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Review

Structure and dynamic regulation of Src-family kinases

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Abstract. Src-family kinases are modular signaling proteins involved in a diverse array of cellular processes. All members of the Src family share the same domain organization, with modular SH3, SH2 and kinase domains followed by a C-terminal negative regulatory tail. X-ray crystallographic analyses of several Src family members have revealed critical roles for the SH3 and SH2 domains in the down-regulation of the kinase domain. This review focuses on biological, biophysical, and computational studies that reveal conformationally distinct active states within this unique kinase family.

Keywords. Src-family kinases, SH3 domain, SH2 domain, hydrogen exchange mass spectrometry, Gaussian Network Model.

Introduction

The Src family of cytoplasmic non-receptor protein-tyrosine kinases was first discovered in the context of the transforming retroviral oncogene v-*src*, which is responsible for the potent sarcoma-inducing activity of the Rous sarcoma virus [1, 2]. The identification of viral Src and the realization that it was captured from the host cell genome led to the discovery of the cellular proto-oncogene c-*src*, which serves as the prototype for this multi-kinase family. The Src family kinase complement of the human genome includes 11 members [3], 8 of which have been well-characterized in mammalian cells: Fyn, Lyn, Hck, c-Yes, Blk, Fgr, and Lck, in addition to c-Src itself. Most cell types express multiple Src family kinases (SFKs). While some family members are ubiquitously expressed (e.g. c-Src, Yes, and Fyn), others show more restricted patterns of expression (e.g., Hck and Fgr in myeloid leukocytes and Lck in T-lymphocytes) [4]. SFKs transduce signals for cell growth, differentiation, and survival, influence cellular adhesion, migration and invasion, and also regulate synaptic transmission [5]. SFK activation has been linked to many cell-surface signaling inputs, including growth factor, cytokine, and immune cell receptors, G-protein-coupled receptors, as well as integrins and other cell adhesion molecules [6, 7]. Thus SFKs represent critical points for the integration and transmission of diverse signals

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in a broad array of cellular contexts. Loss of SFK regulation has been linked to a variety of diseases, including several types of cancer [8], neurodegenerative diseases [9], epilepsy [10], as well as HIV/AIDS [11]. A more complete understanding of SFK structure and dynamics in specific signaling contexts may lead to selective inhibitors with therapeutic utility. In this review, we focus on recent advances in the structural biology and dynamic behavior of SFKs.

Overview of Src family kinase structure

All SFKs exhibit the same N- to C-terminal arrangement of structural domains (Fig. 1A). The extreme N-terminal region of all SFKs contains a signal sequence for myristoylation, with some Src-family members additionally palmitoylated. While lipid modification promotes membrane localization and is often essential for biological activity [12], kinase regulation can be maintained *in vitro* in the absence of this lipid modification. The N-terminal myristoylation signal sequence is followed by a unique domain, which is the only non-conserved region within the kinase family. SFK unique domains are typically 50–80 residues in length and are also dispensable for regulation of kinase activity *in vitro*. Note that unique domains are not found in existing structures of SFKs. The unique domain is followed by modular SH3 and SH2 domains, a regulatory linker, the catalytic or kinase domain (SH1 domain), and a C-terminal negative regulatory tail. In general, SH3 domains are an average of 60 residues in length and bind to target sequences rich in proline and other hydrophobic amino acids. These sequences generally form a polyproline type II (PPII) helix which associates with the hydrophobic surface of the SH3 domain [13, 14]. In the context of SFKs, the SH3 domain contributes to substrate recruitment [15, 16] and is critical for the regulation of kinase activity [17–20]. The SH2 domain also functions in protein-protein interaction(s) by virtue of affinity for phosphotyrosine-containing peptide sequences [14, 21]. The canonical high-affinity peptide motif for interaction with SFK SH2 domains is pTyr-Glu-Glu-Ile (pYEEI) and has been likened to a two-pronged plug (the peptide) engaging a two-holed socket (the SH2). The phosphorylated tyrosine occupies the first hole while the isoleucine residue fills the second, somewhat shallower hole [21, 22]. Phosphorylation of tyrosine residues in SH2 target sequences is critical as unphosphorylated peptides do not bind to SH2 domains (reviewed in [14]). In SFKs, phosphorylation of a highly conserved tyrosine residue in the tail region (Tyr-527 in c-Src, see Fig. 1) induces intramolecular interaction with the SH2 domain, helping to lock the kinase in an inactive conformation. (Note: all residue numbering in this review is based on the crystal structure of human c-Src; PDB 2SRC [23].) The SH2 domains of SFKs are therefore essential for negative regulation of kinase activity.

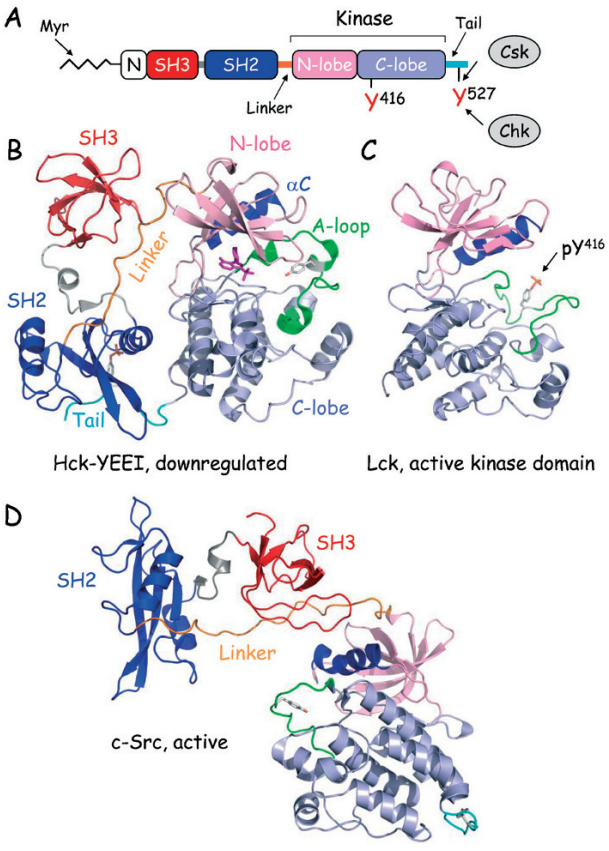

Figure 1. Structural organization of Src-family kinases. (*A*) The overall domain organization includes an N-terminal unique domain, with sites for myristoylation and in some cases palmitoylation; the SH3 domain (red); the SH3-SH2 connector (grey); the SH2 domain (blue); the SH2-kinase linker (orange); and the C-terminal kinase domain. The kinase domain consists of a smaller N-terminal lobe (pink) and a larger C-terminal lobe (light blue). The activation loop tyrosine is located in the large lobe at position 416. The kinase domain is followed by the C-terminal tail (cyan) with a conserved tyrosine residue (Tyr-527) that is phosphorylated by separate regulatory kinases. (*B*) X-ray crystal structure of Hck-YEEI, in the downregulated conformation. The color scheme from (*A*) is mapped onto the structure. Highlighted within the kinase domain are the αC helix (blue) and the activation loop (green). Also shown is the side chain of the activation loop tyrosine (Tyr-416), as well as the pyrazolo-pyrimidine inhibitor PP1 (magenta) which maps the ATP-binding site. Model based on PDB file 1QCF [27]. (*C*) For comparison, the kinase domain of Lck in the autophosphorylated, active conformation is shown. Color scheme is the same as for (*B*) (PDB 3LCK [29]). (*D*) X-ray crystal structure of active c-Src revealing persistence of SH3:linker interaction. Domains and other structural features are colored as per Hck-YEEI in parts A and B (PDB 1Y57 [30]). Note that all numbering in this figure and throughout the text is based on the crystal structure of human c-Src (PDB 2SRC [23]).

At first, the SH2-tail interaction was believed to be the major intramolecular interaction that held SFKs in the downregulated state. It was later discovered in the high-resolution X-ray crystal structures of the inactive forms of c-Src and Hck [23–27] that the downregulated conformation is stabilized by an additional intramolecular interaction of the SH3 domain with a PPII helix formed by the linker connecting the SH2 and kinase domains. Figure 1 shows a molecular model of the SFK Hck in its tail-phosphorylated, downregulated conformation. Note that the SH3 and SH2 domains work together to downregulate kinase activity by engaging their intramolecular ligands (SH3 binds to the linker and SH2 binds to the tail) and pushing on the 'back side' of the kinase domain (the entrance to the active site for substrates and ATP is on the opposing face of the kinase domain, at the far right of the Hck structure shown in Fig. 1B). These interactions cause an alpha helix (the αC helix) in the N-lobe of the kinase domain to swing outward into a conformation incompatible with catalytic activity [28]. In this 'turned-out' conformation, the activation loop of the kinase domain can adopt a partially helical conformation and the autophosphorylation site (Tyr-416) points inward. By contrast, crystal structures of the active conformation of the kinase domain of an SFK (Lck; Fig. 1C [29]) as well as a full-length SFK (c-Src; Fig. 1D [30]) show that the αC helix is re-oriented inward, with the activation loop adopting a different conformation that is more compatible with ATP and peptide substrate binding. This active conformation becomes stabilized upon autophosphorylation of Tyr-416.

The conserved tail tyrosine residues of SFKs are phosphorylated by a distinct regulatory kinase known as Csk (for C-terminal Src kinase) [31] as well as a closely related kinase known as Chk [32]. Tail phosphorylation by these regulatory kinases forces SFKs into the downregulated conformation via SH2:tail interaction as described above. Gene-knockout experiments strongly suggest that Csk is the master regulator of all Src family members [33, 34]. Without Csk, embryonic lethality is observed with a concomitant elevation in overall SFK activity. More recent work has shown that mouse embryonic stem cells express seven members of the Src kinase family, and that pharmacological inhibition of all SFK activity interferes with differentiation of ES cells to embryonic structures known as embryoid bodies *in vitro* [35]. Together with the Csk knockout results, these data suggest that strict temporal and spatial control of SFK activity is critical to early development.

The importance of intramolecular interactions for stabilization of the downregulated state of SFKs has been demonstrated for several Src-family members using site-directed mutagenesis of key regulatory sites. Figure 2 illustrates this point using the myeloid Src-family member, Hck, and a rodent fibroblast transformation assay. Overexpression of wild-type Hck in fibroblasts does not produce a biological effect, because kinase activity is effectively downregulated by endogenous Csk. Thus, fibroblasts overexpressing wild-type Hck grow as a contact-inhibited monolayer in the same manner as a negative control culture expressing a kinase-dead Hck mutant. However, replacement of the tail tyrosine residue with phenylalanine prevents phosphorylation by Csk, releasing the negative regulatory tail from the SH2 domain and permitting upregulation of kinase activity [36]. Constitutive activation of Hck in this manner causes loss of contact inhibition and the formation of many transformed foci that are visible as dark spots in Figure 2A. Alanine replacement of the two proline residues essential for the PPII helical conformation of the SH2-kinase linker also disrupts downregulation, causing kinase activation and a transformed phenotype [36]. In this case, the modified linker is unable to interact with the SH3 domain. Similarly, mutation of a conserved tryptophan residue that is essential for SH3 domain function has also been shown to induce fibroblast transformation, most likely by preventing SH3:linker engagement [37]. Interestingly, this mutant shows lower focus-forming activity compared to the tail mutant, presumably because the disabled SH3 domain cannot recruit substrates essential for fibroblast transformation such as Stat3 [37].

Activation of SFKs by regulatory domain displacement

As described in the preceding section, mutagenesis of key residues involved in SH2:tail and SH3:linker engagement disturbs the downregulatory influence of these interactions on the kinase domain. These observations suggest that interactions between the SH2 and/or SH3 domains of SFKs with other proteins may also lead to SFK activation *in vivo*. This idea was first demonstrated by taking advantage of the SH3-binding properties of Nef, an HIV-1 accessory factor involved in AIDS progression [38–41]. Nef is a small myristoylated protein that, like SFKs, is associated with the plasma membrane [42–44]. Nef has no known catalytic activity, and appears to function through interactions with multiple cellular proteins involved in signal transduction, including both Ser/Thr and Src-family tyrosine kinases [42]. Nef binds to the SH3 domain of Hck with one of the highest reported affinities for an SH3-mediated interaction ($K_d = 250$ nM) [45, 46]. Mutagenesis has established that one

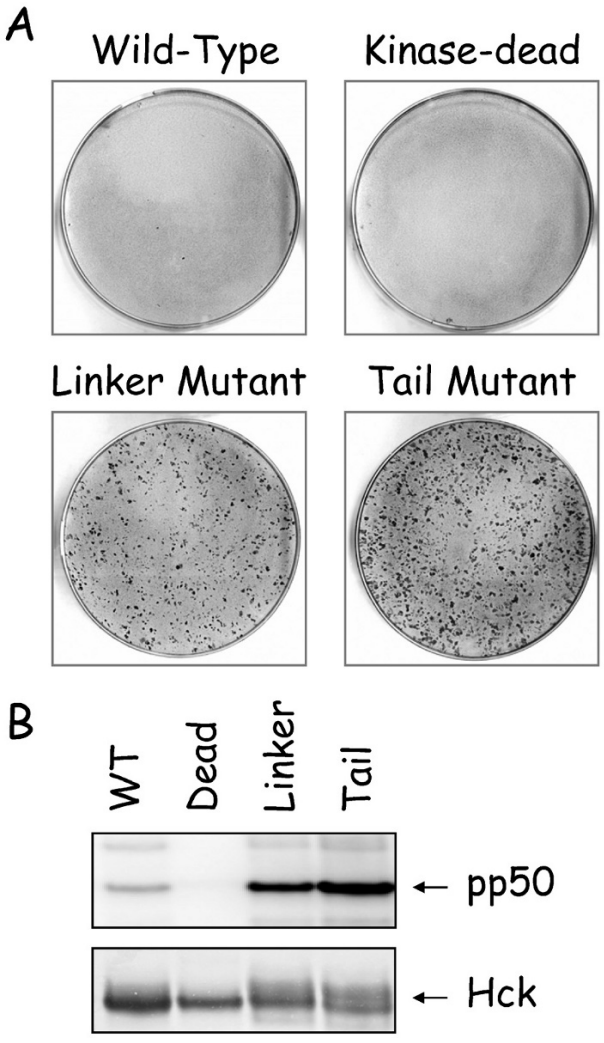

Figure 2. Activation of the SFK Hck by mutation of the SH2-kinase linker or negative regulatory tail tyrosine residue. (*A*) Culture plates of Rat-2 fibroblasts expressing wild-type Hck, kinase-dead Hck, a linker mutant, or a tail mutant were stained for transformed foci, which appear as dark spots. (*B*) Hck was immunoprecipitated from lysates of the cells in (*A*), and incubated *in vitro* with [γ-^{32}P]ATP and the substrate protein p50, which is derived from the natural SFK substrate Dok [90]. Proteins were separated by SDS-PAGE and visualized by autoradiography. Note that the phosphorylated p50 substrate is readily visible in reactions from cells where Hck is activated by tail or linker mutations (pp50). Aliquots of the immunoprecipitates were also probed with a Hck antibody as a positive control (Hck). Figure adapted from Briggs et al. [36]

determinant of Nef:SH3 interaction is the consensus proline-rich motif PQVPxR in Nef [46]. X-ray crystallography and NMR structural studies of Nef:SH3 complexes show that these residues form the critical PPII helix that contacts the SH3 hydrophobic surface [47, 48]. The Nef PxxP motif responsible for SH3 binding is highly conserved among primary HIV isolates [49–51], and is essential for the high-titer replication of HIV in unstimulated peripheral blood mononuclear cells *in vitro* [46] and a murine model of AIDS [11]. Also essential for Nef SH3 binding are conserved residues from the Nef αA and αB helices, which form a hydrophobic pocket that accommodates an Ile residue found in the RT loop of the Hck SH3 domain [45]. This RT loop Ile residue is unique to the Hck and Lyn SH3 domains, providing a structural basis for an important relationship between Nef and Hck (and possibly Lyn) in HIV pathogenesis.

Consistent with the X-ray crystal structure of down-regulated Hck and the SH3:linker mutagenesis experiments outlined above, binding of Nef to full-length Hck induces constitutive kinase activation. This effect of Nef has been demonstrated *in vitro* [52, 53], in cell-based assays using fibroblasts [20, 54] and yeast [53], and also in HIV-infected cells [55]. A representative experiment is shown in Figure 3 using fibroblast transformation as the model system (described above). Here, expression of Nef or Hck alone produces no effect on fibroblast morphology. However, co-expression of Nef with Hck leads to a very strong transformed phenotype, similar to that observed with the tail and linker mutants of Hck (Fig. 2). Kinase assays show that Hck is strongly activated in the presence of Nef in cells expressing both proteins, consistent with the biological effect (Fig. 3B). In addition, mutagenesis of the Nef PxxP motif or replacement of Tyr-120 in the Nef hydrophobic pocket with isoleucine abolishes Nef-induced kinase activation, demonstrating that SH3 engagement by Nef is necessary to cause kinase activation in living cells [20, 56]. These data support a model in which Nef binding causes displacement of SH3 from its downregulatory position on the back side of the catalytic domain, priming the kinase for activation [52] (Fig. 3C). Also noteworthy is the observation that Nef is an oligomeric protein, and may juxtapose multiple Hck molecules simultaneously to promote autophosphorylation in *trans* [57].

HIV-1 Nef provides a powerful example of how domain displacement can lead to SFK activation. A growing number of examples of SH2- and SH3-based SFK activation events have also been described for both receptor systems upstream from SFKs as well as downstream signaling partners and substrates. For example, SFKs have been implicated in signal transduction by both cytokines and growth factors, and in some cases are required for cell-cycle progression in response to factor treatment [7]. SFK activation by growth factor receptor tyrosine kinases involves SH2 domain-dependent recruitment to specific membrane-proximal phosphotyrosine residues on the active, autophosphorylated receptor. SH2 domain binding may induce Src activation by displacing the negative regulatory tail, leading to phosphorylation

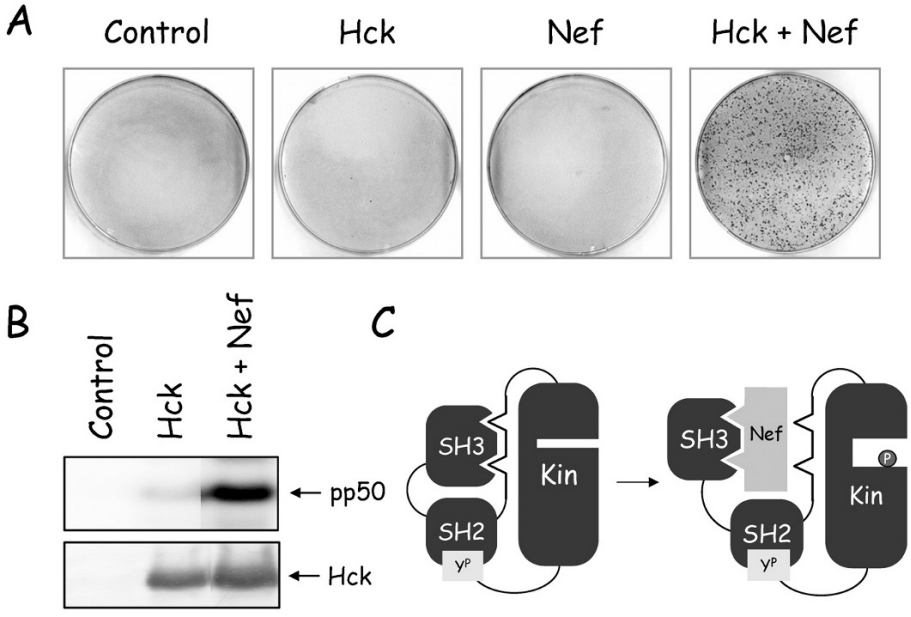

Figure 3. Activation of the SFK Hck by SH3:linker displacement. (*A*) Culture plates of Rat-2 fibroblasts expressing wild-type Hck, HIV-1 Nef (SF2 strain), or a combination of both (Hck+Nef) were stained for transformed foci (dark spots). Cells expressing only the drug selection marker served as negative control. (*B*) Hck was immunoprecipitated from lysates of cell cultures in (*A*) and assayed for kinase activity as described in the legend to Figure 2. Phosphorylated p50 is readily visible in reactions from cells where Hck is co-expressed with Nef (pp50). Assays from cells expressing Nef alone look identical to the negative control lane (not shown). (*C*) Model of Nef-induced Hck activation *in vivo*. Nef engages the Hck SH3 domain, displacing it from the SH2-kinase linker and leading to kinase activation. Evidence described in the text suggests that activation by Nef does not require tail dephosphorylation or release from the SH2 domain. Figure adapted from Briggs et al. [20, 36]

of the receptor in some cases [5, 6]. The mechanism of Src family kinase activation by cytokine and antigen receptors is less clear, but may also involve recruitment to the activated, oligomeric form of the receptor. Recruitment of two or more kinase molecules into close proximity may allow for *trans*-phosphorylation, which is important for kinase activation [58].

Binding of SFKs to substrates or other cellular molecules via their SH3 domains is also sufficient to induce kinase activation in some instances. Examples include the focal adhesion protein p130 Cas [59, 60], the progesterone receptor [61], and the Stat3 transcription factor [37]. These studies suggest that SH3-dependent recruitment of substrates to Src family kinases may induce transient kinase activation, substrate phosphorylation and release, followed by a return of the kinase to its inactive state [62].

Structures of downregulated kinases predict multiple active conformations

Studies outlined in the previous section show that disruption of either SH2:tail or SH3:linker interactions, either by mutation, interaction with other proteins, or tail dephosphorylation, is sufficient to induce SFK activation. The presence of two negative regulatory interactions within the same molecule suggests a number of interesting possibilities with respect to the overall structure of the active conformation. One possibility is that displacement of either interaction alone may be sufficient to induce kinase activation, resulting in more than one active conformation (modeled in Fig. 4). Alternatively, disruption of one regulatory interaction may destabilize the entire regulatory apparatus, leading to only one active-state conformation. Evidence for the latter hypothesis comes from molecular dynamics simulations of Hck which show that the motions of the SH2 and SH3 domains are highly correlated in the repressed state [63], suggesting that displacement of either domain from its internal ligand may cause disruption of the other interaction, producing a single active conformation [64]. Resolution of this issue is important, because it will clarify whether SFKs behave as either simple on-off switches, where a single active state is adopted regardless of the activating input, or if different activating inputs produce unique active states with distinct signaling properties. Recent work suggests that at least in the case of Hck, the latter possibility may be true. These studies are summarized in the next section.

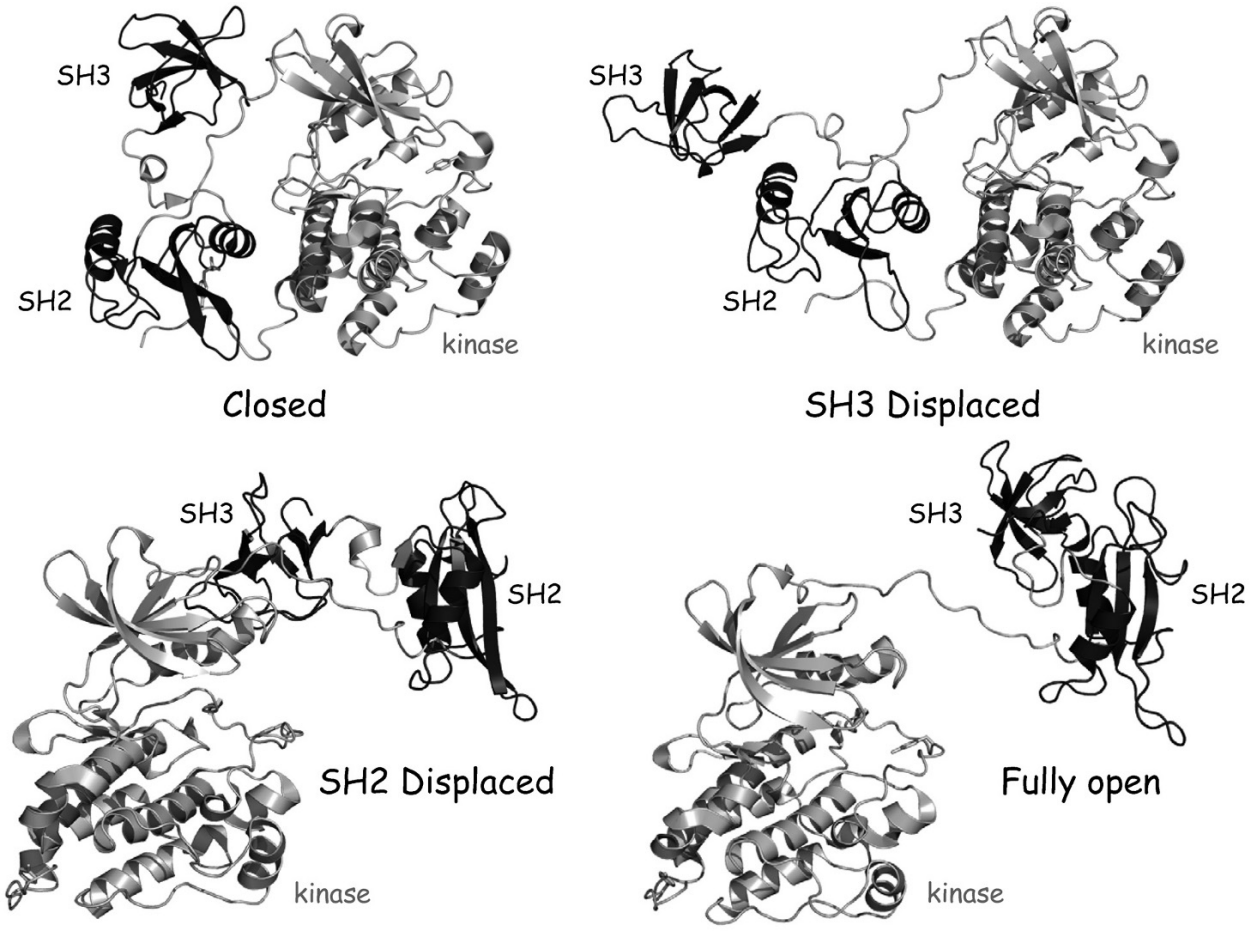

Figure 4. Hypothetical conformations of active SFKs based on activating inputs. The closed, inactive conformation is the crystal structure of Hck-YEEI, as shown in Figure 1 (PDB 1QCF [27]). The model with SH3 displaced was produced by docking the SH2 domain of the Lck SH3-SH2 crystal structure (PDB 1LCK [91]) onto the SH2 domain of Hck-YEEI. The model with SH2 displaced is the crystal structure of dephosphorylated c-Src (PDB 1Y57 [30]; see also Figure 1). The fully open model, in which both SH2 and SH3 displaced from their internal ligands, was produced by docking the SH2 domain of the Lck SH3-SH2 crystal structure onto the SH2 domain of the dephosphorylated c-Src structure. The SH3 and SH2 domains are labeled and colored in black; the kinase domain in each model (grey) is positioned in the same relative orientation.

Biological evidence for alternative active conformations of the SFK Hck

Lerner and Smithgall investigated SH3-based activation of Hck, either by protein ligand binding (HIV-1 Nef) or by SH2-kinase linker mutagenesis [54]. This study addressed whether the negative regulatory tail remained phosphorylated and bound to the SH2 domain when Hck was activated through its SH3 domain. Using rodent fibroblast transformation as a model system, the negative regulatory tyrosine residue was found to remain phosphorylated when Hck was activated either by Nef binding to the SH3 domain or by linker mutagenesis. These data demonstrated that active SFKs can be dually phosphorylated in cells, both on the activation loop in the kinase domain as well as on the negative regulatory tail. The presence of phosphate on the tail tyrosine in the active kinase led to the question of whether the tail remained SH2-bound in the active state. This question was addressed using a form of Hck with a modified tail with a much higher affinity for the SH2 domain than in the wild-type protein. The modified tail sequence is Tyr-Glu-Glu-Ile (YEEI), which represents the core sequence of high-affinity peptide ligands for SFK SH2 domains [22]. Previous structural studies have established that this form of Hck (Hck-YEEI) adopts the same inactive conformation as Csk-phosphorylated wild-type Hck, with the SH2 domain bound to the tail [27]. Surprisingly, Nef activated wild-type Hck and Hck-YEEI equally well in terms of fibroblast transformation and in kinase assays [54, 65]. This result supports the idea that release of the tail from the SH2 domain may not be required when Hck is activated through its SH3 domain, and supports the existence of a novel Nef-induced active Hck conformation. In related

experiments, mutagenesis of the SH2-kinase linker within Hck-YEEI also led to strong kinase activation, further supporting the existence of a unique active conformation in which the SH3 domain is displaced from the linker while the tail remains SH2-bound (modeled in Fig. 4, top right).

While the Hck-YEEI experiments described above suggest that SH2:tail release is not required for SH3-mediated Hck activation, more recent work has shown that disruption of this interaction is essential for SFK signaling in other situations. Using the analogous 'YEEI' form of Lck, Nika et al. demonstrated that SH2:tail displacement is essential for Lck to transduce signals from the T-cell antigen receptor [66]. Using the T-cell line JCaM1, which does not express endogenous Lck, they found that transfection of wild-type Lck but not Lck-YEEI led to reconstitution of ZAP-70 activation and other Lck-dependent signaling events downstream from the T-cell receptor. The status of Lck SH3:linker engagement was not investigated in this study. These experiments suggest that while the normally weak SH2:tail interaction present in Lck is sufficient for negative regulation, competitive displacement by activating proteins or dephosphorylation is essential for effective T-cell receptor signaling. Considered in light the results with Hck, this study also suggests that different SFKs may respond to activating events in unique ways and that extrapolation of the effects observed with one SFK to other family members may not be valid.

To address the complementary question of whether SH2-directed activation of Hck requires disruption of SH3:linker interaction, the wild-type Hck linker sequence was modified to enhance its interaction with the SH3 domain [67]. The natural sequence of the Hck linker represents a sub-optimal SH3 ligand. In the crystal structure of the downregulated conformation of Hck, the key positions of the linker PPII helix that face the SH3 domain are occupied with lysine residues which do not optimally engage the SH3 binding surface [27]. To enhance this interaction, these residues were replaced with prolines. Surface plasmon resonance experiments verified that this modified high affinity linker (HAL) was essentially locked onto the SH3 domain. To evaluate the impact of the HAL substitution on the overall structure of Hck, energy minimization was performed on a 'virtual HAL mutant' of the Hck crystal structure [67]. Of particular concern was the possible impact of the modified linker on the position of Trp-260 relative to the αC helix in the small lobe of the kinase domain. Previous studies have established that Trp-260, which is located at the C-terminal end of the linker, provides an essential stabilizing influence on the αC helix in the down-regulated state [68]. Molecular modeling predicted that the lysine-to-proline substitutions in the Hck-HAL mutant would be well-tolerated, as the overall Hck structure in general and the position of Trp-260 in relation to the αC helix in particular were virtually unchanged. This prediction was borne out in subsequent biological experiments, as Hck-HAL showed no evidence of kinase activity when expressed in rodent fibroblasts, indicating that the modified linker did not disrupt downregulation. The HAL substitution was then combined with a mutation of the negative regulatory tail tyrosine residue. Previous work has shown that substitution of the tail tyrosine causes strong upregulation of Hck kinase activity, producing a very strong transforming signal in fibroblasts [36] (see Fig. 2). Expression of the Hck-HAL-tail mutant also resulted in very strong fibroblast transformation that correlated with a high level of constitutive kinase activity. This result suggests that the SH2-based activation of Hck resulting from mutation of the negative regulatory tail does not necessarily require disruption of SH3:linker interaction.

Experiments with Hck-HAL in fibroblasts described above support the possibility that SH3:linker interaction need not be disrupted for activation of the kinase domain to occur following SH2:tail release. However, this observation does not necessarily imply that SH3:linker interaction persists when wild-type Hck or other SFKs are activated through SH2- or tail-based mechanisms. To test for the persistence of SH3:linker interaction in wild-type Hck, *Saccharomyces cerevisiae* (baker's yeast) was used as a model system. This small eukaryote does not express orthologs of SFKs or Csk, the negative regulatory kinase that phosphorylates SFKs on the key tail tyrosine residue (Fig. 1). Expression of Hck alone, which is unregulated in yeast since there is no Csk, leads to suppression of yeast growth. This effect positively correlates with Hck kinase activity [67] as observed previously with c-Src [17]. Co-expression with Csk, however, leads to Hck inhibition and reversal of growth suppression [53, 67]. Interestingly, when Hck is co-expressed with HIV-1 Nef in the absence of Csk, both kinase activity and growth suppression are further increased in comparison to yeast cultures expressing Hck alone. (Recall that Nef represents a high-affinity ligand for the Hck SH3 domain.) This observation suggests that wild-type Hck may retain some degree of SH3:linker interaction even though the negative regulatory tail is not bound to the SH2 domain and the kinase is active. Recently, a crystal structure of c-Src was reported that supports this idea as well. This structure was determined using an unphosphorylated form of c-Src in which the kinase domain αC helix and activation loop adopt conformations similar to those found in active kinase

domains [30]. Because the structure is not tyrosine-phosphorylated, the tail is released from the SH2 domain, similar to what happens with Hck in yeast. Relevant to this discussion is the observation that the SH3 domain remains bound to the linker [30], despite the active conformation of the kinase domain (Fig. 1D and Fig. 4, lower left). Interestingly, the SH3-SH2-linker assembly is rotated by about 130° relative to its position in the downregulated conformation of c-Src, with the linker retaining a very similar conformation to that observed in the inactive state. Whether this structure represents a fully active conformation or an intermediate in a pathway to full activation will require further investigation.

Biological studies described above provide evidence that multiple active conformations of Hck and possibly other SFKs may exist. What, then, is the significance of such conformational variants to physiological signaling? One possibility is that the type of activating input (e.g., SH2- vs. SH3-directed) may determine the type of downstream signaling that can occur. For example, SH3-based activation such as that produced by HIV-1 Nef binding may not make the SH2 domain accessible for substrate recruitment. Conversely, activation of SFKs through mechanisms disturbing SH2:tail interaction may be independent of SH3-mediated signaling. Data presented in Figure 5 provide preliminary evidence for different patterns of substrate selection that depend upon the Hck activation mechanism. In this experiment, Rat-2 fibroblasts were transformed with either a constitutively active Hck mutant in which the negative regulatory tail tyrosine was replaced with phenylalanine (Hck-YF; SH2-based activation) or by co-expression of wild-type Hck with HIV-1 Nef (SH3-based activation). Aliquots of soluble protein from each transformed cell population were then separated by two-dimensional SDS-PAGE, and analyzed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Figure 5, multiple unique pTyr-containing proteins were observed in each case, supporting the idea that substrate selection by Hck is indeed influenced by the activation mechanism. Future studies will investigate this phenomenon under physiologically relevant signaling conditions.

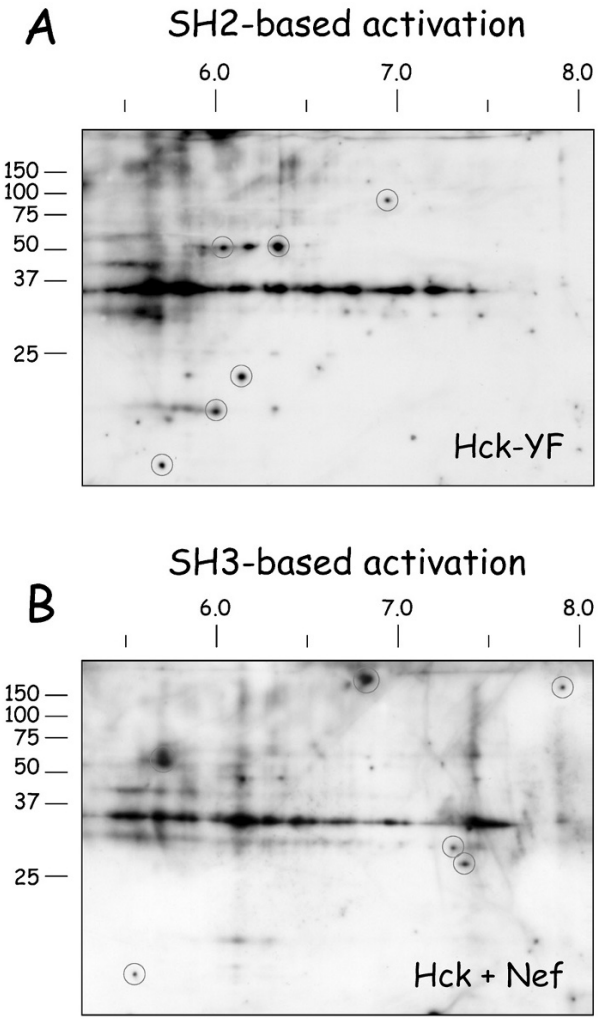

Figure 5. Mechanism of Hck activation affects substrate selection patterns *in vivo.* Rat-2 fibroblasts were transformed with either (*A*) a constitutively active Hck tail mutant (Hck-YF; SH2-based activation) or (*B*) co-expression of wild-type Hck with HIV-1 Nef (Hck+Nef; SH3-based activation). Aliquots of cellular protein extracts were separated by 2D electrophoresis, transferred to PVDF membranes and visualized with anti-phosphotyrosine antibodies. Positions of molecular mass standards are shown on the left in kDa, while the relative position of the pH gradient is shown at the top. Examples of strong signals unique to each sample are circled. Similar analysis of control fibroblasts revealed very few signals, none of which overlapped with the unique phosphoproteins that are circled (not shown). These data provide strong evidence that SH2- vs. SH3-based activation mechanisms affect substrate recruitment by Hck, at least in this model system.

Biophysical studies of SFK dynamics

Biological evidence described in the preceding section strongly suggests that displacement of the SH3 and/or SH2 domains from their regulatory intramolecular interactions is an important mechanism by which SFK kinase activity is regulated *in vivo.* However, until recently, biophysical evidence in support of this hypothesis was lacking. Furthermore, while biological assays and X-ray crystallography support the persistence of SH3:linker and SH2:tail interaction in active forms of Src-family kinases, biophysical evidence supporting the presence of these interactions in solution at physiological kinase concentrations is difficult to produce. It has not been possible to obtain crystallographic or NMR structural data of active forms of full-length SFKs (except as noted above for c-

Src), because the active forms generally behave poorly in solution. This may be related to the propensity of the active kinase proteins to aggregate under the high protein concentrations required for *in vitro* analyses with these biophysical methods.

One solution to this problem involves an alternative biophysical approach, hydrogen exchange mass spectrometry (HXMS), which has provided new insight regarding changes in SFK conformation as a function of activation. Details of HXMS will not be reviewed here, and the following review is recommended for readers less familiar with this technique [69]. In brief, when proteins are incubated in solutions prepared in deuterium oxide, labile backbone amide positions will exchange hydrogen for deuterium. The rate of hydrogen:deuterium exchange varies as a function of both protein dynamics and protein conformation. To detect deuterium incorporation, high-resolution electrospray mass spectrometry is used. Because deuterium has a mass of 2.014 and hydrogen has a mass of 1.008, deuteration causes the mass of a protein to increase. The location of the deuteration events can be determined to a resolution of 5–10 amino acids by digesting the deuterium-labeled protein with a protease after the labeling reaction. HXMS has distinct advantages, particularly for analysis of SFKs, including the ability to work with very small amounts of dilute protein solution and the ability to localize dynamic changes that occur in response to both intramolecular and intermolecular interactions.

Conformational analyses of SFKs with HXMS have been successfully approached in a 'divide-and-conquer' fashion, in which individual recombinant domains were subjected to HXMS first, followed by larger, multi-domain structures and ultimately the near-full-length kinase itself. This strategy provided baseline data for each domain separately, thus allowing assessment of HXMS changes in the presence of additional domains. Initial HXMS studies performed on the Hck SH3 domain yielded a surprising discovery [70]. The Hck SH3 domain was unexpectedly observed to exhibit intrinsic flexibility, manifested as partial unfolding on a slow time scale (minutes) ideally suited to analysis by HXMS techniques. The kinetics of the unfolding event change when the domain is bound to a ligand (Fig. 6), providing an ideal assay to probe the binding status of the domain both in *trans* with other proteins and peptides and in *cis* with covalently attached sequences of Hck. While the physiological relevance of this unfolding event remains unknown, its conservation across a diverse group of SH3 domains suggests an important regulatory function [71].

Subsequent studies showed that changes to HXMS in the SH2 domain as a result of peptide binding could also be monitored [72]. HXMS analysis of a combined construct of the Hck SH3 and SH2 domains, called SH32, showed that the two domains only influence each others' conformation at the interface, and that the interaction between them is minor [73]. Furthermore, ligand binding to one of the domains had little influence on hydrogen exhange at the adjacent domain, suggesting that little interdomain cross-talk exists at least in the isolated SH32 construct [74].

NMR methods have also been applied to the study of SH3-SH2 interdomain communication as a function of ligand binding. In these experiments, chemical shift changes were monitored in the domain not involved in the direct binding of the peptide ligand. NMR studies of Fyn, c-Src, and Lck SH32 proteins revealed that peptide ligand binding to the SH2 domain resulted in either small (c-Src, Fyn) [75–77] or undetectable (Lck) [78] chemical shift changes in the SH3 domain, again suggesting that SH2 ligation is not structurally communicated to the SH3 domain. Together, the HXMS and NMR results are consistent with the idea that SFK SH3 and SH2 domains have independent folding and binding activity which is not grossly influenced by being next to one another in sequence. However, SH3-SH2 coupling is very important when considered in the context of the larger downregulated conformation of SFKs. Here the amino acid sequence connecting the SH3 and SH2 domains plays a key role in conferring sufficient rigidity on the SH3-SH2 apparatus so as to ensure proper assembly of the downregulated state. Indeed, molecular dynamics simulations of the downregulated conformation show strong dynamic coupling between SH3 and SH2, leading to the concept of a 'snap-lock' mechanism that ensures effective inhibition of the kinase domain [63]. Mutations within the SH3-SH2 connector have been shown to induce upregulation of kinase activity, supporting the importance of SH3-SH2 coupling in attaining the downregulated conformation [63].

HXMS also revealed that a peptide based on the natural SH2-kinase linker sequence of Hck did not bind to the SH3 domain, while a peptide derived from the conserved PPII helix of the Nef protein, as well as the full-length Nef protein itself, bound strongly to the SH3 domain (see Fig. 6) [74]. This observation suggested that the local concentration of the linker peptide was not high enough for efficient SH3 interaction and that covalently tethering of the linker sequence to the SH32 protein, as it is in full-length Hck, would confer tight SH3 binding. However, attachment of the linker also was not sufficient to induce SH3 domain binding in the context of the SH32L construct. These results indicated that the linker sequence itself cannot adopt a conformation

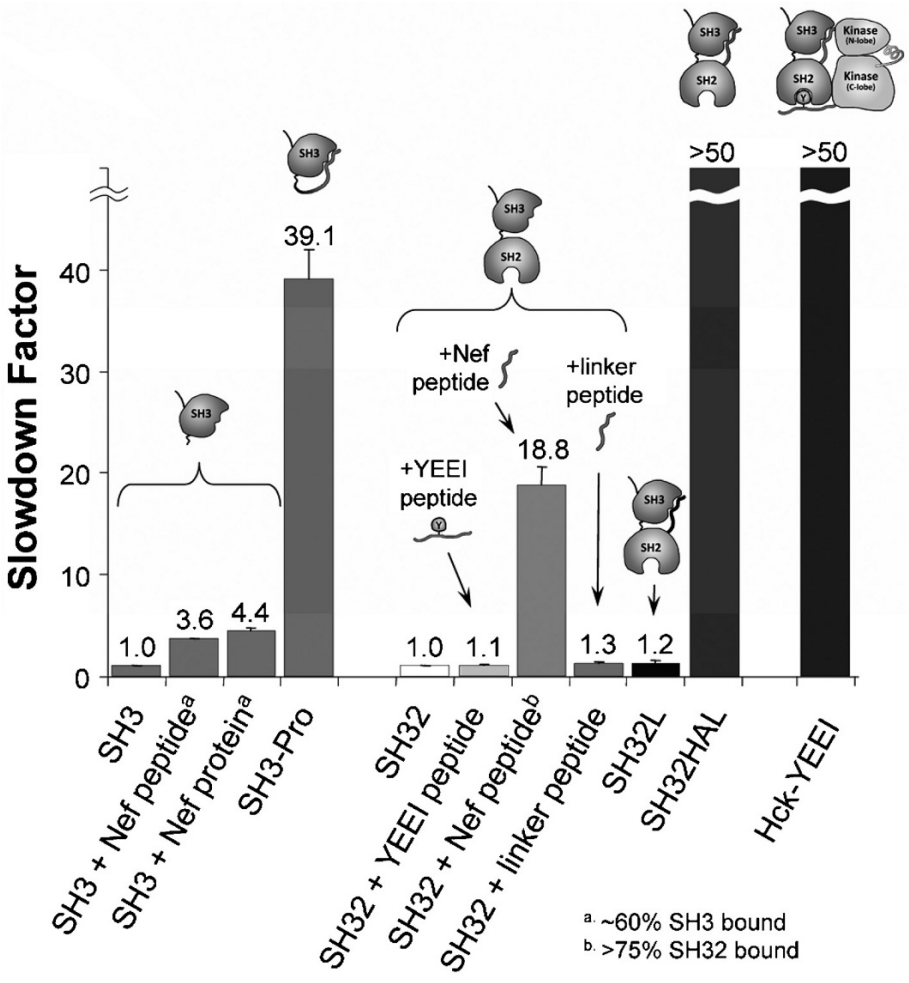

Figure 6. Determination of SH3 interactions in Hck using HXMS. Partial unfolding of the Hck SH3 domain was assayed with HXMS by measuring the appearance of EX1 kinetics in mass spectra of the intact deuterated Hck SH3 and peptic peptides derived from it [70, 71, 73, 74]. The unfolding rate in SH3 alone (far left) was compared to the unfolding rate in the presence of various peptide ligands and in the larger proteins shown. Observed differences in SH3 unfolding were used to calculate a slowdown factor [74] that describes the relative strength of binding to SH3: a small slowdown factor (close to 1) means weak or no binding, while a large slowdown factor means tight binding. The constructs involved in each experiment are shown in cartoon form. In cases of interaction with Nef and the Nef-derived peptide, the percentage of SH3-bound molecules is indicated by the superscripts a and b. Further details on the proteins used to create this figure can be found in Hochrein et al. [74] and in the text.

competent for SH3 binding (i.e., a PPII helix). This conclusion was validated by creating a form of SH32L containing the high-affinity linker (HAL) sequence described in the preceding section. As shown in the biological experiments and in Biacore analysis [67], the HAL form of the linker bound strongly to the SH3 domain. In HXMS experiments, the binding of HAL to the SH3 domain was so tight that all SH3 domain unfolding was abolished (Fig. 6) [74]. A similar effect was also observed when a natural SH3 ligand derived from p120 Ras GAP was covalently attached to the C-terminus of the Hck SH3 domain [79] (SH3-Pro construct in Fig. 6). NMR analysis also revealed tight intramolecular binding in this construct [79].

To test the idea that the intramolecular interactions between the kinase domain and the rest of the regulatory machinery (SH3-SH2-linker) are important, HXMS was measured in the near-full-length Hck protein (lacking only the N-terminal unique domain), an experiment that is not possible with NMR. The YEEI-tailed version of Hck [27] was selected for these experiments because the majority of the molecules must be in one conformation in order to obtain meaningful results. HXMS analysis of the full-length Hck-YEEI protein showed that the C-terminal tail was indeed bound to the SH2 domain. The HXMS signature for peptides in the SH2 domain was similar to that seen when the isolated SH2 was bound to a high-affinity phosphopeptide bearing the phospho-YEEI sequence [72]. Remarkably, the SH3 domain was also bound tightly to its internal ligand, the SH2-kinase linker. Recall that in the absence of the kinase domain (SH32L construct), there was no binding of the SH3 domain to the linker. In HXMS data from the whole protein, however, the SH3 unfolding rate was the same as it was for the SH32HAL construct [74], indicating that SH3 bound tightly to the wild-type linker in the context of the near-full-length Hck protein (Fig. 6). These data strongly suggest that the kinase domain is essential for structuring the linker sequence as a PPII helix competent for SH3 binding. Such a phenomenon has been seen before for proteins bearing PPII helices [76, 80, 81].

Biological data summarized above support the idea that the SH2 domain may remain bound to the C-terminal tail, despite activation of the kinase following SH3 domain displacement by Nef. HXMS analysis of the Nef:Hck-YEEI complex revealed that SH2:tail interaction indeed remained in place, as the exchange of SH2 peptides was identical in the presence or absence of Nef. On the other hand, a slight perturbation in the hydrogen exchange rate was observed in the SH3 domain in the presence of Nef. We interpret this as a change to the dynamics of the SH3 domain as a result of being bound to Nef rather than being bound to the linker. In addition, other regions of Hck, especially those in the linker and the small lobe of the kinase domain near the Nef contact site, displayed altered hydrogen exchange rates as a result of Nef binding that are consistent with kinase activation [Wales, Smithgall and Engen, unpublished results].

HXMS analyses of Lck provided similar conclusions about the general regulatory behavior of SFKs. These experiments also employed a YEEI-tailed version of Lck, which served to lock the protein in a single downregulated conformation prior to HXMS analysis. Binding of a peptide to the Lck SH3 domain could also be detected with HXMS methods [82]. In this case, the peptide in question was Herpesvirus samairi Tip, an intrinsically unstructured protein that has high affinity for Lck [83] and is a potent activator of Lck kinase activity as well [84]. HXMS analyses of the full-length Lck protein in the presence and absence of Tip showed that the binding status of the SH3 and SH2 domains can be monitored in the context of the entire kinase structure. Specifically, the SH3 domain is easily displaced by the Tip protein whereas the SH2 domain remains bounds to the Lck tail [Weis, Smithgall and Engen, unpublished results].

In summary, the biophysical characterization of SFKs with HXMS and NMR has validated a number of hypotheses from the biological data summarized in the preceding sections. The combination of HXMS with biological approaches in model systems allows access to details about SFK regulation, in particular the intramolecular interactions, which are difficult to obtain by other strategies. One new hypothesis that has also come out of HXMS analysis of SFKs (both Hck and Lck) is that kinase activation does not require drastic conformational changes. Rather, in the case of viral activators such as Nef and Tip, the kinase domain may be held in the active state (which is only modestly different in terms of conformation) for a much longer period of time (or indefinitely) than normal cellular activating events. The higher relative affinity of viral activators compared to the affinity of internal regulatory elements, such as the SH2-kinase linker, plays a significant role in this phenomenon.

Computational studies of Hck SH2 and SH3 dymanics

The biological and HXMS data presented above support the conclusion that activation of Hck through its SH3 domain, either by binding of a high-affinity protein ligand (Nef) to the SH3 domain or by mutation of its intramolecular target sequence (the SH2-kinase linker), does not affect the association of the negative regulatory tail with the SH2 domain. To test this idea computationally, the global conformational dynamics of Hck with and without the SH3:linker interaction were examined using the Gaussian Network Model (GNM) [85–87]. Two Hck crystal structures were used as the starting point for these experiments. The first is the original wild-type kinase structure (PDB 1AD5 [25]), while the second is the structure bearing the high-affinity SH2 binding sequence in its C-terminal tail as described above (Hck-YEEI, Fig. 1; PDB 1QCF [27]).

Figure 7 compares Hck flexibility in the presence and absence of SH3:linker interactions. Panel A displays the mean square fluctuations of individual Hck residues as driven by the most cooperative (lowest frequency) mode of molecular motion. These motions are estimated to be at least two orders of magnitude slower than those in the nanosecond regime, operating on a local scale (at the level of individual residues). The fluctuation profiles of Hck in the absence and presence of the SH3:linker have been rescaled based on their lowest frequency modes (lowest eigenvalues) in order to avoid improper mobilities of Hck in the absence of SH3:linker interactions.

Strikingly, the relative mobilities of the SH3 and SH2 domains change in opposite directions following removal of the SH3:linker interaction from the calculation. In the computations based on the crystal structure that include the linker interaction, the SH2 domain exhibits higher amplitude fluctuations than the SH3 domain and the rest of the molecule, with the exception of the negative regulatory tail. In contrast, in the absence of the SH3-linker interaction, the mobility of the SH3 domain is significantly enhanced, with a coordinated decrease in the motions of the SH2 domain and tail region. Figure 7B shows ribbon diagrams corresponding to the wild-type and linker-destabilized Hck structures, which are color-coded according to decreasing flexibility as determined in panel A (i.e., the spectrum of colors red-magenta-blue-cyan-green show the order of decreasing flexibility). This representation clearly illustrates that, on the slowest timescale, removal of the SH3:linker interaction does not result in a coordinated increase in SH2 mobility and subsequent tail release, but instead may stabilize this interaction. Close examination of the ribbon diagrams for the unmodified wild-type structure reveals that the motion of the SH3 domain is

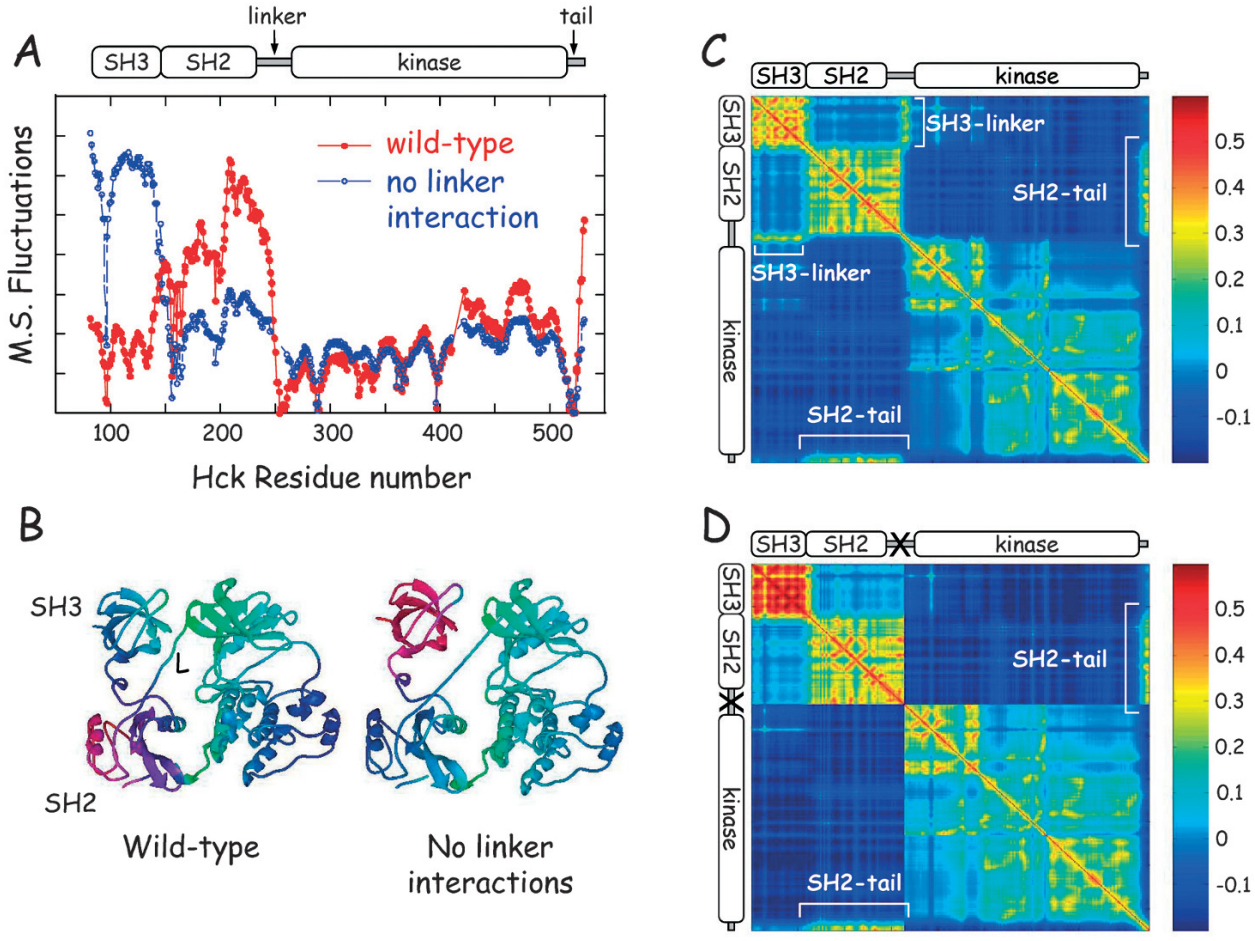

Figure 7. Fluctuation behavior of the wild-type Hck structure and a destabilized form lacking the intramolecular SH3:linker interaction (designated 'no linker interaction'). (*A*) Motions of Hck residues were determined by the Gaussian Network Model of protein dynamics. The ordinate represents the distribution of mean-square (ms) fluctuations ($<\Delta R_i \cong \Delta R_i>$) in the position vectors (R_i) of the individual residues as a function of the residue index, i($82 \leq I \leq 531$; c-Src numbering). Motions for the wild-type Hck crystal structure are shown in red, while those resulting from a hypothetical structure lacking SH3:linker interaction are shown in blue. A scale diagram of Hck is shown at the top for reference. (*B*) Motions illustrated in (*A*) are mapped onto ribbon diagrams of the wild-type Hck crystal structure. The colors used, in order of decreasing flexibility, are red, magenta, blue, cyan, and green. The wild-type Hck structure is shown on the left, while the structure without linker interactions is shown on the right. The positions of the linker (*L*), SH2, and SH3 domains are indicated for the wild-type Hck structure. (*C*, *D*) Correlation maps for residue fluctuations obtained for wild-type Hck (*C*) and the modified form lacking interactions between the SH3 domain and the SH2-kinase linker; the position of linker residue interactions that were removed are indicated by the 'X' (*D*). The correlation map describes the type and strength of the coupling between residue fluctuations. The two axes refer to residue indices, illustrated with scale diagrams of Hck for reference. The value on the map for a given pair of residues i and j is the correlation coefficient C_{ij} between the fluctuations ΔR_i and ΔR_j of these residues, normalized with respect to their ms fluctuations according to $C_{ij} = <\Delta R_i \cdot \Delta R_j> / [<(\Delta R_i)^2> <(\Delta R_j)^2>]^{1/2}$. The maps are symmetric ($C_{ij} = C_{ji}$) and C_{ij} values vary in the range $-1 \leq C_{ij} \leq 1$. The upper limit of 1 represents fully correlated fluctuations (those with the same orientation and sense) while the lower limit of -1 represents fully anti-correlated (same direction, opposite sense) fluctuations. Uncorrelated or orthogonal fluctuations yield $C_{ij} = 0$. Each value was color-coded to produce the maps shown, and the correlation color scale is shown on the right. The squares along the diagonals indicate the structural regions undergoing *en bloc* motions. In the wild-type structure, these include the SH3, SH2, and kinase domains; the N- and C-terminal lobes of the kinase domain can also be distinguished. Positive, off-diagonal correlations between the SH2 domain and the C-terminal tail as well as the SH3 domain and the SH2-kinase linker are readily apparent in the wild-type structure (*C*). The absence of SH3-linker interactions does not impact SH2-tail correlations (*D*). Similar correlation maps for a Hck structure lacking the SH2:tail interaction revealed the persistence of SH3:linker interaction (data not shown).

stabilized by its interaction with the linker in the wild-type structure; as expected, removal of this interaction substantially enhances SH3 mobility. In contrast to SH3, the central β-sheet of the SH2 domain as well as its solvent-exposed α-helix are highly flexible in the unmodified structure. The C-terminal tail exhibits comparable high-amplitude motions that are closely coupled to those of SH2. Motions of both the SH2 domain and C-terminal tail are reduced in the linker-destabilized structure and their dynamic coupling persists. Results of these simulations support our experimental observations that enhanced SH2:tail

interaction does not affect Hck activation by either an SH3 ligand (Nef) or by linker mutation [54]. Identical results were produced following GNM analysis of the Hck crystal structure bearing the high-affinity tail (data not shown).

Figure 7 also shows the correlation maps for residue fluctuations in the wild-type and linker-destabilized structures. The maps reveal strong intra- and inter-domain correlations of motion within the wild-type Hck structure. Along the diagonals, large contiguous blocks of positively correlated residues are readily apparent, which coincide with the sequences of the SH3 and SH2 domains as well as the N- and C-terminal lobes of the kinase domain. Two important interdomain correlations are also readily apparent. The first is the strong correlation between the motions of the SH2 domain and the C-terminal tail, and the second corresponds to the interaction of the SH3 domain with the SH2-kinase linker. These correlations are labeled in Figure 7C, and are expected based on interactions revealed in the crystal structure. Figure 7D shows a correlation map for the Hck structure in which the SH3-linker interaction is removed. Several observations can be made relative to the wild-type structure. First, the SH2:tail correlation remains unchanged. This observation is consistent with both the fluctuation behavior of the linker-destabilized molecule (Fig. 7A, B) as well as experimental evidence showing no change in tail phosphorylation or effect of enhanced SH2:tail engagement upon SH3-directed activating events [54]. Second, correlations of residues within the SH3 domain are dramatically increased in the absence of interactions with the linker, despite enhanced overall motions in the SH3 domain. Whether these changes translate into quantitative or qualitative differences in SH3 function will require further analysis. Finally, inter-domain correlations between SH3 and SH2 are observed, both in the presence and absence of linker:SH3 interactions. This result agrees with molecular dynamics simulations performed by Young et al. which revealed coordinated motions in the SH3-SH2 apparatus that are influenced by the sequence connecting these domains [63]. Coordinated motions of these domains may be essential for effective downregulation of Hck and other SFKs by the 'snap-lock' mechanism described in the preceding section. More recent computational analysis supports and extends this view, by showing that the intrinsic overall capacity of an SH3-SH2 unit to adopt a conformation consistent with assembly of the inactive conformation may be critical to effective downregulation of catalytic activity [88].

Conclusions and future directions

Biological, biophysical, and computational data summarized in this review provide a picture of Src-family kinases as highly dynamic molecular switches that can be regulated by a variety of cellular inputs, particularly in the form of protein-protein contacts of the kinase through its non-catalytic SH3 and SH2 domains. Conformational changes that occur during activation do not appear to be drastic, and slight alteration of the positioning of either regulatory domain can lead to kinase activation. Slight changes, therefore, also imply that downregulation and return to the inactive conformation is not a major conformational event either. This conformational plasticity is consistent with the intrinsic ability of many enzymes to undergo functional changes in conformation [89] and may explain why SFKs have successfully adapted themselves to such a diverse array of cellular signaling environments and inputs from various sources. As key nodes in many signaling pathways, SFKs represent attractive targets for drug design. Blocking aberrant signaling facilitated by SFKs, for example, may provide effective anti-oncogenic therapy. However, SFKs are closely related to one another in terms of structure and regulation, which may make discovery of selective inhibitors quite a challenge. Selectivity in SFK inhibitors may ultimately come from a more complete understanding of conformations adopted by the kinases in specific signaling situations. Such an understanding will require a combined approach involving multiple experimental and computational avenues such as those touched on here. Ultimately, SFKs that adopt specific conformations in signaling pathways associated with oncogenic transformation or other pathologies could be selectively targeted with small molecules that take advantage of the disease-specific state. Some of the current challenges in this field lie in designing experiments to better understand whether conformation-specific signaling does indeed take place *in vivo*, and in development of screening assays that can identify conformationally specific compounds as novel inhibitor leads.

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1 Martin, G. S. (2004) The road to Src. Oncogene 23, 7910–7917.
2 Martin, G. S. (2001) The hunting of the Src. Nat. Rev. Mol. Cell Biol. 2, 467–475.
3 Manning, G., Whyte, D. B., Martinez, R., Hunter, T. and Sudarsanam, S. (2002) The protein kinase complement of the human genome. Science 298, 1912–1934.

4 Lowell, C. A. and Soriano, P. (1996) Knockouts of Src-family kinases: stiff bones, wimpy T cells, and bad memories. Genes Dev. 10, 1845–1857.
5 Brown, M. T. and Cooper, J. A. (1996) Regulation, substrates, and functions of Src. Biochim. Biophys. Acta 1287, 121–149.
6 Parsons, J. T. and Parsons, S. J. (1997) Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. Curr. Opin. Cell Biol. 9, 187–192.
7 Parsons, S. J. and Parsons, J. T. (2004) Src family kinases, key regulators of signal transduction. Oncogene 23, 7906–7909.
8 Ishizawar, R. and Parsons, S. J. (2004) c-Src and cooperating partners in human cancer. Cancer Cell 6, 209–214.
9 Chin, J., Palop, J. J., Puolivali, J., Massaro, C., Bien-Ly, N., Gerstein, H., Scearce-Levie, K., Masliah, E. and Mucke, L. (2005) Fyn kinase induces synaptic and cognitive impairments in a transgenic mouse model of Alzheimer's disease. J. Neurosci. 25, 9694–9703.
10 Kojima, N., Ishibashi, H., Obata, K. and Kandel, E. R. (1998) Higher seizure susceptibility and enhanced tyrosine phosphorylation of N-methyl-D-aspartate receptor subunit 2B in fyn transgenic mice. Learn. Mem. 5, 429–445.
11 Hanna, Z., Weng, X., Kay, D. G., Poudrier, J., Lowell, C. and Jolicoeur, P. (2001) The pathogenicity of human immunodeficiency virus (HIV) type 1 Nef in CD4C/HIV transgenic mice is abolished by mutation of its SH3-binding domain, and disease development is delayed in the absence of Hck. J. Virol. 75, 9378–9392.
12 Resh, M. D. (1994) Myristylation and palmitylation of Src family members: The fats of the matter. Cell 76, 411–413.
13 Musacchio, A., Saraste, M. and Wilmanns, M. (1994) High-resolution crystal structures of tyrosine kinase SH3 domains complexed with proline-rich peptides. Nat. Struct. Biol. 1, 546–551.
14 Kuriyan, J. and Cowburn, D. (1997) Modular peptide recognition domains in eukaryotic signaling. Annu. Rev. Biophys. Biomol. Struct. 26, 259–288.
15 Weng, Z., Thomas, S. M., Rickles, R. J., Taylor, J. A., Brauer, A. W., Seidel-Dugan, C., Michael, W. M., Dreyfuss, G. and Brugge, J. S. (1994) Identification of Src, Fyn, and Lyn SH3-binding proteins: Implications for a function of SH3 domains. Mol. Cell. Biol. 14, 4509–4521.
16 Briggs, S. D., Bryant, S. S., Jove, R., Sanderson, S. D. and Smithgall, T. E. (1995) The Ras GTPase-activating protein (GAP) is an SH3 domain-binding protein and substrate for the Src-related tyrosine kinase, Hck. J. Biol. Chem. 270, 14718–14724.
17 Superti-Furga, G., Fumagalli, S., Koegl, M., Courtneidge, S. A. and Draetta, G. (1993) Csk inhibition of c-Src activity requires both the SH2 and SH3 domains of Src. EMBO J. 12, 2625–2634.
18 Murphy, S. M., Bergman, M. and Morgan, D. O. (1993) Suppression of c-Src activity by C-terminal Src kinase involves the c-Src SH2 and SH3 domains: analysis with *Saccharomyces cerevisiae.* Mol. Cell. Biol. 13, 5290–5300.
19 Okada, M., Howell, B. W., Broome, M. A. and Cooper, J. A. (1993) Deletion of the SH3 domain of Src interferes with regulation by the phosphorylated carboxyl-terminal tyrosine. J. Biol. Chem. 268, 18070–18075.
20 Briggs, S. D., Sharkey, M., Stevenson, M. and Smithgall, T. E. (1997) SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1. J. Biol. Chem. 272, 17899–17902.
21 Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D. and Kuriyan, J. (1993) Binding of a high affinity phosphotyrosyl peptide to the Src SH2 domain: crystal structures of the complexed and peptide-free forms. Cell 72, 779–790.
22 Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J. et al. (1993) SH2 domains recognize specific phosphopeptide sequences. Cell 72, 767–778.
23 Xu, W., Doshi, A., Lei, M., Eck, M. J. and Harrison, S. C. (1999) Crystal structures of c-Src reveal features of its autoinhibitory mechanism. Mol. Cell 3, 629–638.
24 Xu, W., Harrison, S. C. and Eck, M. J. (1997) Three-dimensional structure of the tyrosine kinase c-Src. Nature 385, 595–602.
25 Sicheri, F., Moarefi, I. and Kuriyan, J. (1997) Crystal structure of the Src family tyrosine kinase Hck. Nature 385, 602–609.
26 Williams, J. C., Weijland, A., Gonfloni, S., Thompson, A., Courtneidge, S. A., Superti-Furga, G. and Wierenga, R. K. (1997) The 2.35 Å crystal structure of the inactivated form of chicken Src: a dynamic molecule mith multiple regulatory interactions. J. Mol. Biol. 274, 757–775.
27 Schindler, T., Sicheri, F., Pico, A., Gazit, A., Levitzki, A. and Kuriyan, J. (1999) Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor. Mol. Cell 3, 639–648.
28 Huse, M. and Kuriyan, J. (2002) The conformational plasticity of protein kinases. Cell 109, 275–282.
29 Yamaguchi, H. and Hendrickson, W. A. (1996) Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. Nature 384, 484–489.
30 Cowan-Jacob, S. W., Fendrich, G., Manley, P. W., Jahnke, W., Fabbro, D., Liebetanz, J. and Meyer, T. (2005) The crystal structure of a c-Src complex in an active conformation suggests possible steps in c-Src activation. Structure. (Camb.) 13, 861–871.
31 Nada, S., Okada, M., MacAuley, A., Cooper, J. A. and Nakagawa, H. (1991) Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of $pp60^{c\text{-}src}$. Nature 351, 69–72.
32 Chong, Y. P., Ia, K. K., Mulhern, T. D. and Cheng, H. C. (2005) Endogenous and synthetic inhibitors of the Src-family protein tyrosine kinases. Biochim. Biophys. Acta 1754, 210–220.
33 Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M. and Aizawa, S. (1993) Constitutive activation of Src family kinases in mouse embryos that lack Csk. Cell 73, 1125–1135.
34 Imamoto, A. and Soriano, P. (1993) Disruption of the *csk* gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. Cell 73, 1117–1124.
35 Meyn, M. A., III, Schreiner, S. J., Dumitrescu, T. P., Nau, G. J. and Smithgall, T. E. (2005) SRC family kinase activity is required for murine embryonic stem cell growth and differentiation. Mol. Pharmacol. 68, 1320–1330.
36 Briggs, S. D. and Smithgall, T. E. (1999) SH2-kinase linker mutations release Hck tyrosine kinase and transforming activities in rat-2 fibroblasts. J. Biol. Chem. 274, 26579–26583.
37 Schreiner, S. J., Schiavone, A. P. and Smithgall, T. E. (2002) Activation of Stat3 by the Src family kinase Hck requires a functional SH3 domain. J. Biol. Chem. 277, 45680–45687.
38 Kestler, H., Ringler, D. J., Mori, K., Panicali, D. L., Sehgal, P. K., Daniel, M. D. and Desrosiers, R. C. (1991) Importance of the nef gene for maintenance of high viral loads and for development of AIDS. Cell 65, 651–662.
39 Kirchhoff, F., Greenough, T. C., Brettler, D. B., Sullivan, J. L. and Desrosiers, R. C. (1995) Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. 332, 228–232.
40 Deacon, N. J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D. J., McPhee, D. A., Greenway, A. L., Ellett, A., Chatfield, C. et al. (1995) Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science 270, 988–991.
41 Hanna, Z., Kay, D. G., Rebai, N., Guimond, A., Jothy, S. and Jolicoeur, P. (1998) Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. Cell 95, 163–175.
42 Herna, R. G. and Saksela, K. (2000) Interactions of HIV-1 NEF with cellular signal transducing proteins. Front Biosci. 5, D268-D283.

43 Geyer, M., Fackler, O. T. and Peterlin, B. M. (2001) Structure-function relationships in HIV-1 Nef. EMBO Rep. 2, 580–585.
44 Arold, S. T. and Baur, A. S. (2001) Dynamic Nef and Nef dynamics: how structure could explain the complex activities of this small HIV protein. Trends Biochem. Sci. 26, 356–363.
45 Lee, C.-H., Leung, B., Lemmon, M. A., Zheng, J., Cowburn, D., Kuriyan, J. and Saksela, K. (1995) A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to HIV-1 Nef protein. EMBO J. 14, 5006–5015.
46 Saksela, K., Cheng, G. and Baltimore, D. (1995) Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef^+ viruses but not for down-regulation of CD4. EMBO J. 14, 484–491.
47 Lee, C.-H., Saksela, K., Mirza, U. A., Chait, B. T. and Kuriyan, J. (1996) Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. Cell 85, 931–942.
48 Grzesiek, S., Bax, A., Clore, G. M., Gronenborn, A. M., Hu, J.-S., Kaufman, J., Palmer, I., Stahl, S. J. and Wingfield, P. T. (1996) The solution structure of HIV-1 Nef reveals an unexpected fold and permits delineation of the binding surface for the SH3 domain of Hck tyrosine protein kinase. Nature Struct. Biol. 3, 340–345.
49 Shugars, D. C., Smith, M. S., Glueck, D. H., Nantermet, P. V., Seillier-Moiseiwitsch, F. and Swanstrom, R. (1993) Analysis of human immunodeficiency virus type 1 *nef* gene sequences present in vivo. J. Virol. 67, 4639–4650.
50 Geyer, M. and Peterlin, B. M. (2001) Domain assembly, surface accessibility and sequence conservation in full length HIV-1 Nef. FEBS Lett. 496, 91–95.
51 O'Neill, E., Kuo, L. S., Krisko, J. F., Tomchick, D. R., Garcia, J. V. and Foster, J. L. (2006) Dynamic evolution of the human immunodeficiency virus type 1 pathogenic factor, Nef. J. Virol. 80, 1311–1320.
52 Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C.-H., Kuriyan, J. and Miller, W. T. (1997) Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. Nature 385, 650–653.
53 Trible, R. P., Emert-Sedlak, L. and Smithgall, T. E. (2006) HIV-1 Nef selectively activates SRC family kinases HCK, LYN, and c-SRC through direct SH3 domain interaction. J. Biol. Chem. 281, 27029–27038.
54 Lerner, E. C. and Smithgall, T. E. (2002) SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo. Nat. Struct. Biol. 9, 365–369.
55 Kim, M. O., Suh, H. S., Si, Q., Terman, B. I. and Lee, S. C. (2006) Anti-CD45RO suppresses human immunodeficiency virus type 1 replication in microglia: role of Hck tyrosine kinase and implications for AIDS dementia. J. Virol. 80, 62–72.
56 Choi, H. J. and Smithgall, T. E. (2004) Conserved residues in the HIV-1 Nef hydrophobic pocket are essential for recruitment and activation of the Hck tyrosine kinase. J. Mol. Biol. 343, 1255–1268.
57 Ye, H., Choi, H. J., Poe, J. and Smithgall, T. E. (2004) Oligomerization is required for HIV-1 Nef-induced activation of the Src family protein-tyrosine kinase, Hck. Biochemistry 43, 15775–15784.
58 Johnson, L. N., Noble, M. E.M. and Owen, D. (1996) Active and inactive protein kinases: Structural basis for regulation. Cell 85, 149–158.
59 Pellicena, P. and Miller, W. T. (2001) Processive phosphorylation of p130Cas by Src depends on SH3-polyproline interactions. J. Biol. Chem. 276, 28190–28196.
60 Burnham, M. R., Bruce-Staskal, P. J., Harte, M. T., Weidow, C. L., Ma, A., Weed, S. A. and Bouton, A. H. (2000) Regulation of c-SRC activity and function by the adapter protein CAS. Mol. Cell Biol. 20, 5865–5878.
61 Boonyaratanakornkit, V., Scott, M. P., Ribon, V., Sherman, L., Anderson, S. M., Maller, J. L., Miller, W. T. and Edwards, D. P. (2001) Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases. Mol. Cell 8, 269–280.
62 Pellicena, P. and Miller, W. T. (2002) Coupling kinase activation to substrate recognition in SRC-family tyrosine kinases. Front Biosci. 7, d256-d267.
63 Young, M. A., Gonfloni, S., Superti-Furga, G., Roux, B. and Kuriyan, J. (2001) Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation. Cell 105, 115–126.
64 Lim, W. A. (2002) The modular logic of signaling proteins: building allosteric switches from simple binding domains. Curr. Opin. Struct. Biol. 12, 61–68.
65 Porter, M., Schindler, T., Kuriyan, J. and Miller, W. T. (2000) Reciprocal regulation of Hck activity by phosphorylation of Tyr(527) and Tyr(416). Effect of introducing a high affinity intramolecular SH2 ligand. J. Biol. Chem. 275, 2721–2726.
66 Nika, K., Tautz, L., Arimura, Y., Vang, T., Williams, S. and Mustelin, T. (2007) A weak lck tail bite is necessary for lck function in T cell antigen receptor signaling. J. Biol. Chem. 282, 36000–36009.
67 Lerner, E. C., Trible, R. P., Schiavone, A. P., Hochrein, J. M., Engen, J. R. and Smithgall, T. E. (2005) Activation of the Src family kinase Hck without SH3-linker release. J. Biol. Chem. 280, 40832–40837.
68 LaFevre-Bernt, M., Sicheri, F., Pico, A., Porter, M., Kuriyan, J. and Miller, W. T. (1998) Intramolecular regulatory interactions in the Src family kinase Hck probed by mutagenesis of a conserved tryptophan residue. J. Biol. Chem. 273, 32129–32134.
69 Wales, T. E. and Engen, J. R. (2006) Hydrogen exchange mass spectrometry for the analysis of protein dynamics. Mass Spectrom. Rev. 25, 158–170.
70 Engen, J. R., Smithgall, T. E., Gmeiner, W. H. and Smith, D. L. (1997) Identification and localization of slow, natural cooperative unfolding in the Hck SH3 domain by amide hydrogen exchange and mass spectrometry. Biochemistry 36, 14384–14391.
71 Wales, T. E. and Engen, J. R. (2006) Partial unfolding of diverse SH3 domains on a wide timescale. J. Mol. Biol. 357, 1592–1604.
72 Engen, J. R., Gmeiner, W. H., Smithgall, T. E. and Smith, D. L. (1999) Hydrogen exchange shows peptide binding stabilizes motions in Hck SH2. Biochemistry 38, 8926–8935.
73 Engen, J. R., Smithgall, T. E., Gmeiner, W. H. and Smith, D. L. (1999) Comparison of SH3 and SH2 domain dynamics when expressed alone or in an SH(3+2) construct: the role of protein dynamics in functional regulation. J. Mol. Biol. 287, 645–656.
74 Hochrein, J. M., Lerner, E. C., Schiavone, A. P., Smithgall, T. E. and Engen, J. R. (2006) An examination of dynamics crosstalk between SH2 and SH3 domains by hydrogen/deuterium exchange and mass spectrometry. Protein Sci. 15, 65–73.
75 Ulmer, T. S., Werner, J. M. and Campbell, I. D. (2002) SH3-SH2 domain orientation in Src kinases: NMR studies of Fyn. Structure. 10, 901–911.
76 Tessari, M., Gentile, L. N., Taylor, S. J., Shalloway, D. I., Nicholson, L. K. and Vuister, G. W. (1997) Heteronuclear NMR studies of the combined Src homology domains 2 and 3 of pp60 c-Src: effects of phosphopeptide binding. Biochemistry 36, 14561–14571.
77 Arold, S. T., Ulmer, T. S., Mulhern, T. D., Werner, J. M., Ladbury, J. E., Campbell, I. D. and Noble, M. E. (2001) The role of the Src homology 3-Src homology 2 interface in the regulation of Src kinases. J. Biol. Chem. 276, 17199–17205.
78 Hofmann, G., Schweimer, K., Kiessling, A., Hofinger, E., Bauer, F., Hoffmann, S., Rosch, P., Campbell, I. D., Werner, J. M. and Sticht, H. (2005) Binding, domain orientation, and dynamics of the Lck SH3-SH2 domain pair and comparison with other Src-family kinases. Biochemistry 44, 13043–13050.
79 Gmeiner, W. H., Xu, I., Horita, D. A., Smithgall, T. E., Engen, J. R., Smith, D. L. and Byrd, R. A. (2001) Intramolecular binding of a proximal PPII helix to an SH3 domain in the fusion

protein SH3Hck : PPIIhGAP. Cell Biochem. Biophys. 35, 115–126.
80 Pisabarro, M. T., Serrano, L. and Wilmanns, M. (1998) Crystal structure of the abl-SH3 domain complexed with a designed high-affinity peptide ligand: implications for SH3-ligand interactions. J. Mol. Biol. 281, 513–521.
81 Cobos, E. S., Pisabarro, M. T., Vega, M. C., Lacroix, E., Serrano, L., Ruiz-Sanz, J. and Martinez, J. C. (2004) A miniprotein scaffold used to assemble the polyproline II binding epitope recognized by SH3 domains. J. Mol. Biol. 342, 355–365.
82 Weis, D. D., Kjellen, P., Sefton, B. M. and Engen, J. R. (2006) Altered dynamics in Lck SH3 upon binding to the LBD1 domain of Herpesvirus saimiri Tip. Protein Sci. 15, 2402–2410.
83 Mitchell, J. L., Trible, R. P., Emert-Sedlak, L. A., Weis, D. D., Lerner, E. C., Applen, J. J., Sefton, B. M., Smithgall, T. E. and Engen, J. R. (2007) Functional characterization and conformational analysis of the Herpesvirus saimiri Tip-C484 protein. J. Mol. Biol. 366, 1282–1293.
84 Biesinger, B., Tsygankov, A. Y., Fickenscher, H., Emmrich, F., Fleckenstein, B., Bolen, J. B. and Broker, B. M. (1995) The product of the Herpesvirus saimiri open reading frame 1 (tip) interacts with T cell-specific kinase p56lck in transformed cells. J. Biol. Chem. 270, 4729–4734.
85 Bahar, I., Atilgan, A. R., Demirel, M. C. and Erman, B. (1998) Vibrational dynamics of folded proteins: Significance of slow and fast motions in relation to function and stability. Phys. Rev. Lett. 80, 2733–2736.
86 Haliloglu, T., Bahar, I. and Erman, B. (1997) Gaussian dynamics of folded proteins. Phys. Rev. Lett. 79, 3090–3093.
87 Bahar, I., Atilgan, A. R. and Erman, B. (1997) Direct evaluation of thermal fluctuations in proteins using a single-parameter harmonic potential. Fold. Des 2, 173–181.
88 Faraldo-Gomez, J. D. and Roux, B. (2007) On the importance of a funneled energy landscape for the assembly and regulation of multidomain Src tyrosine kinases. Proc. Natl. Acad. Sci. USA 104, 13643–13648.
89 Bahar, I., Chennubhotla, C. and Tobi, D. (2007) Intrinsic dynamics of enzymes in the unbound state and relation to allosteric regulation. Curr. Opin. Struct. Biol. 17, 633–640.
90 Yamanashi, Y. and Baltimore, D. (1997) Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. Cell 88, 205–211.
91 Eck, M. J., Atwell, S. K., Shoelson, S. E. and Harrison, S. C. (1994) Structure of the regulatory domains of the Src-family tyrosine kinase Lck. Nature 368, 764–769.