.11). Indeed, the P232A substitution decreases the stability of the closed conformation of Crk<sup>SLS</sup> by ~1.1 kcal mol-1, whereas the effect of the L231G substitution is even stronger (Figure 4.21b and 4.22). Interestingly, Pro238 is compatible with SH3<sup>N</sup> binding in both the *cis* and *trans* conformations (Figure 4.21a). Therefore, the prolyl isomerization does not directly regulate binding specificity by the actual conformation of the proline itself but rather indirectly by sculpturing the SH3<sup>C</sup> surface responsible for binding to SH3<sup>N</sup>.



Protein	Open (%)	Closed (%)	∆G (kcal mol <sup>-1</sup> )
WT-Crk <sup>sLs</sup>	10	90	-1.4
Crk <sup>sLs</sup> -P232A	40	60	-0.3
Crk <sup>sLs</sup> -F239A	92	8	1.5
Crk <sup>sLs</sup> -P238A	>95	<5	>1.9
Crk <sup>sLs</sup> -L231G	>95	<5	>1.9

**Figure 4.22** (a) Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of isolated Crk<sup>SLS</sup> (red), Crk<sup>SLS</sup>-F239A (orange), and SH3<sup>N</sup> (cyan). The F239A substitution results in all resonances of the SH3<sup>N</sup> domain in Crk<sup>SLS</sup> shifting to the corresponding chemical shifts of the isolated domain (characteristic shifts are indicated in the figure). (b) Percentage of population of Crk<sup>SLS</sup> molecules adopting the closed and open conformation as assessed by integrating well resolved resonances of the two conformations by NMR.

b

# **4.2.8.** The SH3<sup>N</sup>–SH3<sup>C</sup> intramolecular interaction results in Crk autoinhibition and modulated Abl-mediated Crk phosphorylation

Previous experiments have shown that the binding of PPII peptides to the SH3<sup>N</sup> domain in Crk is much weaker compared to the binding of the PPII ligand to the isolated SH3<sup>N</sup> domain. The present data provide the structural basis for the inhibition. SH3<sup>C</sup> binds to SH3<sup>N</sup> and masks the canonical PPII binding site thereby providing an autoregulatory mechanism. Indeed, Abl kinase, which interacts with Crk SH3<sup>N</sup> through a P-x-L-P-x-K sequence motif, binds to the closed, autoinhibited form of Crk<sup>SLS</sup> with a 10-fold lower

affinity than to the open form of Crk<sup>SLS</sup>, induced by the F239A substitution (**Figure 4.23**). Thus, Abl binding to Crk is inhibited in the *cis* form but it is promoted in the *trans* form.



**Figure 4.23** Effect of the autoinhibitory Crk conformation on its interaction with Abl. Energetics for the interaction of the PxxP motif of Abl kinase with WT-Crk<sup>SLS</sup> (closed) and Crk<sup>SLS</sup>-F239A (open).

## 4.2.9. Novel SH3 binding surfaces mediate linker interactions and Crk autoinhibitory conformation

The linker–SH3<sup>C</sup> and SH3<sup>N</sup>–SH3<sup>C</sup> interactions are mediated by a novel set of SH3 surfaces and contacts not seen previously in SH3 domains. Specifically, the linker interacts extensively with a surface of the SH3<sup>C</sup> domain delineated by  $\beta$ -strands a and e and the n-src loop (**Figure 4.24**).



**Figure 4.24** SH3<sup>C</sup> uses novel binding surfaces. Surfaces used by SH3 domains to interact withnon-canonical ligands are colored. Binding surfaces are colored as follows: PPII ligands, Itk SH237 and ubiquitin-like domain (Ubl)49, red; PINCH-1 LIM450, orange; SAP SH251, yellow;myosin 7 intrapeptide52, green; Crk intrapeptide linker, light blue (this work); Crk SH3<sup>N</sup> domain, dark blue (this work).

Notably, the linker interacts with this  $SH3^{C}$  surface in both the *cis* and *trans* conformers, although a distinct set of contacts stabilize the two conformations. The interaction is quite extensive as it buries ~1,150 Å2 of surface. Moreover,  $SH3^{C}$  uses a novel binding surface to interact with the other SH3 domain in Crk,  $SH3^{N}$  (**Figure 4.24**). The two surfaces are distinct and not mutually exclusive. In the closed form of Crk<sup>SLS</sup>  $SH3^{C}$  is bound to both the linker and  $SH3^{N}$  simultaneously. Thus, the present data further highlight the binding versatility of SH3 domains and expands the list of the non-canonical ligands that may interact with this small modular domain.

#### 4.3. DISCUSSION

Proline *cis-trans* isomerization is an emerging regulatory mechanism implicated in the regulation of a wide range of biomolecular processes. Understanding the underlying

mechanisms of such molecular switches necessitates the availability of structural data on both the *cis* and *trans* conformers. The limited structural information (Andreotti, 2003; Grochulski et al., 1994; Lu et al., 2007; Mallis et al., 2002; Santiveri et al., 2004) on both conformers available so far has been suggestive of a rather local effect caused by proline isomerization. Here we provide atomic details of the structural and dynamic changes elicited by proline *cis*-*trans* isomerization in the Crk signaling protein. The data demonstrate that proline isomerization causes a remarkable reorganization of the interface between the linker and the SH3<sup>C</sup> domain, to the extent that the two resultant conformers are conferred with distinct binding properties, with the *cis* one promoting the intramolecular engagement of the two SH3 domains to form an autoregulatory conformation and the *trans* conformer promoting a dumbbell-like, uninhibited conformation.

How can a 180° rotation about a single prolyl bond cause such a strong effect on the global structure of the protein? Pro238 in Crk is located at a strategic position where the linker connects to the SH3<sup>C</sup> domain. In the *trans* conformer the linker interacts extensively with the SH3<sup>C</sup> domain. *Trans-to-cis* isomerization at Pro238 forces a new conformation to the linker thereby disrupting all contacts between the linker and the SH3<sup>C</sup> present in the *trans* form. Notably, both the linker and the SH3<sup>C</sup> domain readjust their structure and a new set of contacts are formed to stabilize the *cis* conformer. As a result, the SH3<sup>C</sup> domain exposes several residues that form a binding surface capable of mediating the specific interaction between SH3<sup>C</sup> and SH3<sup>N</sup> (**Figure 4.21a**). In the *trans* form this set of residues is buried and thus the SH3<sup>N</sup>-SH3<sup>C</sup> interaction is not possible resulting in a dumbbell-like conformation. Thus, the structural basis for the function of Pro238 as a molecular switch is the surface "sculpturing" afforded by the actual isomerization process. The intrinsic slow kinetics of the *cis-trans* isomerization and the action of cyclophilin A (CypA) afford this molecular switch additional regulatory mechanisms(Sarkar et al., 2007).

To what extent this regulatory mechanism is shared by other Crk family members remains to be addressed. Pro238 is strictly conserved among all Crk proteins (**Figure 4.25**) raising the possibility that proline isomerization may be a conserved feature.



Figure 4.25 Sequence alignment of Crk-family proteins.

Interestingly, our data also indicate that the identity of the residue following the proline determines the population of the *cis* form. For example, the F239A substitution decreases the *cis* population to ~8%, from 50% in the wild type protein. This position is variable among Crk proteins (**Figure 4.25**). Thus, it is possible that proline isomerization is present in other Crk proteins although the relative population of the two conformers may not be as high as in chicken Crk.

A particularly striking result in the present work is related to the versatility of the SH3 domain as a binding partner(Li, 2005). The interaction between  $SH3^N$  and  $SH3^C$  in Crk provides the first example of an SH3–SH3 complex mediated by direct contacts between the two domains. Moreover, the surfaces used by  $SH3^C$  to interact with the linker and  $SH3^N$  are novel binding surfaces for SH3 domains. It is remarkable that almost the entire surface (**Figure 4.24**) of such a small domain can be molded to accommodate a wide range of ligands with no apparent sequence or structure similarity.

### Chapter 5 : Crk is a new substrate for CypA

#### 5.1. Introduction

As discussed in chapter 3 that proline isomerization is emerging as a critical component of certain important biological processes. Prolyl *cis-trans* isomerization is a slow process in the time scale regime of minutes at 25°C due to the relatively high enegy barrier (~20 kcal/mol) (Grathowl and wutrich1981). Interestingly, this slow isomerization process can be catalyzed by a highly conserved and ubiquitous group of enzymes known as peptidyl-prolyl *cis-trans* isomerase (PPIase), including cyclophillins, FK50-binding proteins and parvullins (Schiene and Fischer, 2000).

#### 5.1.1. PPIase

The PPIases family comprises three distinct enzyme families: cyclophilins (Cyp), FK-506 binding proteins (FKBP's), and the parvulins (Schiene-Fischer and Yu, 2001). Both CypA and FKBP12 are the intracellular protein targets of the immunosuppressive drugs cyclosporine A (CsA) and FK506, respectively. These small molecules inhibit PPIase activity and block T cell activation (Andreotti, 2003; Liu et al., 1991; O'Keefe et al., 1992). However, the immunosuppressant activity of these small molecules are not related to inhibition of the isomerase activity but due to interruption of signaling events by formation of inhibitor–PPIase complexes (Walsh et al., 1992). The binary CsA–CypA and FK506–FKBP12 complexes bind to and inhibit calcineurin, the serine-threonine phosphatase activity and thus prevent T-cell proliferation by interfering with downstream signaling event (Liu et al., 1991; O'Keefe et al., 1992).

#### **5.1.2.** Cyclophilins (Cyps)

Cyclophilin A (CypA) was the first identified member of the cyclophilin family (Cyp) as a target of immunosuppressive drug cyclosporine A (CsA), about 25 years ago. Latter they were shown to possess enzymatic acitivity to catalyze *cis-trans* isomerization of peptidyl-proline bond (Fischer et al., 1989; Hunter, 1998; Rutherford and Zuker, 1994). Cyps also possess chaperone activities. These two functions let Cyps to be involved in proper folding of proteins and to transport proteins to proper intracellular locations.

Cyps are highly conserved from *E. coli* to humans throughout evolution. A total of 16 Cyp isoforms have been identified in humans (Galat, 2003). Seven major human Cyp isoforms, namely hCypA, hCypB, hCypC, hCypD, hCypE, hCyp40, and hCypNK, have been well characterized (Anderson et al., 1993). Different Cyps localize themselves at different cellular compartments like endoplasmic reticulm (ER), mitochondria, nucleus and cytosol via its unique domain.

#### 5.1.3. Cyps in various human diseases

Cyps are invoved in various pathological conditions including HIV (Towers et al., 2003), hepatitis B and C viral infection, atherosclerosis (Satoh et al., ; Wohlfarth and Efferth, 2009; Wohlfarth C, 2009), ER stress-related diseases such as diabetes, and neurodegenerative diseases. Cyps are also involved in normal cellular functions of muscle differentiation, detoxification of reactive oxygen species (ROS) (Hong et al., 2002), andimmune response (Wiederrecht et al., 1993). CypA has been found to be overexpressed in small cell lung cancer (Campa et al., 2003; Howard et al., 2005; Li et al., 2005; Howard et al., 2007), pancreatic cancer(Li et al., 2005; Li et al., 2006;

Mikuriya et al., 2007; Shen et al., 2004), breast cancer (Hathout et al., 2002; Zheng et al., 2008), colorectal cancer (Lou et al., 2006; Melle et al., 2005; Wong et al., 2008), squamous cell carcinoma (Chen et al., 2004a; Qi et al., 2008), melanoma (Al-Ghoul et al., 2008), and glioblastoma multiforme (Han et al.). Overexpression of CypB is associated with tumor progression through regulation of hormone receptor expression and gene products involved in cell proliferation and motility (Fang et al., 2009). CypC is primarily located in ER and CyC-osteopontin complex regulates *in vitro* migration and invasion properties of 4T1 and 4T07 breast cancer cells (Mi et al., 2007). Cyp40 mRNA has also been reported to increase in many breast cancer cell lines including MCF-7 (Ward et al., 1999). Several reports have shown that CypD is overexpressed and has an anti-apoptotic effect in various tumors via a Bcl 2 collaborator and an inhibitor of cytochrome c release from mitochondria (Eliseev et al., 2009).

#### 5.1.4. Sturctural insight into CypA mechanism

CypA is the best studied member of the Cyps family of PPIase. CypA is a ubiquitous protein and expressed abundantly in both eukaryotes and prokaryotes.

Active site of CypA is consists of four β-sheets on the surface of the enzyme. Four loops were protruded out of the surface forming a narrow channel, where the substrate binds (Hur and Bruice, 2002). There is very little change in the active site of CypA during the isomerization. Residues of CyPA involved in the interactions with the substrate include Arg55, Ile57, Phe60, Met61, Gln63, Ala101, Asn102, Gln111, Phe113,Trp121, Leu122, and His126. The N-terminal part of the substrate binds between the first two loops L1 and L2 and forms three hydrogen bonds with Gln63, Arg55, and

Asn102. The C-terminal of the substrate binds to L3 and L4 loop and have only hydrophobic interaction with Trp121 (Figure 5.1).



**Figure 5.1** The structure of the cyclophilin A-peptide complex based on the X-ray structure (PDB code 1AWQ64). The protein is shown in ribbon format and is color-coded based on secondary structures (helix, purple; sheet, yellow; loops, blue and white). The substrate peptide (His-Ala-Gly-Pro-Ile-Ala) is shown in ball-and-stick and is color-coded by atom types. Three essential active site residues discussed extensively in the text, Arg 55, Gln 63, and Asn 102, are shown in line forms (Li. G, 2003).

The positively charged Arg55 guanidino group is positioned right above the Pro(N) in all three states (*cis*,transitions state and *trans*). The -NH2 groups in Arg55 maintain stable interactions with the C=O group in the isomerizing proline by forming a tight hydrogen bond with Pro(O) of C=O group. In this geometry Arg55 does not interact with the amide Pro- (N) (sp3 hybridized, pyramidal shape) in the ground state but is ready to interact with the lone pair in TS; the Pro(N)...Arg55 distance ) ~4.0 Å (CyP.*cis*) to ~3.5 Å. This interaction between Pro(N) and Arg55 guanidino group, results in the single bond character to peptide bond (Fischer et al., 1993; Zhao and Ke, 1996). This is in

accordance with the fact that catalysis enhanced by factors that disfavor the double bond character of peptide bond and favor the less polar and free rotating single bond (Stein, 1993). In addition to the Pro(O)...Arg55 interaction, there is another hydrogen bond between Gln63(OE1)...Arg55 that holds the Arg55 guanidino group in the proximity of Pro(N). In absence of a ligand, Gln63 interacts with His54 rather than with Arg55, forming a different conformation of Gln163 in unliganded CypA. Another important Hbond is between X(C=O) and Asn 102(HN). The X(O)...Asn102(HN) interaction is sufficient to stabilize the distortion of the amide bond of the cis-isomer by  $\sim 20^{\circ}$ counterclockwise. This distortion is efficiently performed because, while X(O) is attracted by Asn102(HN), the proline ring is held in position by a contact with the phenyl of Phe113. Also, Bruce.R.Howard .et al. showed that Gly-Pro is the most preferred binding motif for CypA as steric clash from the side chain of the preceeding residue to Pro prevents the optimal binding of the *trans* isomer to CypA and also destabilize ground state in both *cis* and *trans* conformers even in case of Ala-Pro containing peptide(Howard et al., 2003).

#### 5.2. Results

#### 5.2.1.Crk is a substrate for the PPIase, CypA

## **5.2.1.1.** CypA Accelerates the Intrinsically Slow Gly237-Pro238 Interconversion Rate

The presence of a heterogeneous proline residue undergoing *cis-trans* isomerization in Crk, provides a potential binding site for PPIase enzymes. In fact, the Gly237-Pro238-Phe239 motif in Crk provides a favorable recognition site for CypA (Piotukh et al., 2005). To determine whether CypA catalyzes the isomerization process at Pro238, we characterized the fl-SH3<sup>C</sup> polypeptide in the presence of catalytic amounts of CypA using <sup>1</sup>H-<sup>15</sup>N NMR exchange spectroscopy (Farrow et al., 1994).

This NMR experiment identifies conformational exchange processes that take place with exchange rates between 0.1 and 100 s<sup>-1</sup> (Bosco and Kern, 2004). In the absence of CypA, *cis-trans* isomerization about the Gly237-Pro238 prolyl bond is very slow, as indicated by the absence of exchange peaks in the 2D exchange spectrum (**Figure 5.2**). Using line shape analysis, we estimate that the exchange rate,  $k_{ex}$ , for the isomerization at 22°C, in the absence of CypA, is ~0.01 s<sup>-1</sup>. Addition of catalytic amounts of CypA results in the appearance of exchange peaks in the 2D exchange spectrum (**Figure 5.2**). The appearance of the exchange peaks provides direct evidence that CypA catalyzes the Gly237-Pro238 *cis-trans* isomerization process. To determine the rate constant of *cis-trans* interconversion in the presence of CypA, we measured the intensity of the exchange and auto peaks as a function of the mixing time. The fitting procedure yielded an exchange rate of ~80 s<sup>-1</sup> at 22°C, meaning an estimated ~300 s<sup>-1</sup> at physiological temperature, such fast kinetics would enable Crk to switch between the autoinibited and non-inhibited conformation much more rapidly. Thus, CypA catalyzes very efficiently the *cis-trans* isomerization process in Crk by accelerating the rate of interconversion several thousand fold. Increasing amounts of CypA also resulted in specific chemical shift perturbations that are localized to residues surrounding Pro238. The collective data provide strong evidence for a direct, specific, and catalytically productive interaction between CypA and Crk.



**Figure 5.2** 2D <sup>1</sup>H-<sup>15</sup>N heteronuclear (ZZ) NMR exchange spectra of fl-SH3<sup>C</sup> in the absence and presence of catalytic amounts of CypA. Exchange peaks, indicated within the dotted lines, appear when the rate of interconversion between the *cis* and *trans* conformations is relatively fast. The determined uncatalyzed and catalyzed rates of interconversion are included.

#### 5.2.1.2. Presence of a second substrate site on Crk for CypA

Interestingly, we have recently identified a second proline residue (Pro 221) located in the inter SH3 linker region that undergoes *cis-trans* isomerrization (**Figure1.3**). Proline isomerization at Pro221 has a very local effect. In contrast to the long range effect induced by Pro238 isomerization, residues that get affected by G220-P221 peptidyl-prolyl isomerization are from 219 to 224 (**Figure 5.3**). Furthermore, this peptidyl-prolyl isomerization is independent of the isomerization at Pro238. The HSQC spectra of SH3<sup>C</sup>-

hl P238A construct shows only one set of peaks for all the residues except for those six residues from 219-224 (**Figure 5.3**).



**Figure 5.3** Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of hl-SH3<sup>C</sup> (blue) and hl-SH3<sup>C</sup>-P238A (orange). The P238A mutation abolishes the *cis/trans* isomerization of P238 (blue numbers) but not that of the P221.

#### 5.2.1.3. CypA binds to Crk at Gly220-Pro221 site

To test this hypothesis that Gly220-Pro221 act as a second substrate binding site for CypA, due to its sequence similarity to consensus binding motif for CypA (Piotukh et al., 2005), we record <sup>15</sup>N-HSQC spectra of SH3c-hl P238A in presence of catalytic amount of CypA. If CypA will catalyzed the isomerization, then significant line broadening would be expected with increasing amount of CypA. Notably, significant line broadening was observed for the duplicated resonacec of residues 219-224. Addition of CypA also

causes specific chemical shift perturbations that were localized to residues surrounding P221 (Figure 5.3 and 5.4). Therefore, this provides direct evidence that Crk can interact with CypA at two different sites.



**Figure 5.4** Selected regions of overlaid <sup>1</sup>H-<sup>15</sup>N HSQC NMR spectra recorded on free fl-SH3<sup>C</sup> (red), and in the presence of 4 mol% (green) and 8 mol% CypA.

Also, titration of <sup>15</sup>N labeled CypA with SH3<sup>C</sup>-hl P238A in 1:1 ratio results in significant chemical shift perturbations along, suggesting binding interaction between CypA and SH3<sup>C</sup>-hl P238A (**Figure 5.5**). Addition to chemical shift perturbation there are large number of resonances also disapper. Disaapereance of resonances occur due to first interchange between bound the state (SH3<sup>C</sup>-hl P238A bound to CypA) and unound state.The interconversion is on the ps-ns timescale.



**Figure 5.5** Over-laid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of CypA (blue) with CypA in presence of SH3<sup>C</sup>-hl P238A (magenta) in equimolar ratio.



**Figure 5.6** Overlaid <sup>1</sup>H-<sup>15</sup>N HSQC spectra of SH3<sup>C</sup>-hl P238A (blue) with SH3<sup>C</sup>-hl P238A with three times molar excess of CypA (red).

#### 5.2.1.6. CypA reduces the level of phopshorylation at Y222 by c-Abl

The Crk tyrosine residue (Tyr 222) get phophorylated by c-Abl and provides an intramolecular binding motif pTyr222-x-x-Pro225 for the SH2 domain, leading to an autoinhibited state of Crk (Rosen et al., 1995), where both Crk SH2 and SH3 domain were incapable to bind to their signaling partners. Thus the intriguing hypothesis would be that the binding of CypA to the Gly220-Pro221 of the linker region inhibits phosphorylation of the regulatory Y222. To test this hypothesiswe use western blot technique (**Figure 5.7**).

In the western blot experiments we monitored the amount of phosphorylated protein as a function of time by using primary antibody specific for pY222 of chicken Crk. We observed that in presence of CypA in 7:1 ratio to SH3<sup>C</sup>-fl the rate of phosphorylation reduces by 10-fold at ambient temperature. As a reference we carryed out the same reaction in the absence of ATP and MgCl<sub>2</sub>. These result, indeed proves that CypA may play an important role in regulation of the signaling pathway carried out by Crk.



**Figure 5.7** Western blots of SH3<sup>C</sup>-hl P238A of Crk in presence of catalytic amount of c-Abl. Immune complexes were electrophoretically resolved and probed with anti-Crk pY222 antibody (to track amount of phosphorylation). Lanes are described asfollows; 1-empty, 2-SH3<sup>C</sup>-hl P238A with out Abl, 3-SH3<sup>C</sup>-hlP238A with Abl in presence of 10mM MgCl2 and 0.6mMATP,20 sec

reaction, 4-7 reaction from step 3 with increasing amount of time, 40sec, 60sec, 2:00min and 3:00 min. 8- SH3<sup>C</sup>-hl P238A with CypA with no Abl, 9- SH3<sup>C</sup>-hl P238Awith excess CypA with Abl in presence of 10mM MgCl2 and 0.6mMATP,20 sec reaction, 10-13 reaction from step 9 with increasing amount of time, 40sec, 60sec, 2:00min and 3:00 min.

#### 5.3. Discussion

Although activation via shift of the conformational ensemble is believed to be common in signaling, the unique feature of Crk activation process is that the kinetics of interconversion between the closed and open states can be regulated. Toggling between the two conformations is controlled by a molecular switch afforded by prolyl cis-trans isomerization. Because the activation energy for prolyl isomerization is high (~20 kcal mol<sup>-1</sup>), the interconversion rates are intrinsically very slow (Andreotti, 2003). Indeed, the rate of Gly237-Pro238 prolyl bond isomerization in Crk is ~0.01s<sup>-1</sup> at 22°C rendering the kinetics of the molecular switch particularly slow. Nevertheless, CypA accelerates dramatically the rate of the interconversion to an estimated  $\sim 300s^{-1}$  at physiological temperatures enabling Crk to switch between the autoinhibited and noninhibited conformation much more rapidly. Therefore, the CypA-mediated accelerated kinetics of the proline switch will contribute significantly to the rapid activation kinetics that Crk should exhibit in response to ligand binding in certain signaling pathways. On the other hand, if particular circumstances, such as ligand-induced conformational changes, ligand mediated masking, or Crk phosphorylation, render Pro238 non-accessible to CypA, then the kinetics of Crk activation will be particularly slow, resulting in overall slow response to the signal. Therefore, the control by prolyl isomerization of the interconversion rate
between the autoinhibited and noninhibited states provides a means of modulating the kinetics of the response in a signaling process.

Moreover, we also demonstrate the second peptidyl-prolyl isomerization (Gly220-Pro221) and binding site for CypA in the inter SH3 linker region near Y222 site which undergo phosphorylation by c-Abl that play an important role in regulation of Crk in signaling. Here, we exhibit that Crk is a newly identified biological substrate for CypA. Therefore, it is very likely that CypA exerts some of its effects to the function of the cell through signaling pathways that are mediated by Crk.

Our studies also implicate that multiple layers of regulation are present in a simple adaptor protein and it will be interesting to understand how all these regulatory mechanisms integrate within the same protein molecule and how they respond to the presence of the various Crk partners. Also, it raises a question whether phosphorylation of pY221 may inhibit binding of CypA to Crk and may present another mode of regulation of Crk adaptor protein and also more studies are needed to understand if these two sets of peptidyl-prolyl isomerization are somehoe related to each other and how all these mechanism together regulate Crk activity in signaling.

# **Chapter 6 : Materials and methods.**

# 6.1. Protein Preperation of Crk fragments.

The various fragments of Crk with a glutathione *S*-transferase (GST) N-terminally fusion were cloned from a template plasmid that encodes full length Crk, inserted into pGEX6P-1 vector and then transformed into BL21(DE3) cells followed by plating on Lysogeny broth (LB) plate. A single colony was picked from the plate and was transferred into 5 ml of autoclaved LB media (for the preparation of deuterated samples, LB media was dissolved in 75 % D2O). After ~ 8 hour growing, the cells were transferred in 100 mL of LB media. Before transferring, the cells was centrifuged and washed with fresh LB media and kept overnight for growing at 37 °C. Then the cells were transferred into 1L of LB media. The cells were grown at 37 °C in the presence of ampicilin and chloramphenicol. Protein synthesis was induced by the addition of 0.5 mM of IPTG at  $A_{600} \sim 0.4$  and cells were harvested at  $A_{600} \sim 1.0$  (**Figure 6.1**) and centrifuged at 6000 *x g* in a 6-liter JLA-8.100 rotor (Beckman). The cells were re-suspended into buffer containing 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.3), 140mM NaCl, 1 mM β-mercaptoethanol (BME).



Figure 6.1 Growth curve for Crk<sup>SLS</sup> at 37°C. Yellow point indicates point of IPTG addition.



**Figure 6.2** Expression of Crk<sup>SLS</sup> at 37°C. From left to right, lane1-marker, lane2-before induction, induction, lane 3 to 6- samples taken every each hour after induction.

Isotopically labeled samples for NMR studies were prepared by growing the cells in M9 minimal media containing 1 gL<sup>-1</sup> of <sup>15</sup>NH<sub>4</sub>Cl and 2 gL<sup>-1</sup> <sup>13</sup>C<sub>6</sub>-glucose, 2ml of 1M MgSO<sub>4</sub>L<sup>-1</sup>, 100 $\mu$ l of 1M CaCl<sub>2</sub> L<sup>-1</sup>. Protein expressions for all construct were checked by using SDS-page (**Figure 6.2**).

#### 6.2. Protein Purification of Crk fragments.

All cells were sonicated or homogenized followed by centrifugation at  $50,000 \times g$  using a JA-25.50 rotor (Beckman), in order to seperate the cytosolic fraction from the membrane fraction. The lysate was loaded on glutathione Sepharose fast-flow 4% agarose resin (Amersham) pre-equilibrated with phosphate-buffered saline (PBS), 140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.8 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.3) with a flow rate of 0.2ml/min, followed by a wash of 30ml of PBS buffer at 1ml/min flow rate. Washing helped removing any non-specific bound protein from the column. The GST-fused protein was eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) (**Figure 6.3**). The eluted protein was then dialyzed into precission protease cleaving

buffer (50mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM BME, pH 7.0 at 25°C) and was cleaved overnight by the addition of PreScission protease (one unit will cleave 90% of 100 μg of a GST-fusion protein). Cleaved protein was then loaded for the second time on glutathione Sepharose fast-flow 4% agarose resin (Amersham) pre-equilibrated with phosphate-buffered saline (PBS) and the flow-through during loading was collected, which contained the desired protein (**Figure 6.3**). The collected protein was then exchanged into buffer containing 140mM NaCl, 50mM KPi, pH6.5, 1mM BME, 5% glycerol using an amicon stirred cell. Later on, the sample was concentrated and applied to the Superdex-75 size exclusion column, previously equilibrated with the same buffer. Protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 41035, 24075, and 9970 M<sup>-1</sup> cm<sup>-1</sup> for full-length Crk, SH3<sup>N</sup>-L-SH3<sup>C</sup> and SH3<sup>C</sup>-fl, respectively and the protein purity was confirmed by SDS-PAGE.



Figure 6.3 Purification of Crk<sup>SLS</sup> on LP.

All Crk fragments including SH3<sup>C</sup>-hl, SH3<sup>C</sup>-fl, SH3<sup>N</sup>-L-SH3<sup>C</sup> and full length Crk and all of the mutants were prepreared in the same way as mentioned above.

#### **6.3.** Protein preparation and purification CypA

His<sub>6</sub>-tagged CypA was transformed into BL21(DE3) cells and plated on LB plate. A single colony was picked from the plate and was transferred into 5 ml of autoclaved LB media. After ~ 8 hour growing, the cells were transferred in 100 mL of LB media. Before transferring, the cells was centrifuged and washed with fresh LB media and kept overnight for growing at 37 °C. Then the cells were transferred into 1L of LB media. The cells were grown at 37 °C in the presence of ampicilin. Protein synthesis was induced by the addition of 0.5 mM of IPTG at  $A_{600} \sim 0.4$  and cells were harvested at  $A_{600}$ ~ 1.0. The cells were re-suspended into Tris, 400mMNaCl, 30mM immidazole, 10mM BME, pH8.0.

Cell were sonicated and centrifuged at 50,000×g using a JA-25.50 rotor (Beckman). The supernatant was loaded on a Ni-column pre-equilibrated with buffer containing 50mM Tris, 400mMNaCl, 30mM immidazole, 10mM BME with a flow rate of 0.7/ml/min, followed by two- column volume wash with 50mM Tris, 1MNaCl, 30mM immidazole, 10mM BME, pH8.0 (high-salt buffer). In next step column was washed with two column volume of low salt buffer, i.e. 50mM Tris, 400mMNaCl, 30mM immidazole, 10mM BME, pH8.0. Finally the protein was eluted with buffer containing 50mM Tris, 140mM NaCl, 400mM immidazole, 10mM BME with a pH8.0 (**Figure6.4**).



**Figure 6.4** Purification of CypA on Ni-column. Column1- 1-elution of cypA with 400 mM imidazole, 2- diluted fraction of column 1, 3-washing with high salt 1M NaCl, 30mMimidazole, 4- loading with 30mM imidazole with 150mM NaCl.

The eluted protein was then exchange into buffer containig 140mM NaCl, 50mM KPi, pH6.5, 1mM BME, 5% glycerol using an amicon stirred cell. Later on, the sample was concentrated and applied to the Superdex-75 size exclusion column, previously equilibrated with the same buffer. Finally the protein was exchanged into desired salt buffer. Protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 8470 M<sup>-1</sup> cm<sup>-1</sup>.

#### 6.4. c-Abl Protein Preperation and purification

The Abl Kinase domain (229-551) was cloned into a modified Pet16b vector that contains a tobacco etch virus (TEV) protease cleavable N-terminal hexahistidine tag followed by a MBP solubility tag. The gene was cloned into vector using NdeI and XhoI sites. This vector was transformed into Escherichia coli BL21(DE3) that had been already transformed with a vector containing the YopH phosphatase and pREP4-groESL followed by plating on LB plate. A single colony was picked from the plate and was transferred into 5 ml of autoclaved LB media. After ~ 8hour of growing, the cells were

were transferred to 50ml LB. Before transferring, the cells was centrifuged and washed with fresh LB media and kept overnight for growing at 37 °C. Then the cells were transferred to 1L culture of LB. The cells were grown at 37 °C to an  $OD_{600}$  of 0.6. The temperature was then lowered to 16 °C and induced with 0.5mM IPTG.

After 18hrs the cells were harvested. The cell pellet was resuspended in lysis buffer containing 50mM Tris, 500mM NaCL, 5% glycerol and 5mM beta-mercaptoethanol, pH8.0. Cells were lysed and purified using standard nickel affinity purification protocol as described for CypA.

The eluted protein was then extensively dialyzed into tev cleavage buffer (50mM Tris, 150mM NaCl, 0.5mM EDTA, 2mM BME with a pH8.0 and incubated with tev protease for overnight (1  $OD_{280}$  of TEV protease per 100  $OD_{280}$  of substrate) at 4°C. Next morning SDS-page was use to determine complete cleavage followed by exchanging the protein solution back to loading buffer containing 50mM Tris, 150mM NaCl, 3mM immidazole, 2mM BME, and pH8.0. Cleaved protein solution was then loaded on a pre-equilabred Ni-column and the flow through was collected which contain the cleaved protein.

The eluted protein was then exchange into buffer containig 100mM NaCl, 25mM NaPi, pH6.5, 0.5mM EDTA, 1mM BME, 5% glycerol using an amicon stirred cell. Later on, the sample was concentrated and applied to the Superdex-75 size exclusion column, previously equilibrated with the same buffer. Finally the protein was exchanged into desired salt buffer. The protein eluted as a monomer and there was no aggregation.

### 6.5. Polymerase chain reaction (PCR).

To produce the single mutants of Crk, the QuikChange XL Site-Directed Mutagenesis Kit (from Stratagene) was used.

For the following mutants, F239A, P238A, P232A, L231G, the Crk plasmid was used as a template for PCR.

The following steps were used for PCR site directed mutagenesis:

1) Wild-type plasmid was isolated by using Qiagen kit

2) Plasmid template DNA (approximately 0.5 pmol) and 12-20 pmol of forward and reverse primers were then added to a PCR mix (from Strategene site directed mutagenesis kit) containing mutagenesis buffer: (20 mM Tris-HCl, pH 7.5; 8 mM MgCl<sub>2</sub>; 40 μg/mL BSA); dNTP, *Pfu* DNA polymerase.

3) PCR was performed using the following cycle:

Initialization	Denaturation	Annealing	Elongation	Final elongation	Final hold
95 ⁰C	95 ⁰C	60 °C	68 °C	68 <sup>0</sup> C	4 °C
1 min	50 sec	50 sec	7 min	7 min	

4) *DpnI* digestion was performed to remove parental (wild type) methylated DNA. The reaction mixture was incubated at 37°C for 2 h 30 min.

5) The plasmid containing the point mutation was transformed into BL21(DE3) competent cells and plated. Clonies were picked, plasmide were isolated and sequenced.

# 6.6. NMR Spectroscopy

All NMR experiments were performed on Varian 800- and 600-MHz and Bruker 700and 600-MHz spectrometer. Sequential assignment of the <sup>1</sup>H, <sup>13</sup>C, and <sup>15</sup>N protein backbone

chemical shifts was achieved by means of through-bond heteronuclear scalar correlations using the following 3D pulse sequences: 3D HNCO, 3D HN(CA)CO, 3D HNCA, 3D HN(CO)CA, 3D HNCACB, and 3D HN(CO)CACB. Side-chain assignment was performed using 3D C(CO)NH and 3D H(CCO)NH spectra. NOEs were assigned and collected on the basis of 3D <sup>15</sup>N-NOESY-HSQC and <sup>13</sup>C-NOESYHSQC spectra using mixing time of 100 and 80ms respectively. All NMR samples were prepared in 50 mM KPi (pH 5.5), 140 mM potassium phosphate, 1 mM BME and 1g L<sup>-1</sup> NaN<sub>3</sub>. Concentration of all protein NMR sample was 0.6-0.8 mM. Spectra were recorded at 22 °C. 2D <sup>1</sup>H-<sup>15</sup>N HSQC spectra were recorded for all constructs in different concentrations, pH, and temperatures too. All spectra were processed using the NMRPipe software package and analyzed with NMRView.

For all the experiment using CypA and c-Abl we use similar buffer condition with pH6.5 and temperature 25°C.

# 6.6.1. 2D <sup>1</sup>H-<sup>15</sup>N Heteronuclear (ZZ NMR experiments)



Figure 6.5 Schematic representation of CypA catalyzed *cis-trans* isomerization.

The 2D <sup>1</sup>H-<sup>15</sup>N heteronuclear (ZZ) NMR exchange experiments (Farrow et al., 1994) were used to measure the rate of CypA-catalyzed *cis-trans* isomerization ( $k_{ex}$ ) of Gly237-Pro238. This pulse sequence allows observation of the transfer of <sup>15</sup>N-labeled heteronuclear zz-magnetization between the two conformations. The rate constant of *cis-trans* interconversion in the presence of CypA, can be determined by measuring the intensity of the exchange and auto peaks as a function of the mixing time and then to fit to the equations described below (Bosco and Kern, 2004) :

$$I_{tt}(\tau_{m}) = I_{t}(0)[(-(\lambda_{2} - a_{11}) e(-\lambda_{1}\tau_{m}) + (\lambda_{1} - a_{11}) e(-\lambda_{2}\tau_{m})/(\lambda_{1} - \lambda_{2})] (a)$$

$$I_{tc}(\tau_{m}) = I_{t}(0)[(a_{21} e(-\lambda_{1}\tau_{m}) - a_{21} e(-\lambda_{2}\tau_{m})/(\lambda_{1} - \lambda_{2})] (b)$$

In eq a and b,  $I_{tt}$  is the intensity of the X trans of X-P auto peak and  $I_{tc}$  is the intensity of the *trans* exchange peak at mixing time  $\tau_m$ .  $I_t(0)$  is the intensity of the trans peak at  $\tau_m = 0.\lambda_{1,2} = \frac{1}{2}[(a_{11}+a_{22}) \pm [(a_{11}-a_{22})^2 + 4k_{tc}k_{ct}]^{1/2}]$ ,  $a_{11} = R_{1t} + k_{tc}$ ,  $a_{21} = -k_{tc}$  and  $a_{22}$ 

=  $R_{1c} + k_{ct}$ .  $R_{1c}$  and  $R_{1t}$  are the longitudinal relaxation rates for the *cis* and *trans* conformations of X of X-Pro bond which undergoes *cis-trans* isomerization, respectively. Because proline residues lack a backbone amide moiety, CypA-catalyzed *cis-trans* isomerization of X-Pro was measured from exchange curves for the X amide resonance.

The rate of CypA-catalyzed chemical exchange  $(k_{ex})$  in presence of substoichiometric amount of CypA is the sum of the individual rate constants in steps 2-4 in Scheme 1 as shown in eq 2.  $p_c$  and  $p_t$  denotes populations of X in the *cis* and *trans* conformation, respectively, and were determined from the relative intensities of the *trans* and *cis* resonances of X in an HSQC spectrum. The equilibrium constant  $(K_{eq})$  for the *cis* and *trans* conformations can be determined by eq 1. From eq 1, the individual rate constants  $k_{ct}$  and  $k_{tc}$  are simplified to  $p_t kex$  and  $p_c k_{ex}$ , respectively, in eqs 1a and b. Fitting the exchange curves with eqs a and b results in the rate of CypA-catalyzed X-P *cis-trans* isomerization  $(k_{ex})$ (Bosco and Kern, 2004).

$$K_{\rm eq} = \mathbf{p}_{\rm t}/\mathbf{pc} = k_{\rm ct}/k_{\rm tc} \tag{1}$$

#### 6.6.1. Residual Dipolar Coupling (RDC) Measurements.

Alignment of the proteins for RDC measurements was achieved using poly(ethylene glycol)/alcohol mixtures1. A 5%  $C_{12}E_5$ /hexanol (molar ratio=0.96) mixture was prepared.  $C_{12}E_5$  was used to a final concentration of 5% (w/w) in 90% H2O:10% D2O solution. The pH was adjusted using sodium hydroxide. The amount of hexanol was added dropwise, while vigorously shaking, to a final molar ratio  $C_{12}E_5$ :hexanol of 0.96. Air

bubbles were removed by centrifugation at  $5,000 \times g$  for few minutes. The HDO quadrupolar deuterium splitting was checked to confirm the presence of the crystalline phase (a splitting of ~25 Hz was observed). For the measurement of RDCs in the protein, 250 µl of the C<sub>12</sub>E<sub>5</sub>: hexanol stock solution was added into 50 µl of protein in buffer. <sup>15</sup>N-HSQC (IPAP) and HNCO based experiments were used to measure one-bond N-H and C $\alpha$ C' RDCs(Yao et al., 2009). The alignment tensor was determined as described (Clore et al., 1998b).

In order to compare the accuracy of experimentally determined RDC values, the back calculated values were plotted versus the measured ones. The program MODULE was used to properly define the aligment tensor parameters from residual dipolar couplings. The perfect agreement between experimental and calculated RDC values signifies the accuracy and overall quality of our final structure.

# 6.7. Structure calculation

Structure calculations were performed with Xplor-NIH46. The <sup>13</sup>C $\alpha$ , <sup>13</sup>C $\beta$ , <sup>13</sup>C', H $\alpha$ , <sup>15</sup>N and NH chemical shifts served as input for the TALOS program(Cornilescu et al., 1999) to extract dihedral ( $\varphi$  and  $\psi$ ) angles. Distance restraints derived from the NOESY spectra of protonated sample (using 80 and 100ms mixing time for <sup>13</sup>C-NOESYHSQC and <sup>15</sup>N-NOESY-HSQC, respectively) were categorized in 3 bins with upper-bound distances of 2.8, 3.5, and 5.0 Å. RDC restraints were included in the final stages of the calculation. The initial structures were then used as a reference for the assignment of additional NOE cross peaks, particularly from the side chains. For the structure calculations, a simulated annealing (SA) protocol consisting of four stages was used using both torsion angle dynamics (TAD) and Cartesian dynamics. (i) The high temperature TAD stage consisted

of 10,000 steps at 10,000 K. This was followed by (ii) an 8000 steps TAD cooling stage with a final temperature of 2000 K, (iii) a 5000 steps first Cartesian cooling stage to 1000 K, and (iv) a 10,000 steps second Cartesian cooling stage to 50 K. RDC restraints were introduced. During the SA protocol, the force constants for the TAD stages, first Cartesian cooling stage and second Cartesian cooling stage were set to 10, 50 and 50 kcal\*mol<sup>-1</sup>\*Å<sup>-1</sup> for the NOE restraints and to 50, 100 and 200 kcal\*mol<sup>-1</sup>\*rad<sup>-2</sup> for the dihedral restraints, respectively. In the final calculations, the force constants RDC restraints were increased in concert with the other energy terms to ensure a proper minimization. The best 20 structures in terms of restraint energies were selected for analysis with PSVS (Protein Structure Validation Suite) and PROCHECK(Laskowski et al., 1996).

	L-SH3 <sup>c</sup> trans	L-SH3 <sup>c</sup> cis	Crk <sup>sLs</sup>
NMR Restraints			
NOEs	1523	1557	3094
Intra-residue	328	333	760
Inter-residue			
Sequential ( i-j =1)	375	380	752
Medium range (1< i-j <3)	187	194	368
Long range ( i-j >4)	633	650	1214
Hydrogen bonds	22	22	46
Dihedral Angle Restraints			
φ and ψ	104	97	174
Residual Dipolar Couplings			
<sup>1</sup> D <sub>HN</sub>			90

# NMR and Refinement Statistics for Crk structures

Structure Statistics (20 structures)

Violations (mean and SD)				
Distance restraints (Å)	0.06±0.008	0.08±0.014	0.07±0.010	
Dihedral angle restraints (°)	2.15±0.241	3.03±0.369	5.20±0.542	
Residual dipolar couplings (Hz)				
HN			1.00±0.023	
C′Cα			1.20±0.043	
Deviations from idealized geometry				
Bond lengths (Å)	0.012	0.013	0.013	
Bond angles (°)	1.2	1.5	1.5	
Impropers (°)	2.3	2.2	2.4	
Ramachandran plot statistics (%)				
Residues in most favored regions	71	66	65	
Residues in additionally allowed regions	26	32	29	
Residues in generously allowed regions	3	2	6	

	L-SH3 <sup>c</sup> trans	L-SH3 <sup>c</sup> cis	Crk <sup>sLs</sup>
Residues in disallowed regions	0	0	0
Average pairwise rmsd (Å)			
Backbone	0.4	0.3	0.7
Heavy atoms	0.7	0.5	1.2

## 6.8. Relaxation measurements and analysis.

Three relaxation parameters were measured for all backbone amides of Crk proteins: the  ${}^{1}\text{H}{-}^{15}\text{N}$  NOE, the longitudinal relaxation rate R<sub>1</sub> and the transverse relaxation rate R<sub>2</sub>(Tzeng and Kalodimos, 2009).

Three relaxation parameters were measured for all backbone amides of  $SH3^{N}-L-SH3^{C}$  and  $SH3^{C}$ -fl in *cis* and trans the <sup>1</sup>H-<sup>15</sup>N NOE, the longitudinal relaxation rate R<sub>1</sub> and the transverse relaxation rate R<sub>2</sub>. <sup>15</sup>N R<sub>1</sub> values were measured from 2D spectra recorded with relaxation delays 100, 200, 300, 400, 500,600, 700, 800, 1000, 1200 and 1400 ms; <sup>15</sup>N R<sub>2</sub> values were measured from 2D spectra recorded with relaxation delays 7.7, 23.2, 31.0, 46.6, 54.3, 62.1, 77.6, 85.4, 93.2, 100, 107.8 and 115.5 ms. Data sets were acquired as 256 x 1,024 complex points in the t1 x t2 time-domain dimensions. Data points were fitted as a function of the length of the parametric relaxation delay to two-parameter decay curves of the form I(t)=I0e<sup>-Rt</sup>, where I is the intensity of the magnetization. <sup>1</sup>H-<sup>15</sup>N NOE data were obtained by recording, in an interleaved manner, one spectrum with a 4-s recycle delay followed by a 4-s saturation and another spectrum with no saturation and a 8-s recycle delay.

ModelFree (Palmer, A. <a href="http://www.palmer.hs.columbia.edu/software/modelfree.html">http://www.palmer.hs.columbia.edu/software/modelfree.html</a>) was used to optimize the fit of both internal dynamics and global tumbling parameters using the model-free approach(Lipari and Szabo, 1982). Initial estimates for the rotational diffusion tensor were obtained from the ratio of longitudinal and transverse relaxation rates(Tjandra et al., 1995). Residues with <sup>1</sup>H-<sup>15</sup>N NOE value less than 0.65 or with R<sub>1</sub> or R<sub>2</sub> values exceeding one standard deviation from the mean and residues experiencing R<sub>ex</sub> contributions were excluded from the fitting(Hwang et al., 2001).

Overall correlation times and rotational diffusion tensors for isotropic, axially symmetric, and fully anisotropic models were estimated from the R<sub>2</sub>/R<sub>1</sub> ratios of the remaining backbone amide groups and the structures of Crk proteins using the programs quadric\_diffusion (http://www.palmer.hs.columbia.edu/software/ quadric.html) and TENSOR(Dosset et al., 2000). The most appropriate diffusion tensor was selected by a comparison of  $\chi^2$  goodness-of-fit parameters and using F-statistical analysis. Using these rotational diffusion tensors, backbone relaxation data were fit to the five standard Lipari-Szabo model-free formalism models(Mandel et al., 1995). The fitted dynamics parameters for each model are as follows: model 1, order parameter (S<sup>2</sup>); model 2, S<sup>2</sup> and internal correlation time ( $\tau_e$ ); model 3, S<sup>2</sup> and Rex; model 4, S<sup>2</sup>,  $\tau e$ , and Rex; model 5, order parameters for two time scales (S<sub>f</sub><sup>2</sup> and S<sub>s</sub><sup>2</sup>) and  $\tau_e$  for the slower time scale.

Parameters of the model-free formalism were optimized for each residue individually, and the best parameter set identified by model selection. All parameters, including the diffusion tensor, were then optimized. This process was repeated until the solution converged. The quality of the fits between the experimental data and each model were calculated as  $\chi^2$  statistics, and the different models were then compared to each other using F statistics. For both the *cis* and *trans* forms of LSH3<sup>C</sup> ( $\tau_c \sim 6.2$  ns) an axially symmetric diffusion tensor was optimal, with  $D_{II}/D_{\perp} \sim 1.23$  and ~1.25, respectively.

# 6.9. Isothermal titration calorimetry (ITC) experiments.

Calorimetric titrations of the C3G PPII peptide (N-DNSPPPALPPKKRQSAPS-C) with isolated SH3<sup>N</sup> and SH3<sup>N</sup>-fl-SH3<sup>C</sup> were performed on a VP-ITC microcalorimeter (Microcal). Protein samples were extensively dialyzed against the ITC buffer containing 50 mM KPi (pH 5.5), 140 mM NaCl, and 1 mM TCEP. All solutions were filtered using

membrane filters (pore size, 0.45 mm) and thoroughly degassed for 20 min by gentle stirring under vacuum. The 1.35 sample cell was filled with a 50 mM solution of protein, and the 250  $\mu$ l injection syringe was filled with 0.5mM titrating peptide. The ligand solution was prepared by dissolving peptide in the flowthrough of the last buffer exchange. Each titration typically consisted of a preliminary 2  $\mu$ l injection followed by 15 subsequent 16  $\mu$ l injections. Data for the preliminary injection, which are affected by diffusion of the solution from and into the injection syringe during the initial equilibration period, were discarded. Binding isotherms were generated by plotting heats of reaction normalized by the modes of injectant versus the ratio of total injectant to total protein per injection. The data were fitted using Origin 7.0 (Microcal).

Calorimetric titrations of Abl-PxxP (aa 50-535) with  $SH3^{N}$ -fl- $SH3^{C}$ ,  $SH3^{N}$ -fl- $SH3^{C}$ F239A and  $SH3^{N}$  were also performed on an iTC200 microcalorimeter (Microcal). Protein samples were extensively dialyzed against the ITC buffer containing 50 mM KPi (pH 7.0), 140 mM NaCl, and 1 mM TCEP. All solutions were filtered using membrane filters (pore size, 0.45 mm) and thoroughly degassed for 20 min by gentle stirring under vacuum. The sample cell was filled with a 50  $\mu$ M solution of protein, and the injection syringe was filled with 0.5  $\mu$ M titrating peptide. The ligand solution was prepared by dissolving peptide in the flowthrough of the last buffer exchange. Each titration typically consisted of a preliminary injection followed by 15-20 subsequent injections. Data for the preliminary injection, which are affected by diffusion of the solution from and into the injection syringe during the initial equilibration period, were discarded. Binding isotherms were generated by plotting heats of reaction normalized by the modes of injectant versus the ratio of total injectant to total protein per injection. The data were fitted using Origin 7.0 (Microcal).

#### 6.10. Western Blot

We performed western blot by using purified protein. For detection of phophorylated Tyr222 we use primary antibody, phospho-CrkII (Tyr222) antibody from Cell Signaling. We make ~6-7 reaction mixture depending on our need. All reactions are carried out at room temperature and all proteins are exchanged into the same buffer condition (50mM KPi, 140mM NaCl, 1mM BME, pH6.5). First we aliquot SH3<sup>C</sup>-hl P238A into all the ependroff and then in excess (~6-7 times) add CypA for complete saturation of SH<sup>C</sup>-hl P238A. In another ependroff we make c-Abl reaction mixture. First we add c-Abl and then to it we add 1mM MgCl<sub>2</sub>, 0.6mM ATP and immediately add it to the SH3<sup>C</sup>-hl P238A sample, followed by collection of sample at different interval of time and stopping the reaction by using SDS-loading buffer.

All the samples were run on a 10% SDS gel. In the next step we transfer proteins from SDS-gel to nitro-cellulose membrane using gel-electrophoresis [transfer buffer: 700ml H<sub>2</sub>O, 100ml 10X transfer buffer (30.4g Tris, 144.1g glycine),200ml MeOH]. Then we place the nitrocellulose membrane into blocking solution (5% non-fat dry milk in 1X TBST buffer and 0.2% tween). 1X TBST buffer is made from 10X TBST buffer i.e. trisbase 24.2g/L, NACl 80g/L and pH7.5. Then we wash for 2-3 times with 1X TBST buffer. Then we incubate the membrane in primar antibody solution (substrate to antibody ratio; 1:1000) for overnight. Next morning we wash out exces of primary antibody by using 1X TBST buffer for 3-4 times. We then incubate it with secondry antibody in blocking solution for firther one more hour. Secondry antibody was use for detection purpose. In

our case we use secoundary antibody from goat with horseradish peroxidase label. We use Pierce ECL Western Blotting Substrate for the detection of horseradish peroxidase (HRP) on immunoblots. Finally, the blots were exposed to X-ray film to obtain the results. Blots were analyzed using Image J software.

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## **Curriculam Vitae**

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