Domain organization differences explain Bcr-Abl’s preference for CrkL over CrkII

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Abstract

CrkL is a key signaling protein that mediates the leukemogenic activity of Bcr-Abl. CrkL is thought to adopt a structure that is similar to that of its CrkII homolog. The two proteins share high sequence identity and indistinguishable ligand binding preferences; yet they have distinct physiological roles. Here we show that the structures of CrkL and phosphorylated CrkL are drastically different than the corresponding structures of CrkII. As a result, the binding activities of the SH2 and SH3 domains in the two proteins are regulated in a distinct manner and to a different extent. The different structural architecture of CrkL and CrkII may account for their distinct functional roles. The data show that CrkL forms a constitutive complex with Abl thus explaining the strong preference of Bcr-Abl for CrkL. The results also highlight how the structural organization of the modular domains in adaptor proteins can control signaling outcome.

INTRODUCTION

The Crk family of adaptor proteins are important signaling molecules that function downstream of a wide number of receptors and regulate important cellular processes, including cell adhesion, motility, phagocytosis, differentiation, proliferation, transformation and apoptosis1–3. Crk proteins are implicated in many human cancers, including lung adenocarcinoma and glioblastoma4, prostate5, ovarian6, gastric7, and breast cancer8. The Crk family consists of two alternatively spliced protein forms, CrkL and CrkII, expressed by a single gene (crk)9 and the Crk-like (CrkL) protein expressed by a distinct gene (crkl)10.
The CrkII (304 residues) and CrkL (303 residues) proteins each consists of a single SH2 domain, an N-terminal SH3 (SH3\textsuperscript{N}) domain and a C-terminal SH3 domain (SH3\textsuperscript{C}) (Supplementary Results, Supplementary Fig. 1). The SH3\textsuperscript{N} and SH3\textsuperscript{C} domains are tethered by a ~50 residue–long linker, which contains a tyrosine residue (Tyr221 in CrkII, Tyr207 in CrkL) that is phosphorylated by the Abl kinase\textsuperscript{11,12}.

Crk proteins link activated receptors to specific downstream signaling cascades using their SH2 and SH3\textsuperscript{N} domains, which selectively bind to pY-x-x-P and P-x-L-P-x-K motifs, respectively\textsuperscript{13,14}. A large variety of proteins have been identified as binding partners of Crk proteins\textsuperscript{1,15}. In contrast, the SH3\textsuperscript{C} domain lacks the binding determinants of typical polyproline (PP)-II binding SH3 domains\textsuperscript{16} and was shown to function as an autoregulatory element in CrkII\textsuperscript{17–19} or even to promote certain signaling pathways\textsuperscript{20}.

CrkL has received particular attention primarily because it is a preferred substrate of Bcr-Abl\textsuperscript{21,22}, an oncogenic kinase that causes chronic myelogenous leukemia (CML)\textsuperscript{23}. CrkL is indispensable for mediating the aberrant activity of Bcr-Abl\textsuperscript{24,25} and is constitutively phosphorylated in human CML cells\textsuperscript{26,27}. In fact, the level of CrkL phosphorylation is being used as a predictor of clinical outcome in patients treated for CML\textsuperscript{28}. In addition to the relevance of CrkL to cancer, deletion of the \textit{crkl} gene causes cardiovascular and craniofacial defects resulting in embryonic lethality\textsuperscript{29}. Interestingly, CrkL appears to be a rather unique adaptor protein since it has been reported to function in the cytoplasm, the nucleus and the extracellular milieu\textsuperscript{5}.

Although CrkL and CrkII have been shown to compensate for each other in certain cases\textsuperscript{1}, numerous studies have demonstrated that the two Crk proteins have distinct, non-overlapping functions\textsuperscript{1,29–31}. Because of the high sequence identity between CrkL and CrkII (56%, up to 72% in the modular domains; Supplementary Fig. 1a) it is hypothesized that the two proteins adopt very similar structures. Taking also into account the identical binding preferences of their SH2 and SH3\textsuperscript{N} domains, it has been difficult to account for the different functional roles and signaling output of the CrkL and CrkII proteins.

Here we report the structure of CrkL in its unliganded and unphosphorylated state as well as in the phosphorylated (pTyr207) form (pCrkL). The data show that the SH2 and SH3 modular domains in CrkL are organized in a drastically different architecture than in CrkII\textsuperscript{18,19}; (i) the pTyr-binding site of SH2 is partially masked in CrkL whereas it is accessible in CrkII; (ii) the PPII-binding site of SH3\textsuperscript{N} is accessible in CrkL but is completely occluded in CrkII; and, (iii) the SH3\textsuperscript{C} domain is mobile and does not interact with any of the other domains in CrkL, whereas it interacts extensively with the SH2 and SH3\textsuperscript{N} domains in CrkII thereby stabilizing its overall structure. We also show that upon Tyr207 phosphorylation, the linker region interacts in an intramolecular fashion with the CrkL SH2 domain, thereby inhibiting the binding of pTyr-ligands. Very interestingly, this intramolecular association has little effect on the overall structure of CrkL, in sharp contrast to CrkII wherein phosphorylation of Tyr211 results in SH3\textsuperscript{N} autoinhibition. Notably, the present data demonstrate that CrkL forms a constitutive complex with Abl. Thus, despite the very high sequence identity of CrkL and CrkII, the structural organization of the two
adaptors is drastically different and may account for their different functional roles, as well as the preference of Bcr-Abl to interact with CrkL rather than CrkII.

RESULTS

Structure determination of CrkL and pCrkL

Human CrkL (~34 kDa) consists of 303 residues. Gel filtration and multi-angle laser light scattering (MALLS) data show that the protein exists as a monomer in solution (Supplementary Fig. 2). Despite the relatively large size of CrkL, the NMR spectra are of outstanding sensitivity and resolution (Supplementary Fig. 3a). Assignment of CrkL was facilitated by preparing and assigning the isolated SH2 and SH3 modular domains (Supplementary Fig. 4a). Interestingly, overlay of the NMR spectra of the isolated domains with that of full-length CrkL revealed significant chemical shift differences only for a relatively small number of residues, which are located at the SH2 and SH3N domains, while no differences were observed for the SH3C domain (Supplementary Fig. 4b, c). The phosphorylated (pTyr207) CrkL (pCrkL) sample for NMR and structural characterization was prepared by adding catalytic amounts of the Abl kinase as detailed in Supplementary Methods (Supplementary Fig. 2d). Similarly to CrkL, the spectra of pCrkL are also of high quality allowing for complete backbone and side chain assignment (Supplementary Fig. 3b). The structures of both CrkL and pCrkL were determined by combining NOE, paramagnetic relaxation enhancement (PRE) and residual dipolar coupling (RDC) restraints (Supplementary Table 1).

Structural architecture of CrkL

The lowest-energy structure of CrkL is shown in Figure 1a and the conformational ensemble is shown in Supplementary Figure 5a. The structures of the CrkL individual SH2 and SH3 domains are very similar to the corresponding domains of the CrkII protein (Supplementary Fig. 5). As expected on the basis of sequence conservation (Supplementary Fig. 1a), the binding pockets of SH2 and SH3N are almost identical in CrkL and CrkII (Supplementary Fig. 6), thus accounting for the same recognition preferences of CrkL and CrkII. The CrkII SH2 domain has a 17-residue-long insertion between β-strands D and E that forms a flexible loop (DE loop; Supplementary Fig. 6a). The DE loop is enriched in proline residues and has been shown to bind to the SH3 domain of the Abl kinase. This loop is absent in CrkL SH2 (Supplementary Figs. 1a, 6a) and thus the binding mode of CrkL and CrkII to Abl is expected to be different. In agreement with previous studies, the structural data show that the CrkL SH3C domain cannot bind polyproline II (PPII)–type sequences because of the lack of aromatic residues at its canonical binding site.

The structural organization of CrkL is rather unique within the family of adaptor proteins: the SH2 and the first SH3 domain (SH3N) interact directly with each other, whereas the second SH3 domain (SH3C) does not interact with any of the modular domains (Fig. 1a). In agreement with the chemical shift analysis, the SH2–SH3N interaction is mediated by a small surface burying ~600 Å². The binding surfaces on both SH2 and SH3N domains consist primarily of polar and charged residues and thus the interaction is mediated almost
exclusively by polar contacts (Fig. 1b). Substitution of residues located at the SH2–SH3\textsuperscript{N} interface disrupts the interaction between the two domains in CrkL (Supplementary Fig. 4c).

**Dynamic properties of CrkL**

In order to determine the motional properties of CrkL we used NMR relaxation methodologies\textsuperscript{34}. We measured the \textsuperscript{1}H–\textsuperscript{15}N nuclear Overhauser effect (NOE), the longitudinal relaxation rate \( R_1 \) and the transverse relaxation rate \( R_2 \) (Fig. 1c and Supplementary Fig. 7a). The \( R_1 \) and \( R_2 \) relaxation rates are sensitive to and thus report on changes of the diffusion properties of the protein\textsuperscript{34}. If only residues located at rigid parts of the molecule are considered, then the ratio \( R_2/R_1 \) provides a good estimate of the correlation time\textsuperscript{35}. The relaxation analysis demonstrates that the SH2 and SH3\textsuperscript{N} domains tumble as a unit, with a correlation time \( \tau_c \) of \( \sim 11.0 \) ns, whereas the SH3\textsuperscript{C} domain tumbles much faster and in an independent fashion with a \( \tau_c \) of \( \sim 7.8 \) ns (Fig. 1d). Therefore, the relaxation data are in agreement with the structural data (Fig. 1a) showing that the SH2 and SH3\textsuperscript{N} domains interact with each other while the SH3\textsuperscript{C} domain does not interact with any of the other domains. The present results indicate that SH3\textsuperscript{C} play no role in regulating the activity of the SH2 and SH3\textsuperscript{N} domains in CrkL, in sharp contrast to CrkII proteins wherein SH3\textsuperscript{C} was shown to act as a regulatory structural element by stabilizing the autoinhibitory conformation\textsuperscript{18,19}.

Further analysis of the relaxation data demonstrates that most of the residues located at the SH2–SH3\textsuperscript{N} interface exhibit enhanced motions on the micro-to-millisecond (\( \mu \)-ms) timescale (Fig. 1e). This observation suggests that the two domains move relatively to each other giving rise to a dynamic binding interface.

**The binding site of the SH2 domain in CrkL is occluded**

Structural analysis of the SH2–SH3\textsuperscript{N} interface reveals that although the pTyr-binding site of the SH2 domain is largely accessible, the SH3\textsuperscript{N} domain masks the binding sites for the residues immediately downstream of the pTyr residue (Fig. 2). Thus, the structural data raise the intriguing possibility that the SH2–SH3\textsuperscript{N} intramolecular arrangement in CrkL may inhibit the binding activity of SH2 for phosphotyrosine ligands. To test this hypothesis, we used isothermal titration calorimetry (ITC) to directly measure the binding energetics of a phosphorylated peptide containing a consensus sequence for CrkL SH2 (CrkL-pTyr\textsuperscript{207}-peptide) to isolated SH2 and full-length CrkL (Supplementary Fig. 8a). The CrkL-pTyr\textsuperscript{207}-peptide binds to isolated SH2 with a moderate affinity (\( K_d \sim 7 \) \( \mu \)M), whereas the affinity for SH2 in the context of full-length CrkL is more than 3-fold weaker (\( K_d \sim 23 \) \( \mu \)M) (Fig. 2c). Thus, the intramolecular arrangement in CrkL gives rise to an autoinhibitory mechanism that partially occludes the SH2 domain thereby modulating its activity for phosphotyrosine ligands.

In contrast to CrkL, the SH2 domain in CrkII appears not to be inhibited (Fig. 2b)\textsuperscript{19}. Indeed, our ITC experiments show that a phosphorylated peptide containing a consensus sequence for CrkII SH2 (CrkII-pTyr\textsuperscript{221}-peptide) binds with the same affinity to both the isolated SH2 and full-length CrkII (Fig. 2c). It was previously proposed that the SH2 domain of CrkII and CrkL have different sequence specificity, with CrkL reported to bind a phosphorylated peptide...
peptide derived from the FGF receptor (FGFR-pTyr-peptide) with a ~30-fold higher affinity than CrkII. However, ITC data show that the intrinsic affinity of the isolated SH2 domains of CrkII and CrkL for phosphotyrosine ligands (Fig. 2c), even for the FGFR-pTyr-peptide (Supplementary Fig. 9a), is very similar and the two domains have identical specificity properties, as expected on the basis of the structures of the SH2-pTyr complexes (Supplementary Fig. 9b). Taken together, the present data show that any phosphotyrosine ligand will bind preferably to full-length CrkII over full-length CrkL by a factor of ~3–4 (Fig. 2c).

The binding site of the SH3N domain in CrkL is accessible

Structural analysis of CrkII proteins have shown that the canonical PPII-binding site of the SH3N domain is almost completely occluded (Fig. 2b). As a result, PPII ligands, such as the Abl kinase, bind to full-length CrkII with a ~10-fold lower affinity than in the isolated SH3N (Fig. 2c). In sharp contrast to CrkII, the present structural data demonstrate that the PPII-binding site of SH3N in CrkL is completely accessible (Fig. 2a). Indeed, ITC data (Supplementary Fig. 8b) show that a PPII-peptide containing the consensus sequence for SH3N binds to isolated SH3N and full-length CrkL with the same affinity (Fig. 2c). NMR analysis of the titration of the PPII-peptide to CrkL demonstrates that no steric clashes occur between the SH3N-bound peptide and the rest of the protein indicating that PPII-peptide binding to CrkL is unrestricted. In contrast, PPII-peptide titration to CrkII results in drastic conformational rearrangement and relieve of the autoinhibitory conformation. Taken together, our results show that a PPII ligand will bind preferably to CrkL over CrkII by a factor of ~10 (Fig. 2c).

CrkL Tyr207 phosphorylation results in SH2 inhibition

Tyr207 in CrkL is phosphorylated by the Abl kinase. The pTyr207-x-x-Pro210 region in the CrkL linker is a target sequence for CrkL SH2. NMR analysis of pCrkL (Supplementary Fig. 3b) shows that the CrkL SH2 domain interacts with pTyr207. MALLS and gel filtration data demonstrate that pCrkL remains a monomer in solution (Supplementary Fig. 2a, b). The data collectively show that upon phosphorylation of CrkL Tyr207, the SH2 domain interacts in an intramolecular fashion with pTyr207, similarly to CrkII.

We used NMR to determine the solution structure of pCrkL. In agreement with the NMR chemical shift analysis, the structural data show that the phosphorylated linker is bound to the canonical pTyr-binding cleft of the SH2 domain (Fig. 3). The interactions between the linker and the SH2 domain are virtually identical to those reported previously for the structure of CrkII SH2 and a phosphopeptide (Supplementary Fig. 9b). Interestingly, the structural rearrangement elicited to CrkL by the phosphorylation of Tyr207 and the ensuing intramolecular binding to SH2 is minimal. The SH2–SH3N interface adjusts slightly so as to accommodate the binding of the pTyr-linker region to SH2 (Fig. 3b and Supplementary Fig. 5c), whereas the SH3C domain tumbles independently (Fig. 3c) as in the unphosphorylated form.
The intramolecular binding of the phosphorylated linker (pTyr207) to the SH2 domain is expected to prevent SH2 from interacting with other phosphorylated ligands. To test this hypothesis we monitored by NMR the effect of phosphorylating CrkL that is already bound to a phosphorylated peptide encompassing the linker sequence (pTyr-linker; Fig. 4a). Indeed, the NMR data show that following the addition of catalytic amounts of Abl kinase domain (AblKD) the phosphorylated linker binds intramolecularly to SH2 and as a result the pTyr-linker is displaced (Fig. 4b). Pull-down of CrkL and pCrkL with Tyr-phosphorylated paxillin, a focal adhesion protein that interacts specifically with CrkL SH2, show that paxillin forms a complex only with the unphosphorylated CrkL (Fig. 4c). Collectively, the data show that Tyr207 phosphorylation of CrkL by Abl results in intramolecular binding of the linker with the SH2 domain thereby giving rise to CrkL SH2 inhibition.

**pCrkL interacts with signaling partners via SH3\textsuperscript{N}**

Upon Tyr phosphorylation, the SH2 domain in both CrkL (Fig. 4a–c) and CrkII\textsuperscript{11,39} are inhibited for pTyr-ligand binding. CrkII has been shown to undergo a major conformational change upon phosphorylation that results in PPII-ligand binding inhibition to SH3\textsuperscript{N19}. However, the structural rearrangement elicited by phosphorylation and the ensuing intramolecular binding in CrkL is minimal (Fig. 3a, b and Supplementary Fig. 5c). As a result, the PPII-binding site of the SH3\textsuperscript{N} domain in phosphorylated CrkL is completely accessible (Fig. 3b). In agreement with the structural data, ITC experiments (Supplementary Fig. 8b) show that a PPII-peptide binds to the unphosphorylated CrkL (or the isolated SH3\textsuperscript{N}) and pCrkL with very similar affinities (Fig. 3d). Pull-down of CrkL and pCrkL with DOCK1, a guanine exchange factor (GEF) that activates Rac1 and binds specifically to the SH3\textsuperscript{N} domain, shows that DOCK1 associates strongly with both CrkL and pCrkL (Fig. 3e).

Abl binds to the SH3\textsuperscript{N} domain of CrkL and CrkII using a consensus PxxP motif located C-terminally to its kinase domain\textsuperscript{40}. An important implication of SH3\textsuperscript{N} inhibition in CrkII is that Abl binding to and phosphorylation of CrkII will result in pCrkII–Abl complex dissociation\textsuperscript{41}. To test the emerging hypothesis that pCrkL will remain tightly bound to Abl, we titrated Abi\textsuperscript{PxxP}, an Abl construct encompassing the kinase domain and the first PxxP Crk-binding motif, to labeled CrkL (Fig. 4d). NMR analysis indicates that Abi\textsuperscript{PxxP} binds to the SH3\textsuperscript{N} domain of CrkL (Fig. 4e). Addition of ATP-Mg\textsuperscript{2+} results in Abl-mediated phosphorylation of Tyr207. NMR analysis (Fig. 4d) shows that pCrkL adopts the intramolecularly folded conformation but forms a tight complex with Abi\textsuperscript{PxxP} (Fig. 4e). In agreement, pull-down of CrkL and pCrkL with full-length Abl kinase shows that Abl forms complexes with CrkL that are not dependent on the CrkL phosphorylation state (Fig. 4f).

**DISCUSSION**

CrkL is an adaptor protein that regulates important cellular processes ranging from cell adhesion and motility to phagocytosis and apoptosis. CrkL has been recently identified as an essential gene in cancer cell proliferation\textsuperscript{25} and has been shown to be indispensable for mediating the leukemogenic activity of Bcr-Abl\textsuperscript{24,25}. CrkL is constitutively phosphorylated in human CML cells\textsuperscript{26,27} and the level of CrkL phosphorylation is being used as a predictor
of clinical outcome in patients treated for CML\textsuperscript{28}. Despite the important role of CrkL, the lack of its structure has impeded a proper understanding of its function.

Because CrkL shares high sequence identity with CrkII, it has been thought that the structures of the two proteins are very similar. The structural data reported here demonstrate that the structural organization of the two proteins is drastically different. The CrkII structure\textsuperscript{19} is stabilized by a hydrophobic segment in the linker region, part of which is quite different in CrkL (Supplementary Fig. 10). Moreover, several contacts exist between the SH2 DE-loop and SH3\textsuperscript{C} in CrkII. In contrast, the DE-loop is not present in CrkL SH2. The autoinhibited structure of pCrkII is stabilized by a linker region that is totally different in CrkL (Supplementary Fig. 10b). Thus, despite the high sequence identity (up to 72\% in the structured regions), few key sequence differences between CrkL and CrkII appear to modulate the overall structural organization of the two proteins (Supplementary Fig. 10). The distinct structural architectures of CrkL and CrkII determine the signaling input and output giving rise to distinct physiological functions for the two proteins. These results further highlight the notion that adaptors regulate signaling in a dynamic way and do not simply serve to wire signaling components in a passive manner\textsuperscript{42}.

In the resting state, the pTyr-binding site of the SH2 in CrkL is inhibited, whereas the one in CrkII is not and as a result the binding of phosphorylated ligands to CrkII will be favored over binding to CrkL (Fig. 2). The differential SH2 binding activity modulation in CrkL vs CrkII can have important implications, such as in the binding of the p130 Crk-associated substrate (p130CAS), a scaffold protein mediating integrin signaling (Fig. 5)\textsuperscript{3}. Although p130CAS has multiple phosphorylation sites, it is conceivable that the number of such sites may be limited either because of the action of phosphatases or cell conditions\textsuperscript{43}. In this case, competition will favor CrkII over CrkL binding to p130CAS (Fig. 5i).

Eventual association of CrkL/CrkII with p130CAS will have minimal effect on the overall structure of either CrkL (Figs. 1a and 3a) or CrkII\textsuperscript{19}. Therefore, the PPII-binding site of SH3\textsuperscript{N} in CrkII will be inhibited in the p130CAS-bound CrkII\textsuperscript{41}, whereas the corresponding site in CrkL will be accessible. As a result, guanine nucleotide exchange factors (GEFs), such as DOCK1 and C3G, will bind to CrkL with a much higher affinity than to CrkII (Figs. 2c and 5ii). In this case the CrkL-mediated complex is expected to activate more efficiently downstream GTPases giving rise to stronger signaling outcome than the CrkII-mediated complex (Fig. 5iii). Enhanced association between CrkL and DOCK1 will increase cell migration and invasion\textsuperscript{3} and may explain previous observations that CrkL has a much higher oncogenic potential than CrkII in fibroblasts\textsuperscript{21}.

In both CrkL and CrkII complexes, phosphorylation of Tyr207 (Fig. 3) and Tyr221\textsuperscript{11,19,39}, respectively, by Abl will cause intramolecular folding and displacement of the pCrk proteins from p130CAS (Fig. 4a–c), resulting in negative regulation of cell migration (Fig. 5iv)\textsuperscript{19}. Interestingly, pCrkL may form a constitutive complex with DOCK1, in contrast to pCrkII, since in pCrkL the SH3\textsuperscript{N} domain is not inhibited (Fig. 3e). Thus, when phosphatases act to dephosphorylate CrkL and CrkII, the p130CAS CrkL DOCK1 complex will assemble more readily than the corresponding CrkII complex.
A particularly striking result in the present work is that the phosphorylated form of CrkL remains active and may interact via its uninhibited SH3N domain with various ligands, including the Abl kinase (Fig. 4d–f). The interaction of CrkL with Bcr-Abl stimulates substantially the leukemogenic activity of the oncoprotein. The present data demonstrate that CrkL binds and remains tightly bound to Abl even after the kinase has phosphorylated CrkL (Fig. 4e, f), explaining why CrkL is a preferred substrate for Bcr-Abl. This is in sharp contrast to pCrkII, which has been shown to be an entirely inactive protein. Therefore, although the intrinsic affinity of the SH3N domains of CrkL and CrkII for the PxxP motif of Abl is almost identical (Fig. 2c), the overall structural organization of the proteins confers an advantage to CrkL since the binding of CrkII to Abl is suppressed.

Although the SH3C domain has been shown to function as an autoregulatory element in CrkII, in CrkL SH3C does not interact with any of the other domains and thus its role remains elusive. Functional data however indicate that CrkL SH3C is indispensable for fibroblast transformation and hematopoietic cell adhesion. It is likely that SH3C mediates its function by interacting with an as yet identified partner or by being tyrosine phosphorylated, as shown recently for CrkII.

SH2 and SH3 domains have long been thought to mediate sequence-specific interactions involving PxxP and pY motifs, respectively. However, there is now a growing list of examples indicating that these signaling domains can interact with sequences that do not conform to these general rules. Interestingly, our data demonstrate that the two domains can engage each other in a completely unconventional manner. SH2–SH3 interactions have been previously observed in Itk kinase and the SAP–Fyn complex. Comparison of the binding mode in these systems and in CrkL demonstrates the great versatility of the SH2 and SH3 domains in mediating interactions in cell signaling (Supplementary Fig. 11).

METHODS

Protein preparation
The following human CrkL constructs were prepared: SH2 (residues 1–105), SH3N (residues 120–188), linker-SH3C (residues 184–303), SH2-SH3N (residues 1–188), pTyr-linker (188–230), and full-length CrkL (residues 1–303). The constructs were cloned into the pet42a vector using the NcoI and XhoI restriction sites. A Tev protease cleavage site was introduced between the histidine tag and the protein. For NMR studies, the samples were dialyzed in NMR buffer (50 mM KPi (pH 6.8), 140 mM NaCl and 1 mM β-mercaptoethanol) and concentrated using Amicon cell units. All fragments are monomeric in solution at concentrations used for the NMR studies (typically 0.6–1.0 mM), as indicated by gel filtration and light scattering. Phosphorylated CrkL and pTyr-linker were prepared by the addition of catalytic amounts of AblKD. Phosphorylation efficiency is over 99% as judged by mass spectrometry and NMR. The sequence of the CrkL-pTyr207-, CrkII-pTyr221-, PPII- and FGFR-pTyr-peptides used are EPAHApYAQPTT, PEPGPpYAQPSV, YLQAPELPTKTRTS, and AGVSEpYELPEDPR, respectively. The MS-determined molecular mass of the peptides were 1393.1, 1221.2, 1605.3, and 1541.4, respectively. All peptides were >99% pure. More details about the preparation of AblKD, AblKD-PxxP and NMR samples are provided in Supplementary Methods.
**NMR spectroscopy**

NMR experiments were performed on Varian 800- and 600-MHz and Bruker 700- and 600-MHz spectrometers. Complete backbone and side chain assignment for the CrkL proteins studied was achieved using standard triple-resonance pulse sequences. NOEs were measured using two- and three-dimensional $^{13}\text{C}$ and $^{15}\text{N}$ edited NOESY experiments using mixing times of 80 and 100 ms, respectively. All experiments were performed at 32 °C.

**Paramagnetic Relaxation Enhancement Measurements**

Nitroxide spin labels (MTSL; Toronto Research Chemicals Inc.) were introduced via cysteine-specific modification of engineered CrkL derivatives containing single-solvent-accessible cysteine residues. Mutants and MTSL derivatives that did not perturb the CrkL structure, as assessed by $^1\text{H}$-$^{15}\text{N}$ HSQC spectra, were used for measuring PRE rates. PRE-derived distances were determined from $^1\text{H}$-$^{15}\text{N}$-HSQC spectra of CrkL by measuring peak intensities before (paramagnetic) and after (diamagnetic) reduction of the nitroxide spin label with ascorbic acid. More details are provided in Supplementary Information.

**Residual Dipolar Coupling (RDC) Measurements**

Alignment of the proteins for RDC measurements was achieved using poly(ethylene glycol)/alcohol mixtures. More experimental details are provided in Supplementary Information.

**Relaxation measurement and analysis**

Three relaxation parameters were measured for all backbone amides of CrkL: the $^1\text{H}$-$^{15}\text{N}$ NOE, the longitudinal relaxation rate $R_1$ and the transverse relaxation rate $R_2$. Experimental details about data collection and analysis are provided in Supplementary Information.

**Calorimetry**

All calorimetric titrations were performed on a iTC200 microcalorimeter (GE), as described in Supplementary Methods.

**Structure calculation and refinement**

Structure calculations were performed with Xplor-NIH. The $^{13}\text{C}_\alpha$, $^{13}\text{C}_β$, $^{13}\text{C}'$, $^2\text{H}_α$, $^{15}\text{N}$ and NH chemical shifts served as input for the TALOS+ program to extract dihedral ($\phi$ and $\psi$) angles. The initial structure was calculated using NOEs, PREs, dihedral angles and hydrogen bonds. RDC restraints were included in the final stages of the calculation. Ramachandran statistics are as follows: most favored regions: 90%; allowed regions: 8%; disallowed regions: 2%. The summary of NMR restraints and structure refinement statistics is presented in Supplementary Table 1.

**Pull-down assays**

Phosphorylated GST or GST-CrkL was obtained by incubating purified GST or GST-CrkL (~1μM) with purified AblKD (~0.1 μM) overnight at room temperature in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT and 5 mM ATP. Purified GST-CrkL was incubated in the same buffer without AblKD and ATP overnight for the unphosphorylated sample. Lactose-free glutathione agarose beads (Sigma) were then
washed once with PBS containing 0.1% Triton-X-100 and then incubated with the aforementioned samples for 60 minutes at 4°C. Beads were then spun down and washed twice with PBS containing 0.1% Triton-X-100. Beads with bound phosphorylated GST or phosphorylated/unphosphorylated GST-CrkL were each incubated with lysates of 293T cells untransfected or transfected with Flag-Paxillin or human Ab11b for 2 hours at 4°C. Beads were then spun down and washed twice with PBS containing 0.1% Triton-X-100. Samples were then boiled in SDS sample buffer and analyzed by western blotting with anti-Flag, anti-DOCK180, anti-Abl, anti-CrkL and anti-pCrkL(Y207) antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structural and dynamic properties of CrkL
(a) Structure of CrkL. The SH2, SH3$^N$ and SH3$^C$ domains are colored green, magenta, and blue, respectively. The linker regions are colored grey. The SH3$^C$ domain does not interact with the other domains. (b) Close-up view of the SH2–SH3$^N$ interface in CrkL. Only polar/charged residues mediate the interaction between the two domains. (c) Plot of the ratio of R$_2$ over R$_1$ $^{15}$N relaxation rates of the CrkL backbone as a function of residue number. The R$_2$/R$_1$ ratio provides information about the tumbling of the molecule with higher values indicating slower tumbling. (d) Correlation times ($\tau_C$) for the tumbling of CrkL. The SH2–SH3$^N$ module tumbles as a rigid unit whereas the SH3$^C$ domain tumbles much faster and independently of the other domains. (e) Residues undergoing substantial µs-ms time scale motions, as denoted by enhanced R$_{ex}$ values, are mapped on the structure of CrkL. Almost all residues located at the interface between the SH2 and SH3$^N$ domains exhibit relatively high R$_{ex}$ values indicating that the binding interface is dynamic.
Figure 2. Binding of pTyr- and PPII-peptide ligands to CrkL and CrkII

(a, b) Structure of the SH2–SH3N module in (a) CrkL and (b) CrkII. The pTyr-peptide and PPII-peptide are shown as they have been previously determined to bind to the isolated SH2 (PDB 1JU5) and SH3N domains (PDB 1CKA), respectively. The pTyr-peptide binding site in CrkL is partially masked but is completely accessible in CrkII. Conversely, the PPII-peptide binding site in CrkL is completely accessible but is entirely masked in CrkII. (c) Dissociation constants (K_d) of pTyr-peptide and PPII-peptide complexes with CrkL (Supplementary Fig. 8a) and CrkII. The K_d values of PPII-peptide binding to CrkII were obtained from ref. 19.
Figure 3. Structural and dynamic properties of pCrkL
(a) Structure of pCrkL. Phosphorylated Tyr207 (pTyr207) is shown as orange sticks. (b) Close-up view of the pTyr207-binding site. The SH2–SH3N interface adjusts slightly to accommodate the binding of the linker to SH2. (c) Plot of the ratio of $R_2$ over $R_1$ relaxation rates of pCrkL as a function of residue number. As in CrkL, the SH2–SH3N module in pCrkL tumbles as a unit whereas the SH3C domain tumbles much faster and independently of the other domains. (d) $K_d$ values of PPII-peptide complexes with CrkL (Supplementary Fig. 8a) and CrkII$^{19}$ variants. (e) Pull-down of CrkL and pCrkL with DOCK1, an SH3N binding physiological partner of CrkL (Supplementary Fig. 12).
Figure 4. Effect of Tyr207 phosphorylation on CrkL folding and its association with Abl kinase
(a) $^1$H-$^{15}$N HSQC NMR spectra of the linker region of CrkL containing the phosphorylated Tyr207 (pTyr-linker) in the presence of CrkL (orange) and after the addition of catalytic amounts of Abl$^{KD}$ and ATP-Mg$^{++}$ (blue). The pTyr-linker is $^{15}$N-labeled whereas CrkL and Abl$^{KD}$ are unlabeled. (b) Analysis of the NMR experiments in (a) shows that the pTyr-linker binds to the SH2 domain of CrkL. Phosphorylation of Tyr207 in CrkL induces the intramolecular association of pTyr207 and SH2. As a result, the pTyr-linker is displaced. (c) Pull-down of CrkL and pCrkL with paxillin, an SH2 binding physiological partner of CrkL (Supplementary Fig. 12). (d) $^1$H-$^{15}$N HSQC NMR spectra of free CrkL (blue), in complex with Abl$^{PxxP}$ (orange) and after adding ATP-Mg$^{++}$ (magenta). Abl$^{PxxP}$ is a construct that encompasses the kinase domain and the first PxxP motif that binds to CrkL. (e) Analysis of the NMR experiments in (d) shows that CrkL forms a 1:1 complex with Abl$^{PxxP}$ using its SH3$^N$ domain. Phosphorylation of Tyr207 elicits the intramolecular association of pTyr207 and SH2 but the intramolecular folding in CrkL has no effect on the CrkL–Abl$^{PxxP}$ complex, which remains tightly associated. (f) Pull-down of CrkL and pCrkL with full-length Abl (form 1b) (Supplementary Fig. 12).
Figure 5. CrkL versus CrkII in integrin signaling
(i) Integrin activation elicits p130CAS phosphorylation by tyrosine kinases (TK) and as a result CrkL and CrkII are recruited. (ii) GEFs (for example, DOCK1 and C3G) associate with CrkL and CrkII via their SH3 domain giving rise to efficient localized activation (iii) of small GTPases (for example, Rac and Rap) at the membrane. (iv) Abl-induced phosphorylation of CrkL and CrkII forces their dissociation from p130CAS and thus results in signaling suppression. The distinct structural organization of CrkL and CrkII modulates the interactions with their physiological partners to a different extent. The blue and orange shaded regions in SH2 and SH3N denote the pTyr- and PPII-binding sites, respectively. See text for details.