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Reengineering the substrate specificity of Src family kinases

A Dissertation Presented by

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Abstract of the Dissertation

Reengineering the substrate specificity of Src family kinases

by

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Src family kinases (SFKs) are modular signaling proteins possessing SH3, SH2, and tyrosine kinase domains. Their domain architecture is conserved throughout evolution. The SH3 and SH2 domains of SFKs have dual roles: (1) by engaging in intramolecular interactions, they regulate the activity of the kinases, and (2) they target SFKs to their cellular substrates. Ligands for the SH3 and SH2 domains can disrupt the intramolecular associations and activate the kinases. Using purified proteins, and by expression in mammalian cells, we show that proteins containing ligands for both SH3 and SH2 domains can lead to a cooperative activation of Src family kinases.

The relative positions of the domains in SFKs are important for enzyme regulation; they permit the assembly of Src kinases into autoinhibited conformations. We addressed the question of whether the domain arrangement of Src family kinases has a role in substrate specificity by producing mutants with alternative arrangements. Our results suggest that changes in the positions of domains can lead to specific changes in substrate specificity. The placement of the SH2 domain with respect to the catalytic domain of Src appears to be important for proper substrate recognition, while the placement of the SH3 domain is more flexible.

The modular nature of SFKs makes them ideal for studying the evolution of new proteins and signaling networks. We generated a novel SFK by replacing the SH2 domain of Hck with the syntrophin PDZ domain. The negative regulatory tyrosine in the C-terminal tail was replaced with a PDZ ligand sequence. The overall substrate specificity of PDZ-Hck is redirected towards PDZ specific substrates. Using PDZ-Hck, we were able to rewire cellular signaling pathways leading to migration and ErbB2-MAPK activation. The PDZ-kinase shows auto-regulatory properties similar to a natural SFK. Our data highlight the modularity and evolvability of signaling proteins.

Dedication

I would like to dedicate this thesis to my mother Late Mrs. Shail Singh. She has been and will always be a source of strength for me.

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List of Abbreviations

2	Src homology domain 2
3	Src homology domain 3
Abl	Abelson kinase
Ack	Activated cdc42-associated kinase
ADP	adenosine diphosphate
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCR	B-cell receptor;
C	Src catalytic domain;
Cas	Crk-associated substrate
cdc42	Cell division cycle 42
CMV	cytomegalovirus
CO_2	carbon dioxide
DMEM	Dulbecco's modified eagle's medium
DNA	deoxy ribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetate
EGF	Epidermal growth factor
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
GFP	Green fluorescent protein
Grb2	Growth factor-receptor bound protein 2;
Hck	hematopoeitic cell kinase
HPLC	High performance liquid chromatography
HRP	Horse-radish peroxidase
IB	Immuno blot.
IP	Immunoprecipitation
IRES	Internal ribosome entry site
LT	low toxicity
MAPK	Mitogen Activated Protein Kinase
mg/ml	milligram per milliltre
$MgCl_2$	Magnesium chloride
mL	milliliter
mM	millimolar
NaCl	sodium chloride
NADH	Nicotineamide adenine dinucleotide hydrogenase
nm	nanometer
nM	nanomlar
NP40	Nonidet P40
NRTKs	nonreceptor tyrosine kinases
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PDGF	Platelet derived growth factor
PEP	Phosphoenolpyruvate

PK/LDH	Pyruvate kinase/Lactate dehydrogenase
pTyr	phospho-tyrosine
PVDF	polyvinylene difluoride
pY	phospho-tyrosine
Rac	Ras-related C3 botulinum toxin substrate
RACK1	Receptor for activated C-kinase
rpm	revolutions per minute
S.D.	standard deviation
Sam68	Src-associated in mitosis
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sf9	*Spodoptera frugiperda* 9
SFKs	Src family kinases
SH2	Src homology domain 2
SH3	Src homology domain 3
SYF	Src, Yes, Fyn knockout cells
TKs	Tyrosine kinases
U	Src Unique domain
v/v	volume/volume
WB	Western blot;
WCL	whole-cell lysate
WT	Wild type
β-ME	beta-mercaptoethanol
μl	microlitre
μM	micromolar

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CHAPTER 1

Introduction

Introduction

Evolution of signaling networks in multicellular animals

Multicellular animals evolved ~600 million years ago [1]. The evolution of multicellularity is marked by three important features: cellular adhesion, signal transduction and differentiation. In eukaryotes there is a constant need for monitoring and responding to the environment and in metazoans there is an additional need for cell to cell communication [1]. These needs are met by complex signaling pathways. There are seven major signaling components that are unique to eumetazoans: receptor tyrosine kinases (RTK), transforming growth factor β (TGFβ), Wnt, JAK/STAT, Notch, nuclear hormone receptor (NHR) and Hedgehog [2]. These pathways are involved in development, differentiation, proliferation and immunity, all of which are needed for survival of multicellular animals. All of these pathways are comprised of an array of signaling proteins.

The different signaling networks in multicellular animals arose by creation of novel protein-protein interactions and wiring together of diverse proteins. With evolution different levels of complexity were incorporated to regulate these pathways. The closest documented relatives to metazoans are the unicellular/colonial choanoflagellates. Some cell signaling proteins like cell adhesion proteins, RTKs and TKs are found in choanoflagellates [3, 4]. Thus, the basic toolkit for assembly of multicellularity exists in unicellular flagellates. Pre-existing protein families developed new interactions that conferred novel functions to the metazoans. For example, cadherins are linked to the actin cytoskeleton leading to cell adhesion via β-catenin. In choanoflagellates and in sponges, the lowest branch in the phylogenetic tree of metazoans, this interaction is not possible as these organisms lack classical cadherins (Table 1-1) [1]. Similarly, intermediate filaments which form desmosomes are found in bilateria only (chordate and non-chordate bilaterians). Cas, a Src substrate, is also absent in sponges, pointing to a later evolution of the signaling proteins to serve specific complex functions in higher metazoans (Table 1-1) [2]. One well conserved pathway involved in development of multicellular animals is the Wnt signaling pathway. An analysis of evolution of components of this pathway sheds light on the evolution of signaling networks. The ligand (Wnt), receptor (Fz) and downstream effectors (β-catenin, Nemo) are conserved from sponges to humans. But the antagonist to the pathway (Dkk) is found only in vertebrates, suggesting later incorporation of negative regulation in this pathway (Table 1-1) [2].

Evolution of protein kinases

Protein kinases are cell signaling proteins that are exclusive to eukaryotes. Protein kinases (PK's) are classified based on the sequence of their kinase domains. Eukaryotic protein kinases (ePKs) are comprised of two main families: Ser/Thr kinases and Tyr kinases (TKs). ePKs are one of the largest families of proteins and account for 1.5-2.5% of all eukaryotic genes. There are ~500 eukaryotic protein kinases (ePKs), of which 144 ePKs are present in all metazoans [5]. Only thirteen families are unique to humans, suggesting major divergence occurred early in evolution. Families unique to humans include atypical protein kinases (aPKs), DNA-PKs and some tyrosine kinases (TKs), e.g., Tie [5, 6].

The largest and most diverse family of PKs is that of TKs. With the exception of choanoflagellates, their expression appears to be restricted to metazoans, and thus TKs are believed to play a major role in the evolution of multicellular animals. There are 90 TK genes and 5 pseudogenes in humans. Fifty-eight receptor tyrosine kinases (RTKs) are grouped into 20 subfamilies and 32 non receptor tyrosine kinases (nRTKs) are grouped into 10 sub-families based on kinase domain sequence. TK genes are distributed on 19 of 24 chromosomes in humans [7]. Compared to 90 TK genes in humans, there are only 32 in flies, while yeast has none. There has been an expansion of kinase families in humans relative to flies and worms (Table 1-2). There are 11 Src family kinases in humans, but only 2 and 3 in fly and worm respectively (Table 1-2) [6]. These observations are consistent with the more extensive role of TK signaling in higher eukaryotes.

TKs evolved by gene duplication and domain shuffling. If two genes of the same family lie close together on the same chromosome, then that is probably a result of a gene duplication event. An example of a recent gene duplication event for TKs can be traced for the Brk family members Brk and SRMS, both of which lie close together on Chromosome 20 [7]. A phylogenetic analysis of nRTKs suggests that 7 of the 18 nRTKs diverged before the animal-choanoflagellate split by domain shuffling, while the remaining 11 nRTK unique domain structures evolved in each lineage after the split [8]. The architecture of domains is conserved within subfamilies for TKs. Among nRTKs the SH3-SH2-kinase architecture is conserved not only across species (from choanoflagellates to humans) but it is also conserved across several sub-families (Figure 1-1). In contrast all sub-families of RTKs have a different combination of domains. In fact the RTK family has the largest diversity of domain combinations among metazoans. Different domain architectures of TKs points to their evolutionary flexibility. Thus, new TKs could arise by domain recombination (Figure 1-1).

Role of modular domains in evolution of cellular signaling

There are 23,000 genes in humans compared to 14,000 in worms and flies and 9,000 in choanoflagellates [9, 10]. A comparison of metazoan genomes points to the fact that increased complexity of phenotype does not correlate with development of new domains (only 7% of human protein families are vertebrate specific). Complexity rather correlates with an increase in number and type of new domain combinations. Humans have 1.8 fold more distinct arrangements of domains in the primary sequence than do worms and flies [11]. A modeling study where different signaling protein networks were subjected to a change in environments suggested that novel interaction networks evolve spontaneously based on changing environmental needs [12].

By analogy to the importance of modular domains in protein evolution, the structural and functional modularity of transcriptional nodes provides an evolutionary advantage in generating new connections in transcriptional networks [13, 14]. (A transcriptional node is comprised of the gene coding region and flanking promoter and enhancer regions that control transcription from the gene coding region). Structural diversity results from physically separable gene coding regions and cis regulatory elements. Functional modularity arises when the input (regulatory elements) and output (gene) components are still functional upon separation and can be recombined to yield new input output connections. Recombination of transcriptional elements is thought to be a major source of phenotypic diversity during evolution [13, 14]. There is selective pressure to maintain modularity and evolvability. The ability to evolve rapidly is advantageous in adapting to a rapidly changing environment [15].

In contrast to highly modular transcriptional networks, classical regulatory metabolic enzymes like hexokinase and glycogen phosphorylase are very rigid in terms of evolution [15]. They are conserved from prokaryotes to eukaryotes. In these enzymes, the allosteric regulatory sites and the active site are present in the same polypeptide. Apart from the catalytic site, glycogen phosphorylase has binding sites for activators (AMP, ATP) and inhibitors (caffeine and purine) on the same polypeptide chain. Phosphofructokinase (PFK) has binding sites for activators (ATP and Fructose-6-phosphate) and inhibitors (phosphoenolpyruvate, PEP) on the same polypeptide chain [16]. Effector binding to the allosteric site is coupled to specific conformational changes at the active site. Metabolic enzymes rarely show structural and functional modularity. Thus, mutations which would alter regulatory properties would be likely to affect catalytic activity as well.

On the other hand, signaling pathways are comprised of enzymes (e.g. kinases) that led to the evolution of multicellularity. The major difference in these enzymes and metabolic enzymes is that the former are more modular in nature than the latter. Signaling enzymes more often contain additional independently folding domains in addition to core catalytic domains that mediate interactions with other signaling elements [17]. These are found in different combinations with different catalytic domains (Figure 1-1) so insertion and recombination of modular domains is a common mode of evolution of novel proteins [18].

Eukaryotic signaling proteins developed a range of modular strategies for controlling input/output connectivity, all of which have increased functional separation between the core catalytic domain and the connecting elements [15]. Though generally applicable to other classes of signaling proteins as well, we will focus on the evolution of modularity in protein kinases. Protein kinases employ three different mechanisms for establishing novel connections in signaling networks. These are: (1) docking sites, (2) modular interaction domains, and (3) adaptors and scaffolds, each of which appears to be a step in evolution towards increasing flexibility [15]. Docking sites are small recognition motifs present on the surfaces of the catalytic domains of some Ser/ Thr kinases. These motifs are distinct from the kinase active site [19]. Docking interactions decrease the K_m for substrates and increase the efficiency and specificity of Ser/Thr kinases. MAPKs have a docking site termed a D-box on the face opposite to the active site [20, 21]. The addition of a ligand for a MAPK docking site to a protein can target the protein for phosphorylation, thereby providing a means of forming a novel connection [15]. Since docking sites are part of the catalytic domain they are restricted in terms of evolution and cannot be readily transferred. In support of this view, tyrosine kinases do not have docking sites, and they evolved later than Ser/Thr kinases (tyrosine kinases are absent from yeast).

The next step towards evolution of complex metazoan signaling involved usage of domain recombination to generate novel proteins. Tyrosine kinases with a diverse array of domain combinations belong to this class (Figure 1-1) [7]. This arrangement is analogous to the structural and functional modularity of transcriptional networks. The non-catalytic domains and the catalytic core are physically separable and can be recombined in different contexts without loss of basic function. Modular interactions are more flexible than docking interactions on the catalytic domains themselves, as both the motif and domain can be transferred through recombination and insertion. Thus both enzyme and substrate can make new connections by incorporating either the domain or the recognition motif [15].

Modular domains also mediate conditional regulation (for example, alosteric regulation, or regulated binding to sites of post-translational modification). Another advantage provided by the modular arrangement of kinases is the ability to regulate the output by multiple inputs, thereby making the kinases like molecular switches. These qualities provide the advantage of precise control of signaling as well as signal amplification. Allosteric intramolecular regulation is a common feature of tyrosine kinases like Src and Abl [22-25]. This allosteric regulation is not restricted to kinases, but is found in several other classes of signaling proteins with modular architecture, such as the actin regulatory protein WASP [26] and the protein phosphatase Shp2 [27-29]. The modular signaling enzymes are analogous to the logic gates of electronic circuits [18].

From an evolutionary perspective, the ultimate separation of catalytic and connectivity elements is the separation of these functions into distinct proteins [15]. This is achieved by scaffolds and adaptors, which are platforms for organizing signaling complexes. Adaptors like cyclins and Grb2 link together two partners. Scaffolds like Cas link together more than two partners. The most important advantage of scaffold and adaptor proteins is that they allow catalytic proteins like kinases to play several distinct roles, depending upon the complex into which they are assembled. They also provide fine-tuning of signaling, as feedback regulators leading to increases or decreases in signal intensity can be present in the same complex [15].

β-arrestin 2 is a scaffold protein which can bind JNK kinase, and a negative regulator phosphatase (MKP7). Upon stimulation, MKP7 transiently dissociates from β-arrestin to permit MAPK phosphorylation and downstream signaling. MKP7 binds to β-arrestin again after 30-60 minutes post stimulation [30]. Thus, the scaffold provides time dependent pathway activation and inactivation. AKAP79 (A kinase anchoring protein) is a scaffold which binds PKA, PKC and phosphatase, calcineurin, thereby providing negative feedback [31].

Src family kinases (SFKs)

SFKs comprise a sub-family of non receptor tyrosine kinases. SFKs are found from choanoflagellates to humans. They are absent from bacteria, plant, slime molds and fungi, thus they have essential functions in cell-cell communication in multicellular animals. There are 9 SFKs in vertebrates: Src, Yes, Fyn, Fgr, Lyn, Hck, Lck, Blk and Yrk. Some SFKs (Src, Yes and Fyn) are expressed ubiquitously, while others (Hck, Lck and Lyn) are restricted to hematopoeitic cells[32]. The cellular Src gene was the first molecularly defined proto-oncogene, and the first protein to be identified with intrinsic tyrosine kinase activity. The first identified oncogene was v-Src, which is a homolog of cellular Src found in Rous Sarcoma Virus [33]. v-Src has several mutations distributed throughout the protein coding sequence, including a C-terminal truncation[34].

All SFKs have a conserved domain architecture with the following regions (from N to C terminus): an N-terminal 7-residue membrane anchor region (SH4), a poorly conserved 40-70 residue unique region, a 50-80 residue SH3 domain, a 100 residue SH2 domain, a 250 residue kinase domain, and a 15-17 residue C-terminal regulatory region with a conserved Tyr (Y527, in Src)[35].

The SH4 region of SFKs aids in their membrane localization. There are two different post-translational modifications in the SH4 region which are responsible for membrane localization of SFKs [36]. One is myristoylation at Gly2; this modification is present in all members of SFKs [37]. The other is palmitoylation at Cys 3, which is restricted to some members of SFKs like Hck [38, 39]. Src lacks palmitoylation but instead has a patch of basic residues that aids in membrane localization [40]. Because of the weaker affinity of the basic residues for the membrane (as compared with palmitate), only a fraction of myristoylated Src is membrane localized, while the rest is cytosolic [41].

The unique region differs in length as well as sequence among the Src family members. It is believed to play a role in mediating specific protein-protein interactions. The unique region of Lck has two Cys residues that form disulfide bonds and interact with CD4 and CD8 molecules in T cells [42]. The unique regions of Fyn and Lyn mediate interactions with the B-cell receptor (BCR) and the T-cell receptor (TCR) [43, 44]. In some cases, phosphorylation of SFKs within the unique region plays a regulatory role. The Src unique region has phosphorylation sites for PKC, PKA and Cdc2/ cyclin [35]. Ser 49 in the unique region of Lck, when phosphorylated, regulates the specificity of its SH2 domain [45].

The SH3 domain is a small module comprised of 5 antiparallel β-strands that pack together to form two perpendicular β-sheets [46]. The ligand binding site is on one side of the Src SH3 domain. It is a slightly depressed hydrophobic surface with an acidic cluster at one end. SH3 domains bind to proline rich ligands that can adopt a left-handed helical conformation. SH3 domains have ligands belonging to two different classes. The consensus sequence for a Src SH3 Class I ligand is RPLPPLP, while ClassII ligands have the reverse orientation with a consensus ϕPPLPXR, where ϕ is any hydrophobic residue. SH3 domains bind to their ligands with a moderate affinity ($K_d \approx 5\text{-}50\mu M$) [47].

The SH2 domain is also a compact modular domain made up of a large central antiparallel β-sheet (with 7 β-strands) flanked by 2 α-helices. SH2 domains bind to tyrosine phosphorylated peptides and the specificity is determined by the 3 residues C-terminal to the pTyr. For SFKs, the optimal SH2 ligand is pYEEI [48]. The ligand binding site on the Src and Lck SH2 domain is analogous to a "two-pronged plug engaging a two-holed socket" [49]. There are two pockets: one binds to the pTyr, while the other is a deep hydrophobic pocket that is ideally positioned to accept Ile at pY+3 of SH2 ligand [50]. The SH2 domain also binds to its ligand with moderate affinity (nM–μM). For pYEEI peptide, the K_d is 200-600nM [51].

The kinase domain is highly conserved across SFKs and is well conserved with other tyrosine kinases as well [35]. The kinase domain is globular and made up of two lobes, the N and C lobes. The N-lobe is largely made up of β-sheets and one important α-helix whose orientation determines the catalytic activity of the kinase. The C lobe is predominantly α-helical and is involved in binding substrate peptides. The catalytic cleft lies between the two lobes. There is an activation loop with a tyrosine residue (Tyr 416) which, when unphosphorylated, blocks access of substrate to Src catalytic cleft [23, 24, 52, 53]. When the activation loop tyrosine is phosphorylated, kinase activity increases [54].

The C-terminal region has a conserved Tyr 527 and its position is constant among SFKs. Tyr527 is phosphorylated by another kinase designated Csk (C-terminal Src kinase) and this phosphorylation leads to a decrease in activity of the kinase. The C-terminal tail sequence $pY^{527}QPG$ has a low affinity (29μM) for the Src SH2 domain, but its presence on the same polypeptide favors an intramolecular interaction between the SH2 domain and C-terminal pTyr527[35].

Biological roles of SFKs

SFKs play diverse roles in signaling in multicellular animals. They have been implicated in processes as diverse as growth, differentiation, apoptosis, cell-cell adhesion, cell motility and cell-cell communication [32]. They are involved in signaling from RTKs, GPCRs, cadherins, integrins, TCRs, BCRs, JAK/STATs and others [55]. The three ubiquitously expressed SFKs (Src, Yes, and Fyn) are thought to have redundant functions, as single knockout mice survive but have specific defects depending upon the SFK being knocked out. $Src^{-/-}$ mice develop osteopetrosis, which is characterized by decreased bone absorption and overgrowth of bone. Src plays a role in the normal differentiation and response of osteoclasts, a myeloid cell type. Osteoclasts from $Src^{-/-}$ mice fail to form a ruffling border and resorb bone [56]. On the other hand, $Fyn^{-/-}$ mice have neurological abnormalities [57]. Src, Yes and Fyn triple knockout mice die in utero with cardiovascular and developmental defects. SYF mouse embryonic fibroblasts derived from Src, Yes Fyn knockout mice embryos by immortalization with T antigen are defective in cell migration [58]. $Hck^{-/-}$ macrophages are impaired in phagocytosis [59].

Src kinases have been implicated in a variety of cancers where they have elevated levels of expression especially in the case of colon, breast and pancreatic cancer [60-62]. In colon cancer a germline activating mutation in the C-terminal Tyr527 has been identified in SFKs [63]. Due to their involvement in cancer, and in a in a variety of signaling systems, SFKs are important therapeutic targets.

Substrate Specificity of SFKs

The catalytic domain of SFKs in isolation possesses tyrosine kinase activity but has limited substrate specificity[64]. The optimal Src catalytic domain substrate peptide based on degenerate peptide library studies conforms to the sequence EEIYG/EEFD[65]. The substrate specificity of the kinase domain mirrors the binding specificity of its SH2 domain; this might lead to processive phosphorylation of Src substrates by binding of Src SH2 domain to the site phosphorylated by Src[65]. The strict specificity of TKs for Tyr over Ser/Thr is due to a loop (FP^{425}IKWTA in Src) that is conserved across all TKs. The loop lies in close proximity to the main chain of the substrate. The Pro contributes in binding to the phenyl ring of Tyr from the substrate and would not help in binding Ser or Thr [35]. The preference of substrates with acidic residues N terminal to the Tyr is due to ionic contacts with Arg (HRDLR^{338}AAN) and the substrate [35].

The SH3 and SH2 domains of SFKs are major determinants of substrate specificity of SFKs [64]. Substrate peptides with SH3 or SH2 ligands attached to them are better phosphorylated by SFKs in comparison to substrate peptides lacking SH3 or SH2 ligands [66, 67]. Most of the known SFK substrates have ligands for either the SH3 domain, or the SH2 domain or both. In fact best known substrates of SFKs are proteins with ligands for both the SH3 and SH2 domains these include Cas, Sin, AFAP110, Sam68, and Connexin 43 [68-73]. Some Src substrates, such as ErbB2, PDGFR, and RACK1 are capable of interaction via the SH2 domain only [74-76]. Some substrates that interact with Src SH3 domain alone are RasGAP and HIV-Nef [77, 78].

Since the discovery of Src in the 1970's there has been extensive work on identifying bonafide downstream substrates of Src kinases. It has been a challenging problem, since SFKs are present in complex signaling pathways, often containing additional TKs. In addition, there is frequently cross-talk between different pathways. As an example, GPCRs lead to RTK activation and a proliferative response via cross-talk mediated through SFKs [79]. Due to the above mentioned reasons, the specific downstream effect of a specific Src-substrate interaction is also a challenge to study. It is important to recognize direct substrates of SFKs and their downstream effectors. Due to their involvement in cancer it might be beneficial to understand the differences in normal substrates versus tumor cell specific substrates of Src so that targeted therapeutic intervention might be possible.

Several experimental strategies have recently been developed to identify direct Src substrates. In one example, engineered Src kinases capable of utilizing only a specific ATP analog have been utilized. This strategy allows the identification of direct substrates of Src, which are modified by the unique nucleotide [80]. A different approach was taken by Mayer *et al*, who engineered specific interactions between SFKs and proteins to examine downstream effects. This approach (termed a "functional interaction trap") is useful for identifying and studying downstream signaling via specific Src/substrate interactions. Using this strategy Mayer *et al* demonstrated that a specific coiled-coil domain based interaction between Src and Stat3 could lead to transcriptional activation via a Stat3 specific promoter [81]. This strategy provides a valuable research tool to break-down complex pathways into simple interactions.

Regulation of SFKs

In addition to determining substrate specificity, the SH3 and SH2 domains of SFKs play an important role in regulating kinase activity. The structure of down-regulated forms of the SFKs Hck and Src clearly show the importance of these modular domains in making SFKs functional as molecular switches. The crystallographically determined structures are comprised of SH3, SH2 and kinase domains with the C-terminal tail phosphorylated at Tyr527. The SH4 and unique region are absent from the X-ray structures. The structure suggests that SH3-SH2-kinase domains are sufficient to keep the kinase in a repressed state. There are two intramolecular interactions which are responsible for repressing kinase activity. One is between the SH2 domain and C-terminal pTyr527. The other is between the SH3 domain and a PPII helix formed by the linker between the SH2 and the kinase domain [23, 24]. These two interactions bring about three important changes in the catalytic domain which represses its activity. First, the two lobes of the kinase domain are oriented such that the catalytic cleft is closed relative to its open position in an active kinase domain (Lck structure, Nature (1996), 384, 484-489) [52]. Second, the N-terminal lobe has an αC-helix that is rotated outwards along its axis compared to its position in active kinases. This orientation disrupts an important salt bridge interaction that is present in active catalytic domains. A third change involves the position of the activation loop, which includes residues 404-432 in Src [82, 83]. The activation loop blocks substrates from accessing the active site as it occupies the space where peptide substrate should bind, and the unphosphorylated Tyr416 is buried between the kinase lobes. This conformation of the activation loop prevents full formation of the peptide recognition site. In contrast, in an active kinase domain the activation loop is extended out with Tyr416 phosphorylated [84, 85].

Thus Src family kinases are classical modular proteins where the output catalytic domain is flanked by modular regulatory domains [18]. These modular SH3 and SH2 domains not only control the output but also control input by targeting substrates to the output domain. There are three separate inputs governing the output activity of SFKs. One is disruption of the SH2-pTyr 527 interaction either by a higher affinity SH2 ligand or by dephosphorylation of pTyr527 by a phosphatase. The second is by SH3 domain displacement by a SH3 ligand. The third is by autophosphorylation at Y416 within the activation loop [86]. Thus substrates that carry ligands for either SH3 or SH2 domain will lead to outputs of different intensities, which might be relevant for different outcomes from a signaling pathway. Molecular dynamic stimulations predict cooperative activation by ligands for both the SH3 and SH2 domains [87]. Src could be analogous to an AND gate where both SH3 and SH2 ligands together bring about maximal activation of the kinase. Several SFK substrates have ligands for both the SH3 and SH2 domain of SFKs, and in this case potent activation of SFKs would be coupled to substrate phosphorylation. In Chapter 3 of this thesis, we describe experiments aimed at understanding the cooperative function of SH3 and SH2 ligands for SFKs. In Chapter 4, we describe our studies aimed at understanding the role played by the highly conserved domain architecture (SH3-SH2-kinase) in dictating SFK substrate specificity. In Chapter 5, we discuss our studies providing support to the hypothesis that TKs evolved by domain recombination. We show that novel kinases can be made by domain recombination. The novel SFKs are capable of being controlled by desired inputs, and they can be used to rewire cell signaling.

Table 1-1. **Comparison of phylogenetic distribution of some signaling proteins**. Filled black circle denotes presence in the phylum while open black circle represents absence. Open red circles are proteins with domains from β-catenin but are not metazoan orthologs. The phylum Porifera includes sponges, while Cnidaria includes corals, jellyfishes and sea anemones. Table compiled and adapted from [88]

				Metazoa			
	Amoebozoa	Fungi	Choanoflagellates	Porifera (sponges)	Cnidaria (hydra)	Non-chordate bilateraians	Chordates
Cadherin	○	○	○	○	●	●	●
β-Catenin	○	○	○	●	●	●	●
Intermediate Filaments	○	○	○	○	○	●	●
Cas	○	○	○	○	●	●	●
Src	○	○	●	●	●	●	●
Ligand (Wnt)	○	○	○	●	●	●	●
Receptor (Frizzled)	●	○	○	●	●	●	●
Effector (β-Catenin)	○	○	○	●	●	●	●
Anatagonist (Dkk)	○	○	○	○	○	○	●

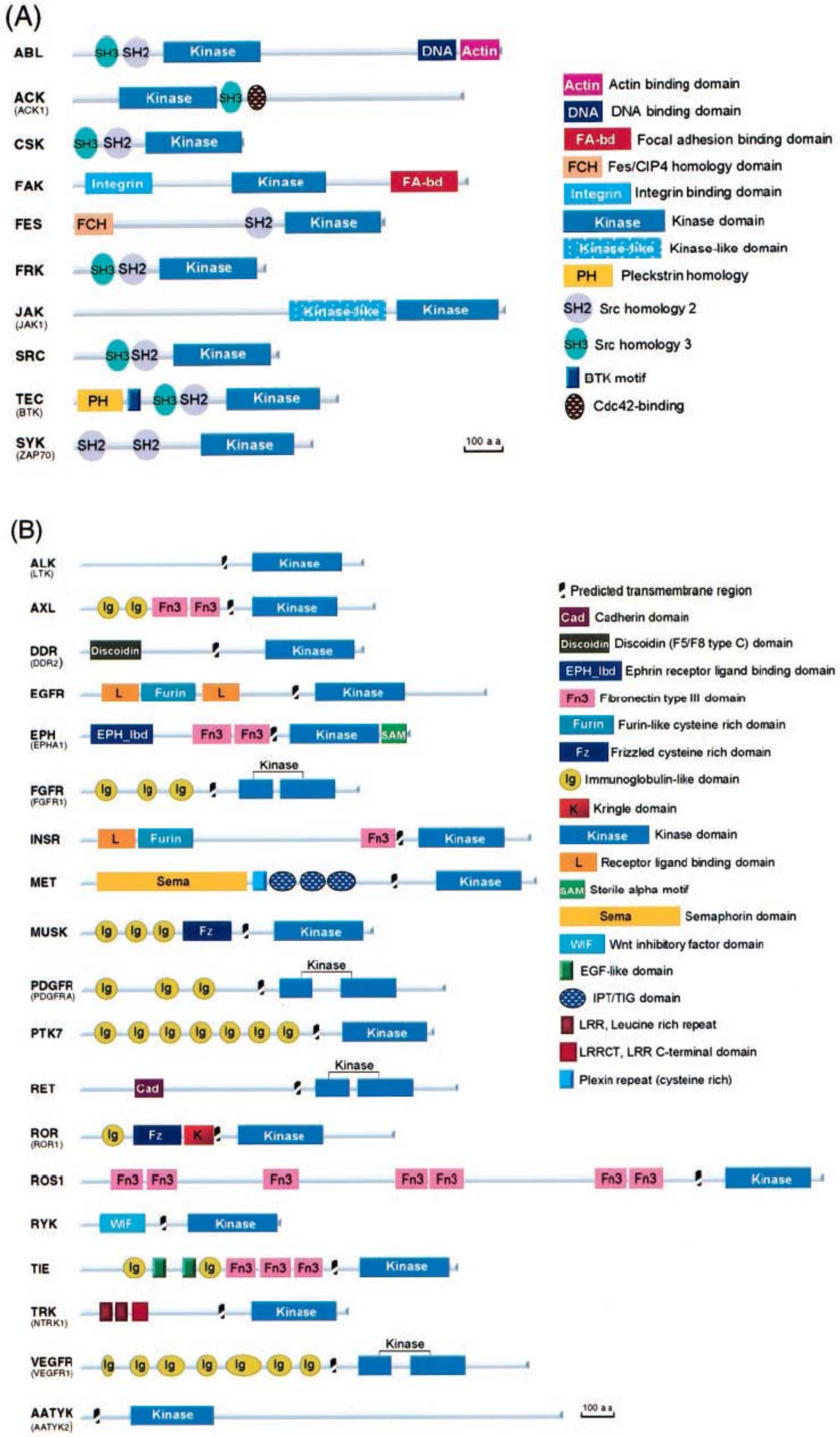
***Table 1-2*. Distribution of Tyrosine Kinases into major groups in humans and a comparison with model organisms**. An expansion of SFKs in humans can be seen from a comparison with fly and worm.

	Yeast	Worm	Fly	Humans	Human pseudogenes	Human unique
All TKs	0	90	32	90	5	5
SFKs	0	**3**	**2**	**11**	-	-

Figure 1-1. **Domain architecture of human tyrosine kinases.** The overall architectures point to various domain combinations that are found in tyrosine kinases pointing to their evolution by extensive domain shuffling.

(A) Families of non-receptor tyrosine kinases. The SH3-SH2-Kinase architecture is the most common. Tandem SH2 domains are found in members of the SYK family. The SH3 domain is typically N-terminal to the SH2+kinase domains, but in Ack it is present C-terminal to the kinase domain.

(B) Families of Receptor tyrosine kinases. Unlike nRTKs, the domain architecture is not conserved.
Figure taken from: Robinson, D. R. Wu, Y. M., and Lin, S. F. Oncogene (2000) 19, 5548-5557 [7].


(A)
ABL
SH3 SH2 Kinase DNA Actin
ACK
(ACK1)
Kinase SH3
CSK
SH3 SH2 Kinase
FAK
Integrin Kinase FA-bd
FES
FCH SH2 Kinase
FRK
SH3 SH2 Kinase
JAK
(JAK1)
Kinase-like Kinase
SRC
SH3 SH2 Kinase
TEC
(BTK)
PH SH3 SH2 Kinase
SYK
(ZAP70)
SH2 SH2 Kinase
100 a a
Actin Actin binding domain
DNA DNA binding domain
FA-bd Focal adhesion binding domain
FCH Fes/CIP4 homology domain
Integrin Integrin binding domain
Kinase Kinase domain
Kinase-like Kinase-like domain
PH Pleckstrin homology
SH2 Src homology 2
SH3 Src homology 3
BTK motif
Cdc42-binding
(B)
ALK
(LTK)
Kinase
AXL
Ig Ig Fn3 Fn3 Kinase
DDR
(DDR2)
Discoidin Kinase
EGFR
L Furin L Kinase
EPH
(EPHA1)
EPH_lbd Fn3 Fn3 Kinase SAM
FGFR
(FGFR1)
Ig Ig Ig Kinase
INSR
L Furin Fn3 Kinase
MET
Sema Kinase
MUSK
Ig Ig Ig Fz Kinase
PDGFR
(PDGFRA)
Ig Ig Ig Kinase
PTK7
Ig Ig Ig Ig Ig Ig Ig Kinase
RET
Cad Kinase
ROR
(ROR1)
Ig Fz K Kinase
ROS1
Fn3 Fn3 Fn3 Fn3 Fn3 Fn3 Fn3 Kinase
RYK
WIF Kinase
TIE
Ig Ig Fn3 Fn3 Fn3 Kinase
TRK
(NTRK1)
Kinase
VEGFR
(VEGFR1)
Ig Ig Ig Ig Ig Ig Ig Kinase
AATYK
(AATYK2)
Kinase
100 a a
Predicted transmembrane region
Cad Cadherin domain
Discoidin Discoidin (F5/F8 type C) domain
EPH_lbd Ephrin receptor ligand binding domain
Fn3 Fibronectin type III domain
Furin Furin-like cysteine rich domain
Fz Frizzled cysteine rich domain
Ig Immunoglobulin-like domain
K Kringle domain
Kinase Kinase domain
L Receptor ligand binding domain
SAM Sterile alpha motif
Sema Semaphorin domain
WIF Wnt inhibitory factor domain
EGF-like domain
IPT/TIG domain
LRR, Leucine rich repeat
LRRCT, LRR C-terminal domain
Plexin repeat (cysteine rich)

CHAPTER 2

Materials and Methods

Materials and Methods

Reagents and antibodies- DMEM, Trypsin-EDTA, SF900II-SFM, Penicillin, Streptomycin and Amphotericin B were from GIBCO (Cellgro). FBS, polybrene, nocodazole, NADH, PEP, ATP, PK/LDH, anti-FlagHRP, and anti-tubulin antibodies were from Sigma. Cas C-20 and Sam68 antibodies were from Santa Cruz Biotech (Santa Cruz, CA). Cas monoclonal and Crk antibody was purchased from BD Biosciences (San Jose, CA). Anti-phosphotyrosine 4G10 mouse monoclonal and anti-ErbB2 antibody were from Millipore biotechnology, and anti-pY416 antibody was from Biosource. ErbB2 hybridoma 4D10 was a kind gift from Dr. Debbie Brown's lab (Stony Brook University). Erk and p-Erk antibodies were from Cell Signaling Technology. HRP conjugated secondary anti-mouse and anti-rabbit antibodies, and ECL and ECL$^+$ kits were from Amersham.

Plasmid construction- The wild-type, Y668F, and PPX mutant forms of Cas were expressed as YFP fusions, as described previously [89].The Y668F/PPX double mutant was made by sequential site-directed mutagenesis of the pCDNA6-YFP-Cas construct using the QuikChange kit (Stratagene).

The domain rearranged mutants were designed based on a previously-described strategy [90]. Plasmid pUSEamp-cSrc (Upstate) was used as the parental template to generate PCR fragments of all the domains of Src. Each domain was flanked by unique restriction sites. The domains were ligated together in various combinations to generate the mutants. A spacer of 4 amino acids (similar to wild-type Src) was used whenever the SH3 and SH2 domains were adjacent in the proteins. The linker sequence (present in wild-type Src between the SH2 and kinase domains) was N-terminal to the catalytic domain in all mutants. In mutant UC32, the C-terminal tail (containing Y527) was maintained at the C-terminus of the protein. The mutants were cloned into the NotI and BamHI sites of p3XFLAG-CMV-7.1 (Sigma). Site-directed mutagenesis was performed on wild-type Src cloned into p3XFLAG-CMV-7.1 to generate the R175L/W44A mutant. Mutants 2UC3 and 3UC2 were also subcloned into the NotI and BamHI sites of pFastBacHTbM to generate baculovirus constructs. pFastBacHtbM was derived from pFastBacHtb (Gibco) by site-directed mutagenesis to create a BamHI site after NotI in the multiple cloning sequence. For retroviral expression, the mutants were subcloned into the EcoRI and XhoI sites of pMSCV-IRES-GFP (a kind gift from Dr. Nicholas Carpino, Stony Brook University).

The PDZ-kinase constructs cloned into pFastBacHta (Gibco) were made by our collaborators Brian J. Yeh and Dr. Wendell Lim at UCSF. For mammalian expression, all the constructs were sub-cloned into pCMV-Tag2B (Stratagene) between the EcoRI and XhoI sites. The nNOS construct was sub-cloned into pXJ-HA between the BamHI and XhoI sites. For retroviral expression, the PDZ-kinase construct (ZK) was sub-cloned into the EcoRI and XhoI sites of pMSCV-IRES-GFP. To generate Cas-VKESLV, site-directed mutagenesis was performed on wild-type Cas cloned into pCDNA6. Cas-VKESLV was further subjected to site-directed mutagenesis to delete the Src binding sequence of Cas. For the ErbB2 construct with a C-terminal PDZ ligand, pCDNA3-ErbB2 kinase-dead (Dr. Len Neckers, NIH) was sub-cloned into pBS-SK (+/-) and site-directed mutagenesis was performed. The ErbB2kinase-deadVKESLV was sub-cloned back into pCDNA3. All the constructs were confirmed by restriction digestion and sequencing.

Cell culture and transient transfection- Cas$^{-/-}$ cells were maintained in low glucose DMEM (Gibco) supplemented with 10% fetal bovine serum and 100U/ml penicillin and 100U/ml streptomycin in a 37° C, 5% CO_2 incubator. The cells were plated at a density of 1X10^6 cells per 100mm plate. The cells were transiently transfected with Src (0.5-1μg) and Cas (2-5μg) using TransIT LT1 (Mirus).

SYF cells were purchased from ATCC (Manassas, VA) and gradually adapted for maintenance in DMEM containing 10% FBS and 1X antibiotic/antimycotic at 37°C in a humidified 5% CO_2 incubator. Cells were transiently transfected using TransIT LT1 transfection reagent (Mirus) following the manufacturer's recommendations. Typically, 0.5 to 1 μg of DNA was transfected for domain rearranged mutants while 1-10 μg DNA was transfected for the PDZ-kinases using a DNA:TransIT ratio of 1:2.

Western blotting and Immunoprecipitation- Cells were transiently transfected and harvested 40 hours post transfection in EB++ buffer [91]. For PDZ-kinases the cells were harvested in RIPA buffer. For Crk immunoprecipitations, cells were harvested in NP40 buffer [92]. Protein concentration was estimated using the Bradford assay (Bio-Rad). For analysis of whole cell lysates, lysates (10-50μg) were separated on 10% SDS-PAGE and transferred onto PVDF membrane. The membranes were probed with anti-Flag, anti-Cas, anti-pY416, anti-tubulin and 4G10 antibodies. For immunoprecipitation, lysates (500μg) were pre-cleared for 1 hour at 4°C, followed by incubation with antibody (1-5μg) for 2 hours or overnight at 4°C on a rotator. Immunoprecipitates were washed three times with buffer, and proteins were eluted in 2.5X Laemmli buffer, separated by SDS-PAGE, transferred to PVDF, and analyzed by Western blotting. For Sam68 immunoprecipitations, 24 hours post transfection cells were serum starved for 12-16 hours, and then the medium was changed to serum containing DMEM plus nocodazole (5μg/100cm plate) to arrest cells in mitosis [93]. Cells were harvested ~16 hours later with EB++ buffer. For analysis of whole cell lysates, lysates (10-50μg) were separated on 10% SDS-PAGE and transferred onto PVDF membrane. For co-immunoprecipitation experiments cells were lysed in 1% NP-40 buffer (50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, 1mM sodium vanadate, protease inhibitors and 1% NP-40). 500μg lysates were pre-cleared for 1 hour at 4°C and then subjected to immunoprecipitation with 2μg of anti-Cas N17 (Santa Cruz) antibody overnight at 4°C. The IP samples were separated on a 7.5% SDS-PAGE and were subjected to immunoblot analysis with anti-Flag (Sigma) and anti-Cas N17 (Santa Cruz) antibodies.

Immunofluorescence analysis- Cas$^{-/-}$ cells or Cos7 cells were grown on cover slips and transiently transfected with Src and YFP-Cas (wild-type or mutants). Cells were washed in 1X phosphate buffered saline and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 and were blocked with 3% bovine serum albumin. anti-pY416 antibody was used at a dilution of 1:200 and Texas Red conjugated anti-rabbit antibody was used as secondary antibody. Nuclei were visualized by staining with DAPI. Cells were analyzed using deconvolution microscopy (Zeiss) using filters for YFP, DAPI and Texas Red. Only YFP positive cells were analyzed for localization of activated

Src in the focal adhesions. DIC (Differential Interference Contrast) and YFP images of $Cas^{-/-}$ cells expressing YFP tagged wild-type Cas or its mutants were taken using 63X oil objective and YFP filter. Approximately 60-70% YFP positive cells had the phenotype presented in Figure 3-5.

Retrovirus generation and infection– Retroviruses were derived as described previously [94, 95]. Phoenix cell producer cell lines were obtained from Dr. Nicholas Carpino (Stony Brook) and were maintained in DMEM containing 10% FBS and 1X antibiotic/antimycotic solution. Cells were transiently transfected with 10μg of the retroviral constructs along with 10μg helper plasmid. Retroviruses were collected at 32°C for 2 days at 12 hours intervals. Aliquots were frozen at -80°C. $Cas^{-/-}$ cells were transiently transfected with engineered Cas and selected with 5μg/ml blasticidin (Sigma) for one week. For infection, $2x10^5$ SYF cells or $Cas^{-/-}$ were plated per 6 well plate and spin infected with the retroviruses along with polybrene (4μg/ml). Two days post-infection >90% cells were GFP positive. The cells were harvested and used for wound healing and migration assays.

Wound healing and Migration assays- Wound healing assays were performed essentially as described [96]. Retrovirally infected SYF or $Cas^{-/-}$ cells were plated on 60mm dishes and grown to confluency. The plates were washed with 1X PBS and multiple wounds were scratched using a P200 pipette tip across the plate. Phase contrast images of 8 separate wounds were imaged at 10 minute intervals for 10-12 hours using a Carl Zeiss inverted microscope with 20X objective. The width of the wound was measured at 30 minute to 1 hour intervals. Rates for all 8 positions were determined and an average rate of wound closure was plotted for each mutant.

For migration assays, Cas$^{-/-}$ cells were transiently transfected with engineered Cas along with PDZ-kinase or Hck. The cells were harvested 24 hours post transfection and 50,000 cells were placed in the Boyden chamber of a 96 well Transwell Migration assay plate or $2X10^5$ cells were placed in a 12 well transwell migration assay plate. Cells were allowed to migrate for 12 hours in a 5% CO2, humidified incubator maintained at 37°C. For 96 well plates cell migration was measured using Cell-Gro Assay (Promega) according to manufacturer's instructions. For 12 well plates the cells were fixed with a 1:1 acetone:methanol solution and manually counted under a light microscope.

Protein purification and kinase assay- Down-regulated Hck was purified from Sf9 cells as described [54]. The following peptides were used in the studies described in Chapter 3. Substrate peptide: AEEEIYGEFEAKKKKG, SH3 ligand: SPPTPKPRPPRP , SH2 ligand: EPQpYPEEIPIKQ (Table 2-1) [54, 97]. Kinase assays were performed using a continuous spectrophotometric assay, where production of ADP is coupled to oxidation of NADH and is measured as a decrease in absorbance at 340nm. For the kinase reactions, autophosphorylated down-regulated Hck was generated by pre-incubation with 500μM ATP at 4°C for 30 minutes. The autophosphorylated Hck was used at a final concentration of 15nM and varying concentrations of SH3 ligand peptide (10μM-620μM) and SH2 ligand peptide (0.5μM-64μM) were used as activators in the assay. For assays with two ligands, Hck was pre-incubated with a fixed concentration of the first ligand for 2 minutes at room temperature, then the second ligand was added at varying concentrations in the spectrophotometric assay. The substrate peptide was added in a cocktail along-with ATP, NADH, PEP and PK/LDH to start the reaction. The activation constant, K_{act}, was determined by non-linear regression analysis of the rates as a function of ligand concentration as described [54]. The equation used for analysis was

$$V_a = V_{act} [L] / (K_{act} + [L])$$

where V_a = velocity measured in the presence of variable ligand minus the velocity measured in the absence of ligand, V_{act} = maximal activated velocity in the presence of variable ligand minus the velocity measured in the absence of ligand, and [L] is the ligand concentration for which K_{act} is being determined. For experiments with two ligands, the velocity measured in the absence of the variable ligand was determined in the presence of the other ligand.

Mutants 2UC3 and 3UC2 were expressed using the Bac-to-Bac baculovirus expression system using the manufacturer's guidelines (Gibco). Sf9 cells (1 liter) were infected with the viruses at a multiplicity of infection of 10. Cells were harvested 48 hours post-infection. For purification, all steps were carried out at 4°C. Cell pellets were lysed in Buffer A (20mM Tris pH8.0, 10% (v/v) glycerol, 5mM β-mercaptoethanol (β-ME), 0.5M NaCl and 20mM imidazole) plus protease inhibitors using a French pressure cell at 750psi. The lysate was centrifuged at 18,000 rpm for 30 minutes at 4°C. The lysate was then applied to a 2-3ml column of nickel-nitrilotriacetic acid resin (Qiagen) pre equilibrated in 3 column volumes of Buffer A, and the column was washed with 10 column volumes of Buffer A. The column was further washed with 2 column volumes of Buffer B (20mM Tris pH8.0, 10 % (v/v) glycerol, 5mM β-ME, 1M NaCl and 20mM imidazole). Before elution, the column was given a final wash with Buffer C (20mM Tris pH8.0, 10% (v/v) glycerol, 5mM β-ME). The protein was eluted in 3 column volumes of Buffer D (20mM Tris pH8.0, 10% (v/v) glycerol, 5mM β-ME, and 100mM imidazole) and analyzed by 10% SDS-PAGE. The peak fractions were pooled and concentrated using an Amicon concentrator (15ml) and buffer was exchanged to Buffer C. The pure proteins were stored at -20°C in 40% (v/v) glycerol. Protein concentration was estimated using the Bradford assay. The proteins were >90% pure.

The activity of mutants 2UC3 and 3UC2 was measured using a continuous spectrophotometric assay [97, 98]. Reactions were carried out at 30°C in a final volume of 50 μl. The SH3-substrate, SH3-control, SH2-substrate, and SH2-control peptides have been described previously [66, 99]. The peptides were HPLC purified and their identity confirmed by mass spectrometry. The kinase reactions contained 100mM Tris pH 7.5, 10mM $MgCl_2$, 500μM ATP, 1mM phosphoenolpyruvate (PEP), 2.5mg/ml NADH, 89 units/ml pyruvate kinase and 124 units/ml lactate dehydrogenase. The enzymes were used at a concentration ranging from 10nM to 30nM and peptides were used at 50μM. The sequences of peptides used in the study are tabulated in Table 2-1.

IP kinase assays- The kinases were expressed in SYF cells and immunoprecipitated with Flag-agarose beads (Sigma) by incubation of 500μg- 1mg lysates either overnight (for domain rearranged mutants) or for 1 hour (for PDZ-kinases) at 4°C on a rotator. The IP samples were washed 2 times with RIPA buffer and once with PBS+0.1mM sodium ortho vanadate. The beads were finally washed with kinase assay buffer (100mM Tris pH 7.5, 10mM $MgCl_2$ and 0.5mM Na_3VO_4) and divided equally into three parts. The kinase reactions were performed in duplicate for 4 minutes at 30°C. The reactions consisted of enzyme bound to the beads, kinase assay buffer, 500 μM ATP, 0.1μM [γ-^{32}P] ATP and

600μM peptide substrate. For PDZ–kinases the enzyme bound beads were first pre-incubated with GST-GVKESLV protein for 3 minutes before the final reactions, which were carried out at 30°C for 5 minutes. The reactions were stopped with 0.1% trifluoroacetic acid (TFA) and spotted onto P-81 Whatman filter circles. The filters were washed with 0.1% H_3PO_4 3 times for 10 minutes each. The filters were finally rinsed with acetone and air dried. Radioactivity was determined in a scintillation counter. The specific activity was determined by spotting 1μl ATP containing cocktail onto a P-81 filter. The rate either in counts per minute (cpm) or pmol of phosphate incorporated were plotted for each mutant.

Table 2-1. **Synthetic peptides used in these studies.**

The Src substrate sequence is in red and SH2 and SH3 ligand sequences are in blue and green respectively. Nomenclature: SH3L=ligand sequence for SH3 domain, S=substrate sequence, SH3C=mutated SH3 ligand sequence, SH2L=ligand sequence for SH2 domain and, SH2C=mutated SH2 ligand sequence. The order in the name indicates the orientation of the sequences from amino terminus, i.e., SH3L-S has SH3 ligand amino terminal to the substrate sequence

Peptide name	Peptide sequence	Reference
Src substrate	AEEIYGEFEAKKKKG	[65]
SH2 ligand	EPQpYEEIPIKQ	[97]
SH3 ligand	SPPTPKPRPPRP	[99]
S-SH3L	AEEEIYGEFGGRGAAPPPPPVPRGRG	[67]
S-SH3C	AEEEIYGEFGGRGAAAAAAAVARGRG	[67]
SH3L-S	KKRPLPSPPKF(G)$_6$EEEIYGEFG	[67]
S-SH2L	RRLEDAIYAAGGGGGEPPQpYEEIG	[66]
S-SH2C	RRLEDAIYAAGGGGGEPPQFEEIG	[66]
SH2L-S	EPQpYEEIGGGGGEDAIYARRG	[66]
SH2C-S	EPQFEEIGGGGGEDAIYARRG	[66]

CHAPTER 3

SH3 and SH2 ligands act synergistically to activate Src family kinases[*]

[*] The contents of this chapter have been published in Cancer Letters, **7**, 583 - 589 (2007)

Abstract

Src family nonreceptor tyrosine kinases are kept in a repressed state by intramolecular interactions involving the SH3 and SH2 domains of the enzymes. Ligands for these domains can displace the intramolecular associations and activate the kinases. Here, we carried out in vitro activation experiments with purified, downregulated hematopoietic cell kinase (Hck), a Src family kinase. We show that SH3 and SH2 ligands act cooperatively to activate Src family kinases: the presence of one ligand lowers the concentration of the second ligand necessary for activation. To confirm the findings in intact cells, we studied Cas, a Src substrate that possesses SH2 and SH3 ligands. In contrast to wild-type Cas, mutant forms of Cas lacking the SH3- or SH2-ligands were unable to stimulate Src autophosphorylation when expressed in Cas-deficient fibroblasts. Cells expressing the Cas mutants also showed decreased localization of activated Src at focal adhesions. The results suggest that proteins containing ligands for both SH3 and SH2 domains can produce a synergistic activation of Src family kinases.

Introduction

Eukaryotic signaling proteins are typically composed of multiple modular domains. Communication between domains of a signaling protein is often necessary for proper regulation, and for precise and timely responses to stimuli. One mechanism for inter-domain communication is cooperativity, i.e., two structurally and functionally independent domains acting together synergistically to activate or inhibit the final output of the protein [100]. A well-studied example of a signaling protein that exhibits cooperativity is the Wiskott-Aldrich Syndrome protein (WASP). WASP contains a verprolin cofilin acidic (VCA) domain that binds and activates the Arp 2/3 complex to nucleate actin filaments. In the absence of stimulation, the VCA domain is kept in an auto-inhibited state by intramolecular interactions with the G protein binding/switching domain (GBD) and a basic domain (B). The ligands for the GBD and B domains (Cdc42 and PIP_2, respectively) are relatively poor activators of WASP individually. When both ligands are bound to WASP, they synergistically stimulate the actin polymerization activity of the VCA domain [26].

Src family tyrosine kinases are allosteric enzymes with conserved modular domain architecture. The structures of the down-regulated Src family kinases Hck and Src show that the kinase domain is kept in a repressed state by a series of intramolecular interactions [23, 24, 83] . The SH2 domain forms an intramolecular interaction with the C-terminal phospho-tyrosine 527, while the SH3 domain interacts with a polyproline type II helix in the SH2-kinase linker region. Binding of SH3 or SH2 ligands leads to release of these interactions, autophosphorylation of activation loop tyrosine 416, and increased kinase activity [54, 78, 97]. When the SH3 and SH2 domains are bound to their intramolecular ligands simultaneously, the connector between them forms an "inducible snap lock" that couples the domains together [69, 101, 102]. This suggests that SH3 and SH2 ligands could produce cooperative activation of Src kinases [85]. Consistent with this, a peptide containing both SH3- and SH2-binding sites binds to Src with higher affinity than a peptide containing an SH2 ligand alone [87]. Furthermore, several Src family kinase substrates have ligands for both the SH3 as well as the SH2 domain of the kinase. Cas, Sam68, Sin, AFAP110 and Connexin43 are Src substrates known to have ligands for both SH3 and SH2 domains of Src [68-73]. For the Src substrates Cas and Sin, interaction with both the SH3 and SH2 domains leads to maximal downstream signaling as reported by substrate phosphorylation and transcriptional activation [86, 103, 104]. In contrast to WASP, where either ligand alone is a poor activator of WASP, the SH3 ligand alone is a more potent activator of Src family kinases than the SH2 ligand alone. This suggests that the SH3 domain plays a dominant role in regulating kinase activity

We tested the hypothesis that SH3 and SH2 ligands act cooperatively to activate Src family kinases. Even though predicted based on structural analysis it has not been formally tested, i.e., it has not been determined whether the presence of one ligand lowers the concentration of the second ligand necessary for activation. We carried out kinetic studies using purified down-regulated Hck and synthetic SH3 and SH2 domain ligand peptides as activators. We observed a decrease in the K_{act} of an SH2 ligand in the presence of SH3 ligand, and vice-versa. To study the importance of cooperativity in intact cells, we measured Src autophosphorylation in cells expressing wild-type Cas, or mutants lacking SH3 and SH2 domain binding sequences.

Results

Cooperative activation by SH3 and SH3 ligands: To test for cooperativity, we expressed and purified the down-regulated form of the Src kinase Hck. In this form of the enzyme, the SH3 and SH2 domains are engaged with their intramolecular ligands [23, 24, 83, 85, 101, 105]. Hck can be activated by incubation with a synthetic peptide containing the optimal SH2-binding sequence pYEEI, or with a proline-rich SH3 binding peptide [54, 78].

We performed spectrophotometric kinase assays to determine values of K_{act}, the concentration of ligand required for half-maximal activation of Hck. When measured individually, the K_{act} values for the SH2-binding and SH3-binding peptides were 17.6μM and 158.6μM, respectively (Tables 3-1 and 3-2). If the SH3 and SH2 domains function cooperatively to inhibit kinase activity, disrupting one intramolecular interaction should weaken the other one. Thus, addition of one ligand (e.g., the SH2 ligand) should decrease the concentration of the other ligand (the SH3 ligand) needed for half-maximal activation of Hck. In our experiments, the K_{act} for SH3 ligand decreased from 158.6μM to 30.8μM in the presence of increasing concentrations of SH2 ligand (Table 3-1, Figure3-1A.). No additional decrease in the K_{act} for SH3 ligand was observed with concentrations of SH2 ligand higher than 32 μM. Next, we carried out the reciprocal measurements of K_{act} for SH2 ligand in the presence of increasing concentrations of the SH3-binding peptide. As shown in Table 3-2 and Figure 3-1B, we measured a gradual decrease in the K_{act} of SH2 ligand as the concentration of SH3 ligand was increased. The K_{act} decreases to a minimum of 3.3μM in the presence of 320μM SH3 ligand peptide. Our kinetic analysis supports the hypothesis that SH3 and SH2 ligands act cooperatively to activate the kinase. Therefore, for a protein or peptide capable of binding to both the SH3 and SH2 domains of Src family kinases, a lower concentration would be required to activate the kinase than for a protein with a single ligand sequence.

Importance of the SH2/SH3 ligand sequences in Cas for Src activation: Next, we wished to test for activation of Src kinases by a combination of SH3 and SH2 ligands in intact cells. Cas (Crk-associated substrate) is a 130kD adaptor protein that is involved in cell migration and integrin signaling [64, 89, 104, 106, 107]. It is colocalized in focal adhesions with activated Src, and it has been extensively characterized as a substrate of Src family kinases [103, 108-111]. Phosphorylation of Cas leads to binding of the SH2 domain of the adaptor protein Crk. This leads to cell migration through activation of RacI and survival signaling via JNK, Ras and Rac [112]. Cas knockout mice die *in utero* with cardiovascular defects and growth retardation [113]. The fibroblasts derived from Cas knockout mice have defects in actin filament organization and migration [113]. Cas has a Src binding sequence (SBS) near its C-terminus that contains a ligand for the SH3 domain (RPLPSPP), followed by a ^{668}YDYV motif which, when phosphorylated at Y668, can interact with the SH2 domain (Figure 3-2). It has been shown that Src binds to Cas through its SH3 and SH2 domains, and the most important interaction is between the SH3 domain and the polyproline sequence [110, 114]. This binding leads to processive phosphorylation of 15 tyrosine residues in the central substrate region of Cas by Src family kinases [68, 110, 115]. Since Cas has the potential to disrupt both SH3 and SH2 domain mediated intramolecular interactions in Src family kinases, it can function as an activator of Src family kinases [66, 103, 110].

In order to study the cellular interactions between Src and Cas, we employed three Cas mutants that are defective in interaction with Src (Figure 3-2): (1) CasY668F, in which the phosphotyrosine residue necessary for SH2 interaction is removed; (2) CasPPX, in which two proline residues critical for SH3 binding are mutated to alanine; and (3) a CasY668F/PPX double mutant which can bind neither the SH3 nor the SH2 domain of Src. To eliminate the contribution of wild-type Cas, we expressed the mutant forms of Cas in fibroblasts derived from Cas-knockout mice.

We co-expressed Src together with Cas (wild-type or mutants) in Cas$^{-/-}$ cells and analyzed for Src activation using a phosphospecific antibody for Y416 in the activation loop. In the presence of wild-type Cas, Src was significantly autophosphorylated as indicated by anti-pY416 immunoblotting (Figure 3-3). There was a small decrease in autophosphorylation of Src in cells expressing the Y668F form of Cas, and a more significant decrease in cells expressing Cas-PPX (Fig. 3-3). The Y668F/PPX double mutant was similar to vector control, with no detectable autophosphorylation of Src (Figure 3-3). These results provide further support to our hypothesis that the SH3 and SH2 domains act cooperatively in regulating Src family kinases. To analyze Src-Cas binding directly in these cells, we immunoprecipitated Cas and measured the amount of associated Src (Figure 3-4). The PPX and Y688F/PPX mutants were defective in their ability to bind Src, consistent with our previous *in vitro* experiments and yeast two-hybrid studies showing the importance of SH3 mediated interactions between Src and Cas [66].

Localization of activated Src to focal adhesions: Integrin signaling leads to activation of Src and Src-Cas interactions at focal adhesions [92, 116, 117]. We performed immunofluorescence analysis with anti-pY416 antibody to examine the activation status of Src in focal adhesions in Cas$^{-/-}$ cells co-expressing Src and wild-type Cas or its mutants. We analyzed YFP positive cells to identify Cas expressing cells. Similar to wild-type Cas, the mutant formas of Cas showed a general cytoplasmic distribution (Figure 3-5). Cells expressing YFP alone had no localization of activated Src in focal adhesions (Figure 3-5A), while in WT Cas expressing cells Src was active and localized to focal adhesions (Figure 3-5B). The localization was somewhat less pronounced for Y668F compared to wild type Cas (Figure 3-5B and 3-5C). The decrease in activated Src in the focal adhesions was more dramatic for Cas-PPX compared to WT Cas (Figure 3-5B and 3-5D). Cells expressing the Y668F/PPX double mutant were similar to YFP expressing cells with minimal localization of active Src in focal adhesions (Figure 3-5A and Figure 3-5E). We also observed similar results in Cos7 cells (data not shown).

Discussion

Our results provide support to the hypothesis that the SH3 and SH2 domains of Src family kinases act in a cooperative manner to repress the kinases. Src family kinases are regulated by intramolecular SH3 and SH2 domain mediated interactions, and it has been shown previously that the kinase can be activated by disruption of these SH3-linker and SH2-pTyr527 interactions [54, 97]. The individual SH2 and SH3 ligands can activate Src kinases; here, we show that if one ligand is prebound to Hck, the autoinhibited conformation is destabilized and binding of the second ligand is enhanced. This is reflected in a decrease in the concentration of the second ligand necessary for half maximal activation of Hck (Tables 3-1 and 3-2, Figure 3-1). Our results are in accord with targeted molecular dynamics simulations of the closed form of Src, which suggest that the SH3 and SH2 domains act in concert to repress kinase activity [102]. In contrast, the activities of mutant forms of Hck expressed in fibroblasts have led to the suggestion that SH3-based activation and SH2-based activation are independent events that lead to distinct activated states [118, 119]. It is possible that the SH3-activated and SH2-activated states, which are presumably transient in our *in vitro* studies, are populated more fully in the cell due to the presence of SH2- and SH3- associated proteins. In the cellular context, many natural activators of Src family kinases contain tandem SH3 and SH2 ligands. A sub-group of these proteins are SFK substrates which activate Src by disruption of the intramolecular interactions, and are subsequently targeted for phosphorylation while bound to the SH3 and SH2 domains [105]. A few examples of such SFK substrates are Cas, FAK, Sam68, Connexin 43 and Sin [68-73]. The autophosphorylation of FAK at Y397 is increased by integrin-dependent cell adhesion

[117, 120]. This autophosphorylation site acts as a ligand for the SH2 domain of Src. In addition, residues 368-378 of FAK serve as a ligand for the SH3 domain of Src [104]. Thus, these two sequences in FAK cooperate to generate activated Src at the focal adhesions. In the case of Cas and FAK, co-expression with Src leads to enhanced phosphorylation of paxillin and other downstream targets [87, 103], but this effect was not observed for mutants deficient in Src binding. Similarly, expression of wild-type Sin (but not mutants defective in Src binding) led to increased Src-mediated transcriptional activation [86].

To test for cooperative activation of SFKs in intact cells, we examined the ability of Cas to promote Src autophosphorylation. Expression of wild-type Cas led to an increase in Src autophosphorylation (Fig. 3-3). Mutations of the SH3- or SH2-binding sequences interfered with the ability of Cas to activate Src. This effect was most pronounced for the PPX and Y668F/PPX mutants, consistent with previous studies showing that the poly-proline sequence of Cas is the major determinant for Src binding [121]. In the previous studies, removal of the polyproline sequence from Cas prevented tyrosine phosphorylation (including, presumably, phosphorylation of Y668). Cells expressing the PPX and Y668F/PPX mutants also showed low levels of activated Src in focal adhesions (Figure 3-5). The higher potency of the combined SH3-SH2 ligand sequences for Src activation suggests that many cellular activators will contain dual ligands. Consistent with this, activated Src is associated with subcellular sites (e.g., focal adhesions) that are known to contain a high local concentration of SH3 and SH2 ligands [35].

As noted by Lim [18], cooperativity between modular domains in a signaling protein provides a mechanism for signal integration and amplification, allowing precise and specific control of output. Co-operativity is a well established phenomenon in metabolic enzymes and it has also been observed in receptors and ion channels . For example, the presence of ligands for both the GBD and B domains of WASP leads to maximal signal amplification, while either ligand alone leads to sub-maximal activation [26]. The synergistic activation of WASP is therefore an example of “coincidence detection” [122]. Coincidence detection is also seen in the case of protein kinase C, where the C1 and C2 domains require diacylglycerol and calcium, respectively, to bind membranes. In general, co-operativity is maintained by a delicate balance of interactions between the domains and their ligands, and the pattern of regulation can be altered by slight changes in ligands and domains. Thus, cooperativity provides an evolutionary mechanism to adapt to changing environments [18]. Autoinhibition is widespread among signaling proteins, and the repressed states of many of the proteins depend on multiple binding sites, suggesting that many signaling systems may be regulated by cooperative interactions.

***Table 3-1*. Activation constants for SH3 ligand binding to Hck in the presence of fixed concentration of SH2 ligand**

SH2 ligand concentration (μM)	Kact (μM) for SH3 ligand
0	158.6 (+/- 22.7)
2	125.6 (+/- 16.0)
8	65.6 (+/- 6.1)
16	37.7 (+/- 5.9)
32	30.8 (+/- 1.7)

***Table 3-2*. Activation constants for SH2 ligand binding to Hck in the presence of fixed concentration of SH3 ligand**

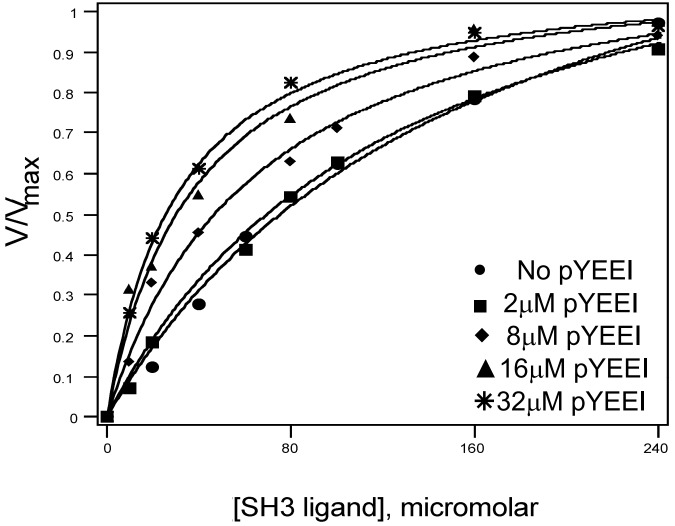
SH3 ligand concentration(μM)	Kact (μM) for SH2 ligand
0	17.6 (+/- 1.4)
20	8.6 (+/- 0.6)
80	7.4 (+/- 0.4)
160	5.9 (+/- 0.8)
320	3.3 (+/- 0.5)

Figure 3-1. **Co-operativity between SH3 and SH2 domain of Hck.**

[A.] Hck activity was measured using the continuous spectrophotometric assay. Assays were carried out in the presence of varying concentrations of SH3 ligand at fixed concentrations of SH2 ligand. K_{act} of SH3 ligand for Hck progressively decreases in the presence of a fixed and increasing concentration of SH2 ligand.

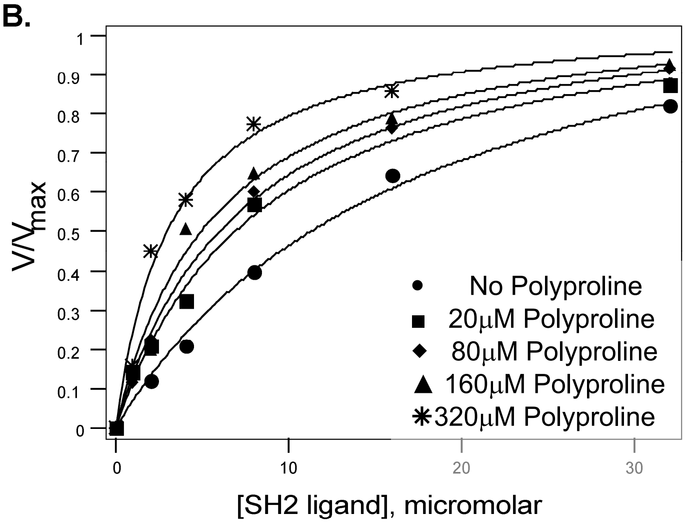
[B.] Hck activity was measured in the presence of varying concentrations of SH2 ligand at fixed concentrations of SH3 ligand. K_{act} of SH2 ligand for down-regulated Hck progressively decreases in the presence of a fixed and increasing concentration of SH3 ligand. Activation constants were calculated as described in Chapter 2.

A.



1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
V/V$_{max}$
0
80
160
240
[SH3 ligand], micromolar
● No pYEEI
■ 2μM pYEEI
◆ 8μM pYEEI
▲16μM pYEEI
✳32μM pYEEI

B.



1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
V/V$_{max}$
0
10
20
30
[SH2 ligand], micromolar
● No Polyproline
■ 20μM Polyproline
◆ 80μM Polyproline
▲ 160μM Polyproline
✳320μM Polyproline

Figure 3-2. **Domain arrangement of Cas showing the Src Binding Sequence (SBS) at the C-terminus**. Cas contains (from N- to C-terminus) a SH3 domain, a proline rich region (Pro), a substrate region containing 15 repeats of YXXP, a serine rich region (Ser), a Src binding sequence (SBS), and a helix-loop-helix region (HLH). Within the SBS, the sequences of the Src SH3 ligand (RPLPSPP) and SH2 ligand (YDYV) are shown. The PPX mutant carries mutations in the proline rich sequence (underlined) that impair interaction with the SH3 domain of SFKs. The Y668F mutation (underlined) blocks interaction with the SH2 domain of Src. The Y668F/PPX double mutant has both the binding sequences mutated.

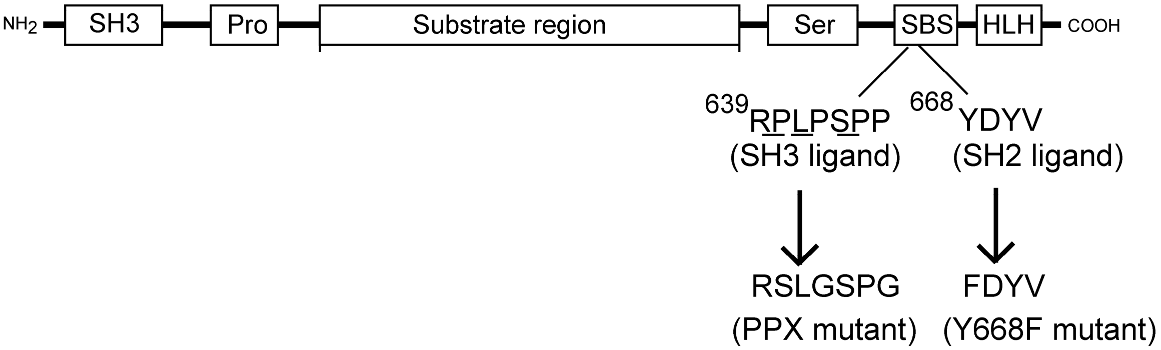

NH2
SH3
Pro
Substrate region
Ser
SBS
HLH
COOH
639RPLPSPP
(SH3 ligand)
668YDYV
(SH2 ligand)
RSLGSPG
(PPX mutant)
FDYV
(Y668F mutant)

Figure 3-3. **Auto-phosphorylation of Src in Cas$^{-/-}$ cells.** Cells expressing wild-type or mutant forms of Cas (or vector control) were lysed and analyzed by SDS-PAGE with Western blotting. The membrane was probed with antibodies for autophosphorylated Src (pY416), then stripped and reprobed with antibodies against total Src, Cas and tubulin as a loading control.

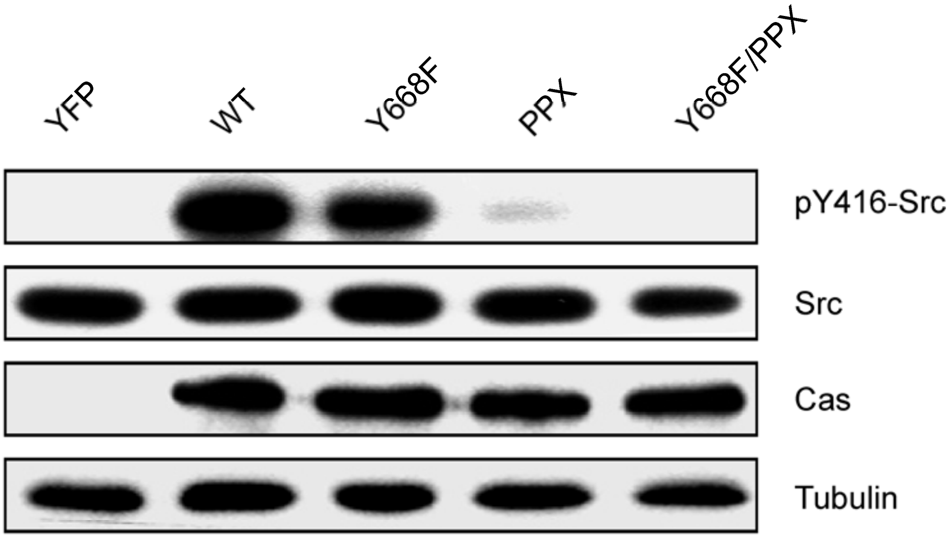

YFP
WT
Y668F
PPX
Y668F/PPX
pY416-Src
Src
Cas
Tubulin

Figure 3-4. **Co-immunoprecipitation of Src and Cas.** Cas$^{-/-}$ cells expressing wild-type or mutant forms of Cas (or vector control) were lysed and subjected to anti-Cas immunoprecipitation reactions. Proteins in the precipitates were transferred to membrane and detected by anti-Flag Western blotting (for Src). The membrane was probed with anti-Cas antibody (the arrowhead indicates the position of Cas). YFP denotes Cas$^{-/-}$ cells transfected with empty vector alone.

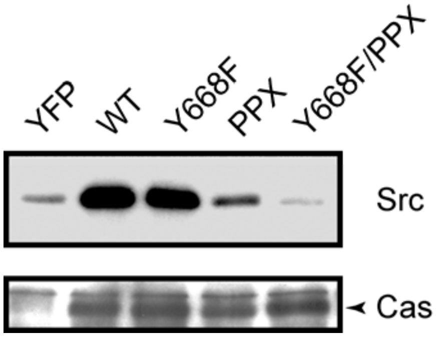
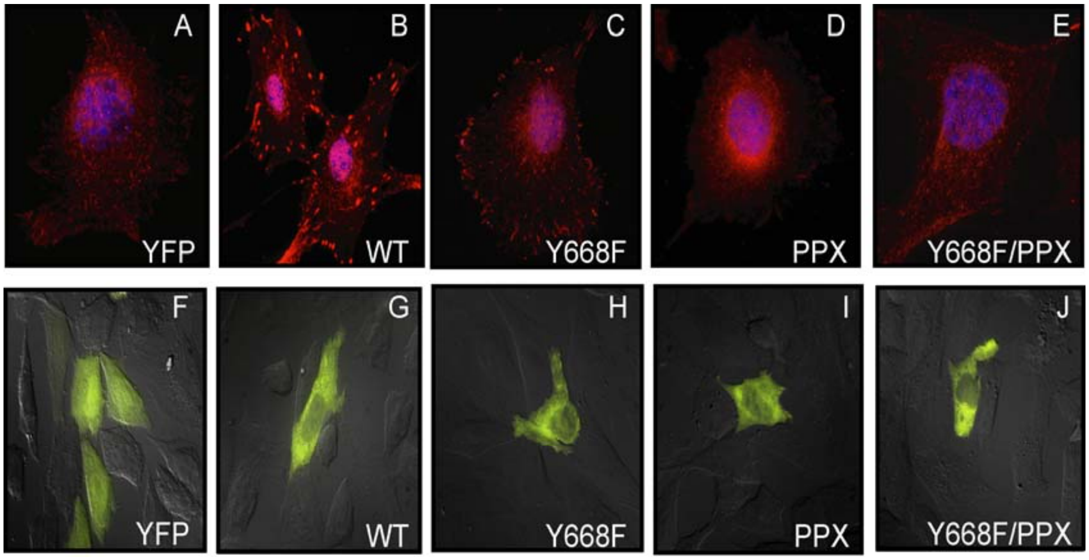

YFP
WT
Y668F
PPX
Y668F/PPX
Src
Cas

Figure 3-5. **Decreased amounts of activated Src in cells expressing Cas mutants.** Cas$^{-/-}$ cells were co-transfected with Src and Cas (wild-type or mutants). Cas expressing cells were identified as YFP positive and analyzed for Src activation. Cells were immunostained 24 h after transfection with anti-pY416 antibody to visualize activated Src. Nuclei were visualized with DAPI [A] YFP vector control. [B] wild-type Cas. [C] Y668F Cas mutant. [D] PPX mutant. [E] Y668F/PPX double mutant. [F-J] Superimposed DIC and YFP images of Cas$^{-/-}$ cells expressing YFP-tagged WT or mutant forms of Cas. A similar cytosolic distribution was observed for all forms of Cas.


A
B
C
D
E
YFP
WT
Y668F
PPX
Y668F/PPX
F
G
H
I
J
YFP
WT
Y668F
PPX
Y668F/PPX

CHAPTER 4

The evolutionarily conserved arrangement of domains in Src family kinases is important for substrate recognition

Abstract

The SH3-SH2-kinase domain arrangement in nonreceptor tyrosine kinases has been conserved throughout evolution. For Src family kinases, the relative positions of the domains are important for enzyme regulation; they permit the assembly of Src kinases into autoinhibited conformations. The SH3 and SH2 domains of Src family kinases have an additional role in determining the substrate specificity of the kinase. We addressed the question of whether the domain arrangement of Src family kinases has a role in substrate specificity by producing mutants with alternative arrangements. Our results suggest that changes in the positions of domains can lead to specific changes in the phosphorylation of Sam68 and Cas by Src. Phosphorylation of Cas by several mutants triggers Cas/Crk coupling and downstream signaling leading to cell migration. The placement of the SH2 domain with respect to the catalytic domain of Src appears to be especially important for processive phosphorylation of substrates with multiple phosphorylation sites. The placement of the SH3 domain is more flexible. The results suggest that the involvement of the SH3 and SH2 domains in substrate recognition is one reason for the strict conservation of the SH3-SH2-kinase architecture.

Introduction

Nonreceptor tyrosine kinases consist of kinase domains flanked by various modulatory domains. Src family kinases (SFKs) have a conserved domain architecture consisting of (from N- to C- terminus) unique, SH3, SH2, and catalytic domains [7, 35]. The SH3-SH2-kinase domain arrangement is conserved in other families of nonreceptor tyrosine kinases as well [6, 123, 124]. In fact, across all species, whenever the three domains are present together they have the same arrangement [125]. Recent genome analyses show that the SH3-SH2-kinase arrangement is also conserved in unicellular choanoflagellates, the most primitive organisms possessing SFKs; thus, this arrangement evolved before the origin of animals [126]. New signaling proteins arise by domain recombination, but clearly selective pressure exists to maintain this arrangement of domains in nonreceptor TKs. The importance of this conserved domain arrangement has not been explicitly addressed by previous studies.

The SH3 and SH2 domains of SFKs play major roles in kinase regulation. The SH3 domain interacts intramolecularly with a polyproline type II helix in the SH2-kinase linker region, while the SH2 domain interacts with the C-terminal pTyr527 [23, 24]. These two interactions occur on the face opposite the kinase active site, and they keep the kinase in an inhibited state until an activating signal is received [78, 85, 127]. Thus, one purpose of the conserved SH3-SH2-kinase arrangement is to permit the assembly of the autoinhibited kinase. The SH3 and SH2 domains also play important roles in targeting SFKs to cellular substrates [35, 64, 74, 128-130]. Some SFK substrates, such as the EGF and PDGF receptors and RACK1, possess phosphorylation sites that bind the SH2 domain [131-134]. Many Src substrates contain ligands for both the SH3 and SH2 domains [68, 70, 72, 104, 135]. One such substrate is the adaptor protein p130Cas, which contains 15 potential tyrosine phosphorylation sites and C-terminal ligands for the SH3 and SH2 domains of Src [68, 106]. Src binding to the C-terminus triggers processive phosphorylation of Cas [89, 115], followed by binding of the SH2 domains of the Crk adaptor protein [106, 136]. Cas/Crk complex formation leads to downstream Rac and Cdc42 activation and increased cell migration [137-139].

Another SFK substrate that depends on SH3/SH2 interactions is the mitotic Src substrate Sam68 [72, 140]. The SH3 binding site of Sam68 resides in the N-terminus, while the SH2 binding ligand and phosphorylation sites are in the C-terminus [140, 141]. For both Cas and Sam68, SFK phosphorylation is driven primarily by SH3/SH2 interactions [89, 141]. Because the intrinsic specificity of tyrosine kinase catalytic domains is not high enough to explain selective signaling, it has been proposed that substrate specificity is governed by the flanking domains [35, 64, 142]. Evidence for this hypothesis comes from studies on the Abl nonreceptor tyrosine kinase, where it was shown that replacing the SH2 domain with exogenous SH2 domains leads to alterations in substrate specificity [90].

In this study, we address the question of whether the evolutionarily conserved domain arrangement of SFKs plays a role in determining substrate specificity. We produced a series of Src mutants in which the positions of the domains were rearranged. We reasoned that these changes might create new substrate interactions or alter existing interactions. As expected, most of the mutants were more active than wild-type Src due to a loss of autoinhibition. In addition, we observed that rearranging the domains led to specific changes in substrate specificity. Furthermore, the relative positions of the domains and the positions of ligand sequences in potential substrates can modulate phosphorylation efficiency. Our results suggest that the ability of SFKs to recognize key cellular substrates is one explanation for the strict conservation of the SH3-SH2-kinase architecture.

Results

Generation of mutants- To study the role of Src domain arrangement in determining substrate specificity, we generated a panel of mutants in which the positions of the domains were altered. We introduced unique restriction sites in the sequences flanking the domains, to preserve the folded structures of the individual domains. We also maintained the natural 4 amino acid distance found between SH3 and SH2 domains of Src. We made three groups of mutants. In the first group, the position of the unique domain was maintained at the N-terminus, but other domains were rearranged. Thus, in mutant U23C the position of the SH3 and SH2 domains is reversed with respect to wild-type Src, and in mutant UC32 the SH3 and SH2 domains are C-terminal to the kinase domain (Figure 4-1). (The mutants were named according to the positions of their domains from N- to C-terminus, with unique=U, SH2=2, SH3=3, and catalytic=C. Thus, wild-type Src is U32C). UC32 therefore resembles Ack family kinases, the only naturally-occurring group of kinases in which the SH3 domain is immediately C-terminal to the kinase domain [6, 124]. In the second group of mutants, we changed the position of the unique domain. The unique domain was positioned between the SH3 and SH2 domains (mutant 3U2C), N-terminal to the kinase domain (mutant 32UC) or C-terminal to the kinase domain (mutant 32CU) (Figure 4-1). In the third group of mutants, we placed the SH3 and SH2 domains on opposite ends of the protein (mutants 2UC3 and 3UC2). The SH3 and SH2 domains often cooperate to recognize substrates, so these mutants were designed to test the importance of proximity between the SH3 and SH2 domains. We also tested two control constructs. First, the mutant R175L/W44A has point mutations in the SH2 domain (R175L) and SH3 domain (W44A) of wild-type Src; these mutations have been previously reported to abrogate SH2 and SH3 domain mediated interactions [99, 143, 144]. We also used the catalytic domain alone with no flanking domains as a control.

Mutants are activated relative to wild-type Src- We expressed the mutants and wild-type c-Src in Src/Yes/Fyn triple knockout (SYF) cells, which lack all Src family kinases [91]. We carried out anti-pY416 immunoblotting of cell lysates as a measure of kinase autophosphorylation and activity (Figure 4-2). We immunoprecipitated the kinases from SYF cells and performed kinase assays as another measure of kinase activity (Figure 4-3). We observed increased kinase activity in the mutants, particularly those in which the position of the unique domain was altered (Figure 4-2 and Figure 4-3). Kinase activation is presumably due to the disruption of the autoinhibitory contacts in Src. Consistent with this, the isolated catalytic domain displayed high activity (Figure 4-2 and Figure 4-3). Mutants U23C and UC32 were autophosphorylated to a similar level as wild-type c-Src. In wild-type c-Src, the SH3 and SH2 domains act in concert to keep Src in an auto-inhibited state. Our results suggest that some repression can be exerted when the SH3 and SH2 domains are present together in either orientation (U32C vs. U23C) or when the SH3 and SH2 domains are C-terminal to the kinase (UC32). The loss of autoinhibition was most pronounced when the distance between the SH3 and SH2 domains was altered, or when they were not directly adjacent to the catalytic domain (Figure 4-2 and Figure 4-3). We confirmed the heightened activity of the mutants by focus-forming assays for transformation (data not shown). In general, our results suggest that domain re-arrangement leads to loss of auto-regulation of the kinase. Our data also support previous findings that the SH3 and SH2 domains of Src act as unit to repress the kinase [83, 87]. Our results from these and subsequent experiments on the mutants are summarized in Table 4-1.

Mutants display no global changes in substrate specificity- To examine phosphorylation of cellular proteins by the mutants, we expressed them in SYF cells and measured overall tyrosine phosphorylation by anti-phosphotyrosine Western blotting of lysates. We observed that the overall tyrosine phosphorylation pattern of the mutants was very similar to wild-type c-Src (Figure 4-4). Consistent with the results in Figure 4-2 and 4-3, most of the mutants showed enhanced phosphorylation as compared to c-Src (Figure 4-4). The R175L/W44A mutant was defective in recognition of many proteins (Figure 4-4), indicating that the observed phosphorylation pattern was due to Src substrates targeted by the SH3 and SH2 domains. The isolated catalytic domain was highly active and phosphorylated a slightly different spectrum of proteins than constructs containing SH2 and SH3 domains (Figure 4-4). Because the patterns were not radically altered by rearranging the domains, at this level of analysis the data suggested that the positions of the domains did not play a dominant role in determining substrate specificity.

Domain rearrangement leads to changes in substrate specificity towards Cas- To test for any specific changes in phosphorylation that might not be visible in the overall analysis of whole cell lysates, we studied the Src substrate Cas. We expressed the mutants and c-Src in SYF cells and immunoprecipitated Cas, and examined its phosphorylation using anti-pTyr Western blotting. SH3 and SH2 interactions are crucial for Src recognition of Cas [68, 89]. Consistent with this, the R175L/W44A mutant and the isolated catalytic domains were unable to phosphorylate Cas (Figure 4-5). We observed that altering the positions of the domains led to specific changes in Cas phosphorylation (Figure 4-5). The U23C and UC32 mutants phosphorylated Cas to a similar level as wild-type Src (Figure 4-5). We observed increased phosphorylation of Cas by the three mutants in which the unique domain was repositioned (3U2C, 32UC and 32CU) (Figure 4-5). This could be due to increased overall activity of the mutants (Figures 4-2 and 4-3) or to a specific enhancement of Cas phosphorylation. On the other hand, while the 2UC3 and 3UC2 mutants had higher overall activity than wild-type Src (Figures 4-2, 4-3 and 4-4), they showed less activity towards Cas (Figure 4-5). This suggests that separation of the SH3 and SH2 domains resulted in decreased Cas recognition.

Since Cas was phosphorylated by the mutants, we tested for activation of downstream signaling. Phosphorylation of the substrate domain of Cas promotes binding to the SH2 domain of the adaptor protein Crk [106, 136]. We observed that Cas and Crk co-immunoprecipitated from SYF cells expressing c-Src or the mutants (Figure 4-6). Complex formation was highest for the mutants in which the unique domain was repositioned. Thus, the domain re-arranged mutants are capable of phosphorylating Cas in a manner that promotes formation of Cas/Crk complexes.

Mutants restore the migration defect of SYF cells- SYF cells show low levels of Cas phosphorylation and are deficient in cell migration [91]. We carried out retroviral expression of the mutants in SYF cells and tested whether they could restore functional migration signaling by wound healing assays. Mutants that were able to phosphorylate Cas (Figure 4-5) were active in these assays (Figure 4-7). Two of the mutants in which the unique domain was repositioned (3U2C and 32CU) were more active than wild-type c-Src, consistent with their high levels of Cas phosphorylation (Figure 4-5). Mutants 2UC3 and 3UC2, which phosphorylated Cas poorly compared to c-Src (Figure 4-5), were also not as efficient as wild-type Src in wound healing (Figure 4-7 and Table 4-1). Thus, the mutants displayed changes in Cas phosphorylation, and there were corresponding changes in their function in this cell signaling pathway.

Specific changes in substrate specificity towards Sam68- Next, we tested for specific changes in recognition of another protein: the mitotic Src substrate Sam68. We expressed mutant or wild-type forms of c-Src in SYF cells and arrested them in mitosis with nocodazole. We immunoprecipitated Sam68 from the mitotic cells and examined tyrosine phosphorylation using anti-pTyr Western blotting. The R175L/W44A mutant and isolated catalytic domain were deficient in Sam68 phosphorylation. Rearrangement of the domains led to changes in phosphorylation of Sam68 as compared to c-Src (Figure 4-8). The U23C and the UC32 mutants were comparable to c-Src in their ability to phosphorylate Sam68. Mutants in which the unique domain was shifted (3U2C, 32UC and 32CU) showed enhanced phosphorylation of Sam68 compared to c-Src (Figure 4-8). Since these three mutants also showed increased phosphorylation of Cas (Figure 4-5 and Table 4-1), the enhanced phosphorylation of Sam68 by this group of mutants is probably due to their increased activity rather than any change in specificity. On the other hand, although mutant 2UC3 phosphorylated Sam68 more strongly than c-Src, mutant 3UC2 showed very weak activity (Figure 4-8). This contrasts with results for Cas, in which both 2UC3 and 3UC2 had lower activity than wild-type c-Src (Figure 4-5 and Table 4-1). The enhancement of Sam68 phosphorylation by 2UC3 points to a specific change in substrate specificity, suggesting that the arrangement of domains can influence Src substrate recognition.

***In vitro* kinase assays with mutants-** We tested whether the changes we observed in Sam68 phosphorylation by 2UC3 and 3UC2 could be observed in vitro. We expressed mutants 2UC3 and 3UC2 in Sf9 insect cells using baculovirus expression vectors. We purified the proteins and compared their properties to the Src family kinase Hck. Our initial studies showed that the kinetic parameters k_{cat} and K_m (ATP) were similar for the mutants and Hck (data not shown). Next, we compared phosphorylation of a synthetic peptide containing a Src phosphorylation site and the SH3 binding sequence from Sam68 (SH3L-S). Consistent with our previous findings [67], Hck phosphorylated this peptide three times more efficiently than a control peptide with amino acid substitutions in the SH3-binding sequence (SH3C-S) (Figure 4-9). Mutant 2UC3 showed a more pronounced effect: it phosphorylated the SH3 substrate 6-7 times better than the control peptide. Mutant 3UC2 phosphorylated the SH3 substrate 2 times better than the control, a slightly smaller effect than Hck (Figure 4-9). This trend is consistent with Sam68 phosphorylation in SYF cells (Figure 4-8). We also tested a peptide containing a Src phosphorylation site and the SH3 binding sequence in reverse orientation (S-SH3L, for sequences of peptides see Table 2-1). Wild-type Hck and mutants 2UC3 and 3UC2 all phosphorylated this peptide more efficiently than the control.

Next, we carried out similar studies on a synthetic Src substrate containing the pYEEI recognition sequence for the SH2 domain. As reported previously [66], the activity of Hck was higher toward the SH2 substrate peptide (S-SH2L) than a control peptide lacking p-Tyr (S-SH2C) (Fig. 4-10). In contrast, 2UC3 and 3UC2 showed no difference between (S-SH2L) and (S-SH2C). We tested a peptide in which the SH2 ligand was N-terminal to the substrate sequence (SH2L-S). Hck, 2UC3, and 3UC2 all phosphorylated this peptide more strongly than a control (SH2C-S); for Hck, the rate was 3-fold higher, while for the mutants the rate was 1.5-2 fold higher. Thus, for Hck SH2-mediated targeting occurs regardless of the orientation, while the mutants displayed targeting only for a peptide with an N-terminal pTyr. This suggests that the positioning of the SH2 domain relative to the kinase domain is especially important for proper substrate recognition.

Discussion

Our results suggest that the SH3-SH2-kinase architecture of nonreceptor tyrosine kinases has been maintained through evolution in part because this position of domains is important in dictating specificity. Thus, we postulate that the adjacent placement of domains in the correct orientation can lead to simultaneous interactions with potential substrates and enhance phosphorylation. These findings are consistent with earlier studies on the Src kinase Fyn, in which it was demonstrated that the presence of the N-terminal region and SH3 domain enhanced the binding of cellular phosphoproteins to the SH2 domain [145]. In another study, repositioning the domains of Abl led to changes in transformation potential without changes in activity, which might be explained by a change in specificity of the kinase [90].

In our study, repositioning the domains of Src led to differential effects in recognition of Src substrates. For example, the mutants 2UC3 and 3UC2 showed very little phosphorylation of Cas (Fig. 4-5). These two mutants were also less effective than c-Src at promoting cell migration, a downstream effect of Cas (Fig. 4-7). In contrast, 2UC3 phosphorylated Sam68 more effectively than c-Src, while 3UC2 had decreased activity toward Sam68 (Table 4-1). This may be due to the different placement of the Src SH3 and SH2 binding sequences in Cas and Sam68. In Cas, the SH3 and SH2 ligands are clustered in the C-terminus, while the substrate region is in the central portion of the molecule. Because the SH3 and SH2 domains are distant from each other in the 2UC3 and 3UC2 mutants, these proteins might not be able to benefit from the cooperative binding to Cas seen in wild-type c-Src [68, 146]. In contrast, Sam68 possesses an N-terminal SH3 ligand, and the substrate and SH2-binding sites are in the C-terminus of the protein. It has previously been shown that SH3-polyproline interactions are the major determinant of Src phosphorylation for Sam68 [141]. Phosphorylation of Sam68 by 2UC3 suggests that the SH3 domain can still target the kinase domain, even when present on the C-terminal side of the kinase domain. The lower activity of 3UC2 could be due to the fact that the SH3 and kinase domains are separated by the unique domain in that construct.

Neither 2UC3 nor 3UC2 phosphorylated Cas significantly (Figure 4-5). In Cas, the SH2-binding sequence is C-terminal to the phosphorylation sites. This is the same orientation as peptide S-SH2L, which was not phosphorylated more than controls. In contrast, SH2L-S showed enhanced phosphorylation by both 2UC3 and 3UC2 relative to a control. Thus, the placement of the SH2-binding sequence relative to the phosphorylation sites seems to be particularly important for targeting. The reason that both 2UC3 and 3UC2 displayed this tendency is not clear at present. In natural NRTKS, SH2 domains are invariably found immediately N-terminal to the kinase domain, and the two domains appear to have co-evolved [125]. There is a correlation between the substrate specificity of NRTKs and the specificity of the associated SH2 domains [48]. In the case of the 3UC2 and 2UC3 mutants, the presence of a C-terminal SH2-binding sequence may make bound proteins inaccessible to the kinase domain. Such proteins might also be more readily dephosphorylated by cellular phosphatases [147].

The structures of nonreceptor tyrosine kinases make it clear that the positions of the domains are critical for enzyme regulation. Our studies point to the importance of domain arrangement in determining substrate specificity. They also shed light on the relative preference of SFK substrates: depending upon the orientation of SH3 and SH2 ligand sequences relative to phosphorylation sites in substrates, different signaling outcomes might ensue. This may also be relevant to the aberrant signaling observed in some cancers that express mutant forms of tyrosine kinases. Many insertions, mutations, or deletions lead to constitutively active kinases. These alterations could also produce changes in substrate specificity. For example, fusion of the BCR segment to Abl in chronic myelogenous leukemia leads to phosphorylation of Tyr177 in BCR. This phosphotyrosine residue binds to the Grb2 SH2 domain; mutation of Tyr177 decreases the transforming potential of BCR-Abl[148]. It will be important to understand how the proper positioning of domains in tyrosine kinases confers the ability to recognize key cellular substrates.

***Table 4-1.* Comparison of properties of domain re-arranged mutants with wild-type Src.** A comparison of activity, autophosphorylation, substrate phosphorylation (Sam68 and Cas) and migration by wild-type Src and the domain rearranged mutants. A (–) indicates a signal that is either undetectable or similar to empty vector (EV) control. A (+) indicates a signal that is comparable to wild-type Src. Increasing numbers of plus signs indicate stronger signals than wild-type. ND indicates the data were inconsistent and more experiments are needed before any conclusion can be made. Highlighted in pink are the mutants 2UC3 and 3UC2, which displayed specific changes in substrate specificity towards the Src substrates Cas and Sam68.

	Activity	Auto-P	Sam68 P	Cas P	Migration
EV	-	-	-	-	-
U32C (WT)	+	+	+	+	+
U23C	+	+	+	+	+
UC32	+	+	+	+	+
3U2C	+++	+++	+++	+++	+++
32UC	+++	+++	+++	+++	+++
32CU	+++	+++	+++	+++	+++
2UC3	++	+++	+++	-	+/-
3UC2	++	+++	-	-	+/-
R175LW44A	ND	+	-	-	-
KINASE DOMAIN	+++	+++	-	-	-

Figure 4-1. **Domain arrangements in c-Src and mutants.** The mutants are named according to the position of the domains from N to C terminus. The unique domain is denoted by U, the SH3 domain by 3, SH2 domain by 2, and catalytic domain is denoted by C. The position of Tyr527 in the C terminal tail of wild-type Src is indicated. The R175L/W44A construct contains point mutations that block ligand binding to the SH2 and SH3 domains.

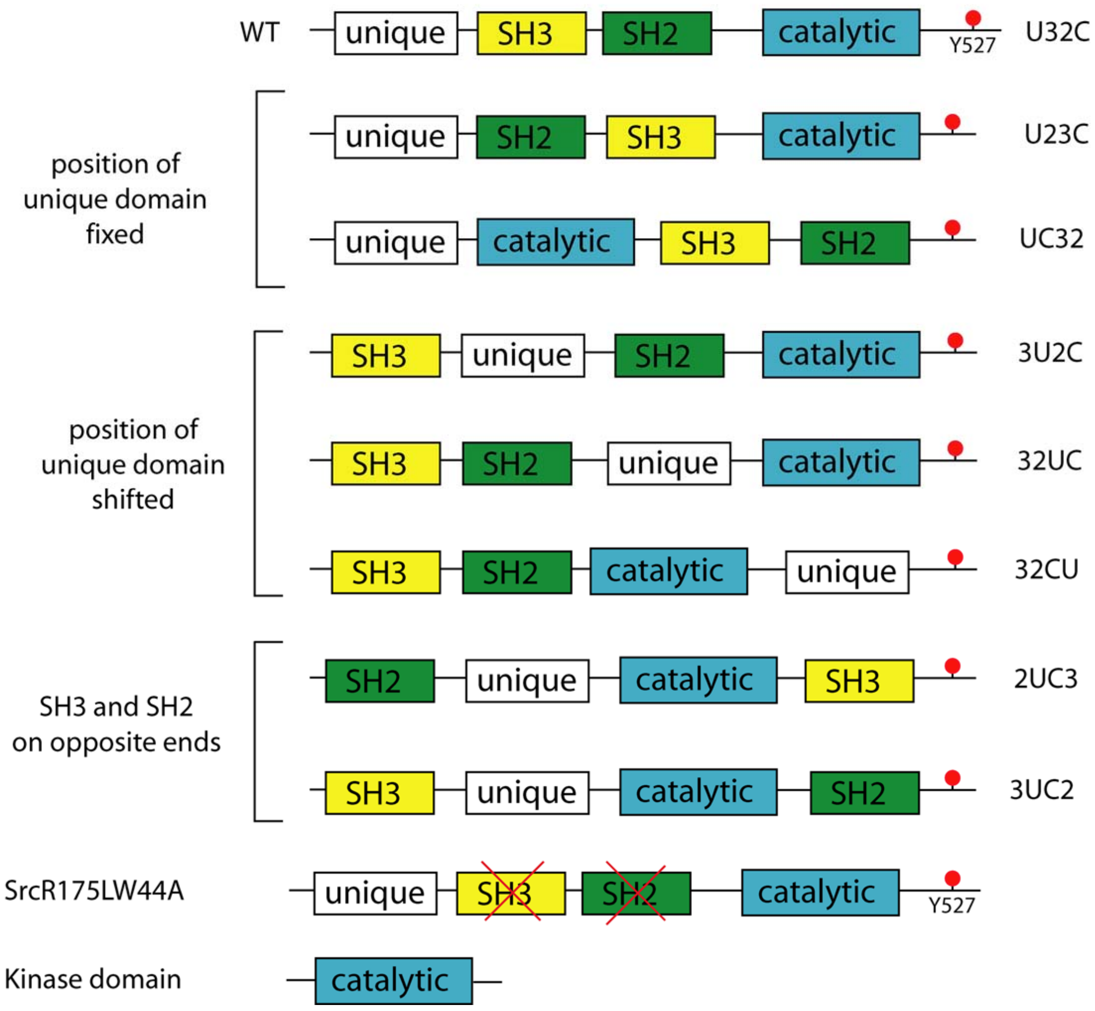

WT
unique
SH3
SH2
catalytic
Y527
U32C
position of unique domain fixed
unique
SH2
SH3
catalytic
U23C
unique
catalytic
SH3
SH2
UC32
position of unique domain shifted
SH3
unique
SH2
catalytic
3U2C
SH3
SH2
unique
catalytic
32UC
SH3
SH2
catalytic
unique
32CU
SH3 and SH2 on opposite ends
SH2
unique
catalytic
SH3
2UC3
SH3
unique
catalytic
SH2
3UC2
SrcR175LW44A
unique
SH3
SH2
catalytic
Y527
Kinase domain
catalytic

Figure 4-2. **Activation loop phosphorylation of c-Src and the mutants.** SYF cells were transiently transfected with wild-type c-Src or the mutants. The domain arrangements in the constructs are displayed as in Figure 1. The unique domain is denoted by U, the SH3 domain by 3 , the SH2 domain by 2 and the kinase catalytic domain by C. The cells were harvested 40 hours post-transfection and whole cell lysates (10μg) were separated on a 10% SD-PAGE and transferred onto PVDF membranes. The membranes were probed with anti-pY416 antibody. To measure expression, membranes were probed with anti-Flag antibody. Anti-tubulin antibody was included as a loading control. Vector represents SYF cells transfected with empty vector alone. The figure is representative of 3 separate experiments.

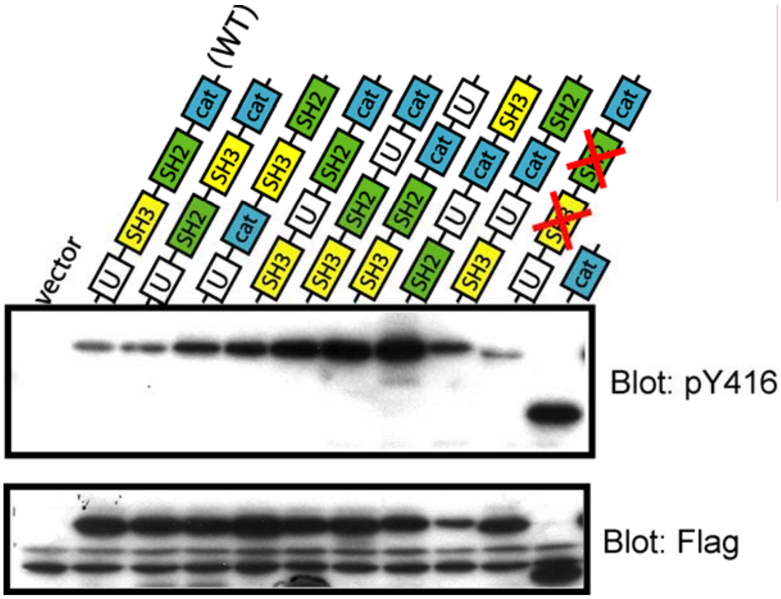
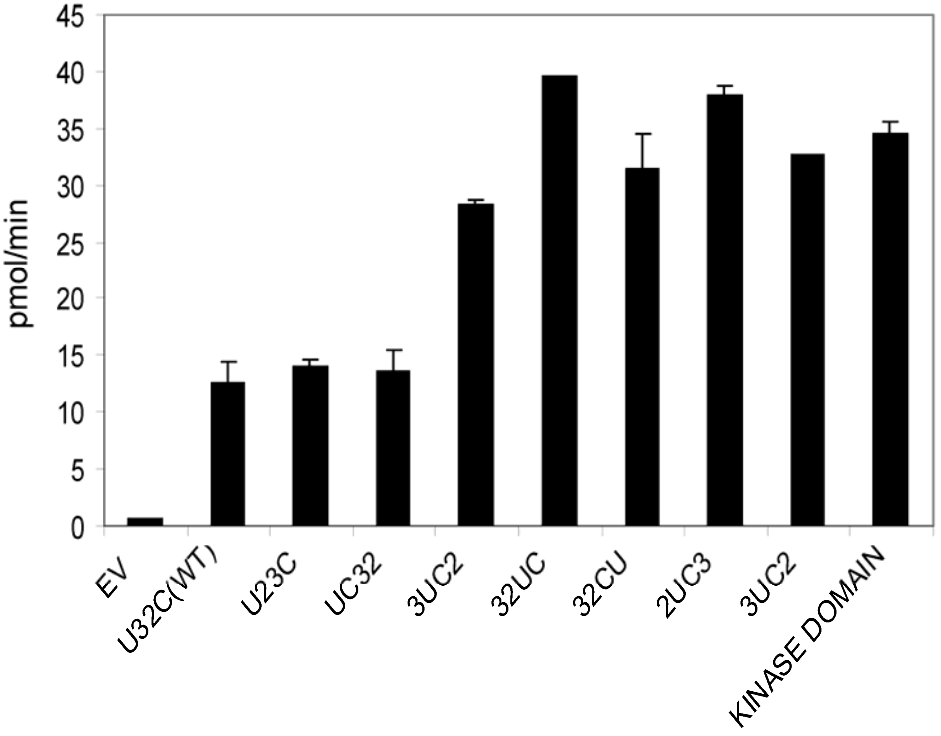
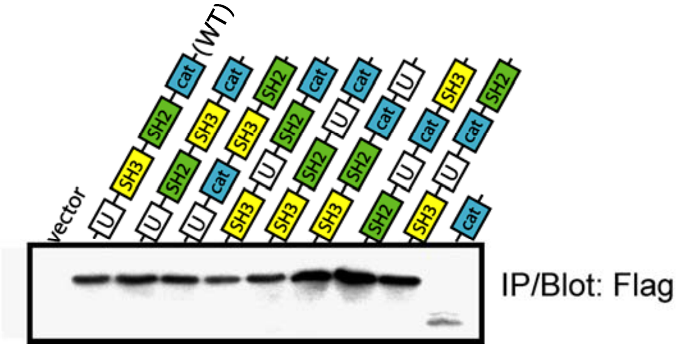

vector
(WT)
U
SH3
SH2
cat
Blot: pY416
Blot: Flag

Figure 4-3. **Activity of c-Src and the mutants.** SYF cells expressing wild-type Src or the mutants were lysed in RIPA buffer. The lysates were subjected to immunoprecipitation of the kinases with Flag-agarose beads, overnight at 4°C. The IP samples were divided into three equal parts. One part was subjected to Western blotting with anti-Flag antibody for measuring amount of Src or the mutants immunoprecipitated (see blot at the bottom of the figure). The other two portions were subjected to kinase assays with substrate peptide (Table 2-1) and [γ–^{32}P] ATP for 4 minutes at 30°C. Reactions were carried out in duplicate and pmol of phosphate incorporated was computed for each mutant.



pmol/min
45
40
35
30
25
20
15
10
5
0
EV
U32C(WT)
U23C
UC32
3UC2
32UC
32CU
2UC3
3UC2
KINASE DOMAIN



vector
U SH3 SH2 cat (WT)
U SH2 SH3 cat
U cat SH3 SH2
SH3 U SH2 cat
SH3 SH2 U cat
SH3 SH2 cat U
SH2 U cat SH3
SH3 U cat SH2
cat
IP/Blot: Flag

Figure 4-4. **Global tyrosine phosphorylation analysis of Src and mutants.** SYF cells were transiently transfected with c-Src or mutants. Cells were harvested 40 hours post transfection and lysates (50µg) were separated on a 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed with anti-pTyr antibody and reprobed with anti-Flag antibody to check expression. The membrane was probed with anti-tubulin antibody as a loading control. Vector represents SYF cells transfected with empty vector alone. The figure is representative of 3 separate experiments.

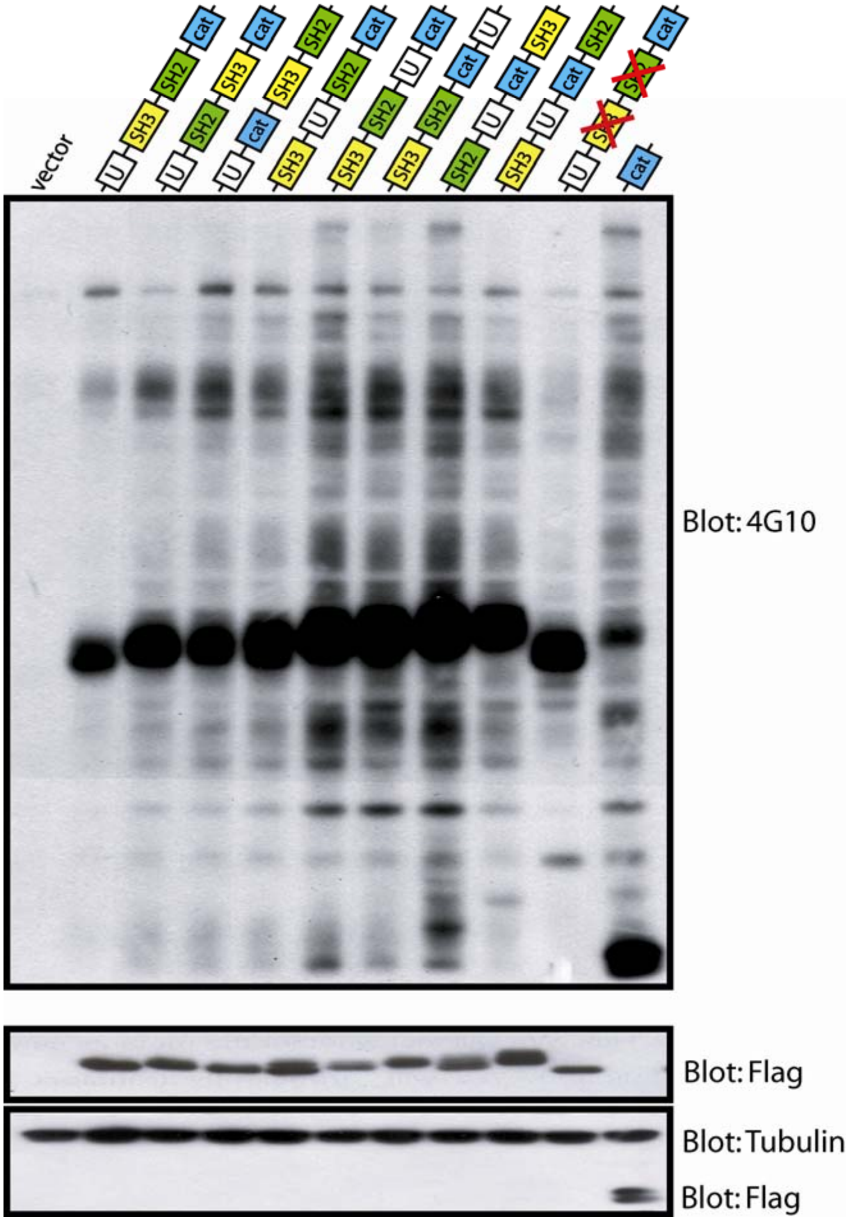

vector
U SH3 SH2 cat
U SH2 SH3 cat
U cat SH3 SH2
SH3 U SH2 cat
SH3 SH2 U cat
SH3 SH2 cat U
SH2 U cat SH3
SH3 U cat SH2
U SH3 SH2 cat
cat
Blot: 4G10
Blot: Flag
Blot: Tubulin
Blot: Flag

Figure 4-5. **Phosphorylation of Cas by Src and the mutants.** SYF cells were transiently transfected with wild-type Src or the mutants. The cells were harvested 40 hours post-transfection and lysates were subjected to immunoprecipitation using anti-Cas antibody. The immunoprecipitates were separated by 7.5% SDS-PAGE, and Western blotting was carried out with anti-pTyr antibody. The membrane was stripped and re-probed with anti-Cas antibody to ensure equivalent Cas immunoprecipitation. The figure is representative of 3 separate experiments.

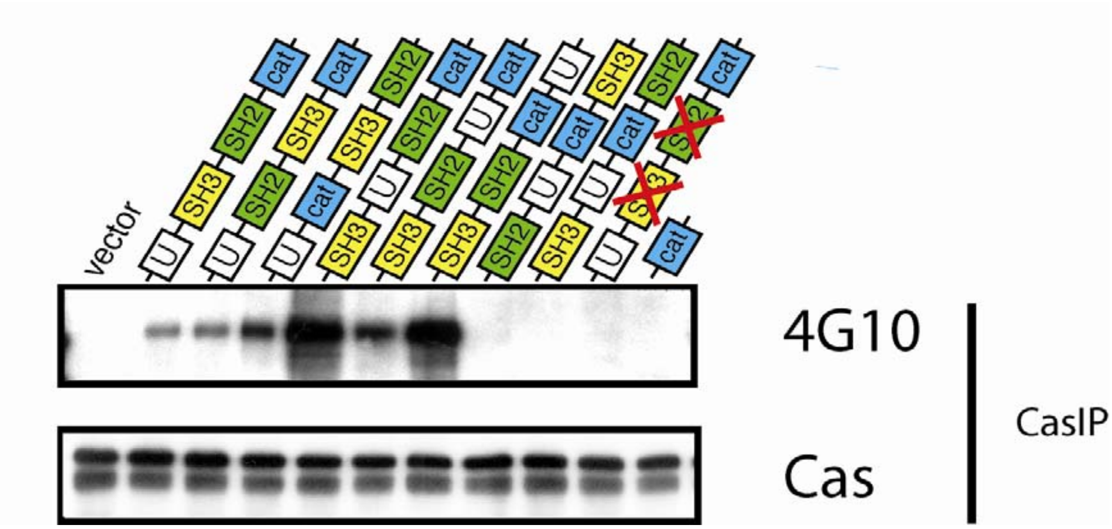

vector
U
SH3
SH2
cat
4G10
Cas
CasIP

Figure 4-6. **Cas/Crk complex formation by mutants and wild-type Src.** SYF cells were transiently transfected with wild-type Src or the mutants. The cells were harvested 40 hours post-transfection and lysates were subjected to immunoprecipitation using anti-Crk antibody. The immunoprecipitates were separated by 10% SDS-PAGE and immunoblotted with anti-Cas and anti-Crk antibodies.

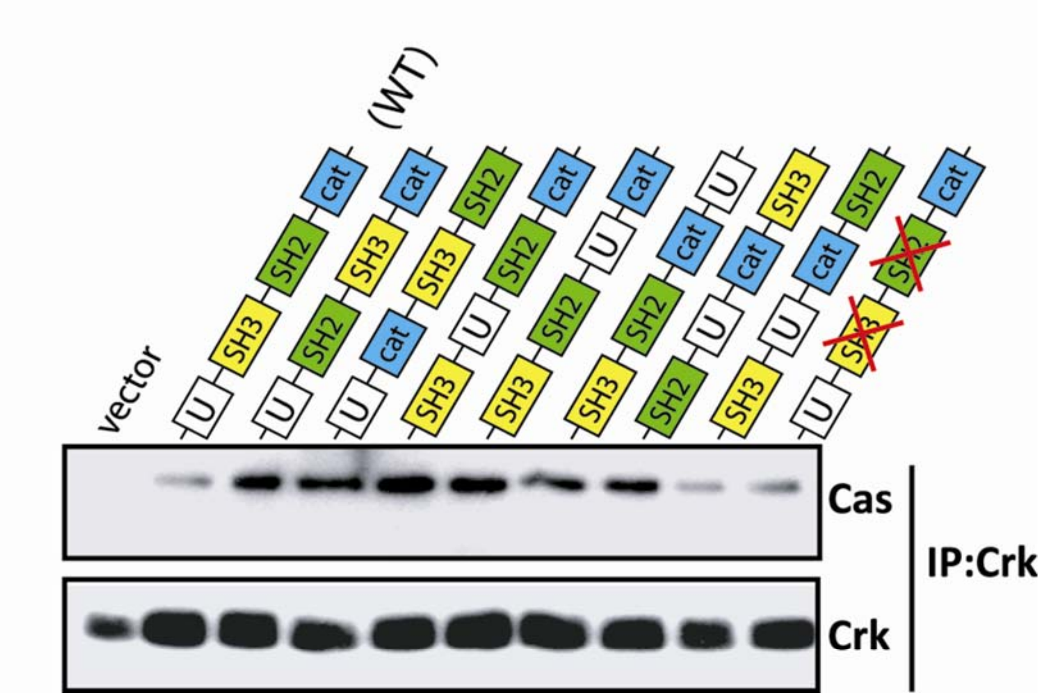
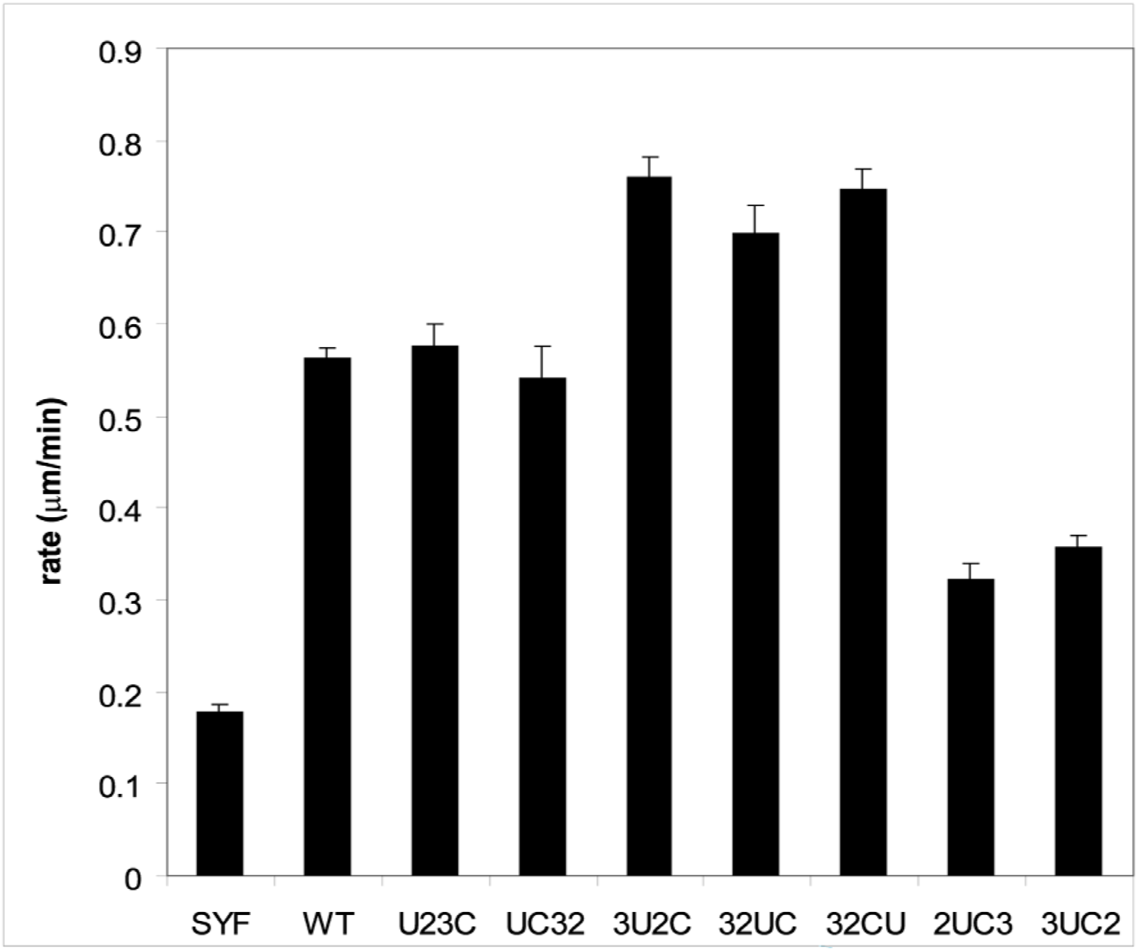

vector
(WT)
U SH3 SH2 cat
U SH2 SH3 cat
U cat SH3 SH2
SH3 U SH2 cat
SH3 SH2 U cat
SH3 SH2 cat U
SH2 U cat SH3
SH3 U cat SH2
U SH3 SH2 cat
Cas
Crk
IP:Crk

Figure 4-7**. Wound healing assays.** SYF cells were infected with retroviruses expressing c-Src or the domain re-arranged mutants. The cells were more than 90% infected as measured by GFP fluorescence (data not shown). Multiple wounds were scratched on the 60mm dishes and 8 positions were photographed for 10-12 hours. The rate of wound closure was calculated by plotting the width of the wound versus time. The rate of closure shown above is average of 8 separate positions. The standard errors represent all 8 positions. Expression of Src and the mutants used for the wound healing assays is shown in the blot at the bottom.


rate (μm/min)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
SYF
WT
U23C
UC32
3U2C
32UC
32CU
2UC3
3UC2

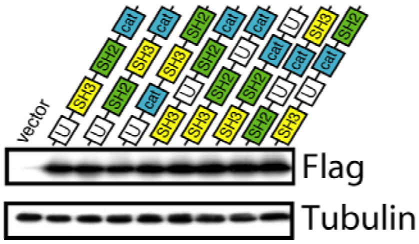

vector
U SH3 SH2 cat
U SH2 SH3 cat
U cat SH3 SH2
SH3 U SH2 cat
SH3 SH2 U cat
SH3 SH2 cat U
SH2 U cat SH3
SH3 U cat SH2
Flag
Tubulin

Figure 4-8. **Phosphorylation of Sam68 by Src and the mutants.** SYF cells were transiently transfected with wild-type Src or the mutants, and arrested in mitosis using nocodazole 24 hours after transfection. The mitotic cells were harvested 40 hours post-transfection and lysates were subjected to immunoprecipitation using anti-Sam68 antibody. The immunoprecipitates were separated by 10% SDS-PAGE and Western blotting was carried out with anti-pTyr antibody. The membranes were stripped and re-probed with anti-Sam68 antibody to ensure equivalent Sam68 immunoprecipitation. The figure is a representative of 3 separate experiments. The two different wild-type lanes in the figure denotes two different levels of expression (low and high) of wild-type. Quantification was done using the Odyssey software from LICOR. The phosphorylation intensity was normalized against amount of nNOS that was immunoprecipitated. The numbers represent fold changes over ZKV-1.

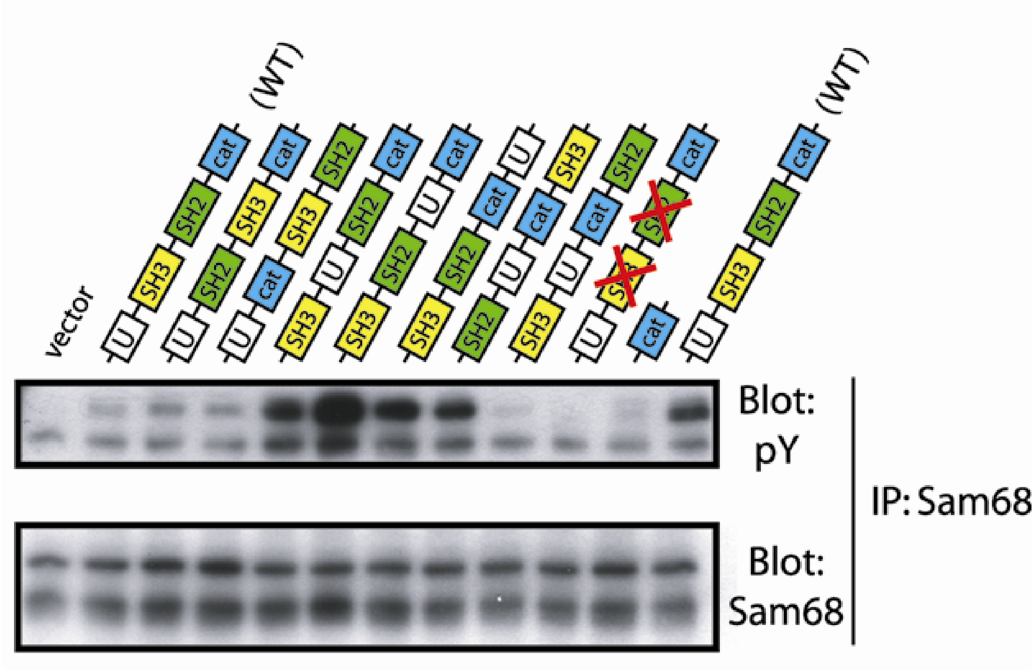

vector
U SH3 SH2 cat
U SH2 SH3 cat
U cat SH3 SH2
SH3 U SH2 cat
SH3 SH2 U cat
SH3 SH2 cat U
SH2 U cat SH3
SH3 U cat SH2
U SH3 SH2 cat
cat
U SH3 SH2 cat
(WT)
(WT)
Blot: pY
Blot: Sam68
IP: Sam68

Figure 4-9. **In vitro kinase assays using substrates with SH3 ligands.** Kinase assays were performed using purified proteins and 50μM of the SH3 substrate peptide (SH3L-S), the reverse orientation SH3 substrate peptide (S-SH3L) or a control substrate peptide lacking the SH3 binding sequence (SH3C-S). Initial rates were measured in mOD/min and they are plotted relative to SH3C-S. Experiments were performed in duplicate and fold activation is shown +/- standard error. Sequences of the peptides are given in Table 2-1.

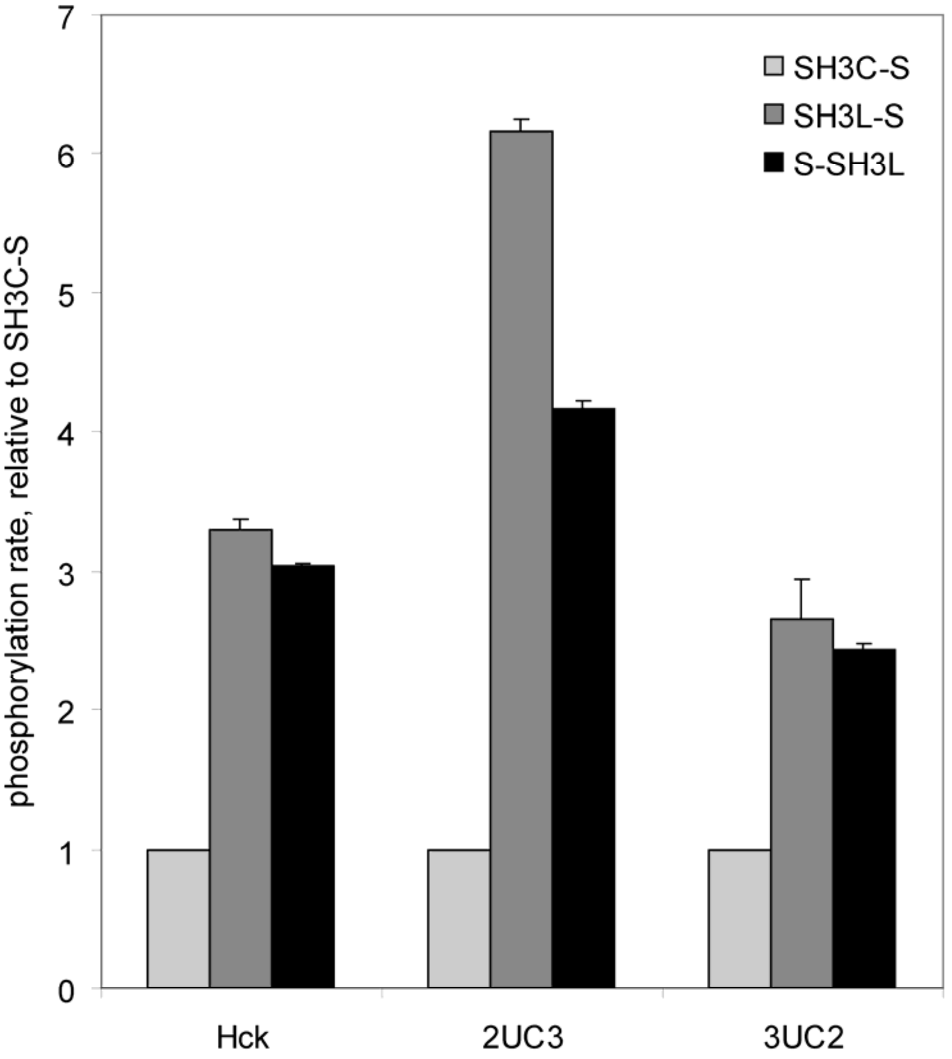
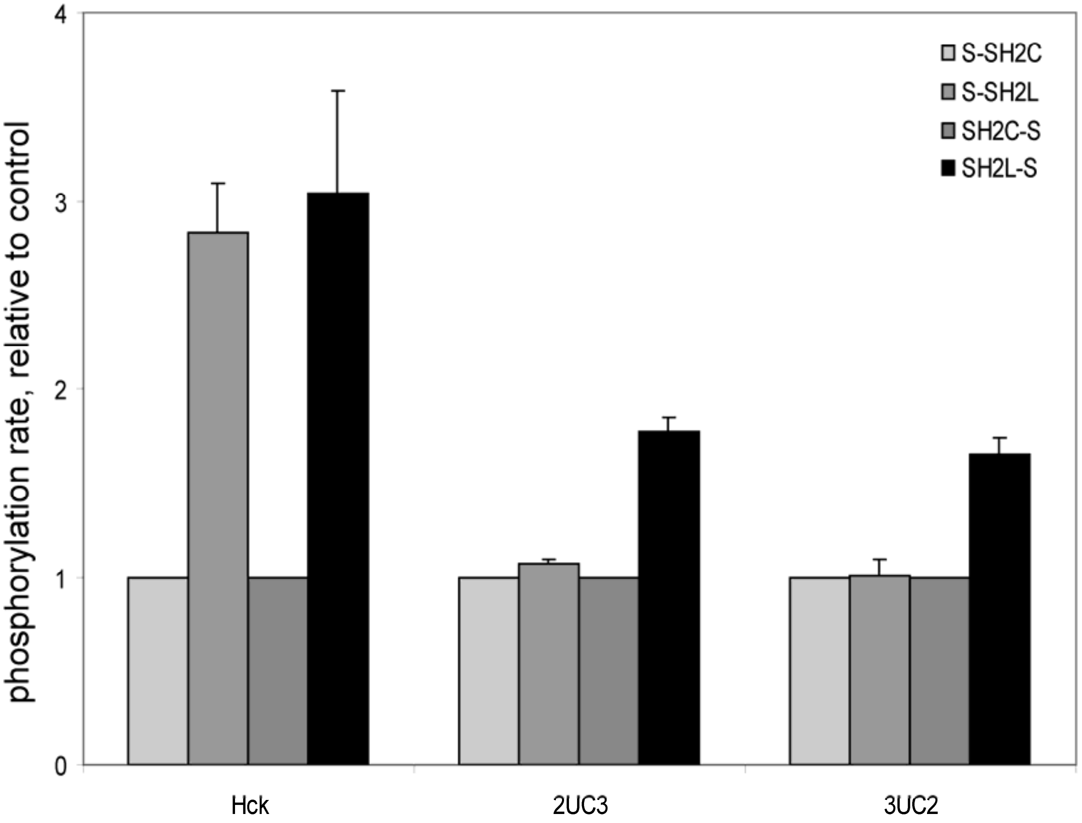

7
6
5
4
3
2
1
0
phosphorylation rate, relative to SH3C-S
SH3C-S
SH3L-S
S-SH3L
Hck
2UC3
3UC2

Figure 4-10. **In vitro kinase assays using substrates with SH2 ligands.** Kinase assays were performed as described in the legend to Figure 4-9. The reactions were carried out in duplicate. Peptides were used at a final concentration of 50μM.


phosphorylation rate, relative to control
0
1
2
3
4
Hck
2UC3
3UC2
S-SH2C
S-SH2L
SH2C-S
SH2L-S

CHAPTER 5

Reengineering the substrate specificity of a Src family kinase

Abstract

Src Family Kinases (SFKs) are modular signaling proteins possessing SH3, SH2, and tyrosine kinase domains. The SH3 and SH2 domains of SFKs have dual roles: they regulate the activity of the kinases, and also target SFKs to their cellular substrates. To test the importance of the associated domains in governing substrate recognition, we generated a novel SFK by replacing the SH2 domain of Hck with the syntrophin PDZ domain. The negative regulatory tyrosine in the C-terminal tail was replaced with a PDZ ligand sequence. When expressed in mammalian cells, the overall substrate specificity of PDZ-Hck is redirected towards PDZ specific substrates. In contrast to wild-type Hck, PDZ-Hck phosphorylates nNOS due to a specific interaction between the PDZ domains of nNOS and PDZ-Hck. We also engineered a PDZ ligand in the Src substrate Cas and observed phosphorylation of the engineered Cas by the PDZ-kinase. The engineered Cas and PDZ-kinase together restored the migration defect of Cas$^{-/-}$ cells. The PDZ-kinase was also functional in rewiring MAPK signaling via an engineered ErbB2 construct containing a PDZ ligand sequence. The PDZ-kinase shows auto-regulatory properties similar to a natural SFK. Addition of a PDZ ligand to PDZ-Hck increases tyrosine kinase activity to a similar degree as observed for activation of Hck by an SH2 ligand. Thus, the PDZ-ligand interaction is able to functionally replace the normal SH2-pY527 interaction that regulates SFKs. Our data highlight the modularity and evolvability of signaling proteins.

Introduction

Many signaling proteins are molecular switches comprised of physically and functionally separable modular domains [15]. There are three important features that can be present in such molecular switch like proteins. First, the modular domains determine the input, that is, targeting of substrates to the output enzymatic domain is via the flanking modular domains. Second, intramolecular interactions mediated by the modular domains that regulates the output activity [18, 149]. Third, these switches are versatile enough to function in diverse signaling pathways depending upon the targeting via the modular domains. SFKs are classical modular signaling proteins that possess all of the above three characteristics [32, 35, 55]. The modular domain architecture of SFKs (consisting of unique, SH3, SH2 and kinase domains) is conserved across all species, including choanoflagellates (the most primitive organism to possess SFKs) [126]. The modular architecture of SFKs is crucial for substrate targeting as well as kinase regulation. In the basal state, SFK catalytic domains are inhibited by intramolecular interactions involving the SH2 and SH3 domains [23, 24]. Disruption of these interactions leads to SFK activation [54, 78]. Thus, SFKs function as a switch in which the input and output functions are physically separable: the input is governed by the SH3/SH2 domains, while the output is by the catalytic domain [18].

There is cooperative activation of the kinase by both SH3 and SH2 ligands, and a large number of SFK substrates like Cas have ligands for both the SH3 and SH2 domains [68, 146]. The dual function of the SH2 and SH3 domains (substrate targeting and auto-regulation) raises the question of which role emerged first in evolution.

There are a fixed number of modular domains and they recombine to generate novel proteins during the course of evolution [15]. Thus in principle domains can be recombined to create novel switch like proteins. Dueber *et. al.* recombined the actin polymerization domain of N-WASP with combinations of nonnative PDZ and SH3 domains and their respective ligands. This led to a variety of novel modular proteins which activated actin polymerization in the presence of exogeneous PDZ/SH3 ligands. The synthetic modular proteins also displayed different levels of input-controlled output behavior, some being activated by both inputs others by only one input, analogous to logic gates. This study points to the evolutionary flexibility of modular signaling proteins [150]. Similarly Yeh *et al* utilized the Dbl family of Rho GEFs and recombined the output DH-PH domain with a PDZ domain and a natural PKA activatable PDZ ligand. These synthetic GEFs also showed PKA dependent activation and switch like behavior [151]. All these studies point to the potential of generating novel switch like proteins that could be used as biosensors or future therapeutic tools to rewire signaling in defective cells [100].

Another important function of the modular domains found in signaling proteins is that of substrate targeting [128, 152-154]. In SFKs the modular SH3 and SH2 domains target substrates to the kinase domain for phosphorylation [129]. Another way of achieving targeting is the use of adaptors and scaffolds, especially in the case of proteins that are highly conserved and not very flexible [15, 155]. MAPKs utilize different scaffolds to assemble different signaling complexes [156], and 24 Rho GTPases utilize 80 different GEFs to assemble various signaling complexes and lead to diverse downstream signaling [157]. Consistent with this, most of the GEFs have a modular architecture. Modular domain mediated substrate targeting has been used to rewire signaling pathways. In one study, normally proliferative EGFR signaling was re-wired to lead to cell death via an artifical adaptor protein comprised of the Grb2 SH2 domain and a death effector domain, DED of Fadd (Fas associated death domain) [158]. In yeast, the MAPK pathway was rewired to generate an osmotic stress response upon mating pheromone treatment via an altered scaffold protein, Ste5 [159]. In yet another study REF52 cells were microinjected with PKA activatable synthetic Dbl family GEFs. The REF52 cells showed a dose dependent customized lamellipodial or filopodial response depending upon the GEF used. The authors also combined two GEFs in a series to generate an artificial cascade with improved signaling response [151].

We sought to generate a novel tyrosine kinase with altered substrate specificity and possible switch like behavior. We used this novel kinase to rewire desired signaling pathways. We generated a novel kinase where the kinase domain of SFK Hck was tethered to a non native syntrophin PDZ domain. We replaced the native SH2 domain and C-terminal pTyr527 sequence by PDZ domain and C terminal PDZ ligand sequence respectively. We observed changes in substrate specificity of the PDZ-kinase. The PDZ-kinase phosphorylated the PDZ domain interacting protein nNOS. The PDZ-kinase also restored migration defect of Cas knock-out cells when co-expressed along with Cas engineered to bind PDZ domains. The PDZ-kinase was also functional in re-wiring Ras-MAPK signaling via an engineered ErbB2 with a C-terminal PDZ ligand. The kinase also showed auto-regulatory properties like SFKs.

Results

Domain architecture of novel kinases- We generated a panel of novel kinases in which the SH2 and SH3 domains of the SFK Hck were replaced by the syntrophin PDZ domain. In constructs ZKV-1 and ZKV-2, the SH2 domain of Hck was replaced by the PDZ domain, and the C-terminal pTyr sequence that normally interacts with the SH2 domain of Hck was replaced by a PDZ ligand (Figure 5-1). Construct ZKV-1 contains the PDZ ligand sequence VKESLV, which binds to the syntrophin PDZ domain with a K_d of 8μM [160]. ZKV-2 contains the sequence VKQSLL, which binds with a K_d of 100μM. In construct 3ZKV, the SH3 domain was retained, while the SH2-pY527 interaction was replaced by the nonnative PDZ-ligand pair. Because 3ZKV contained the polyproline linker sequence of WT Hck between the PDZ and catalytic domains, this kinase potentially has two inputs, one natural (via the SH3 domain) and one artificial (via the PDZ domain). We also generated two control constructs; ZK, has the PDZ and kinase domains but lacks the C-terminal PDZ ligand sequence, and KV, possesses a kinase domain and C-terminal PDZ ligand sequence but lacks the PDZ domain (Figure 5-1).

PDZ domain redirects specificity of Hck- We first tested whether the presence of the PDZ domain altered Hck substrate specificity. To examine phosphorylation of cellular proteins, we expressed the PDZ-kinases in Src/Yes/Fyn triple-knockout (SYF) cells, and measured overall tyrosine phosphorylation by anti-phosphotyrosine immunoblotting of lysates. The pattern of cellular phosphoproteins was markedly different when the PDZ domain was attached to the catalytic domain, as compared to WT Hck or the KV control (Figure 5-2). The data suggest that the PDZ domain retargeted Hck to an alternative group of substrates. We also observed that the ZKV-1construct with a C-terminal PDZ ligand was less active than the ZK construct lacking the ligand, raising the possibility that the engineered PDZ-ligand interaction was functioning to repress kinase activity (Figure 5-2).

Syntrophin PDZ domain directs phosphorylation of nNOS- One known binding partner of the syntrophin PDZ domain is the PDZ domain of nNOS (neuronal nitric oxide synthase) [161]. To test whether the substrate specificity of the PDZ-Hck constructs would mirror the specificity of the associated PDZ domain, we expressed the PDZ-kinases along with nNOS in SYF cells and immunoprecipitated nNOS, and examined its phosphorylation using anti-pTyr Western blotting. All kinase constructs containing a PDZ domain promoted nNOS phosphorylation, while the kinase domain alone (KV) did not (Figure 5-3). As described above in whole cell lysates, construct ZK (lacking a C terminal PDZ ligand) phosphorylated nNOS most efficiently (Figure 5-3). ZKV-1 phosphorylated nNOS less efficiently than ZK, while ZKV-2 was comparable to ZK (Figure 5-3). 3ZKV, with an intact SH3 domain as well as a PDZ domain and high-affinity PDZ ligand, phosphorylated nNOS more weakly. The activity of the PDZ-Hck constructs in this experiment is what would be expected for repression of kinase activity by the artificial PDZ interaction. To confirm these results, we immunoprecipitated PDZ-kinases from SYF cell lysates and performed anti-pY416 immunoblotting as a measure of kinase autophosphorylation and activity. Constructs ZKV-1 and 3ZKV, with high-affinity PDZ ligands at the C-terminus, were inhibited relative to ZK (Figure 5-4). The autophosphorylation of 3ZKV was comparable to WT Hck (Figure 5-4), suggesting that transplantation of the PDZ ligand can yield a kinase with similar autoregulatory properties as WT Hck.

PDZ-kinase leads to phosphorylation of engineered Cas- Next, we tested whether the PDZ-kinase could be directed towards a known SFK substrate. We added the PDZ ligand VKESLV to the C-terminus of Cas and removed the SH3 ligand sequence of Cas, which is the most important feature of Src binding (Figure 5-5A.) [89]. We co-expressed Cas-VKESLV and the PDZ-kinases in Cas$^{-/-}$ cells (cells lacking endogenous Cas) and immunoprecipitated Cas-VKESLV from the lysates. The PDZ domain directs phosphorylation of Cas-VKESLV while the kinase domain alone (KV) does not (Figure 5-5B). Thus, PDZ-mediated targeting can position the catalytic domain for phosphorylation of a substrate that is usually driven by SH3/SH2 interactions. Wild-type Hck phosphorylated Cas-VKESLV weakly, probably due to residual binding between the SH2 domain of Hck and the Src binding sequence of Cas-VKESLV. The order of activity of the synthetic constructs toward Cas-VKESLV (ZK>ZKV-1>ZKV-2>3ZKV) is consistent with the existence of auto-regulatory properties in the constructs.

Reengineered Cas and PDZ-kinase restore migration defect- Cas$^{-/-}$ cells are defective in cell migration [109, 113]. We tested whether the synthetic connection between Cas-VKESLV and PDZ-kinase could restore migration signaling in these cells (Figure 5-6A). Co-expression of PDZ-kinase (ZK) and Cas-VKESLV gave migration activity that was comparable to the activity of WT Hck and Cas (Figure 5-6B). Cas-VKESLV alone gave some migration activity, probably due to its phosphorylation via SH2 ligand binding by endogenous SFKs present in Cas$^{-/-}$ cells (Figure 5-6B). Removal of the polyproline sequence of Cas-VKESLV (construct CasPPX-VKESLV in Figure 5-5A), the major determinant for Src binding, confirmed that interaction was due to the PDZ domain (Figure 5-6B). These results show that functional interaction between SFKs and Cas can be renewed using non-native interactions. A similar strategy using Src kinase catalytic domain tethered to one of a pair of coiled-coil amphipathic helices ZipA and Cas molecule fused to the other heterodimerization partner ZipB led to alteration of morphology of NIH3T3 cells towards a transformed phenotype [81].

PDZ-Hck and engineered ErbB2 restores MAPK signaling- To test the generality of these observations, we studied PDZ-driven substrate targeting in another system. Phosphorylation of Y877 in the activation loop of the RTK ErbB2 by SFKs creates a binding site for Grb2, which promotes downstream Ras-MAPK signaling (Figure 5-7B) [162]. We engineered a kinase-dead form of ErbB2 and placed a PDZ ligand (VKESLV) at its C-terminus (Figure 5-7A and 5-7B). We co-expressed Kd-ErbB2-VKESLV and PDZ-kinase (ZK) in SYF cells, immunoprecipitated Kd-ErbB2-VKESLV, and examined phosphorylation of Tyr877 by Western blotting. Whereas WT Hck was unable to phosphorylate Y877 of ErbB2, the PDZ-kinase promoted phosphorylation (Figure 5-8A). Co-expression of Kd-ErbB2-VKESLV and PDZ-kinase also led to an increase in Erk phosphorylation, while the phosphorylation of Erk in cells expressing Kd-ErbB2-VKESLV alone or Kd-ErbB2-VKESLV plus Hck was comparable to SYF cells (Figure 5-8B). The results point to the flexibility of the targeting function in the associated domains of SFKs and show that cell signaling can be re-wired by engineering protein-protein interactions.

***In vitro* kinase assay-** We tested whether the difference in activation state of the PDZ-kinase (ZK) and PDZ-kinase-VKESLV (ZKV-1) could be seen *in vitro* as well. We expressed ZK and ZKV-1 in SYF cells and immunoprecipitated the kinases. We performed kinase assays in the presence of increasing concentration of GST-GVKESLV protein as an activator. We observed a modest 1.4 fold activation of PDZ-cat-VKESLV (ZKV-1) in the presence of increasing concentration of activator GST-GVKESLV (Figure 5-9A). In contrast PDZ-cat (ZK) lacking a C-terminal PDZ ligand was not activated be GST-GVKESLV (Figure 5-9A). The 1.4 fold activation that we observe is comparable to activation of Hck by pYEEI peptide, which is generally 1.5- 2 fold (data not shown). We also confirmed that both ZK andZKV-1 bind to the activator by GST pull-down (Figure 5-9B). Our results are consistent with the activation states of PDZ-kinases that we observe in cells. This observation was activator specific; our attempts to observe activation with nNOS protein were unsuccessful. We also expressed and purified the proteins from Sf9 insect cells and performed kinase assays, but we did not observe any reproducible activation of ZKV-1 by PDZ ligand peptides as compared to KV or ZK (data not shown).

Discussion

Extensive domain shuffling occurred during the course of evolution of metazoans [1, 7]. This is readily apparent from the structures of modern signaling proteins, which are constructed with a modular architecture that makes them highly evolvable [3-5, 88]. The prevalence of domain shuffling is also apparent in choanoflagellates, unicellular organisms that are closely related to metazoans. The genome of the choanoflagellate *Monosiga brevicollis* contains a number of signaling proteins with domain combinations not found in higher eukaryotes [126].

During the course of evolution, it appears that there has been a gradual separation of catalytic and targeting functions in signaling enzymes [15]. Many metabolic enzymes like phosphofructokinase and hexokinase have catalytic and regulatory functions integrated in the same domain [16]. Eukaryotic MAP kinases as well as other Ser/Thr kinases like PDK1 possess substrate docking sites in the catalytic domain [19, 163-165]. In tyrosine kinases, which arose more recently, the catalytic and specificity-determining modules are separated into independently folding domains. This arrangement makes tyrosine kinases more flexible in terms of evolution, and a variety of domain combinations are found in the various families of tyrosine kinases [5-7, 17, 166]. Adaptor and scaffolding proteins are among the most recently evolved signaling proteins, indicative of a complete separation of the targeting and catalytic functions on different polypeptides [15, 156].

The modular design of SFKs is crucial for substrate recognition, but it has an additional functional consequence: the SH3 and SH2 domains participate in autoinhibitory interactions that allow SFKs to act as complex switches [23, 24, 83, 85]. The need to maintain the repressed state of SFKs has been fine-tuned through evolution for cell survival [167]. In this study, we tested whether new functionality could be imparted to such a complex switch by replacing the conserved domains. Precedent for these studies comes from work on WASP and on GEFs, in which synthetic autoregulatory switches gave gated outputs [150]. We replaced the regulatory apparatus of a SFK with a heterologous PDZ domain and PDZ-binding ligand, and tested effects on substrate targeting and regulation.

We observed clear changes in the substrate specificity of the PDZ-kinases, with the PDZ domain acting as the dominant element in substrate recognition (Figures 5-2 to 5-4 and Figure 5-8). The phosphorylation of Cas-VKESLV by the PDZ-kinases was compatible with the normal cellular function of Cas in promoting migration (Fig. 5-6) [106]. Thus, the normal connections in this signaling pathway can be replaced by heterologous connections. This result is consistent with earlier studies in which Src was retargeted to Cas using a coiled-coil dimerization strategy [81]. We also show that the PDZ-kinase can be targeted to Kd-ErbB2-VKESLV, and that phosphorylation occurs in a manner consistent with downstream Ras-MAPK signaling. These findings are consistent with the role of domain shuffling in establishing new connectivities in signaling [15].

Our results also suggest that it is possible to engineer a novel mode of allosteric regulation in a SFK. PDZ and SH2 domains are similar in size and shape [168, 169], and the preference for PDZ domains binding to protein C-termini suggested that the natural interaction between the SH2 domain and pY-containing tail might be functionally replaced by a PDZ C-terminal tail interaction. Indeed, construct ZKV-1 had lower activity than ZK, which lacks the PDZ ligand (Figures 5-2 to 5-5B). Construct ZKV-2, with a low-affinity tail, showed higher levels of activity than ZKV-1. Although the repression is not as tight as that observed for natural SFKs, these results are consistent with the studies on a form of Src with a high-affinity SH2 ligand (pYEEI) engineered in the C-terminus; this form of Src is incapable of being activated by SH2 ligands [54]. The highest level of repression was observed in 3ZKV, which has two possible modes of regulation; in fact, its autoinhibition (as measured by autophosphorylation and Cas phosphorylation) is comparable to that observed for the SFK Hck (Figures 5-4 and 5-5B). On the other hand, our attempts to show autoinhibition in the purified ZKV-1 and 3ZKV proteins were unsuccessful (data not shown).

Our studies shed light on how the substrate targeting and regulatory functions in modular signaling proteins might have evolved. The ease with which substrate specificity was reengineered (Figures 5-2, 5-3, 5-5B, and 5-8) suggests that substrate targeting may have evolved first as a function of the accessory domains in tyrosine kinases. Further evolutionary fine-tuning would then be necessary to develop the mature allosteric regulation observed in modern SFKs. Consistent with this, a recent study from our lab on choanoflagellate SFKs shows that even though *Monosiga* Src is phosphorylated at the C-terminus regulatory Y527 by Csk, the regulation observed in mammalian SFKs has not yet evolved in this organism [167]. Our results also suggest that novel signaling kinases with engineered targeting and regulation might be created.

Figure 5-1. **Domain architecture of Hck and PDZ-kinases.** The kinases are named according to the position of the domains from N to C terminus. The PDZ domain is denoted by Z, the SH3 domain by 3, the SH2 domain by 2, kinase domain by K and C-terminal PDZ ligand by V. For PDZ-kinase constructs with different C-terminal PDZ ligand sequence the names also have a number at the end. ZKV1 is PDZ-Kinase-VKESLV, where the affinity of VKESLV sequence for syntrophin PDZ domain is 8 mM while ZKV2 has a C-terminal PDZ ligand VKQSLL with an affinity of 100mM.

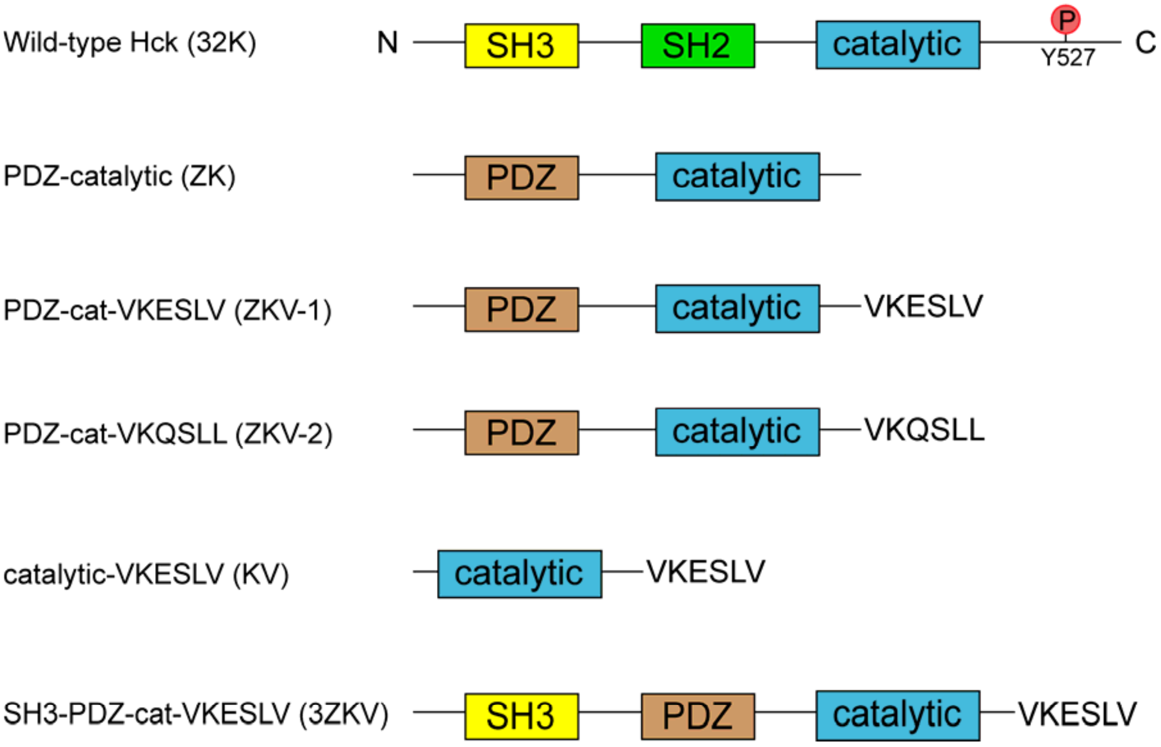

Wild-type Hck (32K)
N
SH3
SH2
catalytic
P
Y527
C
PDZ-catalytic (ZK)
PDZ
catalytic
PDZ-cat-VKESLV (ZKV-1)
PDZ
catalytic
VKESLV
PDZ-cat-VKQSLL (ZKV-2)
PDZ
catalytic
VKQSLL
catalytic-VKESLV (KV)
catalytic
VKESLV
SH3-PDZ-cat-VKESLV (3ZKV)
SH3
PDZ
catalytic
VKESLV

Figure 5-2. **PDZ domain redirects specificity of the kinase.** SYF cells were transienty transfected with Hck or the PDZ-kinases. The cells were harvested 40 hours post-transfection and whole cell lysates (50μg) were separated on a 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with anti-pTyr antibody, 4G10. To measure expression, the membranes were probed with anti-Flag antibody. Anti-tubulin antibody was included as a loading control.

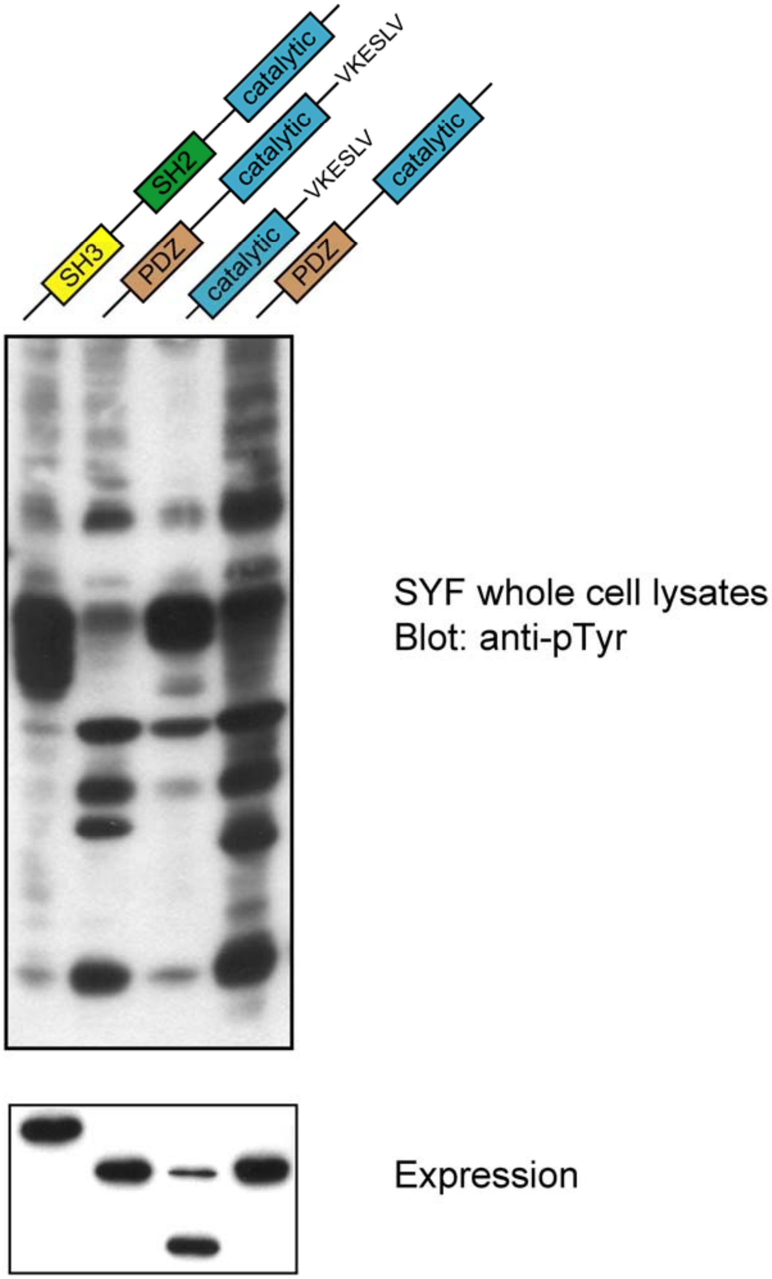

SH3
SH2
catalytic
PDZ
catalytic
VKESLV
catalytic
VKESLV
PDZ
catalytic
SYF whole cell lysates
Blot: anti-pTyr
Expression

Figure 5-3. **Syntrophin PDZ domain directs phosphorylation of nNOS.** SYF cells were transiently transfected with the PDZ-kinases along with nNOS PDZ domain. The cells were harvested 40 hours post-transfection and lysates were subjected to immunoprecipitation using anti-HA antibody. The immunoprecipitates were separated by 10% SDS-PAGE, and Western blotting was carried out with anti-pTyr antibody. The membranes were stripped and re-probed with anti-HA antibody to ensure equivalent nNOS PDZ immunoprecipitation. The figure is a representative of three separate experiments.

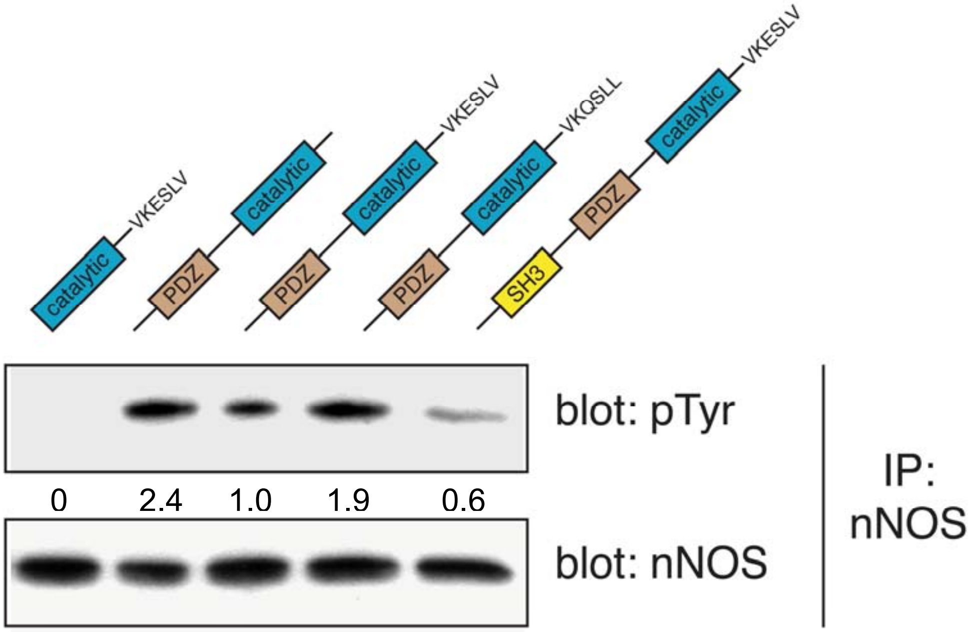

catalytic
VKESLV
PDZ
catalytic
PDZ
catalytic
VKESLV
PDZ
catalytic
VKQSLL
SH3
PDZ
catalytic
VKESLV
blot: pTyr
0
2.4
1.0
1.9
0.6
blot: nNOS
IP: nNOS

Figure 5-4.**Activation state of the PDZ-kinases.** SYF cells were transiently transfected with wild-type Hck or the PDZ-kinases. Cells were harvested 40 hours post transfection and the kinases were immunoprecipitated using anti-Flag antibody. The immunoprecipitates were separated on a 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed with anti-pTyr416 antibody and with anti-Flag antibody.Vector represents SYF cells transfected with empty vector alone.

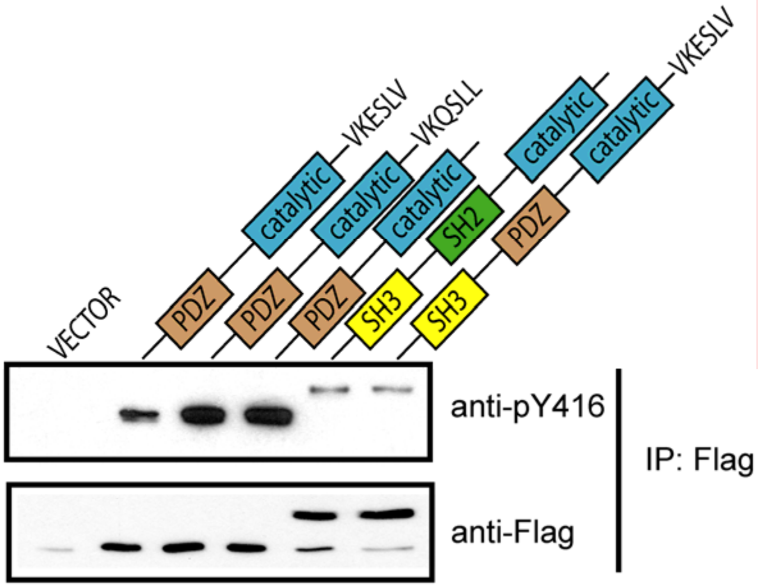
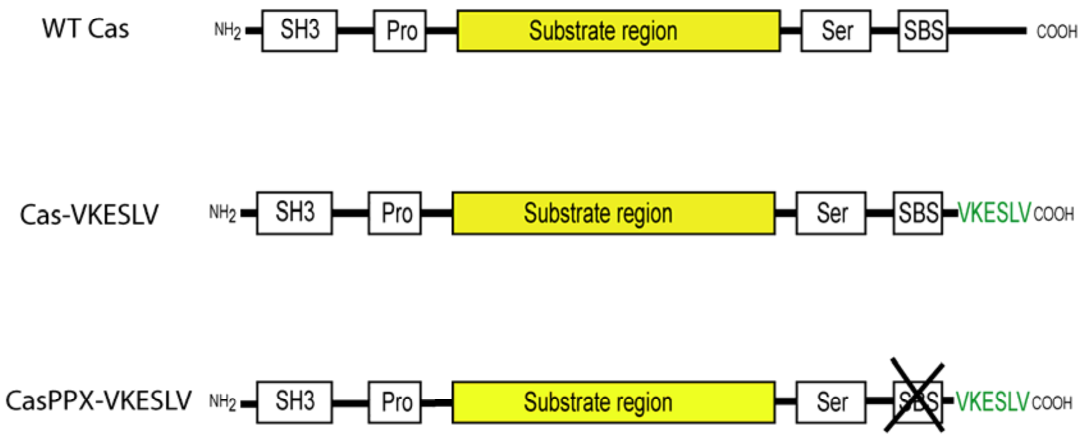

VECTOR
PDZ
catalytic
VKESLV
PDZ
catalytic
VKQSLL
PDZ
catalytic
SH3
SH2
catalytic
SH3
PDZ
catalytic
VKESLV
anti-pY416
anti-Flag
IP: Flag

Figure 5-5**. Reengineered Cas is phosphorylated by PDZ-kinase.** (A). A schematic diagram depicting domain architecture of wild-type Cas and reengineered Cas. Shown in wild-type Cas are the SH3 domain, the substrate region with 15 potential tyrosine phosphorylation sites and the Src binding sequence (SBS) which has a polyproline rich region for Src SH3 domain binding and Y688, which when phosphorylated binds Src SH2 domain. Rengineered Cas-VKESLV was made by changing the C-terminus of Cas to the PDZ ligand sequence VKESLV. In order to abrogate interaction with endogenous SFKs the polyproline rich region of SBS was mutated, to generate CasPPX-VKESLV. (B). Cas-/- cells were transiently co-transfected with the kinases and engineered CasPPX-VKESLV. The cells were harvested 40 hours post-transfection and lysates were subjected to immunoprecipitation using anti-Cas antibody. The immunoprecipitates were separated on a 7.5% SDS-PAGE and immunoblotted with anti-pTyr antibody. The same membrane was stripped and re-probed with anti-Cas antibody to ensure equivalent Cas pull-down.

A.


WT Cas
NH2
SH3
Pro
Substrate region
Ser
SBS
COOH
Cas-VKESLV
NH2
SH3
Pro
Substrate region
Ser
SBS
VKESLV
COOH
CasPPX-VKESLV
NH2
SH3
Pro
Substrate region
Ser
SBS
VKESLV
COOH

B.

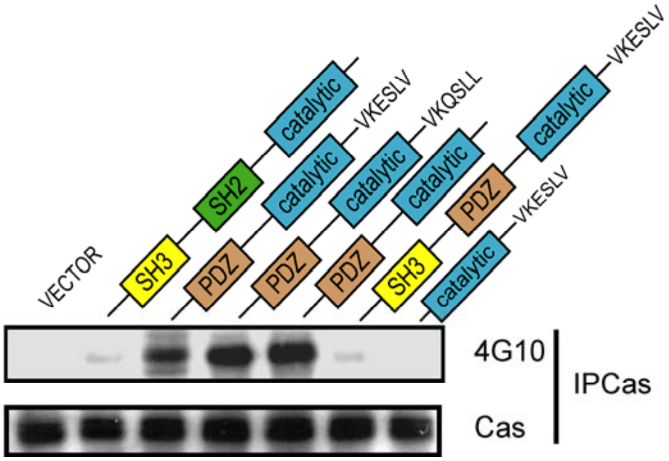
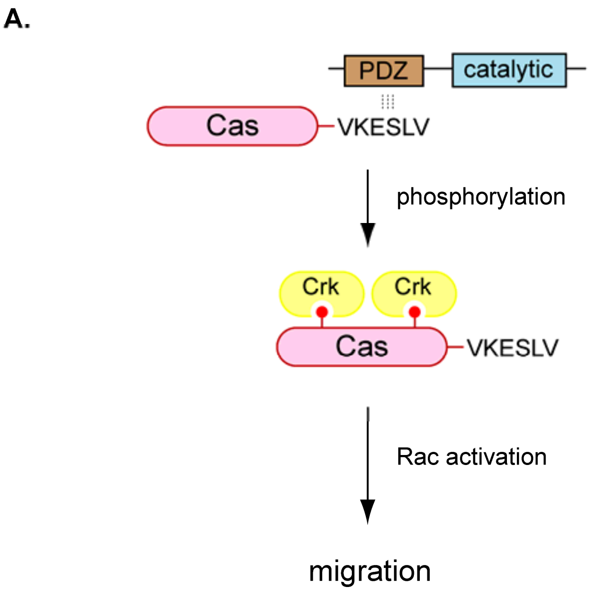

VECTOR
SH3
SH2
catalytic
PDZ
catalytic
VKESLV
PDZ
catalytic
VKQSLL
PDZ
catalytic
SH3
PDZ
catalytic
VKESLV
catalytic
VKESLV
4G10
Cas
IPCas

Figure 5-6. **PDZ-kinase and reengineered Cas restore migration defect of Cas-/- cells.** (A). A schematic diagram depicting rewired signaling of Cas-/- cells leading to migration. Cas-/- cells lack the adaptor protein Cas and as a result are defective in migration. The schematic depicts interaction of engineered Cas and PDZ-kinase via the PDZ domain leading to phosphorylation of engineered Cas. The phosphorylation of Cas creates docking sites for Crk leading to Cas/Crk complex assembly and restoration of downstream signaling leading to cell migration. (B). Cas-/- cells were transiently transfected and 24 hours post-transfection cells were harvested and used for setting up migration assay in a 96well transwell migration assay chamber. Cell migration was quantitated using the Cell-Gro cell counting kit according to the manufacturer's recommendations.


A.
PDZ
catalytic
Cas
VKESLV
phosphorylation
Crk
Crk
Cas
VKESLV
Rac activation
migration

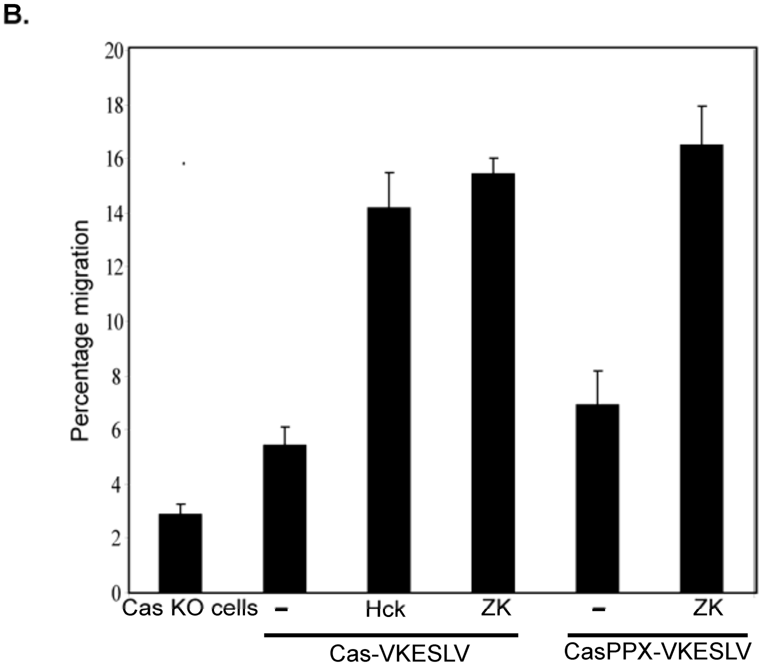
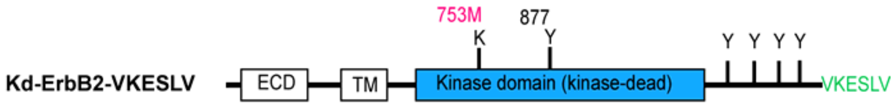

B.
Percentage migration
0
2
4
6
8
10
12
14
16
18
20
Cas KO cells
–
Hck
ZK
–
ZK
Cas-VKESLV
CasPPX-VKESLV

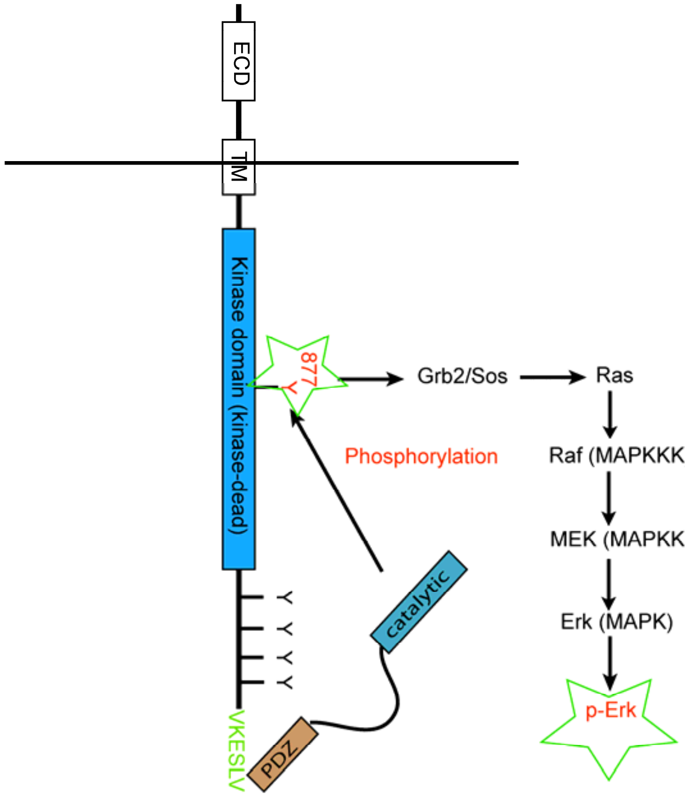
Figure 5-7**. Rewiring of Ras-MAPK signaling.** (A). Domain architecture of engineered kinase dead ErbB2. Shown are the extracellular domain (ECD), kinase-domain with a point-mutation (K753M) making it kinase-dead, and the C-terminal tail with sites of tyrosine autophosphorylation. Y877 is the activation loop tyrosine of ErbB2, and is the site of phosphorylation by Src. Also shown is the mutated C-terminal tail sequence VKESLV, a consensus syntrophin PDZ domain binding sequence. (B). A schematic showing rewired signaling via engineered ErbB2 and PDZ-kinase. The PDZ domain of the kinase leads to interaction with the C-terminus of engineered Kd-ErbB2-VKESLV. Src is normally recruited to ErbB2 via its SH2 domain binding to the C-terminal autophosphorylated ErbB2. As ErbB2 is kinase-dead there will be no auto-phosphorylation and the interaction with Hck will not occur. PDZ-kinase then phosphorylates Y877 in the activation loop of ErbB2. pTyr877 recruits the Grb2/Sos complex via the Grb2 SH2 domain binding to pTyr877. This in turn leads to downstream Ras/MAPK signaling and an increase in Erk phosphorylation.

A.



Kd-ErbB2-VKESLV
753M
K
877
Y
ECD
TM
Kinase domain (kinase-dead)
Y Y Y Y
VKESLV

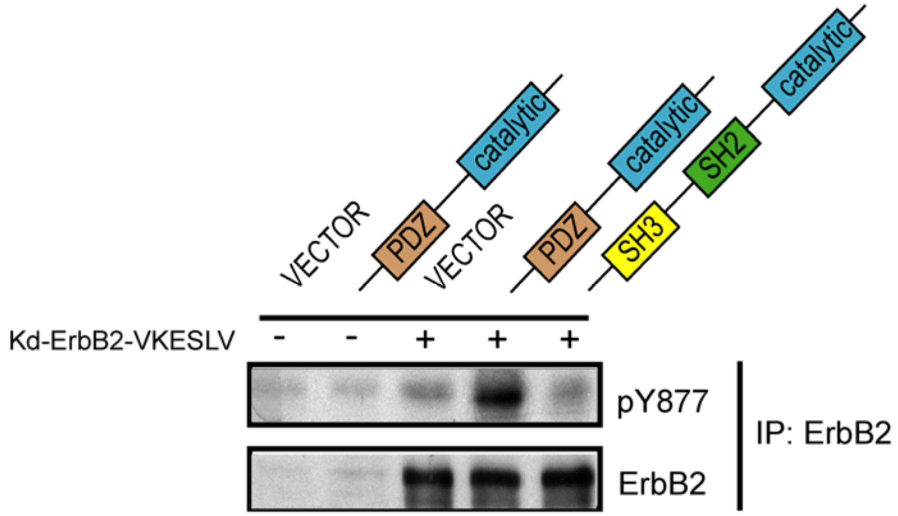
B.



ECD
TM
Kinase domain (kinase-dead)
877
Y
Grb2/Sos
Ras
Raf (MAPKKK)
Phosphorylation
MEK (MAPKK)
Erk (MAPK)
catalytic
Y
Y
Y
Y
VKESLV
PDZ
p-Erk

Figure 5-8**. ErbB2 and Erk phosphorylation** (A) SYF cells were transiently transfected with the kinase and Kd-ErbB2-VKESLV. The cells were harvested 40 hours post-transfection and lysates were subjected to immunoprecipitation using anti-ErbB2 antibody. The immunoprecipitates were separated by 10% SDS-PAGE and Western blotting was carried out with anti-pTyr877 antibody. The membranes were stripped and re-probed with anti-ErbB2 antibody to ensure equivalent ErbB2 immunoprecipitation.
(B). The cell lysates were separated on a 10% SDS-PAGE and probed with anti-pErk and anti-Erk antibodies. Expression was checked by anti-Flag and anti-ErbB2 immunoblotting.

A.


VECTOR
PDZ
catalytic
VECTOR
PDZ
catalytic
SH3
SH2
catalytic
Kd-ErbB2-VKESLV
- - + + +
pY877
ErbB2
IP: ErbB2

B.

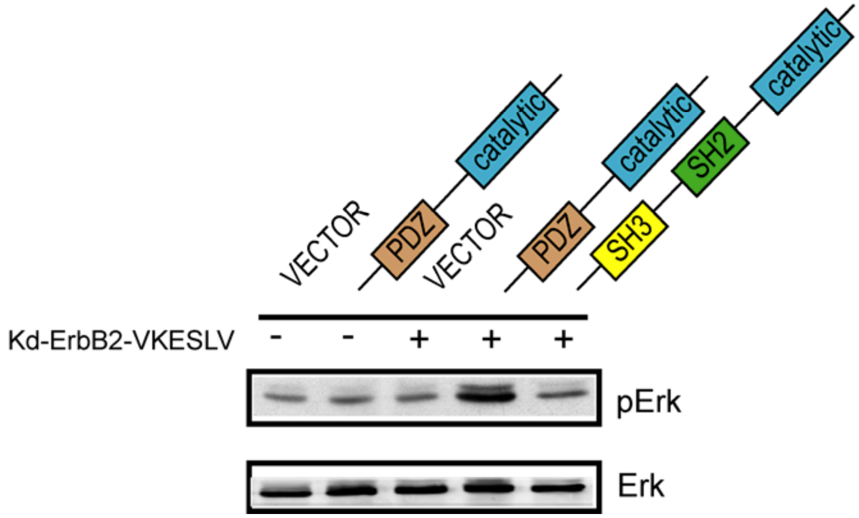
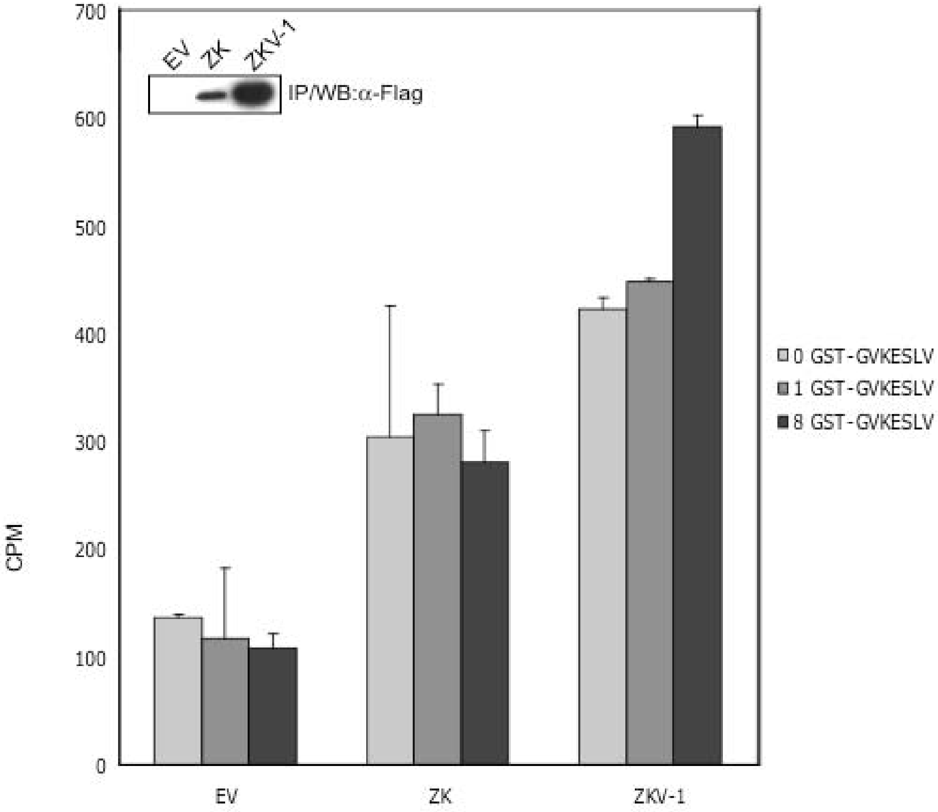
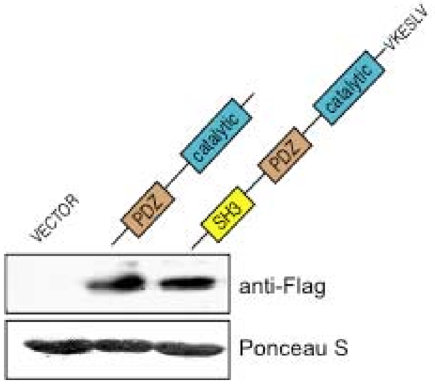

VECTOR
PDZ
catalytic
VECTOR
PDZ
catalytic
SH3
SH2
catalytic
Kd-ErbB2-VKESLV
- - + + +
pErk
Erk

Figure 5-9**. IP kinase assay**. (A). SYF cells were transiently transfected with PDZ-kinases and 40 hours post-transfetion cells were harvested in RIPA buffer. The lysates were subjected to immunoprecipitation with Flag-agarose beads for 1 hour. The immunoprecipitated kinase was subjected to kinase reactions using [γ-^{32}P]ATP and peptide substrate. GST-GVKESLV protein was used as an activator in the reaction at concentrations of 1-25μM. The reactions were carried out for 10 minutes at 30°C. Rates are given in CPM (counts per million) for each kinase in the presence and absence of activator. Experiments were performed in duplicate and rates are given +/- standard error. The figure in the inset shows amount of immunoprecipitated PDZ-kinases used for the IP kinase assay (B). Lysates were incubated with GST-GVKESLV beads for 1 hour at 4°C and the beads were washed with RIPA buffer. The bound proteins were eluted in 2.5X Laemmli buffer and separated on a 10% SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed with anti-Flag antibody for bound PDZ-kinase and stained with Ponceau S to ensure that equivalent GST-GVKESLV bound beads were used for each pull-down.

A.


EV
ZK
ZKV-1
IP/WB:α-Flag
700
600
500
400
300
200
100
0
CPM
EV
ZK
ZKV-1
0 GST-GVKESLV
1 GST-GVKESLV
8 GST-GVKESLV

B.


VECTOR
PDZ
catalytic
SH3
PDZ
catalytic
VKESLV
anti-Flag
Ponceau S

CHAPTER 6

Concluding discussion

Concluding discussion and future directions

Our studies in Chapter 3 provide evidence that Src family kinases are regulated in a cooperative manner by their SH3 and SH2 domains. Cooperativity is a means of providing regulation, signal amplification and most importantly adaptability in changing environments. It provides a way to integrate multiple inputs leading to amplification in a regulated and specific manner. Probably for these reasons, cooperativity has been observed in other signaling proteins as well [18]. In the case of the phosphatase Shp2, there are two tandem SH2 domains preceding the phosphatase domain. The activity of the phosphatase is kept repressed under basal conditions with the first SH2 domain blocking access to the active site [27-29]. The most potent activators of the phosphatase are properly spaced biphosphotyrosine motifs [170]. In the case of Abl kinase it has been shown that activation of Stat5 requires both the SH3 and the SH2 domain of the kinase [171].

Our studies in Chapter 4 highlight the importance of the domain architecture of SFKs in determining substrate specificity in addition to regulating the activity of the kinases. Here, we summarize results on substrate targeting by Hck and mutants 2UC3 and 3UC2. Synthetic peptides containing SH3 ligands in either orientation were phosphorylated more than controls by Hck, 2UC3 and 3UC2 (Figure 4-9). Our studies suggest that the oritentation of SH2 ligand containing peptides is important for the mutants but not wild-type. Unlike wild-type, the mutants were unable to differentiate between SH2 ligand peptides with the orientation SH2L-S and control peptides lacking the SH2 ligand. The reverse orientation S-SH2L was phosphorylated better than the control peptide by Hck and the two mutants 2UC3 and 3UC2 (Figure 4-10)

Our *in vivo* studies show that mutant 2UC3 can phosphorylate Sam68 better than wild-type but it is inefficient at phosphorylating Cas (Table 4-1). This difference could be a result of the different orientation of SH3 and SH2 ligand sequences in Cas and Sam68. In Sam 68 the SH3 ligand sequence is followed by the substrate and SH2 ligand sequence (SH3L-S-SH2L) while in Cas the orientation is substrate followed by SH3 ligand followed by SH2 ligand (S-SH3L-SH2L) [112, 140]. This difference in phosphorylation of Cas and Sam68 by mutant 2UC3 could be studied by synthetic peptides based on Cas and Sam 68. A peptide S-SH3L-SH2L (based on Cas) should be less efficiently phosphorylated than a peptide SH3L-S-SH2L (based on Sam68) by 2UC3. On the other hand, the mutant 3UC2 is defective in phosphorylating both Sam68 and Cas when compared to wild-type Src (Table 4-1). This observation raises the question of whether the C-terminally placed SH2 domain in mutant 3UC2 is functional in targeting *in vivo*. SH2 domains have co-evolved with tyrosine kinases and are invariably found N-terminal to the kinase domains [125, 128]. It is possible that the C-terminal placement in 3UC2 might be disrupting targeting via the SH2 domain. Our *in vitro* studies with synthetic peptides suggest that the SH2 domain might still be functional in targeting, but is selective for the S-SH2L orientation (Figure 4-10). The role of SH2 domain in targeting in mutant 3UC2 could be tested by using a Src substrate which is dependent upon SH2 mediated targeting. For example, Src is recruited to the PDGFR via its SH2 domain only and it phosphorylates a single tyrosine residue (Y934) in PDGFR. The SH2 ligand is C-terminal to Y934 [134]. Based on our *in vitro* studies, we predict that mutant 3UC2 should be as efficient as wild-type in phosphorylating PDGFR. We attempted to test this, but our experiments were inconclusive because the pY845 antibody cross-reacts with other autophosphorylation sites in PDGFR (data not shown). Our studies point to the fact that alteration of domain architecture could lead to differential recognition and phosphorylation of Src substrates. This lends support to our hypothesis that domain architecture is conserved for maintaining substrate specificity of SFKs.

The modular arrangement of domains in SFKs makes them evolutionarily flexible. Despite such flexibility, the domain architecture of SFKs has been conserved from choanoflagellates to humans [126]. The SH3-SH2-kinase architecture is also present in other families of NRTKs, such as the Abl and Csk families [7]. One possibility is that the domain architecture of SFKs arose early in evolution primarily as a means of repressing kinase activity. Studies from our lab on *Monosiga brevicollis* MbSrc1 suggest that this is not the case: phosphorylation of the MbSrc1 C-tail by the *M. brevicollis* Csk homolog fails to inhibit activity [167]. An alternative view is that the substrate targeting function arose first, and autoregulatory properties were added later. Consistent with this view, our studies in Chapter 5 and several other studies demonstrate that it is relatively easy to rewire signaling pathways. Our studies provide support to the evolutionary flexibility of SFKs and we demonstrate that both substrate targeting as well as regulation of SFKs is evolvable by domain recombination. This is not surprising, as different modular enzymes use different modes for achieving repression. For example the phosphatase Shp2 has tandem SH2 domains at its N-terminus, and the N-terminal SH2 domain blocks access of substrates by binding to the catalytic domain, thereby achieving repression [27-29]. It is interesting to note that Abl tyrosine kinase, though it shares the

SH3-SH2-kinase architecture with Src, has a different mode of achieving down-regulation. Unlike SFKs, which have two signaling inpus (SH2 and SH3 ligands), Abl family kinases function as a three-input switch [22, 25, 172, 173]. In Abl there is no equivalent to the C terminal pTyr 527 of SFKs. Repression is achieved by docking of the SH3 and SH2 domains on the kinase domain. The Abl SH3 domain interacts with a polyproline helix in the SH2 kinase linker. There is an N-terminal cap region of Abl that provides inhibition instead of the SH2-pTyr of SFKs. The N-terminally myristoylated cap binds to a hydrophobic groove in the C-terminal lobe of catalytic domain [172]. The two lobes of the kinase are more closed relative to each other in Abl as compared to Src, and consistent with this Abl is more repressed than Src [22, 25, 172, 173]. Thus different kinases are repressed to different degrees, and depending upon flanking domains and interaction motifs can be activated by multiple inputs. Our results in Chapter 5 confirm that novel SFKs with desired regulatory and targeting properties can be designed.

In an attempt to mimic evolution, synthetic signaling switch proteins based on the N-WASP protein were designed which displayed various signaling behaviors. In electronic circuits there are logic gates which respond to various inputs giving a precise output. As an example, an AND gate needs all the inputs for relaying signal while an OR gate needs either one input for an output. Analogous to the logic gates, some proteins behaved like AND gates, where both ligands were required for full activation of WASP mediated actin polymerization. Other proteins behaved like OR gates, where either ligand alone was sufficient for activation [150]. Similarly, synthetic GEFs have been designed with activity under the control of synthetic ligands [151]. One approach to test the evolvability of modular signaling components is to attempt to mimic evolution by using them to create new synthetic pathways. The yeast MAPK pathway has been rewired using a synthetic scaffold such that mating pheromone led to a high osmotic stress response [159]. In mammalian cells, the proliferative response of EGFR signaling was changed to a death response by using a synthetic adaptor protein [158]. The ease with which signaling components can be re-wired is exploited by pathogens and cancer cells to evade recognition by immune cells. The intracellular pathogen *Yersinia sp.* encodes a protein (YopM) that acts as an adaptor and connects two unrelated kinases RSK1 and PRK2 [174]. HIV uses Nef protein to interact with SFKs and this interaction has a role in infection by the virus [175].

In another study a domain insertion strategy was used to recombine β-lactamase protein with maltose binding protein, and a novel allosteric enzyme was made where ampicillin resistance by β-lactamase was maltose dependent [176]. In another study, a novel biosensor was made that could lead to ligand dependent increase in growth of temperature sensitive yeast. Here dihydrofolate reductase (DHFR) enzyme was recombined with the ligand binding domain of estrogen receptor-α or FKBP (forskolin binding protein) [177]. In another study a two-domain protein was designed where ubiquitin was inserted into a loop of barnase, and ubiquitin mediated cell death could be controlled by a temperature switch or by ligand binding [178].

The modular organization of signaling proteins allows for a synthetic rewiring of signaling components to yield new pathways and biological responses [100]. This synthetic approach allows us to understand the basic principles used by multicellular animals to generate complex signaling pathways. The understanding of this basic framework might in the future make possible the rational design of engineered cells with new and specific therapeutic uses.

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